Proc. Natl. Acad. Sci. USA
Vol. 76, No. 1, pp. 521–525, January 1979
Neurobiology

Cholecystokinin octapeptide-like immunoreactivity: Histochemical localization in rat brain

(peptide/indirect immunofluorescence/central nervous system)

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Communicated by David Bodian, October 25, 1978

ABSTRACT **Cholecystokinin octapeptide-like (CCK-OP-like) immunoreactivity was localized in the rat brain by using the indirect immunofluorescence method. Specificity in immunohistochemical studies was demonstrated by the virtual elimination of staining with either preimmune sera or sera preadsorbed with CCK-OP and by the achievement of similar fluorescent patterns with two different primary anti-CCK-OP sera. CCK-OP-like fluorescence was localized in neuronal cell bodies, fibers, and varicose terminals. The most dense collections of CCK-OP cells occurred in the periaqueductal gray and in the dorsomedial hypothalamus. Substantial numbers of cells and fibers also were present in the medial/dorsal and perirhinal cortex; more limited groups of cells were found in the pyramidal layer of the hippocampus and in the dorsal raphe.**

Immunoreactivity related to cholecystokinin (CCK), a 33-amino acid polypeptide originally isolated from the duodenum (1), has been found in the brains of several vertebrate species including man (2, 3) and shown to be attributable largely to the COOH-terminal octapeptide (CCK-OP), although native cholecystokinin is also present in the brain (3, 4). In rat brain, 75% of immunoreactive CCK represents CCK-OP (unpublished data). In preliminary histochemical studies, CCK immunoreactivity has been observed in rabbit cerebral cortex (5). We now report detailed immunohistochemical localizations of CCK-OP immunoreactivity in the rat brain.

MATERIALS AND METHODS

Immunofluorescence. CCK-OP was visualized by the indirect immunohistofluorescence method of Coons (6) as described (7). Sprague–Dawley male rats (100 g) were injected intracerebroventricularly with 50 μg of colchicine (Sigma) dissolved in 20 μl of 0.9% NaCl 48 hr before sacrifice. Animals were perfused through the heart for 10 sec with ice cold isotonic saline and then for 5–10 min with ice cold phosphate-buffered 4% depolymerized paraformaldehyde solution prepared according to Pease (8). Brains were postfixed for 90 min in this perfusate and then soaked for at least 24 hr in 7% sucrose/0.6 M phosphate buffer, pH 7.4. Brain slabs approximately 0.5 mm thick were rapidly frozen onto cryostat chucks by using liquid nitrogen. Sections (16 μm) were cut at −20°C in a Harris cryostat and thaw-mounted onto gelatin/chrome alum-coated slides. Sections were stained at 37°C for 30 min with primary antisera diluted 1:25 with phosphate-buffered saline (P_i/NaCl) containing 0.2% Triton X-100. After three 5-min washes with P_i/NaCl/0.5% Triton, the sections were exposed for 15 min at 37°C to fluorescein-conjugated guinea pig antibody against rabbit IgG (Cappel Laboratories) diluted 1:40 with P_i/NaCl/0.1% Triton. The sections were then washed three times (5 min each) in P_i/NaCl/0.2% Triton, dipped in H_2O, and mounted with 0.5 M sodium bicarbonate buffer (pH 8.4) diluted 1:1 with glycerol.

Each section was examined under dark-field conditions by two independent observers using a Zeiss Universal fluorescence microscope. Each level described in detail here was examined in at least three brains. Adjacent sections from each brain were stained with preimmune sera and with control sera [primary sera preadsorbed overnight at 4°C with 15 μM CCK-OP (generous gift of Squibb Laboratories)]. In all descriptions of immunoreactivity, we refer only to immunofluorescence that was essentially eliminated by this preadsorption. Adjacent sections were stained with cresyl violet, and the drawings of Konig and Klippel (9) and Palkovitz and Jacobowitz (10) were consulted for anatomic localization.

Preparation of Antisera. Antibodies were raised in rabbits to desulfated CCK-OP coupled to bovine serum albumin with glutaraldehyde under conditions similar to those used by Vance *et al.* for corticotropin (11). The antisera used in this study were characterized by radioimmunoassay using ^{125}I-labeled desulfated CCK-OP as tracer. For antiserum 6 at a titer of 1:6000, the sensitivity was 4–5 pg of CCK-OP per ml. For antiserum 10 at a titer of 1:35,000 the sensitivity was 2–3 pg of CCK-OP per ml. For both antisera, equivalent inhibition of binding of ^{125}I-labeled desulfated CCK-OP to antibody was observed with CCK-OP (both intact and desulfated), human gastrin I, and porcine CCK. Boiling water extracts of rat brains gave inhibition curves parallel to those of CCK-OP standards. [Met]Enkelphalin, [Arg]vasopressin, somatostatin, corticotropin, gonadotropin-releasing hormone, and thyrotropin-releasing hormone all failed to inhibit the binding of ^{125}I-labeled desulfated CCK-OP to the antibody (unpublished data).

RESULTS

Several characteristics of the observed immunohistofluorescence indicated that it was primarily associated with CCK-OP. Control experiments using preimmune sera or sera preadsorbed with 10–400 μM CCK-OP, pentagastrin, or gastrin (all of which have identical COOH-terminal pentapeptide sequences) showed negligible fluorescence (Fig. 1*B*). By contrast, preadsorption with 400 μM bradykinin, neurotensin, [Met]enkephalin, [Leu]enkephalin, angiotensin II, substance P, prolactin, or secretin or with 1 μM bovine serum albumin failed to eliminate specific fluorescence.

Throughout the rat brain, CCK-OP-like immunofluorescence was localized to neuronal cells, fiber-like processes, and ap-

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Abbreviations: CCK, cholecystokinin; CCK-OP cholecystokinin octapeptide; P_i/NaCl, phosphate-buffered saline.

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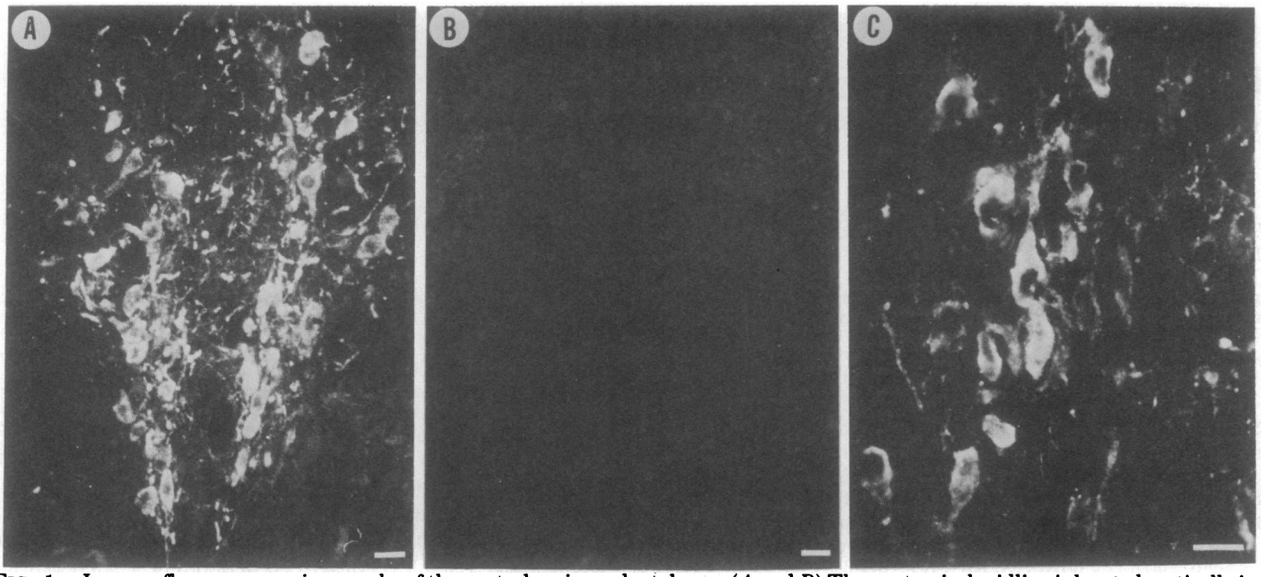

FIG. 1. Immunofluorescence micrographs of the ventral periaqueductal area. (*A* and *B*) The anatomical midline is located vertically in the center of the picture with the aqueduct above the photographic field. These are serial sections, but in *B* the primary antiserum was preadsorbed with CCK-OP. (*C*) Higher magnification of the cluster. Bars = 20 μm.

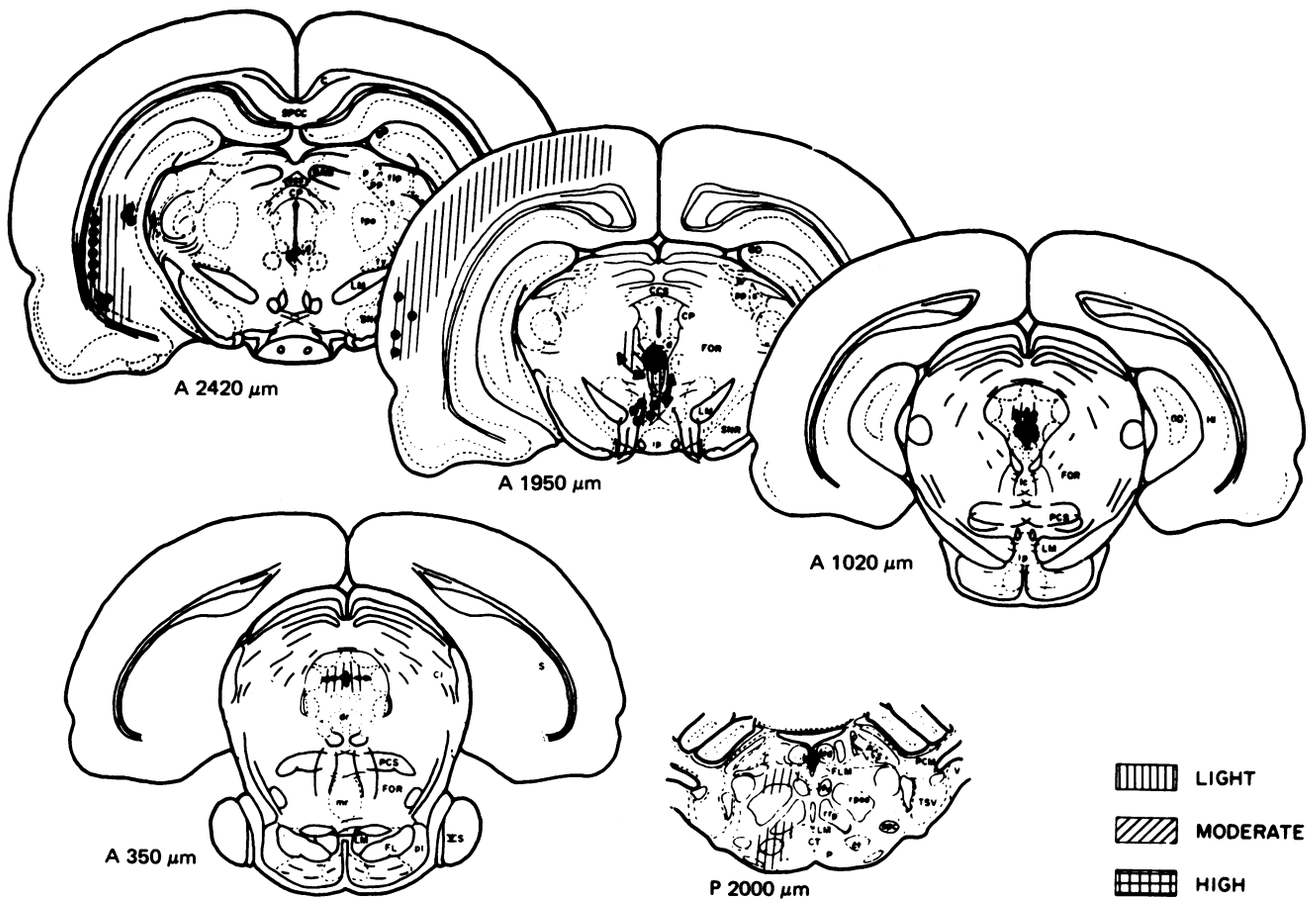

FIG. 2. Distribution of CCK-OP immunofluorescence. Abbreviations are as in Fig. 3 and as follows: CCS, commissura colliculorum superiorum; CI, colliculus inferior; CP, commissura posterior; CT, corpus trapezoideum; FL, fasciculus longitudinalis; FLM, fasciculus longitudinalis medialis; FOR, formatio reticularis; PCM, pedunculus cerebellaris medius; PCS, pedunculus cerebellaris superior; P, pyramidal tract; S, subiculum; SAM, stratum album mediale colliculi superioris; SNR, substantia nigra, zona reticulata; SPCC, splenium corporis callosi; TSV, spinal tract of the trigeminal nerve; V, trigeminal nerve; VS, trigeminal nerve, radix sensoria; ct, nucleus corporis trapezoidei; d, nucleus of Darkshevich; dr, nucleus dorsalis raphes; i, nucleus interstitialis (Cajal); ip, nucleus interpeduncularis; lc, nucleus linearis, pars caudalis; mr, nucleus medianus raphes; ntd, dorsal tegmental nucleus of Gudden; ntV, nucleus spinal tract of trigeminal; pl, nucleus pontis, pars lateralis; rpoo, nucleus reticularis pontis oralis; rrp, nucleus reticularis tegmenti pontis; s, nucleus suprageniculatus. Levels are from refs. 9 and 10. Black dots stand for cell bodies and are not used to represent the actual number of cells observed. In the periaqueductal region and the floor of the fourth ventricle, the dense cellular clusters are marked with coalescing black dots. Arrow indicates orientation of fibers.

parent terminal varicosities. Within neuronal perikarya, the fluorescence was limited to cytoplasmic regions and never involved the nucleus. Fluorescence in perikarya was more pronounced in animals pretreated with colchicine; thus, colchicine-pretreated rats were used routinely for mapping cell bodies.

We examined numerous areas of the rat central nervous system and pituitary for CCK-OP immunoreactivity. In all cases, only immunofluorescence that could be eliminated by preadsorption with CCK-OP is described, and the CCK-OP-like immunoreactivity is referred to simply as CCK-OP.

No CCK-OP fluorescence was observed in the middle or upper cervical spinal cord, cerebellar cortex and nuclear regions, pituitary, or pineal gland. The medulla/oblongata was examined at 0.2-mm intervals and no evidence of CCK-OP fluorescence was detected in fibers or cells.

Metencephalon and Mesencephalon. In the metencephalon, a dense group of CCK-OP cells occurred in the midline of the caudal portion of the dorsal raphe nucleus and extended laterally for a short distance just ventral to the dorsal tegmental nucleus of Gudden (Fig. 2). A band of moderately fluorescent fibers ran from the cells laterally in an area overlying the medial

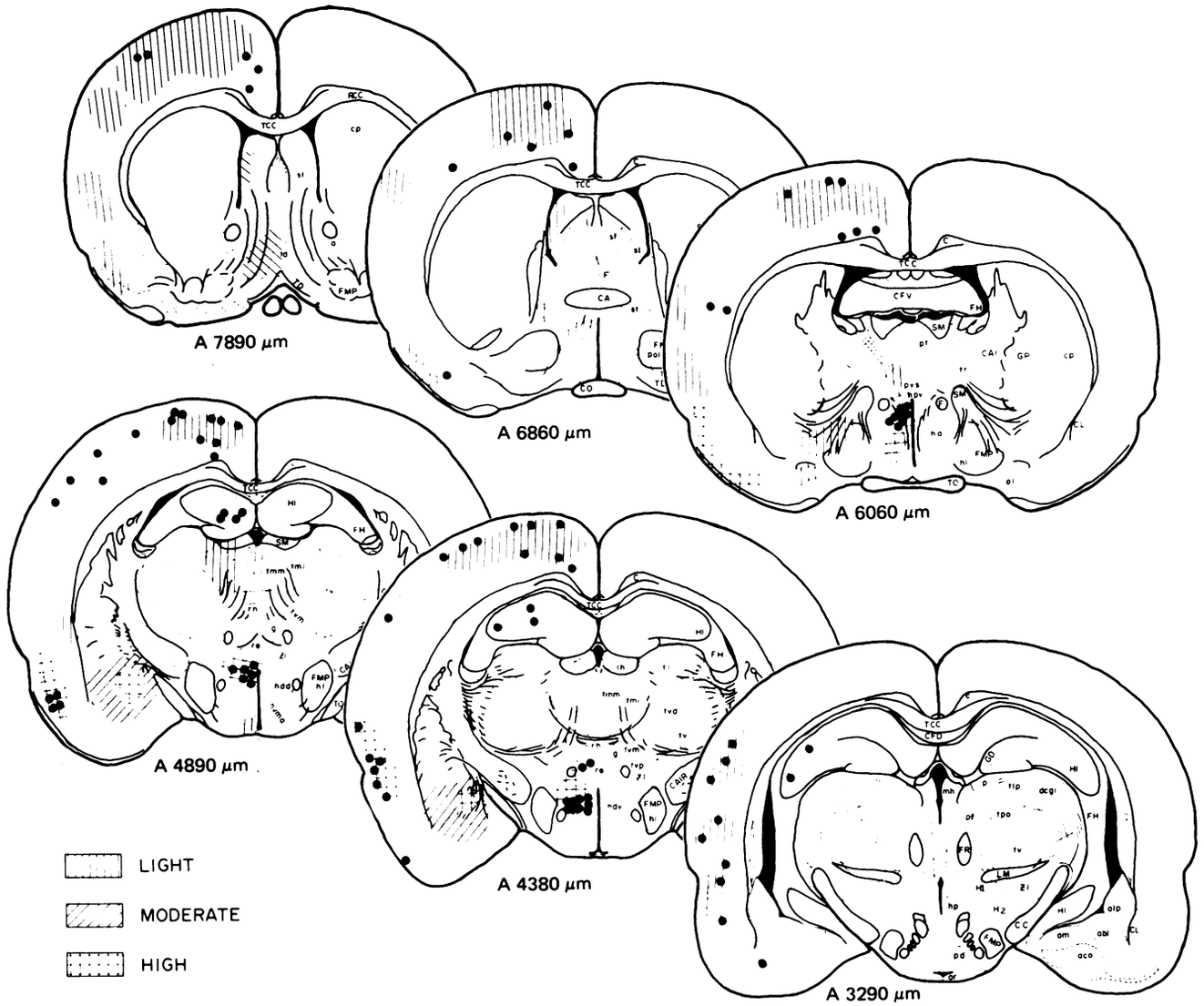

FIG. 3. Distribution of CCK-OP immunofluorescence. Abbreviations are as follows: C, cingulum; CA, commissura anterior; CAI, capsula interna; CAIR, capsula interna, pars retrolenticularis; CC, crus cerebri; CFD, commissura fornicis dorsalis; CFV, commissura fornicis ventralis; CL, claustrum; CU, chiasma opticum; F, columna fornicis; FH, fimbria hippocampi; FMP, fasciculus medialis prosencephali; FR, fasciculus retroflexus; GD, gyrus dentatus; GP, globus pallidus; HI, hippocampus; H_1, H_2, Forel's fields; LM, lemniscus medialis; RCC, radiato corporis callosi; SM, stria medullaris thalami; TCC, truncus corporis callosi; TD, tractus diagonalis (Broca); TO, tractus opticus; ZI, zona incerta; a, nucleus accumbens; abl, nucleus amygdaloideus basalis, pars lateralis; aco, nucleus amygdaloideus corticalis; alp, nucleus amygdaloideus lateralis, pars posterior; am, nucleus amygdaloideus medialis; ar, nucleus arcuatus; cp, nucleus caudatus putamen; dcgl, nucleus dorsalis corporis geniculati lateralis; g, nucleus gelatinosus; ha, nucleus anterior hypothalami; hdd, nucleus dorsomedialis hypothalami, pars dorsalis; hdv, nucleus dorsomedialis hypothalami, pars ventralis; hl, nucleus lateralis hypothalami; hp, nucleus posterior hypothalami; hpv, nucleus periventricularis hypothalami; hvma, nucleus ventromedialis hypothalami, pars anterior; lh, nucleus habenulae lateralis; mh, nucleus medialis habenulae; pl, nucleus tractus olfactorii lateralis; p, nucleus pretectalis; pd, nucleus premamillaris dorsalis; pf, nucleus parafascicularis; pol, nucleus preopticus lateralis; pt, nucleus paratenialis; pvs, nucleus periventricularis stellato cellularis; re, nucleus reuniens; rh, nucleus rhomboideus; sf, nucleus septalis fimbrialis; sl, nucleus septi lateralis; st, nucleus interstitialis striae terminalis; td, nucleus tractus diagonalis (Broca); tl, nucleus lateralis thalami; tlp, nucleus lateralis thalami, pars posterior; tml nucleus medialis thalami, pars lateralis; tmm, nucleus medialis thalami, pars medialis; tpo, nucleus posterior thalami; tr, nucleus reticularis thalami; tv, nucleus ventralis thalami; tvd, nucleus ventralis thalami; pars dorsomedialis; tvp, nucleus ventralis medialis thalami, pars parvocellularis. Levels are from ref. 9.

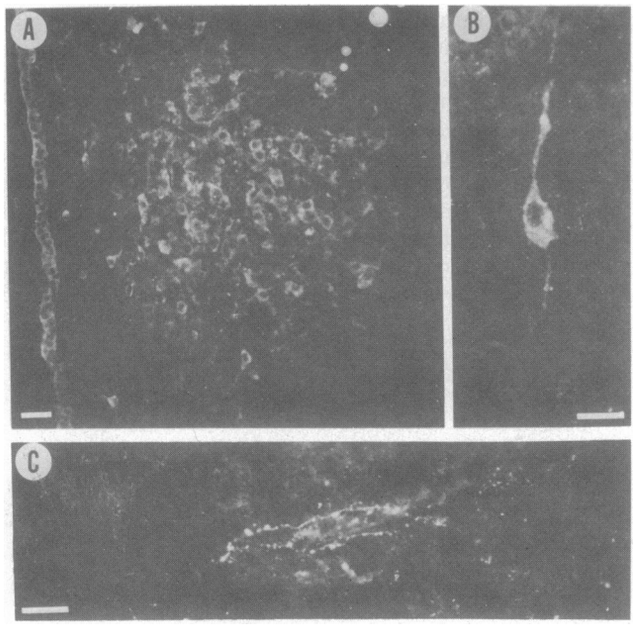

FIG. 4. Immunofluorescence micrographs from the hypothalamus. (*A*) Dense cluster of cells in the dorsomedial nucleus lies close to the ependymal layer of the third ventricle (at left). The cells of the ependymal layer show nonspecific fluorescence—i.e., the fluorescence did not disappear with preadsorption of the antiserum with CCK-OP. (*B*) Higher magnification of a hypothalamic cell with two prominent processes. (*C*) Cluster of fibers with two particularly prominent varicose fibers oriented horizontally. Bars = 20 μm.

longitudinal fasciculus. Another area of fibers and terminals was observed at this level at the ventral boundary of the midbrain.

The most densely packed collection of CCK-OP cells in the brain occurred in the periaqueductal gray (Fig. 2). At the level of cranial nerve IV nucleus (A 350 μm), a sparse number of fluorescent fibers lay just lateral to the Sylvian aqueduct. Superior to this level, the highest concentration of CCK-OP cells lay on either side of the midline just ventral to the cerebral aqueduct along the upper third of the pons to the level of exit of cranial nerve III (A 1950 μm). In the midline itself, no cells were detected although some fibers were observed. This cluster of cells surrounding the midline had a droplet-like shape (Fig. 1*A*) with some fibers oriented toward the ventral portion of the brain stem. These cells had variable shapes but usually were round or spindle-shaped with an average length of 15–20 μm (Fig. 1*C*). A smaller group of cells with less-bright fluorescence overlay the interstitial nucleus of the ventral tegmental decussation.

Diencephalon. Serial sections of the hypothalamus showed a dense and apparently continuous collection of cells and fibers in the dorsomedial nucleus, coursing anteriorly and medially to the periventricular nucleus (Figs. 3 and 4). Dense fibers appeared to project ventrally and dorsally from the periventricular nucleus.

A limited amount of CCK-OP fluorescence was observed over the lateral thalamus. No fluorescence was observed in the posterior hypothalamus.

Telencephalon. CCK-OP cells were observed in the cerebral cortex and hippocampus. The most dense collections of CCK-OP cells and fibers occurred in the pyriform cortex in the vicinity of the rhinal sulcus and in the medial/dorsal cortex (Fig. 3). These cells were noted throughout layers II–VI but were most concentrated in layers II and III. They were usually spindle shaped, bipolar, and large, about 20–30 μm long (Fig. 5). Their processes were oriented radially with respect to the surface of the cortex.

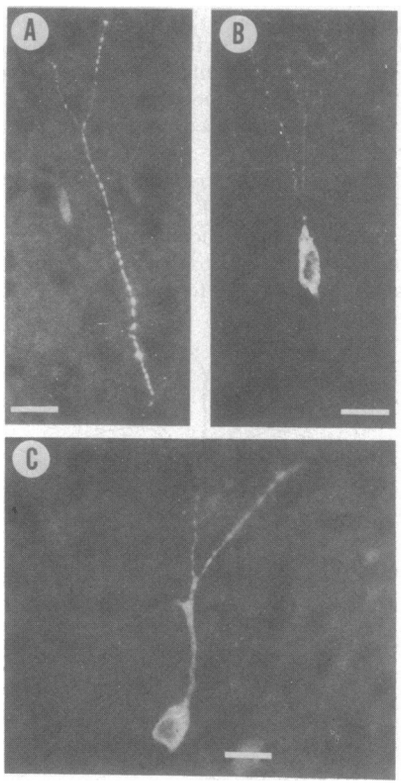

FIG. 5. Fluorescence micrographs from the telencephalon. (*A*) Varicose fiber oriented radially to the surface of the brain, traversing cortical layer III and then branching in layer II. (*B*) Typical radially oriented cortical neuron. There is a single inferior process, and the superior process branches close to its contact with the cell body. (*C*) Cortical neuron from layer III of anterior cingulate cortex. Bars = 20 μm.

A moderate collection of CCK-OP cells and fibers was noted in the hippocampus, with cells confined to a narrow zone in the pyramidal cell layer. No CCK fluorescent cells or terminals occurred in the dentate gyrus.

Dense fibers were seen in the amygdala, with the highest concentration in the central nucleus of the amygdala. However, no fluorescent cells were located in the amygdala.

A moderate collection of fibers occurred in the lateral septal area and more ventrally in the area of the diagonal tract of Broca.

No CCK-OP cells or fibers were observed in the basal ganglia.

DISCUSSION

Several factors suggest that the immunoreactive CCK-OP observed histochemically represents authentic CCK-OP. Essentially the same pattern of CCK-OP distribution is obtained with two different CCK-OP antisera. Preadsorption with CCK-OP and gastrin (which share identical COOH-terminal pentapeptide sequences) eliminates fluorescence, and preadsorption with various unrelated peptides is without effect. Moreover, immunoreactive gastrin in the brain has been shown to be attributable to CCK (3, 4). Although both CCK and CCK-OP have been demonstrated in brains of several species, in rat brain 75% of the immunoreactive CCK represents CCK-OP (unpublished data), suggesting that the preponderance of the immunoreactivity observed in our histochemical studies represents CCK-OP. However, none of these controls completely rules out crossreactivity with related antigens, so that variants or precursors of CCK-OP might be detected in staining experiments.

In both our present study of the rat brain using the indirect immunofluorescence technique and a preliminary study of rabbit cortex using a peroxidase-antiperoxidase technique (5), numerous immunoreactive perikarya were observed in the cerebral cortex. A higher proportion of cells were stained in the work of Straus *et al.* (5) than in our study. Moreover, they obtained "diffuse staining in subcortical white matter," which was not detected in our study. Whether these differences represent species variations or derive from technical differences is not clear.

There are many parallels between the observed histochemical distribution and that determined by radioimmunoassay. However, it is difficult to compare these two techniques in detail because histochemical procedures are not quantitative and it is not clear to what extent histofluorescence in cells or fibers contributes differentially to endogenous levels. Radioimmunoassay of brain regions of rats similar to those used in histochemical studies reveals negligible or extremely low levels of CCK-OP in the cerebellum, spinal cord, medulla oblongata, and pons (unpublished data), where we detect no CCK-OP fluorescence. Highest levels by radioimmunoassay occur in the cerebral cortex, where extensive distributions of cells and fibers occur, whereas somewhat lower levels are detected by radioimmunoassays in most other regions of the brain.

In some respects the distribution of CCK-OP immunoreactivity resembles that of other peptides. Cell bodies and fibers in the hypothalamus have been found to be positive for several peptides, including enkephalin (12), somotostatin (13), thyrotropin-releasing hormone (14), and luteinizing hormone-releasing hormone (15). In the periaqueductal gray, cell bodies and fibers have been positive for neurotensin (16) and enkephalin (17). However, only vasoactive intestinal polypeptide (18) and CCK-OP have been localized to cerebral cortical neurons.

Possible functions of the CCK-OP neuronal systems described here are unclear. CCK-OP is concentrated in the medial hypothalamus which has been implicated in feeding behavior (19). Peripheral administration of CCK-OP alters feeding behavior (20, 21), although this may result from peripheral effects of the peptide (22). Conceivably, the CCK neurons in the periaqueductal gray might be related to the role of this brain area in integrating pain perception (23).

We thank Naomi Taylor for expert technical assistance and Billie Little for assistance with the manuscript. R.B.I. is a recipient of a grant from the Insurance Medical Scientist Scholarship Training Fund sponsored by Mutual of Omaha. F.M.A.C. is a recipient of an international fellowship from Fundação de Amparo a Pesquisa do Estado de São Paulo, Brazil, processo biologicus 77/0627. B.S. is a recipient of a Rockefeller Grant for research in reproductive biology and Grant AM 18325 from the National Institutes of Health. This work was supported by U.S. Public Health Service Grant DA-00266 and a grant from the McKnight Foundation. CCK-OP was the generous gift of Squibb.

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