Proc. Natl. Acad. Sci. USA
Vol. 78, No. 2, pp. 1260–1263, February 1981
Neurobiology

Dynorphin immunocytochemical localization in brain and peripheral nervous system: Preliminary studies

(opioid peptides/endorphins/anatomy/immunohistochemistry)

S. J. WATSON*, H. AKIL*, VARTAN E. GHAZAROSSIAN†, AND AVRAM GOLDSTEIN†‡

*Mental Health Research Institute, University of Michigan, Ann Arbor, Michigan 48109; and †Addiction Research Foundation and ‡Stanford University, Palo Alto, California 94304

Contributed by Avram Goldstein, October 20, 1980

ABSTRACT **Using antisera specific for the opioid peptide dynorphin, we have carried out immunocytochemical studies of the distribution in rat brain and periphery. In the central nervous system, cells that stain positively for dynorphin are found in the supraoptic nucleus, with less-well-stained cells in the paraventricular nucleus of the hypothalamus. Few positive fibers were detected in brain, suggesting problems with fixation and preservation of antigenicity. In pituitary no staining was seen in the anterior and intermediate lobes but heavy staining was detected in the posterior lobe. In the guinea pig, adrenal chromaffin cells stained with dynorphin antisera. Staining of these cells could be blocked with excess of dynorphin-(1–13) or either enkephalin. Radioimmunoassays revealed a great excess of the enkephalins in the adrenal, suggesting cross competition between dynorphin antiserum and adrenal medullary enkephalin. Finally, the dynorphin antiserum stained a complex of fibers in guinea pig ileum. Staining of these fibers could be blocked by moderate amounts of enkephalin as well as by smaller amounts of dynorphin-(1–13). We conclude that in some places (brain and pituitary) dynorphin exists separately from leucine-enkephalin. In other parts of brain and in the periphery the relationship between dynorphin and the enkephalins is very complex and requires further study and improved antisera.**

Dynorphin is a recently discovered peptide belonging to the family of endogenous opioids (1). The first five amino acids at the NH_2 terminus of dynorphin are identical to leucine-enkephalin; however, from position 6 to 13 it has a unique structure (the remainder of the peptide is currently unknown). While the anatomical localization of β-endorphin and the enkephalins have been well described (2–19), little is currently known about the distribution of dynorphin. This peptide was extracted from porcine pituitaries and was purified by means of a bioassay using the guinea pig ileum. We therefore elected to begin our studies on dynorphin immunocytochemistry by looking at rat pituitary and brain and at guinea pig ileum. We also extended our studies to guinea pig adrenal because of the previous reports of an enkephalin-like immunoreactivity in that organ.

MATERIALS AND METHODS

Tissue preparation for immunocytochemistry was as described (17). Antisera against dynorphin-(1–13) were prepared, with the resulting antigenic specificity largely directed toward a COOH-terminal region (20). Several antisera were produced, with similar specificities; that of highest titer (Lucia) was chosen, because it has been the most thoroughly characterized for radioimmunoassay (RIA). As published elsewhere (20), the Lucia antiserum is used in RIA at titers of from 1:50,000 to 1:200,000 and crossreacts with leucine-enkephalin less than 10^{-8}, and [^{3}H]leucine-enkephalin does not appear to bind even in dilutions as low as 1:30. For peroxidase-antiperoxidase immunocytochemistry this antiserum was used at a 1:600 dilution for a 24-hr incubation at 4°C (peroxidase-antiperoxidase and related reagents were from Sternberger–Meyer Immunocytochemical, Jarrettsville, MD).

An important issue in the immunocytochemical study of dynorphin distribution in brain is the potential confusion with leucine-enkephalin-containing structures. The antiserum used in this study (Lucia) was raised against dynorphin-(1–13) (Peninsula Labs, San Carlos, CA) and its antigenic determinant includes residues 4 and 5 of dynorphin and leucine-enkephalin. While there is little crossreactivity with leucine-enkephalin under RIA conditions, the Lucia antiserum was used in a much more concentrated form for immunocytochemistry. It is therefore possible that the lower-affinity populations of antibody may come into play under our immunocytochemical conditions, and that the crossreactivity with other peptides, including leucine-enkephalin, may be different from that observed in RIA. For that reason complete control blocking studies were carried out on all tissues, using 2–20 μM concentrations of methionine- and leucine-enkephalin, β-endorphin, oxytocin, vasopressin, and dynorphin-(1–13).

In experiments in which relative concentrations of dynorphin and enkephalin were determined, a combined high-performance liquid chromatography (HPLC)–RIA procedure was employed. All tissue was obtained fresh after decapitation, kept chilled on ice during dissection, then frozen immediately on dry ice and kept at −70°C until extraction. Peptides were extracted with acetone/0.2 M HCl (3:1, vol/vol) in the presence of peptidase inhibitors (0.01% phenylmethylsulfonyl fluoride and 0.01% iodoacetamide). Addition of labeled materials showed recovery of all opioid peptides, including dynorphin-(1–13), to exceed 90%. The extracts were concentrated in a Savant Speed Vac evaporator and applied to a reverse-phase column (octadecylsilica Ultrasphere; Altex, Berkeley, CA), using a pH 2.7 40 mM potassium phosphate buffer/acetonitrile gradient for peptide separation. Fractions from HPLC were collected, evaporated, and subjected to radioimmunoassay. Enkephalin RIA was carried out as described (21). The leucine-enkephalin antiserum employed showed less than 2% crossreactivity with dynorphin-(1–13) or with methionine-enkephalin. Dynorphin RIA (using antiserum Lucia) was performed according to Ghazarossian *et al.* (20).

RESULTS

Immunocytochemistry of *rat brain* revealed one heavily stained cell group (supraoptic nucleus; see Fig. 1*A*) and a much more lightly stained cell group (paraventricular nucleus), with a faint suggestion of staining in the suprachiasmatic nucleus. These three nuclei are classically associated with the oxytocin/vaso-

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Abbreviations: RIA, radioimmunoassay; HPLC, high-performance liquid chromatography.

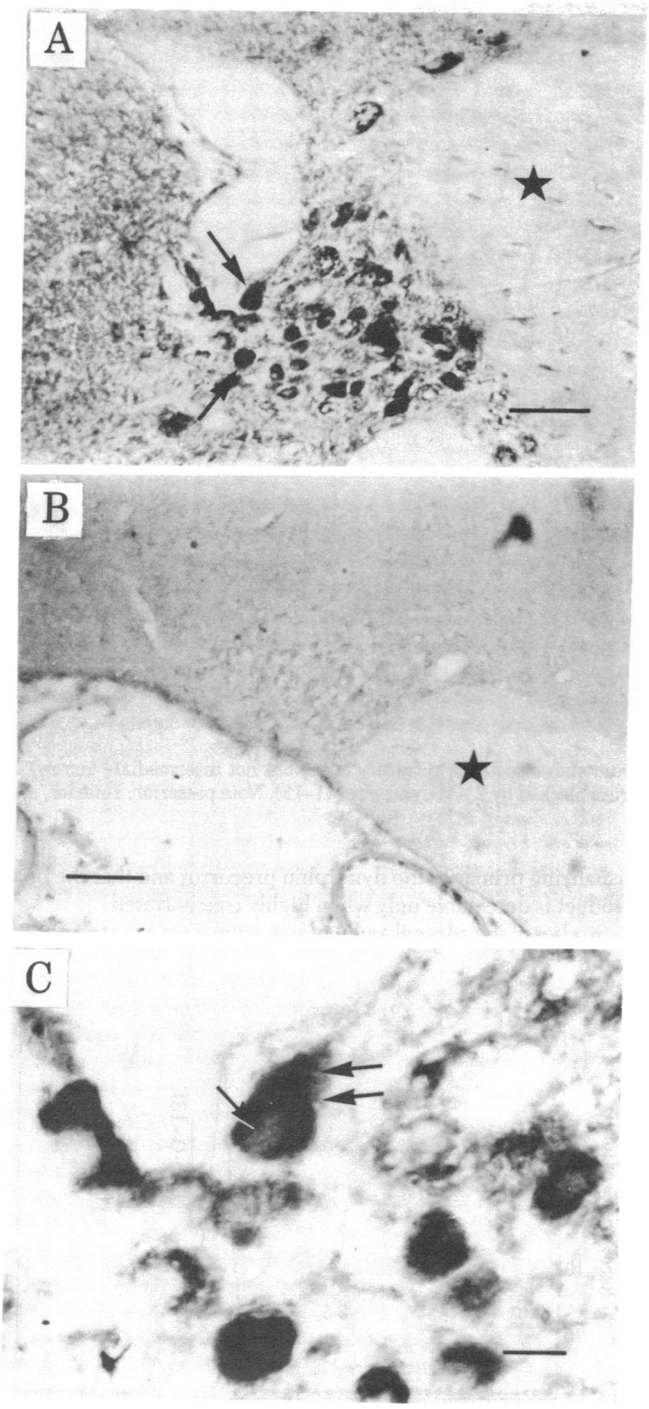

FIG. 1. Supraoptic nucleus of a normal rat. (*A*) Dynorphin immunoreactive cells (arrows). Only a few of the magnocellular neurons are positive. Star is in optic tract. Bar = 50 μm. (*B*) Blocked control of dynorphin antiserum, using 2 μM dynorphin-(1–13). Same animal as seen in *A*. Star is in optic tract. Bar = 100 μm. (*C*) High magnification of same field as seen in *A*. Note the lightly stained nucleus (arrow) with heavy staining around it and moderately stained cytoplasm (two arrows). Bar = 10 μm.

pressin/neurophysin systems in the hypothalamus. All of these demonstrations could be blocked by dynorphin-(1–13) at 2 μM (Fig. 1*B*) but not by any of the other peptides mentioned above at concentrations of 20 μM. Of particular interest was the observation that very few fibers could be seen under these staining and fixation conditions. While it is usually the case that the magnocellular neuronal systems have abundant fiber systems throughout the hypothalamus, in the case of dynorphin no fibers could be detected. A more careful observation of the staining pattern in perikarya of the supraoptic nucleus suggested that the dynorphin staining in those cells was in the perinuclear area (Fig. 1*C*).

This pattern suggested a staining of nascent dynorphin. Taken together with the absence of fiber staining in brain and the inability to trace dynorphin fibers from supraoptic nucleus to median eminence and posterior pituitary, this may indicate that dynorphin precursor was being stained preferentially over the completely processed peptides. Only where the fiber concentration of the final product is very high—as would be expected in the neurosecretory endings of the posterior pituitary—would we expect adequate visualization of the processed dynorphin.

Rat *posterior pituitary* was heavily stained by dynorphin antiserum while intermediate lobe and anterior lobe were left unstained (Fig. 2*A*). Dynorphin-(1–13) (2 μM) was capable of blocking the posterior pituitary demonstration (Fig. 2*B*). However, in contrast to the result with supraoptic nucleus, leucine-enkephalin at very high concentration (20 μM) could produce a decrease of dynorphin staining of posterior pituitary; however, it could not eliminate it entirely. Because of recent work demonstrating the presence of leucine-enkephalin-like immunoreactivity in posterior pituitary (22) and the partial blockade of dynorphin staining by extremely large amounts of leucine-enkephalin, we carried out a biochemical characterization of the dynorphin and enkephalin immunoreactivity (21) in posterior pituitary. The results are presented in Fig. 3. Virtually all the dynorphin immunoreactivity is contained in material that is more polar than leucine-enkephalin. None of the minor peaks coincide with authentic leucine-enkephalin. On the other hand, leucine-enkephalin RIA (not shown) does detect material that cochromatographs with authentic leucine-enkephalin. Thus, it is possible to conclude that both peptides exist in posterior pituitary, a result consonant with that of Goldstein and Ghazarossian (23). The relatively equal concentrations of leucine-enkephalin and dynorphin immunoreactivities suggest that our dynorphin demonstration is not due to crossreactivity with leucine-enkephalin, because a large excess of leucine-enkephalin was required to block the demonstration.

Immunocytochemical studies on *guinea pig adrenal* (Fig. 4*A*) were carried out because of the previous report of enkephalin immunoreactivity in the chromaffin cells of that gland (24). In preliminary staining studies using dynorphin antiserum it was possible to demonstrate the adrenal medullary cells in guinea pig adrenal. These are the same cells that have been reported to produce leucine-enkephalin and the monoamines (24). Control blocking studies, however, were quite different from what had been found in brain and pituitary, in that modest amounts (2 μM) of methionine- and leucine-enkephalin were capable of blocking cellular staining. We assayed dynorphin and leucine-enkephalin content by RIA in a crude synapotosomal (P2) preparation of bovine adrenal prepared according to Holz (25), then extracted as described above. There was approximately 425 times more leucine-enkephalin immunoreactivity than dynorphin immunoreactivity in this preparation (leucine-enkephalin immunoreactivity: 8.5 pmol/mg of P2; dynorphin immunoreactivity: 20 fmol/mg of P2). These results seem to suggest that the staining seen with Lucia antibody could have been from enkephalin crossreactivity.

Immunocytochemical staining of *guinea pig ileum* showed a heavy concentration of dynorphin-positive fibers in the submucous plexus (Fig. 4*B*). Some of the fibers from the submucous plexus penetrated the circularis muscle toward the myenteric plexus located between the two muscle layers. This demonstration could be blocked by dynorphin-(1–13) and also

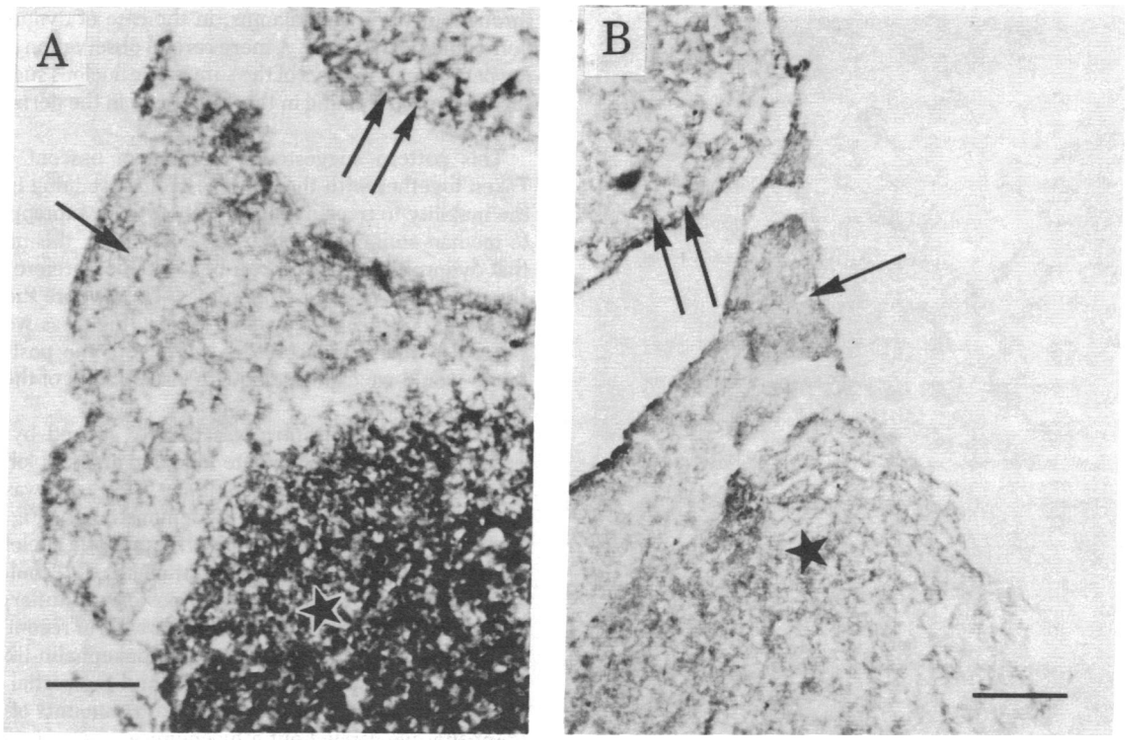

FIG. 2. Rat pituitary staining with dynorphin antiserum. (*A*) The antiserum stains posterior pituitary (star) but not intermediate (arrow) or anterior (two arrows) lobes. Bar = 100 μm. (*B*) Control with dynorphin antiserum blocked by 2 μM dynorphin-(1–13). Note posterior, anterior, and intermediate are similarly unstained. Same rat as *A*. Bar = 100 μm.

by moderate amounts (10 μM) of methionine- and leucine-enkephalin. No biochemical studies have as yet been carried out on guinea pig ileum and therefore it is not possible yet to differentiate dynorphin from enkephalin staining in this organ.

DISCUSSION

We have demonstrated dynorphin-positive cell groups in hypothalamus, with probable projection to posterior pituitary. In the pars nervosa, it is clear from the present and previous work (23) that dynorphin and leucine-enkephalin exist in approximately equal amounts. This hypothalamic–pituitary dynorphin-positive system may be related to other magnocellular peptides, and appears to be separable from the enkephalin and the β-endorphin systems. Thus, a third major opioid peptide system has been demonstrated in the central nervous system.

However, there are several facts that suggest that our demonstration of dynorphin immunoreactivity remains incomplete. We have observed very few fibers in brain, although the posterior pituitary fibers were easily visualized. While the difference may be due to the higher concentration of dynorphin-like material in pituitary fibers, the generally poor fiber demonstration in brain is unusual. Typically, peptide immunocytochemistry reveals primarily fiber patterns, and often requires treatment with colchicine (to block axonal flow) in order to visualize cells of origin. Further, the study by Goldstein and Ghazarossian (23) employing extraction and radioimmunoassay techniques reveals a widespread distribution of dynorphin immunoreactivity in the central nervous system. It is therefore likely that, for technical reasons, our map is incomplete, and has only revealed a subset of the dynorphin-containing pathways. The nature of the technical problem is unclear, but the difficulty may result from a pattern of dynorphin antigenicity in fixed tissue that is different from the pattern obtained in tissue extracts. The observation of perinuclear staining, along with the difficulties discussed above, has led us to suggest that, under our conditions, we are visualizing primarily the dynorphin precursor and that the final product is detectable only when highly concentrated.

Studies of the adrenal and ileum in guinea pig are at an earlier

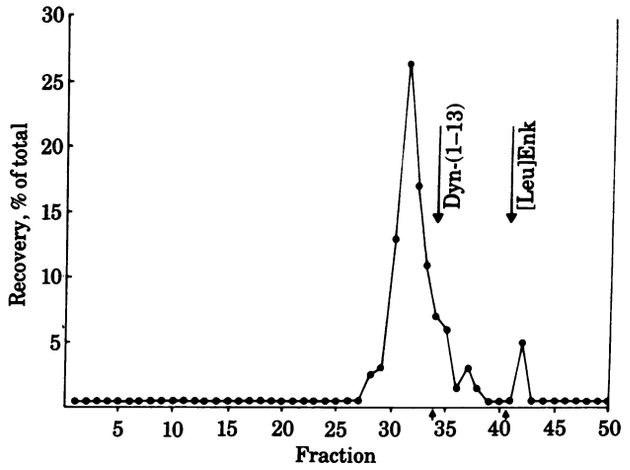

FIG. 3. Profile of dynorphin immunoreactivity in rat posterior–intermediate pituitary. Tissue was extracted as described in the text. The extract was applied to a HPLC column (Altex octadecylsilica Ultrasphere) and eluted with a linear gradient of 20–45% (vol/vol) acetonitrile in 25 min. Fractions (0.5 ml) were collected and evaporated in a Savant Speed Vac concentrator. They were then resuspended in RIA buffer and assayed. Recovery throughout extraction and HPLC exceeded 80%. The major peak of immunoreactive dynorphin is distinguishable from dynorphin-(1–13), which elutes in fraction 34. Leucine-enkephalin is found in fraction 41, just short of the second dynorphin peak in fraction 42. The immunoreactivity in the major peak is 750 fmol of dynorphin-(1–13) equivalents per posterior lobe (uncorrected for recovery). The reported values of leucine-enkephalin immunoreactivity vary between 1.043 (22) and 1.8 (23) pmol per posterior pituitary.

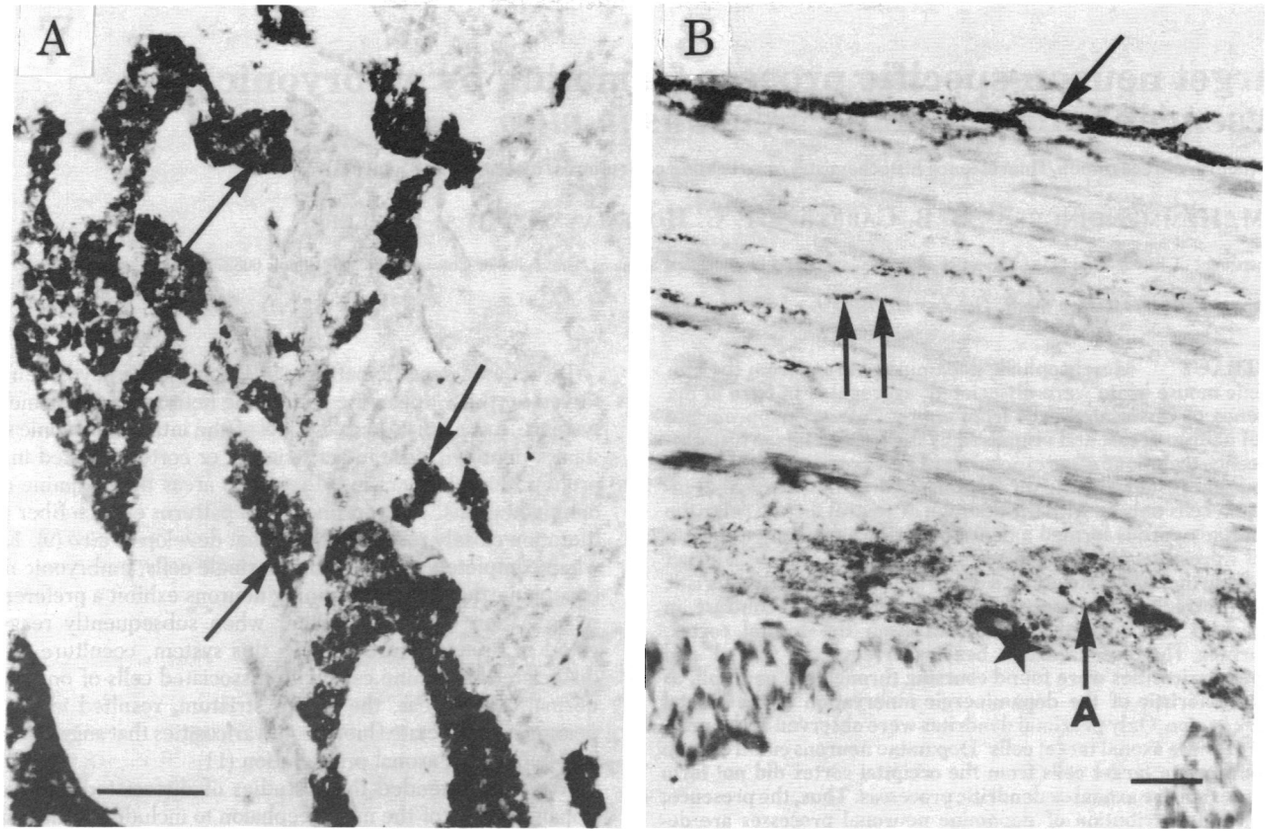

FIG. 4. Dynorphin antiserum staining of guinea pig organs. (*A*) Staining of adrenal medullary cells (arrows). This demonstration was blocked by 2 μM dynorphin or 2 μM methionine- or leucine-enkephalin. Bar = 100 μm. (*B*) Dynorphin-positive fibers in the submucous plexus (arrow). These are heavy bundles of fibers. Between the submucous plexus and myenteric plexus (*A*) are several positive fibers in the circularis muscle layer (two arrows). Finally myenteric plexus has many fine positive terminals in it. These demonstrations are blocked by 2 μM dynorphin-(1–13) but not by 1 μM methionine- or leucine-enkephalin. Blocking was obtained with either enkephalin at 10 μM. The cell marked by the star is nonspecifically stained, because staining was not blocked by any of the peptides. Bar = 20 μm.

stage but suggest a need for very careful biochemical evaluations in parallel with the immunocytochemistry until such time as immunocytochemically useful antibodies with even greater specificity for dynorphin are available.

The authors express their gratitude to P. Erskine for manuscript preparation and to R. Beer, R. Thompson, S. Burke, Y. Ueda, and H.-L. Lin for technical assistance. This work is supported by National Institute on Drug Abuse Grants DA 02265-02 (to H.A. and S.J.W.) and DA 1199 (to A.G.)

1. Goldstein, A., Tachibana, S., Lowney, L. I., Hunkapiller, M. & Hood, L. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 6666–6670.
2. Bloch, B., Bugnon, C., Fellman, D. & Lenys, D. (1978) *Neurosci. Lett.* **10**, 147–152.
3. Bloom, F. E., Battenberg, E., Rossier, J., Ling, N. & Guillemin, R. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 1591–1595.
4. Bloom, F. E., Battenberg, E., Rossier, J., Ling, N., Leppaluoto, J., Vargo, T. M. & Guillemin, R. (1977) *Life Sci.* **20**, 43–48.
5. Cuello, A. C. & Paximos, G. (1978) *Nature (London)* **271**, 178–180.
6. Elde, R., Hökfelt, T., Johansson, O. & Terenius, L. (1976) *Neuroscience* **1**, 349–351.
7. Hökfelt, T., Elde, R., Johansson, O., Terenius, L. & Stein, L. (1977) *Neurosci. Lett.* **5**, 25–31.
8. Moon, H. D., Li, C. H. & Jennings, B. M. (1973) *Anat. Rec.* **175**, 524–538.
9. Nilaver, G., Zimmerman, E. A., Defendni, R., Liotta, A., Krieger, D. A. & Brownstein, M. (1979) *J. Cell Biol.* **81**, 50–58.
10. Pelletier, G., Desy, L., Lissitszky, J.-C., Labrie, F. & Li, C. H. (1978) *Life Sci.* **22**, 1799–1804.
11. Pelletier, G., Leclerc, R., Labrie, F., Cote, J., Chretien, M. & Les, M. (1977) *Endrocrinology* **100**, 770–776.
12. Simantov, R., Kuhar, M. J., Uhl, G. R. & Snyder, S. H. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 2167–2171.
13. Sofroniew, M. V. (1979) *Am. J. Anat.* **154**, 283–289.
14. Uhl, G. R., Kuhar, M. J. & Snyder, S. H. (1978) *Brain Res.* **149**, 223–228.
15. Watson, S. J., Akil, H., Richard, C. W. & Barchas, J. D. (1978) *Nature (London)* **275**, 226–228.
16. Watson, S. J., Akil, H., Sullivan, S. O. & Barchas, J. D. (1977) *Life Sci.* **25**, 733–738.
17. Watson, S. J., Barchas, J. D. & Li, C. H. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 5155–5158.
18. Watson, S. J., Richard, C. W. & Barchas, J. D. (1978) *Science* **200**, 1180–1182.
19. Zimmerman, E. A., Liotta, A. & Krieger, D. T. (1978) *Cell Tissue Res.* **186**, 393–398.
20. Ghazarossian, V. E., Chavkin, C. & Goldstein, A. (1980) *Life Sci.* **27**, 75–86.
21. Akil, H., Watson, S. J., Sullivan, S. & Barchas, J. D. (1978) *Life Sci.* **23**, 121–126.
22. Rossier, J., Battenberg, E., Pittman, Q., Bayon, A., Koda, L., Miller, R., Guillemin, R. & Bloom, F. (1979) *Nature (London)* **277**, 653–655.
23. Goldstein, A. & Ghazarossian, V. E. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 6207–6210.
24. Schultzberg, M., Lundberg, J. M., Hökfelt, T., Terenius, L., Brandt, J., Elde, R. & Goldstein, M. (1978) *Neuroscience* **3**, 1169–1186.
25. Holz, R. W. (1980) *J. Biol. Chem.* **255**, 7751–7755.