Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 1528–1531, March 1986
Neurobiology

Melanin-concentrating hormone: Unique peptide neuronal system in the rat brain and pituitary gland

(immunohistochemistry/radioimmunoassay)

NADAV ZAMIR*, GERHARD SKOFITSCH†, MICHAEL J. BANNON‡, AND DAVID M. JACOBOWITZ†

*Hypertension-Endocrine Branch, National Heart, Lung and Blood Institute, Bethesda, MD 20892; †Laboratory of Clinical Science, National Institute of Mental Health, Bethesda, MD 20892; and ‡Department of Psychiatry, Yale University School of Medicine, New Haven, CT 06508

Communicated by Seymour S. Kety, October 7, 1985

ABSTRACT **A unique neuronal system was detected in the rat central nervous system by immunohistochemistry and radioimmunoassay with antibodies to salmon melanin-concentrating hormone (MCH). MCH-like immunoreactive (MCH-LI) cell bodies were confined to the hypothalamus. MCH-LI fibers were found throughout the brain but were most prevalent in hypothalamus, mesencephalon, and pons–medulla regions. High concentrations of MCH-LI were measured in the hypothalamic medial forebrain bundle (MFB), posterior hypothalamic nucleus, and nucleus of the diagonal band. Reversed-phase high-performance liquid chromatography of MFB extracts from rat brain indicate that MCH-like peptide from the rat has a different retention time than that of the salmon MCH. An osmotic stimulus (2% NaCl as drinking water for 120 hr) caused a marked increase in MCH-LI concentrations in the lateral hypothalamus and neurointermediate lobe. The present studies establish the presence of MCH-like peptide in the rat brain. The MCH-LI neuronal system is well situated to coordinate complex functions such as regulation of water intake.**

Many lower vertebrates exhibit integumental color changes in response to variations in environmental coloration. A dual hormonal control of color change by two antagonistic pituitary melanophorotropic hormones was first suggested in 1931 by Hogben and Slome (1). The melanotropins, α- and β-MSH, are responsible for pigment dispersion in the integumentary melanophore of lower vertebrates (2). These peptides are derived from a common precursor—proopiomelanocortin—by proteolysis within the intermediate lobe of the pituitary gland (2). A putative melanin-concentrating hormone (MCH) (3) was recently isolated from the salmon pituitary gland and characterized (4). This cyclic heptadecapeptide (H-Asp-Thr-Met-Arg-Cys-Met-Val-Gly-Arg-Val-Tyr-Arg-Pro-Cys-Trp-Glu-Val-OH) was reported to stimulate aggregation of melanosomes within melanophores of several species of teleost fishes, both *in vitro* and *in vivo* (4–6), while paradoxically dispersing melanosomes within melanophores in frogs and lizards (6, 7). MCH is synthesized in the teleost hypothalamus and stored and released by the neurohypophysis (8–10). Little is known about the existence and localization of MCH in the mammalian nervous system (10). We now present evidence from RIA and immunohistochemical studies that a peptide resembling the fish neuropeptide MCH occurs in the central nervous system of the rat. This material is distinguishable in immunoreactive properties and distribution from all presently known peptides.

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.

MATERIALS AND METHODS

Peptides and Antisera. Antisera to salmon MCH were obtained by immunization of rabbits with synthetic salmon MCH (Peninsula Laboratories, San Carlos, CA) coupled to thyroglobulin by carbodiimide and glutaraldehyde (11). All other peptides used in this study were from Peninsula Laboratories. In an RIA with ^{125}I-labeled salmon MCH as a tracer, the antiserum failed to show significant affinity (<0.01% relative to MCH) for the following peptides: leucine enkephalin, methionine enkephalin, dynorphin A, β-endorphin, α melanotropin, substance P, oxytocin, and vasopressin.

Radioimmunoassay. Male Sprague–Dawley rats (200–250 g) were killed by decapitation, and their brains were removed. Tissue samples were microdissected from 300-μm sections of the brain (12). At the onset of these studies, three different extraction methods of hypothalamic tissue were examined, (*i*) 2 M acetic acid in boiling water bath for 10 min, (*ii*) 98% methanol in 0.2 M acetic acid, or (*iii*) 0.1 M HCl in boiling water bath for 10 min. Since 0.1 M HCl yielded the greatest amount of MCH-like immunoreactivity (MCH-LI), this method of extraction was adopted. To test the efficiency of the extraction procedure, we added salmon ^{125}I-MCH to lateral hypothalamic tissues just before extraction. About 80–90% of the salmon ^{125}I-MCH was recovered in the extract. Tissue samples were placed in Eppendorf tubes containing 200 μl of 0.1 M HCl and homogenized by sonication. Aliquots (20 μl) of the homogenates were taken for protein determination (13). The homogenates were centrifuged at 2000 × g for 10 min at 4°C. The supernatant fluids were transferred to 12 × 75 mm polystyrene tubes and evaporated to dryness in a vacuum centrifuge. Double-antibody RIA was performed as described here. Samples were rehydrated in phosphate-buffered saline (pH 7.6). The assay buffer consists of 62 mM Na_2HPO_4, 13 mM Na_2EDTA, 0.1% bovine serum albumin, 0.1% gelatin, 0.1% Triton X-100, and 0.01% merthiolate. Antisera were used at a final dilution of 1:60,000, which resulted in 30–40% noncompetitive binding of the trace. Each sample was incubated in a 500-μl volume containing 300 μl of sample in assay buffer, 100 μl of synthetic salmon ^{125}I-MCH (≈6000 cpm), and 100 μl of diluted antisera. Normal rabbit serum was also added to the first antibody solution to give a total normal rabbit serum concentration of 2% (vol/vol). One hundred microliters of this solution was added to all except "nonspecific binding" tubes, which received 100 μl of 2% normal rabbit serum in assay buffer. Reagents were mixed, and the tubes were incubated for 16–24 hr. One hundred microliters of goat anti-rabbit gamma globulin diluted with assay buffer to a concentration sufficient for maximal precipitation was added to all tubes. Reagents were mixed, and

Abbreviations: MCH, melanin-concentrating hormone; MCH-LI, MCH-like immunoreactivity; MFB, medial forebrain bundle; RP-HPLC, reversed-phase high-performance liquid chromatography.

the tubes were incubated for 12–24 hr. After incubation with the second antibody, all tubes were centrifuged for 20 min at 2000 × g at 4°C. The supernatant fluid was aspirated, and the pellet was assayed in a γ counter. The RIA sensitivity was <8 pg per tube.

Immunohistochemistry. For immunohistochemistry, rats were used either without treatment or 2 days after intracerebroventricular injection of 100 μg of colchicine in 25 μl of saline. The rats were anesthetized with sodium pentothal and were perfused through the ascending aorta with 10% formalin in 0.1 M sodium phosphate buffer (pH 7.4). The brains were removed, postfixed in the same fixative for 30 min, and then rinsed in 0.1 M sodium phosphate (pH 7.4) containing 20% sucrose for 48 hr. Brain sections (20 μm) were cut in a cryostat and processed by an indirect immunofluorescence procedure (14). Briefly, the sections were incubated at 4°C for 72 hr with the primary antiserum diluted 1:1000 in 0.1 M sodium phosphate (pH 7.4) containing 0.3% Triton X-100 and 1% normal goat serum, then washed three times (5 min each) with 0.1 M sodium phosphate (pH 7.4) containing 0.2% Triton X-100, and further incubated for 30 min with fluorescein isothiocyanate conjugated with goat anti-rabbit IgG (1:400 in 0.1 N sodium phosphate (pH 7.4) containing 0.3% Triton X-100). Fluorescence was monitored with a Leitz orthoplan fluorescence microscope equipped with a Ploem illuminator. Immunofluorescent cell bodies were seen in colchicine-treated rat brains only.

Osmotic Stimulus. Male Sprague–Dawley rats (220–250 g in body weight) were given saline [2% (wt/vol) NaCl] to drink for 120 hr. Rats given free access to tap water served as controls.

RESULTS AND DISCUSSION

In a RIA with salmon ^{125}I-MCH, the concentration of synthetic MCH needed for 50% inhibition of binding of label was between 60 and 70 pmol/liter (Fig. 1). The serial dilution curve obtained from extracts of rat hypothalamic medial forebrain bundle showed a dose-dependent and parallel displacement of the salmon MCH tracer (Fig. 1).

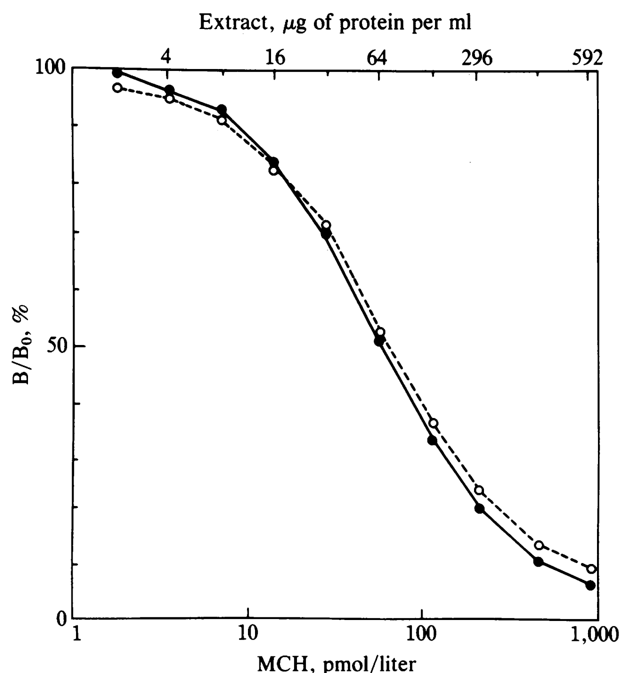

FIG. 1. Inhibition of binding of ^{125}I-MCH to antiserum by graded concentrations of synthetic salmon MCH standard (●) or hypothalamic MFB extract (○).

The distribution of MCH-LI was studied by an indirect immunofluorescence method (14). Rat brain showed an extensive system of immunofluorescent nerve cell bodies containing MCH-LI, extending from the medial forebrain bundle (MFB) medially to a region dorsal to the paraventricular and dorsomedial nuclei and ventral to the zona incerta of the subthalamus (Fig. 2*A*). A few cell bodies were seen in the posterior hypothalamic nucleus. Cell bodies with MCH-LI were confined only to the hypothalamus. Dense networks of fibers and terminals with MCH-LI were identified in the posterior hypothalamic MFB, nucleus of the diagonal band (Fig. 2*C*) and medial lemniscus. Moderate numbers of fibers with MCH-LI were detected in several hypothalamic areas (including the posterior hypothalamic, anterior hypothalamic, medial mammillary, preoptic periventricular, paraventricular, and arcuate nuclei). The median eminence (Fig. 2*D*) and pituitary stalk (Fig. 2*B*) were moderately innervated. Mesencephalic and pontine regions such as the central gray, parabrachial, and dorsal tegmental nuclei contain a moderate number of fibers. Sparse fibers with MCH-LI were present in the cerebral cortex, hippocampus, dentate gyrus, olfactory bulb, caudate-putamen, nucleus accumbens, amygdala, septum, thalamus, epithalamus (habenula), and several nuclei of the medulla oblongata (e.g., substantia gelatinosa, nucleus of the solitary tract, the lateral-reticular and gigantocellular nuclei) and spinal cord. The positive MCH-immunostaining in brain was completely abolished by preabsorption of the antiserum with salmon MCH (1 μM).

The concentration of MCH-LI in a selection of brain areas, as determined by radioimmunoassay, is shown in Table 1. These results are in agreement with the immunohistochemical study and show that MCH-LI is concentrated particularly in the hypothalamus, especially in the MFB, anterior and posterior hypothalamic nuclei, medial mammillary nucleus, and median eminence. High concentrations of MCH-LI are present in discrete telencephalic, diencephalic, and mesencephalic areas, which have dense networks of MCH-LI fibers (e.g., in the nucleus of the diagonal band, bed nucleus of the stria terminalis, and medial lemniscus). The neurointermediate lobe of the pituitary gland contains a relatively low concentration of the peptide (Table 1).

Analysis of rat brain MFB extracts by reversed-phase high-performance liquid chromatography (RP-HPLC) and RIA showed that MCH-LI is eluted at a position different from that of a synthetic salmon MCH (Fig. 3). Gel chromatography of rat hypothalamic extract (using P-6 and P-2 polyacrylamide gels, Bio-Rad) revealed that the material with

Table 1. Distribution of MCH-LI in the rat brain

Region	MCH-LI, fmol/mg of protein
Olfactory tubercle	241.0 ± 28.0
Frontal cortex	263.0 ± 18.0
Nucleus of the diagonal band	1951.0 ± 147.0
Central amygdaloid nucleus	261.0 ± 15.0
Medial septum	423.0 ± 15.0
Bed nucleus of the stria terminalis	622.0 ± 32.0
Subzona incerta	2923.0 ± 244.0
Anterior hypothalamic nucleus	727.0 ± 38.0
Posterior hypothalamic nucleus	1588.0 ± 125.0
Medial forebrain bundle (hypothalamic)	1638.0 ± 198.0
Medial mammillary nucleus	1188.0 ± 105.0
Median eminence	733.0 ± 57.0
Medial lemniscus	757.0 ± 97.0
Nucleus of the solitary tract	257.0 ± 12.0
Cervical spinal cord (dorsal horn)	192.0 ± 16.0
Neurointermediate lobe	172.0 ± 16.0

Values in the table represent the means ± SEM (n = 6).

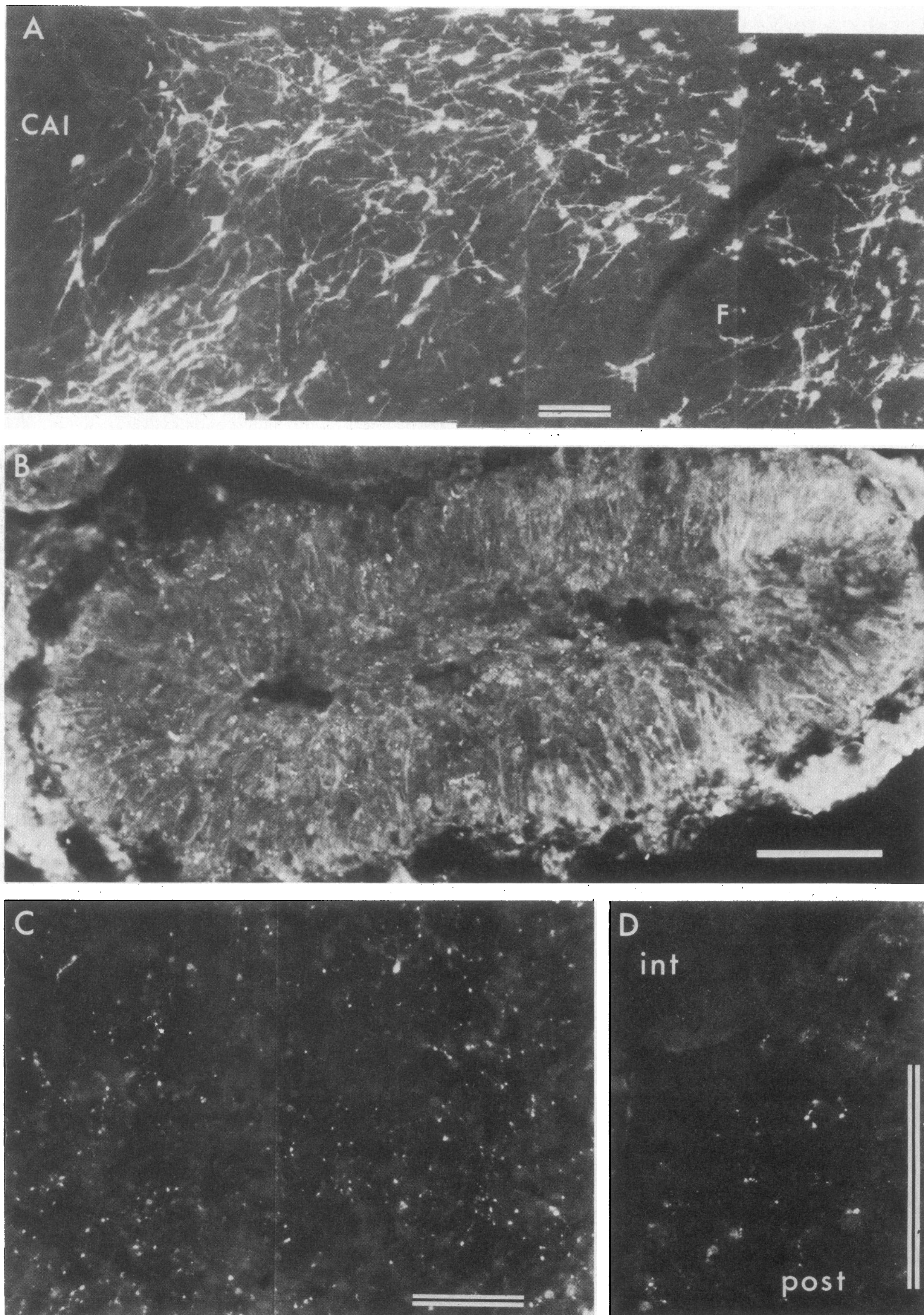

FIG. 2. Fluorescence photomicrographs of the immunoreactive MCH–neuronal system in the rat brain. (*A*) MCH-immunoreactive cell bodies with long multipolar processes in the posterior hypothalamic MFB; F = fornix; CAI = internal capsule. (*B*) MCH-immunoreactive fibers in the pituitary stalk. (*C*) Dense network of MCH-immunoreactive fibers and terminals in the nucleus of the diagonal band. (*D*) MCH-immunoreactive fibers innervating the posterior pituitary. (Bar = 50 μm.)

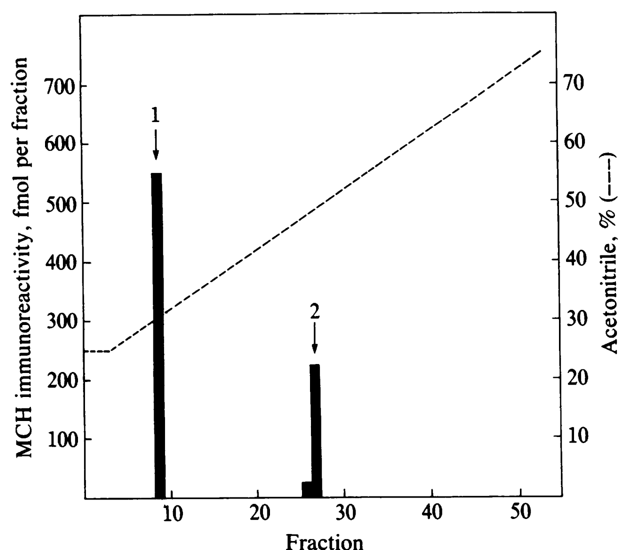

FIG. 3. RP-HPLC of rat hypothalamic MFB extract. Tissues were homogenized in 0.1 M HCl by sonication. The homogenate was centrifuged (10,000 × g for 10 min), and the supernatant fluid was removed and subjected to chromatography. Synthetic salmon MCH was subjected to the same procedures under identical conditions and the immunoreactivity profiles were compared. The RP-HPLC column (μBondapak C_{18} column) was eluted with a 25–75% linear gradient of acetonitrile (as indicated by the broken line) in water containing 0.1% trifluoroacetic acid at a flow rate of 1 ml per min. Fractions (1 ml) were collected, dried, and assayed for MCH after resuspension in assay buffer. Top arrows indicate elution positions of synthetic salmon MCH (column 1) and MCH-like material in MFB extract (column 2).

MCH-LI does not have the same apparent molecular mass as synthetic salmon MCH (2100 daltons) but instead is eluted in several peaks of <1800 daltons (data not shown). The precise nature of these peptides with MCH-LI awaits amino acid sequence analysis. Thus, the mammalian MCH-like substance is recognized by salmon MCH-directed antiserum in RIA and immunohistochemistry and diluted in parallel with synthetic MCH, yet exhibits distinct chromatographic properties on both RP-HPLC and gel chromatography. Both MCH-LI from hypothalamic extract and synthetic salmon MCH were completely destroyed by incubation with a protease (20 μg of Pronase for 1 hr at 37°C) but were unaffected by heating in boiling water, providing evidence for the peptidergic nature of MCH-LI. Therefore, full characterization of the substance identified here must await its isolation.

MCH bioactivity was found in hypothalamus in the nucleus lateralis tuberis and the dorsal hypothalamus of fish. The equivalent regions in the rat hypothalamus also contain large numbers of fibers and cell bodies with MCH-LI. The physiological role of MCH-like material in mammalian brain is unknown. However, its persistence in the mammalian hypothalamo–neurohypophyseal system suggests a role in posterior pituitary function, distinct from the mediation of color change seen in lower vertebrates. Indeed, an osmotic stimulus (2% NaCl as drinking fluid for 120 hr) that is associated with enhanced secretory activity from the neurohypophysis (15) caused significant increases in MCH-LI concentrations in the lateral hypothalamic (area which includes the MFB) and in the neurointermediate lobe (Table 2).

Table 2. MCH stimulation by drinking 2% NaCl

Region	MCH, fmol/mg of protein	
	Control	2% NaCl
Lateral hypothalamus	379.0 ± 55.0	770.0 ± 91.0*
Neurointermediate lobe	294.0 ± 39.0	732.0 ± 63.0*

MCH concentrations (fmol/mg of protein) in rat lateral hypothalamus and neurointermediate lobe of pituitary gland after 120 hr of 2% NaCl as drinking fluid. Values are means ± SEM.
*$P < 0.01$ ($n = 8$) compared to the appropriate control group (Student's two-tailed t test).

Since MCH-containing neurons originate exclusively from the hypothalamus to innervate the pituitary gland, hypothalamus, and many extra hypothalamic brain regions, MCH may be well-situated to coordinate complex functions such as regulation of food and water intake.

The technical assistance of Mrs. Reingard Resch is gratefully acknowledged. G.S. is an International Research Fellow of the Fogarty International Center (Fellowship 3F05 TWO 3293-01S2 BI-5).

1. Hogben, L. P. & Slome, D. (1931) *Proc. R. Soc. London Ser. B* **108**, 10–53.
2. Eipper, B. A. & Mains, R. E. (1980) *Endocrinol. Rev.* **1**, 1–27.
3. Enami, M. (1955) *Science* **121**, 36–37.
4. Kawauchi, H., Kawazoe, I., Tsubokawa, M., Kishida, M. & Baker, B. I. (1983) *Nature (London)* **305**, 321–323.
5. Gilham, I. D. & Baker, B. I. (1984) *J. Endocrinol.* **102**, 237–245.
6. Wilkes, B. C., Hruby, V. Z., Castrucci, A. M. L., Sherbrooke, W. C. & Hadley, M. E. (1984) *Science* **224**, 1111–1114.
7. Wilkes, B. C., Hruby, V. Z., Sherbrooke, W. C., Castrucci, A. M. L. & Hadley, M. E. (1984) *Biochem. Biophys. Res. Commun.* **122**, 613–619.
8. Baker, B. I. & Ball, J. N. (1975) *Gen. Comp. Endocrinol.* **25**, 147–152.
9. Rance, T. A. & Baker, B. I. (1978) *Gen. Comp. Endocrinol.* **37**, 64–73.
10. Baker, B. I. & Rance, T. A. (1983) *Gen. Comp. Endocrinol.* **50**, 423–431.
11. Goodfriend, T. L., Levine, L. & Fasman, G. D. (1964) *Science* **144**, 1344–1346.
12. Palkovits, M. (1973) *Brain Res.* **59**, 449–450.
13. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275.
14. A. H. Coons (1958) in *General Cytochemical Methods* (Academic, New York), pp. 399–422.
15. Zamir, N., Zamir, D., Eiden, L., Palkovits, M., Brownstein, M. J., Eskay, R., Weber, E., Faden, A. & Feuerstein, G. (1985) *Science* **228**, 606–608.