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Immunohistochemical localization of cholecystokinin- and gastrin-like peptides in the brain and hypophysis of the rat

(neurodigestive peptides/limbic system/substantia nigra/dopamine/oxytocin)

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ABSTRACT **The distribution of gastrin–cholecystokinin-like peptide(s) is reported in brain and hypophysis of the rat. The unlabeled peroxidase-antiperoxidase complex immunohistochemical technique was used. Controls of specificity for various peptides were studied with solid-phase absorption. Colchicine treatment was necessary to obtain positivity in many neuronal cell bodies. In addition to their already known distribution, gastrin–cholecystokinins containing neural cell bodies and fibers were present in olfactory structures, in various preoptic and hypothalamic nuclei (except in mamillary bodies), in mesencephalic nucleus linearis rostralis, and in A-10, A-9, and A-8 regions of Dahlström and Fuxe, which include substantia nigra. From previous investigations and the present distribution study, it can be inferred that, although most of the brain material consists of cholecystokinin, gastrins may also be present in hypothalamo-posthypophyseal magnocellular cells, in nucleus tractus solitarii, and in the dorsal horn of the spinal cord. The distribution of positive cell bodies in the peripheral part of the paraventricular nucleus and in the dorsal part of the supraoptic nuclei in the hypothalamus is similar to that of oxytocin neurons. The localization of positive cell bodies in A-10, A-9, and A-8 regions of Dahlström and Fuxe is similar to that of dopaminergic neurons. The mesencephalic concentration of cell bodies and the wide distribution of fibers in striatal, hypothalamic, septal, and other hemispheric structures together with thick positive fibers in the medial forebrain bundle is consistent with the existence of ascending mesencephalic pathways, including the nigrostriate pathway.**

Gastrinlike peptidic material of lower molecular weight than gastrin heptadecapeptide has been located by radioimmunoassay in the central nervous system of various vertebrates (1). This first demonstration that some peptides previously known only in the digestive tract endocrine cells may also be present in the central nervous system has been confirmed and extended to other peptides (2).

By use of immunological techniques combined with fractionation procedures, the gastrinlike peptidic material has been shown to be more closely related to cholecystokinins (2–5), especially to the biologically active COOH-terminal octapeptide of cholecystokinin in its complete sulfated form (CCK8-S) (2). Indeed, cholecystokinins share a common COOH-terminal pentapeptide with gastrins. Recently, the presence of gastrin heptadecapeptide has been shown by radioimmunoassay in the hypothalamus, in the hypophysis (6), and in the vagal nerves (7). Its release after peripheral nerve stimulation has also been demonstrated (8).

By use of immunohistochemical methods to localize gastrin–cholecystokinin (G–CCK)-like peptides, numerous positive cell bodies but no positive nerve terminals have been observed in the rabbit brain cortex (9). In the rat, rare positive cell bodies and fibers have been located in the cerebral cortex, especially in Ammon's horn, and numerous positive fibers have been located in the amygdala and in the hypothalamus (10, 11). In addition, positive cells have also been demonstrated in the paraventricular (12, 13), supraoptic (12), and circularis (13) hypothalamic magnocellular nuclei, in the hypothalamic dorsomedial nucleus (14), and in some brain stem nuclei (12, 14). Positive fibers have been shown, too, in posterior hypophysis (12, 13), spinal cord (11, 13), and spinal ganglia (11).

The present investigation reports a detailed immunohistochemical study of G–CCK-like peptides in the central nervous system and hypophysis of the rat as well as original data on brain stem and some other localizations. Special attention is also paid to the control of specificity.

MATERIALS AND METHODS

G–CCK-like peptides were visualized in the rat central nervous system and hypophysis by using the unlabeled peroxidase–antiperoxidase complex technique (15). Serum was obtained from a rabbit after three intradermal injections of CCK8-S (Squibb) that was coupled with thyroglobulin (Sigma) (16) and mixed with Freund complete adjuvant (Miles). This serum was used for radioimmunoassay as described (1, 2) with ^{125}I-labeled gastrin hexadecapeptide (Imperial Chemical Industries, Cheshire) as tracer, the serum being used at a final dilution of 1:400,000. The sensitivity of the radioimmunoassay was 5 pM CCK8-S. Under these conditions the serum recognized equally well on a molar basis gastrin hexadecapeptide, CCK8-S, and the unsulfated COOH-terminal octapeptide of cholecystokinin. Water-boiled extracts of rat brains (1) gave inhibition curves parallel to those obtained with the three peptides cited above. The serum did not recognize thyroglobulin, [Met]enkephalin, [Leu]enkephalin, angiotensins I and II, neurotensin, substance P, somatostatin (Union Chimique Belge), oxytocin, [Arg]vasopressin, and bovine neurophysins I and II (F. Vandesande) at concentrations up to 1 μM.

For immunohistochemistry, serum was used at a dilution of 1:5000. Eight 3-month-old Wistar rats were used; half of them were injected intracisternally with 200 μg of colchicine (Sigma) dissolved in 20 μl of 0.1% NaCl 48 hr before killing. The rats were perfused under chloral anesthesia with the fixative "Bouin Hollande Sublimé" (17) through the heart for 30 min after a 15-sec wash with 0.9% NaCl. Brains and spinal cords were removed together with hypophysis, postfixed overnight in "Bouin Hollande Sublimé," and embedded in paraffin. Sagittal and coronal sections of 7-μm thickness were taken at 100-μm intervals and used for immunohistochemistry.

Controls were performed on adjacent sections by using solid-phase absorption (18) of the serum with the above cited peptides coupled to activated Sepharose 4B (Pharmacia). We

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Abbreviations: CCK8-S, sulfated COOH-terminal octapeptide of cholecystokinin; G–CCK, gastrin–cholecystokinin.

refer to positive G-CCK-like material when negative results are obtained with serum absorbed with CCK8-S, unsulphated COOH-terminus of cholecystokinin, or gastrin hexadecapeptide and when positive results are obtained with serum absorbed with the other peptides cited above at the same molar concentration.

Structures were identified by using adjacent sections stained with luxol fast blue/cresyl violet stains and according to the nomenclature of König and Klippel (19) and Palkovits and Jacobowitz (20).

RESULTS

Positively stained material was present only in neurons. Positive cell bodies were either diffusely distributed or concentrated into classical nuclei. They were of variable size, ranging from 10 to 30 μm in diameter. They generally gave off one thick process (Fig. 1*A*), which could be followed up to 200 μm, and it branched (Fig. 1*I*) several times before vanishing. The positive material filled the cytoplasm of the cell bodies, was absent from the nuclei (Fig. 1*L*), and had a granular appearance. Some cell bodies were more densely stained than others.

Groups of thick positive fibers that run parallel and have a beaded appearance can be traced for some distance. Transversely sectioned, they appear as numerous granules of variable size. Thin fibers running between neuronal cell bodies or processes were the most frequent ones. Transversely sectioned, they appear as fine granules surrounding cell bodies (Fig. 1 *G* and *N*). Colchicine was used to enhance positive staining in neuronal perikarya by blocking axonal flow (21). The number of positive cells in the cortex and amygdala was not influenced by colchicine treatment. On the contrary, positive cells in the magnocellular hypothalamic system and in brain stem nuclei were detected only after colchicine treatment. Fibers were more numerous in rats not treated with colchicine. These differences could be explained by variability of colchicine penetration, or action, or both throughout the brain.

Cortex. Although the majority of cortical neuronal cell bodies were negative, positive cells were regularly detected throughout the cortex except for layer I and were also present in the claustrum. In general, positive cell bodies were more numerous in the limbic structures, such as the cingulate gyrus (mesocortex), in the prepyriform, periamygdaloid, and entorhinal cortex (paleocortex), and in the hippocampus (archicortex) (Fig. 1*A*). They were present in hippocampal cornu Ammonis 1–4 regions (22), in the anterior hippocampal region and in the gyrus dentatus, in the pyramidal layer of the cornu Ammonis, and in the large area beneath this structure. They were also numerous in the presubiculum, the subiculum, and the frontal pole (neocortex). These cell bodies gave off thick processes. Thick beaded fibers were occasionally seen in the various cortical areas. In some of the neocortical areas, but especially in the prepyriform, amygdaloid, and entorhinal cortex, and in the pyramidal layer of cornu Ammonis 1–3, thin fibers were seen forming a delicate network around cell bodies.

Amygdala. Numerous positive cell bodies were present in the nucleus amygdaloideus corticalis and a few could be detected in the other amygdaloideus nuclei. A high concentration of thick and thin fibers was present in the nuclei amygdaloideus medialis, centralis, corticalis, and lateralis and in the massa intercalata; they frequently were arranged in a network surrounding cell bodies.

Thalamus. No positive cells were detected in this region. A network of thin fibers surrounding cell bodies was present in the nucleus periventricularis and in the nuclei dorsalis and ventralis corporis geniculati lateralis.

Basal Ganglia. No positive cells were detected in this region. Thin positive fibers were present in the medial and ventral parts of the middle and caudal portions of the caudate putamen, in the middle and caudal portions of the nucleus accumbens (Fig. 1*G*), in the nucleus interstitialis striae terminalis, and in the ventral and lateral parts of the nucleus septilateralis.

Prehypothalamic Structures. Thick fibers and rare positive cell bodies were present in the pars dorsalis, lateralis, medialis, and posterior of the nucleus olfactorius anterior and in the nucleus tractus olfactorii lateralis. A few positive cell bodies and thick and thin positive fibers were present in nucleus preopticus, especially in its suprachiasmatic and periventricular parts. A few positive fibers, but no positive cell bodies, were detected in the tuberculum olfactorium.

Hypothalamus and Hypophysis. The highest concentration of positive cell bodies occurred in the periventricular nucleus, in the peripheral part of the paraventricular nucleus (Fig. 1*B*), in the circular nucleus (Fig. 1*F*), and in the dorsal part of the supraoptic nucleus (Fig. 1*E*). A few positive cell bodies were present in the nucleus dorsomedialis. Scarce positive cells and fibers could be detected in the other hypothalamic nuclei except for the corpus mamillaris. A dense network of positive thin fibers was present in the nuclei dorsomedialis and ventromedialis. Thick positive fibers were present in both the inner and external zones of the median eminence and in the posterior hypophysis (Fig. 1 *C* and *D*).

Mesencephalon. The highest concentration of positive cell bodies occurred in various areas of this region. Rostrally, in the tegmental gray, a group of large positive cells up to 30 μm in diameter intermingled with thick beaded positive fibers was present in the region of the nucleus linearis rostralis (Fig. 1 *I*–*L*). More caudally in the periaqueductal gray a group of smaller positive cell bodies was present in the anterior part of the tegmental gray (Fig. 1*H*). In region A-10 of Dahlström and Fuxe (23) large positive cell bodies and thick beaded positive fibers were visible in the midline extending more laterally in a butterfly-like fashion (Fig. 1 *J* and *N*). Laterally, large positive cell bodies were present in the substantia nigra pars compacta and lateralis (A-9 and A-8 regions of Dahlström and Fuxe) (23). Caudally, a few positive cells and a dense network of positive fibers were present in the nucleus parabrachialis dorsalis. A dense network of positive fibers was also present in the nucleus interpeduncularis (Fig. 1*N*).

Metencephalon. A few positive cells and a rich network of thick positive fibers were present in the nucleus parabrachialis dorsalis; positive fibers were present in the nuclei dorsalis and ventralis lemniscus lateralis and in the nucleus raphe dorsalis and raphe magnus. A group of large positive cell bodies was present in the caudal part of the nucleus raphe dorsalis (Fig. 1 *M* and *O*) extending laterally ventral to the nucleus tegmenti dorsalis of Gudden. A few positive fibers but no positive cell bodies were detected in the locus ceruleus. No positive fibers or positive cell bodies were detected in the cerebellum.

Medulla Oblongata. Small positive cell bodies were present in the nucleus gracilis. A rich network of positive thin fibers was present in the nucleus tractus solitarii, in the nucleus commissuralis, and in some parts of the inferior olivary complex. A few large positive cell bodies were present in the nucleus reticularis medullae pars paramedianus and ventralis.

Spinal Cord. Thin positive fibers were concentrated in layers I and II of the posterior horn extending along their inner border and in the posterior gray commissures. A few positive fibers were present throughout the posterior horn.

White Tracts. Thick beaded positive fibers, which could be traced for some distance, were seen in white tracts when suitably sectioned. On transverse sections, these fibers were de-

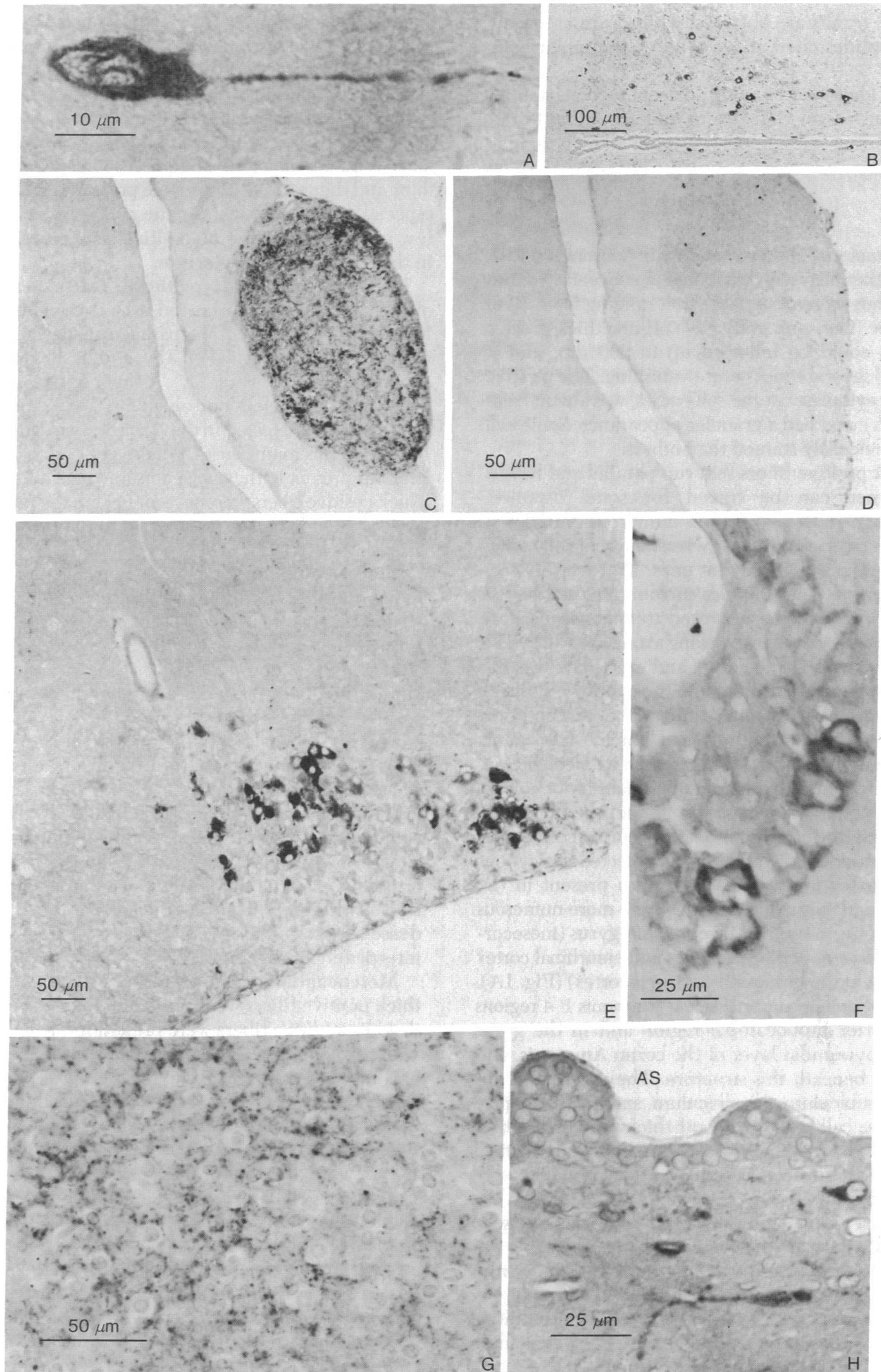
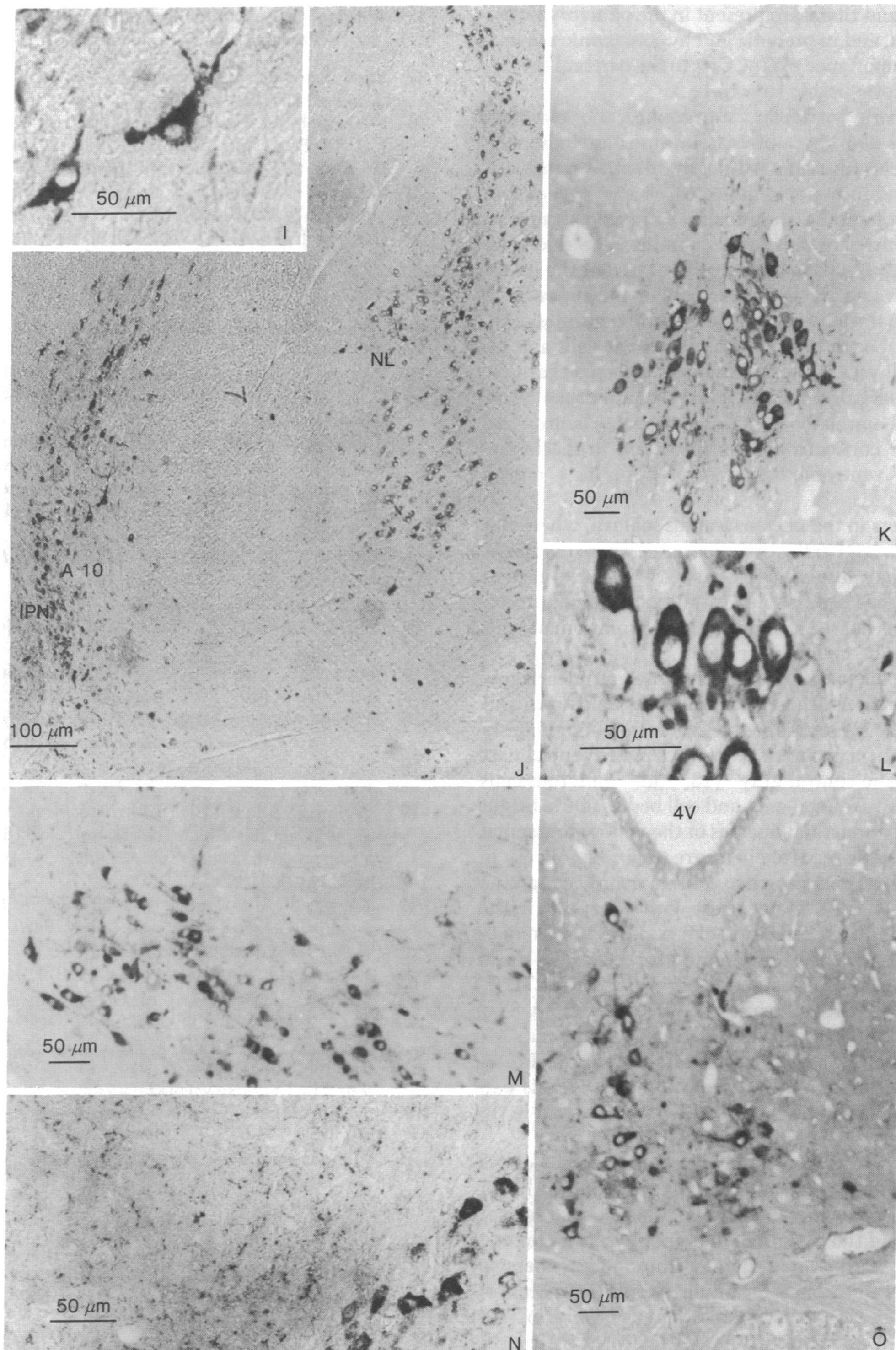


FIG. 1. Unlabeled peroxidase–antiperoxidase complex technique with anti-CCK8-S antiserum at a dilution of 1:5000. Positive G–CCK-like material appears in black. *A, B, E, F, H,* and *O* are taken from transverse sections; the others were taken from sagittal sections. Positive neuronal cell bodies are seen in the Ammon's horn (*A*), in the dorsal part of the supraoptic (*E*), in the paraventricular (*B*) and circular (*F*) hypothalamic nuclei, in the mesencephalic subependymal gray (*H*), nucleus linearis rostralis (*I–L*), and A-10 region (*J* and *N*), and in the metencephalic caudal

tected in the rostral portion of the corpus callosum, in the paraventricular hypothalamic tract, in the tractus diagonalis, and in the tractus striohypothalamicus. On sagittal section they were detected in fasciculus medialis prosencephali (medial forebrain bundle) up to the mesencephalon in various parts of the stria terminalis and in the lateral part of the reticular formation of the brain stem.

DISCUSSION

This work demonstrates the wide distribution of G–CCk(s)-containing nerve cells in the rat brain. The immunohistochemical techniques used in this work and previously do not permit the differentiation between gastrins and cholecystokinins; however, previous immunochemical studies (2, 5) suggest that most of the positive material consists of CCK8-S.



part of the nucleus raphe dorsalis (*M* and *O*). Positive neuronal fibers are seen in posterior hypophysis (*C*), in nucleus interpeduncularis (*J* and *N*), and in nucleus accumbens (*G*). *D* is a negative control of *C* and was obtained with serum absorbed with CCK8-S coupled to Sepharose 4B. 3V, Third ventricle; AS, aqueduct of Sylvius; IPN, interpeduncular nucleus; A-10, A-10 region of Dahlström and Fuxe; NL, nucleus linearis rostralis; 4V, fourth ventricle.

Some differences between our results and other immunohistochemical studies (9–14) can be explained by methodological variations. In this work, colchicine treatment, "Bouin Hollande Sublimé" perfusion, high sensitivity of the peroxidase–antiperoxidase complex technique, and use of a highly diluted serum in order to achieve minimal background may explain our results. Future technical improvements could result in other hitherto unrecognized localizations.

In addition to cerebral cortex and hippocampal localizations, positive cell bodies and fibers are present in the olfactory bulb, in various amygdala, and in preoptic and hypothalamic nuclei. This confirms the importance of G–CCKs in the cerebral cortex and in the hemispheric limbic structures.

Hypothalamic paraventricular, supraoptic, and circular nuclei project to the neurohypophysis. Because gastrin heptadecapeptide has been immunochemically identified in neurohypophysis (6) and in hypothalamus (6), at least part of the material present in hypothalamic magnocellular cells might consist of gastrins instead of cholecystokinins. The selective localization of positive cells in the peripheral part of the paraventricular nucleus and in the dorsal part of the supraoptic nucleus is similar to the localization of oxytocin neurones (24); in view of the number of positive magnocellular cells and of positive posthypophyseal fibers, it can be suggested that this positive material is located mainly in oxytocin neurons. Such a localization of two unrelated peptides in the same neuron has been described for corticotropin and prolactin in the hypothalamic arcuate, ventromedial, and premamillary nuclei (25).

The positive fibers in the nucleus tractus solitarii, where the vagus nerve projects, and in the posterior horn, where the spinal ganglia project, may also contain gastrins, because gastrin heptadecapeptide has been shown immunochemically to be present in the vagus nerve (7) and its release from somatic peripheral nerves has been demonstrated (8).

The mesencephalic localizations in the nucleus linearis rostralis and in the A-10, A-9, and A-8 regions of Dahlström and Fuxe, which include substantia nigra (23), were not previously described, although "a group of cell bodies in the central part of the mesencephalic central gray" (13) and "numerous cells in the ventral periaqueductal gray and cell bodies of less bright fluorescence in the interstitial nucleus of the ventral tegmental decussation" (14) were reported. The great number of cells in A-10, A-9, and A-8 regions known to contain mainly dopamine (26) suggests that G–CCK may coexist with dopamine in the same neuron. Such dual localization of a peptide and a monoamine in the same cell has been reported for substance P and serotonin in the central and peripheral nervous system (27, 28). The presence of G–CCK cell bodies in A-10, A-9, and A-8 regions, together with fibers in striate structures like caudate putamen, accumbens, or tuberculum olfactorium, in limbic structures, and in the medial forebrain bundle suggests participation of G–CCKs in the nigrostriate pathways and in the mesencephalic part of the limbic system (22).

In conclusion, immunohistochemical localization of G–CCK in the rat brain suggests important roles in the cortex, the limbic structures including the mesencephalon, the posterior horn of the spinal cord, and the hypothalamohypophyseal system.

Further studies with double-staining immunohistochemical procedures and localizations after surgical lesions are necessary to establish the G–CCK pathways and their relationships with other peptides and neurotransmitters. In view of their localizations, G–CCKs deserve further investigation in degenerations of the nigrostriate system, as in Parkinson disease, in mental disorders associated with limbic system dysfunction, as well as in hypothalamohypophyseal function.

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