Intracellular dye-marked enkephalin neurons in the magnocellular preoptic nucleus of the goldfish hypothalamus
(electrophysiology/immunocytochemistry/Lucifer Yellow-CH/neuropeptides/opioid peptides)

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ABSTRACT A method that combines intracellular recording, dye marking, and immunocytochemistry makes the study of functional and morphological aspects of enkephalin neurons in the magnocellular preoptic nucleus of the goldfish hypothalamus feasible. By use of multiple techniques, enkephalin neurons can be distinguished from other brain cells and can be reconstructed from drawings of serial sections containing the dye-injected opioid cells. These enkephalin cells and their processes measure 14–42 μm in somata diameter and are unipolar, bipolar, or multipolar. Their electrophysiological properties match those of other mammalian and fish magnocellular endocrine cells. This report confirms the one neuron–one hormone (peptide) hypothesis, supports synaptic over electrotonic coupling between enkephalin and adjacent hypothalamic neurons, and suggests that chemical and functional classification of single electrophysiologically and neuroanatomically studied central neurons can be achieved.

Over the past 20 years, electrophysiological analysis of single cells in the hypothalamus has provided important data on neuronal responses to discrete physical, chemical, and behavioral stimuli (1). Despite these maneuvers, investigators have been unable to identify or directly classify these functionally studied cells chemically. The recent discovery of enkephalin in the magnocellular nucleus, the hypothalamus, and the pituitary gland of the goldfish (2–5) confirms earlier studies in mammals (6–10) and suggests a role for endogenous opioid peptides in neurophysiological regulation in fish and mammals.

The combination of cell identification by immunocytochemistry with a previously developed intracellular dye-injecting approach in the goldfish (11) provides a working model for chemical typing of single cells. This investigation offers an approach to the analysis of neural networks by use of the current technology of cell identification by dye marking (12), peptide biochemistry (13), and immunocytochemistry (6) for the study of single, electrophysiologically defined, morphologically reconstructed magnocellular neurons that contain enkephalin.

MATERIALS AND METHODS

Adult goldfish (Carassius auratus), weighing 290–850 g and measuring 25–35 cm, were anesthetized, immobilized, suspended, and perfused with water, and the brain was exposed according to earlier methods (11, 14).

Glass micropipettes were filled with a 3% aqueous solution of Lucifer Yellow-CH (LY; see ref. 12) and beveled (13) to impedances of 6–40 MΩ for intracellular recordings. Electrophysiological recording and stimulating apparatus and the pituitary stimulating electrodes have been described (11). Intracellular electrophoretic injection (5–10 nA of hyperpolarizing or depolarizing current, 0.3–6 min) of LY (an anionic fluorescent dye) marked the physiologically studied, antidromically identified preoptic neurons for subsequent localization. In most fish, only one cell per nucleus was filled with LY. At the conclusion of each experiment, the fish was perfused with teleost physiological solution (16) followed by Bouin’s fixative (picric acid/formalin/acetic acid, 15:5:1) before the brain was removed, blocked, and immersed in fixative overnight at 4°C. Hypothalamic blocks were embedded in paraffin, sectioned serially at 10 μm in the frontal plane, mounted on glass slides, and dried (2).

In order to see the yellow LY-filled preoptic neuron somata and processes, sections were deparaffinized and examined under a Leitz Orthoplan microscope with Ploem illumination from an HBO 200 mercury vapor lamp with a BG-12 exciter filter and emitted light barrier filters (>495 nm). Selected serial sections containing parts of a dye-marked preoptic neuron were identified chemically by the indirect immunofluorescence technique (4, 6, 17). Three adjacent serial sections of each LY-filled cell were incubated separately at room temperature in high humidity with one of three specific rabbit antisera diluted 1:100 against [Met]enkephalin (anti-[Met]enkephalin, lot 22Tu8, Immuno-Nuclear, Stillwater, MN), vasotocin ([Arg]oxytocin) (antivasotocin, lot G2, a gift from John Fernstrom, Massachusetts Institute of Technology, Cambridge, MA), or oxytocin (antioxytocin, lot K22, a gift from R. R. Dries, Ferring Pharmaceuticals, Kiel, W. Germany) for 15–24 hr. Sections were rinsed in 10 mM phosphate-buffered saline with 1% normal goat serum and then incubated for 30 min at room temperature in high humidity with rhodamine-conjugated goat anti-rabbit IgG antisera (lot 10968, Cappel Labs, Cochrenville, PA) diluted 1:10 with 10 mM phosphate-buffered saline. Finally, sections were rinsed in phosphate-buffered saline, dehydrated, and placed on a coverslip with a fluorescence-free medium (Entellan, Merck, Darmstadt, W. Germany). Antiserum specificity was determined (2, 4) by substituting normal rabbit serum for the primary antiserum, omitting the primary antiserum step, and by liquid- or solid-phase absorption of the primary antibodies with excess [Arg]vasopressin (lot 9459, Bachem Fine Chemicals, Torrance, CA), β-endorphin (lot R9925, Bachem), isotocin ([Ser, Ile]oxytocin) (lot JJ-IV-65, from M. Manning), [Leu]enkephalin (lot R1160, Bachem), [Met]enkephalin (lot R12394, Bachem), mesotocin ([Ile]oxytocin) (lot JJ-IV-70, M. Manning), and oxytocin (lot 5969, Bachem). Anti-[Met]enkephalin reacted only with [Leu]enkephalin and [Met]enkephalin. Antivasotocin reacted only with vasopressin and vasotocin, whereas antioxytocin reacted equally with isotocin, mesotocin, and oxytocin.

The chemical identity of these physiologically studied,
dye-marked, immunocytochemically stained neurons was established by observing the three adjacent serial sections of each LY-filled neuron first under incident UV light at 495 nm for LY fluorescence and then at 580 nm for rhodamine fluorescence. By alternating the selection of exciter and barrier filters, correspondence of unique cell profiles could be noted between LY and rhodamine fluorescence from only one of the three immunocytochemically stained sections in each series. Corresponding LY and rhodamine-fluorescence were photographed at 495 and 580 nm, respectively, with Kokak Tri-X Pan film. The soma and processes of chemically identified neurons were reconstructed with camera lucida drawings from adjacent sections.

RESULTS

A total of 19 neurons in the preoptic nucleus (NPO) pars magnocellularis were recorded intracellularly, identified antidromically by electrical stimulation of the pituitary gland, and identified histologically by dye marking with LY. Six (30%) of these LY-filled cells were identified immunocytochemically as enkephalin-containing magnocellular neuroendocrine cells (MgC). The remaining 13 (70%) LY-filled neurons, reported elsewhere (3, 4), were identified chemically as vasotocin- or isotocin-containing MgC.

The chemical identity of LY-filled, physiologically studied neurons was established immunocytochemically by comparing cell location, size, contour, and processes on the same section first at 495 nm (Fig. 1A) for LY fluorescence and then at 580 nm (Fig. 1B) for rhodamine fluorescence. Rhodamine-stained enkephalin neurons showed less morphological detail than the same cell with LY visible (Fig. 1A and B; see ref. 4). The rhodamine-stained enkephalin cell bodies and processes shone bright red against the brownish-green hypothalamic neuropil background at 580 nm.

The six LY-filled and physiologically studied preoptic neurons stained positively for enkephalin whereas the adjacent serial sections (which also contained the LY-filled enkephalin cell soma) tested negatively for isotocin or vasotocin. The enkephalin neurons were in the pars magnocellularis of the right and left NPO, 52–240 μm deep to the ependymal lining of the preoptic recess of the third ventricle. The LY-filled enkephalin cell bodies and processes shone brilliant yellow against the yellow-green hypothalamic neuropil background at 495 nm. Cell somata ranged from 14 to 42 μm in diameter and appeared multipolar, with polygonal perikaryon (Fig. 1 A–C), unipolar, or bipolar. Dye coupling between LY-filled enkephalin neurons and adjacent or distant cells was not observed. Some cell bodies showed a prolonged fleshy extension from which one or more axonal processes extended (Fig. 1 A and C). Axons were usually multiple, coarse, and vesiculated and streamed downward and laterally in twisted pathways over the preoptic-hypophyseal tract toward the pituitary gland (Fig. 1C). The axonal processes of some cells could be followed for more than 2 mm down through the hypothalamus to the anterior neurohypophysis and the pituitary gland.

Enkephalin neuronal spontaneous action potentials ranged up to 80 mV and were of long duration, up to 5 msec (Fig. 1D). Cells were either silent or fired at slow rates up to 8 impulses per sec. Resting membrane potentials ranged from −20 to −50 mV. Pituitary gland (neurohypophysis) stimulation above

![Fig. 1](image-url)
threshold produced antidromic potentials in enkephalin neurons with multiple, stimulus-strength-dependent latencies (range 4-23 msec; Fig. 1D). Stimulation of the pituitary gland at threshold levels yielded responses of longer latencies. Stepwise increases in the stimulus strength of 2- to 30-fold above threshold caused the invasion latency to shorten in an all-or-none fashion (range, 1-4 latencies; Fig. 1D). These results suggest that some enkephalin neurons have multiple, variable-sized branching of their axonal processes (Fig. 1C; see ref. 11).

DISCUSSION

The electrophysiological properties of enkephalin neurons from this study are consistent with previous findings of MgC of the goldfish (3, 4, 11, 14) and mammalian hypothalamus (18) and in other mammalian central neurons (19). On the basis of these initial electrophysiological and morphological data, enkephalin neurons cannot be distinguished either functionally or structurally from the isotocin or vasotocin MgC also present in the NPO of the goldfish (2, 4, 20). Further studies are required to delineate the specific input-output pathways and cellular electrophysiological mechanisms for enkephalin neurons. The absence of dye coupling between cells in this study supports synaptic over electrotonic coupling between enkephalin and surrounding cells (12). The one neuron—one hormone (peptide) hypothesis is supported by these data.

Immunocytochemical studies of the goldfish hypothalamus and pituitary (2, 4, 20) report the presence of three interspersed peptidergic cell types (enkephalin, isotocin, and vasotocin) and fibers, resembling the distribution of enkephalin, oxytocin, and vasopressin found in the mammalian hypothalamus and posterior pituitary (6-10). Electrophysiological studies on putative chemically specific MgC neurons (vasopressin and oxytocin) in the mammalian hypothalamus have been based on purely physiological criteria (see ref. 1) derived from extracellular recordings of antidromically identified neurons. Such studies have not anticipated the presence of a third type of chemically specific MgC neuron (enkephalin). Until future studies using intracellular recording and dye-marking techniques with immunocytochemical identification of the dye-marked neuron confirm the results of those studies that used only physiological criteria for cell identification, most of the earlier data must be critically reevaluated to account for the presence of enkephalin neurons.

Reconstructions of LY-filled enkephalin neurons fulfilled the criteria for morphological types I and III of goldfish preoptic neurons defined by an earlier study (11) of dye-marked, antidromically identified, morphologically reconstructed preoptic MgC. Type I cells, "olfactory input neurons," are large, multipolar-shaped neurons having multiple-branched axons and fine dendritic projections in the NPO and to the lateral hypothalamus. Type III cells (see Fig. 1), "undefined neurons," are smaller, multipolar-shaped neurons with fine dendritic processes distributed within the NPO and with limited axonal branching. None of the six enkephalin cells resembled the type II "CSF projecting neurons" which reside adjacent to and have "dendritic" connection to the ependymal lining of the third ventricle.

Considerable indirect evidence suggests an important role for endogenous opioid peptides in the regulation of the neurohypophysis. The depletion of enkephalin from the pars nervosa by lesions of the supraoptic and paraventricular nuclei and by dehydration stimuli in the rat suggests a close connection between vasopressin secretion and enkephalins (8). Exogenous morphine can produce inhibitory and excitatory effects on vasopressin in mammals (21, 22). Intravenous infusion of [Leu]enkephalin and its analogs produces mild elevation of blood pressure and antidiuresis in ethanol-anesthetized rats (23).

Our finding of enkephalin neurons in the goldfish NPO with projections of enkephalin axons to the pars nervosa (2, 3, 5) is one more indication of a major role for enkephalins in the regulation of neurohypophysial function in teleosts and mammals. Further delineation of the functional relationships between enkephalin cells and other MgC will require these intracellular, dye-marking, chemical cell identification techniques.

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