Proc. Natl. Acad. Sci. USA
Vol. 80, pp. 2400–2404, April 1983
Neurobiology

Cholecystokinin-immunoreactive neurons in rat and monkey cerebral cortex make symmetric synapses and have intimate associations with blood vessels

(peptide neurotransmitter/nonpyramidal neuron)

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Communicated by W. M. Cowan, January 17, 1983

ABSTRACT **Neurons displaying cholecystokinin-like immunoreactivity (CCK neurons) in rat and monkey cerebral cortex were examined by light and electron microscopic immunocytochemistry. CCK neurons were found to be mainly bipolar cells present in all layers and in all areas of the rat cerebral cortex. CCK neurons were also found in all regions examined in monkey cortex (pre- and post-central gyri and superior parietal lobule). The somata and the dendritic processes of CCK neurons receive relatively few synapses but both symmetric and asymmetric axosomatic and axodendritic synapses were found. The majority of axon terminals displaying CCK-like immunoreactivity formed symmetric synapses, most frequently with the somata and proximal dendrites of pyramidal and nonpyramidal neurons. The somata and processes of CCK neurons were also found to establish very close nonsynaptic associations with blood vessels and with other neurons, suggesting possible roles for the peptide in the maintenance of neuronal excitability and cerebral blood flow.**

The octapeptide cholecystokinin (CCK) is one of several neuropeptides that has been identified in the mammalian cerebral cortex (1–3). The localization of CCK in synaptic vesicle fractions of brain homogenates (4), its release from *in vitro* preparations in a calcium-dependent manner (4), and its excitatory effect on hippocampal (5) and cortical neurons (6) are consistent with the suggestion that the peptide functions as a neurotransmitter in the cortex and elsewhere. However, CCK has yet to be localized at the fine structural level in the cerebral cortex. Cortical neurons displaying CCK-like immunoreactivity have been identified light microscopically in the rat (7, 8) but the nature of their pre- and post-synaptic relationships with other neurons are unknown. In the present study, we have used a well-characterized antiserum against CCK-8 (9) to examine the distribution, morphology, and synaptology of neurons that have CCK-like immunoreactivity (CCK neurons) in the cerebral cortex of both rats and monkeys. We show that, in addition to having many of the characteristics of typical local circuit neurons, CCK neurons in the cortex have unusual nonsynaptic relationships with other neurons and with blood vessels.

MATERIALS AND METHODS

Two cynomolgus monkeys (*Macaca fascicularis*; Hazelton Research Primates, Reston, VA) and eight Wistar rats (bred in our colony) were used. One monkey and three rats received intraventricular injections of colchicine (10 μg/μl; 110 μl for the monkey, 10 or 15 μl for the rats) and were allowed to survive for 2 days. All animals were perfused through the heart with 4% paraformaldehyde/0.1% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). The rat brains and blocks of monkey brains including the pre- and post-central gyri and the superior parietal lobule were sectioned with either a Vibratome or a freezing microtome at 40–50 μm. After several washes in 0.1 M phosphate buffer, the sections were incubated overnight in primary antiserum at dilutions of 1:1,000, 1:250, or 1:100 in the same phosphate buffer with 2% normal goat serum added to block nonspecific staining. The incubation medium for frozen sections also included 0.3% Triton X-100. Incubation with goat anti-rabbit IgG (a 1:50 dilution for 1 hr) and rabbit peroxidase-anti-peroxidase complex (a 1:100 dilution for 1 hr) followed, with several washes in phosphate buffer separating each step. Finally, the sections were treated with 3,3′-diaminobenzidine and hydrogen peroxide. Incubation with primary antiserum previously absorbed with CCK-8 or with nonimmune serum produced no staining. Frozen sections were mounted on gelatin-subbed glass slides. Small wedges from the sections cut with a Vibratome were excised, postfixed in osmium tetroxide, and processed for electron microscopy. Thin sections were cut on a Porter-Blum MT2-B ultramicrotome, collected on 200-mesh or single-slot grids, and examined with or without heavy metal staining in a Zeiss EM-9S electron microscope.

RESULTS

CCK neurons are present in small numbers throughout the rat neocortex and in monkey motor, somatic sensory, and parietal cortex. A greater number of CCK neurons are present in the piriform and entorhinal cortex and in the hippocampus. In the neocortex, labeled somata are found in all layers but the majority are in the superficial layers (I–III). Often CCK neurons are clustered together in groups of two to eight cells, but the locations of these clusters vary widely from section to section and from animal to animal.

The majority of CCK neurons in both rat and monkey cortex have relatively small somata, 8–12 μm in diameter, and are markedly bipolar (Fig. 1). One or two long thin processes originate from either pole and either ascend toward the pial surface or descend through two or more layers, usually with little branching. A few CCK neurons in layers II and III and most of those in deeper layers have more branches and appear multipolar or bitufted. Processes of CCK neurons are of variable thickness but usually thin and beaded, without obvious spines. Light microscopically, it is not possible to distinguish the axon or dendrites from one another. Some of the processes have a spray of terminal branches (Fig. 1) and thus resemble axons; these and other shorter side branches of parent processes oc-

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Abbreviation: CCK, cholecystokinin.

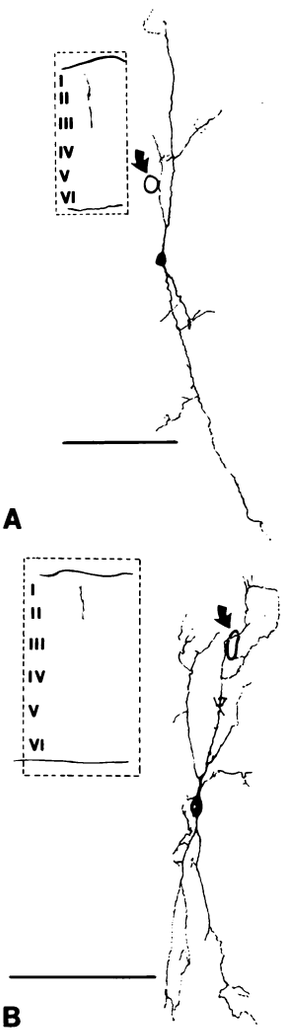

FIG. 1. Camera lucida drawings of CCK-immunoreactive neurons in layer II of rat parietal cortex. Both neurons give rise to processes from their upper and lower poles. The processes shown in *A* give off few branches while those shown in *B* branch profusely. Some branches are very close to blood vessels (arrows). (*Insets*) Positions of the neurons in the cortex. [Bars = 100 μm (*Main figure*) or 1 mm (*Insets*).]

casionally diverge toward the proximity of blood vessels (Fig. 2). In addition to the processes arising from the somata of CCK neurons, a concentration of fibers in layer VI of all areas of rat cortex displays CCK-like immunoreactivity.

The ultrastructure of CCK neurons is typical of many small nonpyramidal cortical neurons. They possess the usual complement of organelles but are often distinct from neighboring unlabeled neurons in having a deeply crenulated nucleus (Fig. 3). The somata of CCK neurons receive relatively few synapses but these have either symmetric or asymmetric membrane thickenings (Fig. 4). All synapses received by CCK neurons involve terminals that are not immunoreactive. The processes of CCK neurons have irregular outlines that, at the electron microscopic level, can be seen to give rise to protrusions, some of which resemble dendritic spines. Most processes receive symmetric and asymmetric synapses and are interpreted as dendrites (Fig. 4). Other processes contain localized concentrations of vesicles, often quite close to their somata of origin, and are interpreted as axons.

Axon terminals displaying CCK-like immunoreactivity are present in all cortical layers. The terminals are small (1 to 2 μm in diameter) (Fig. 5). Each contains a large number of clear

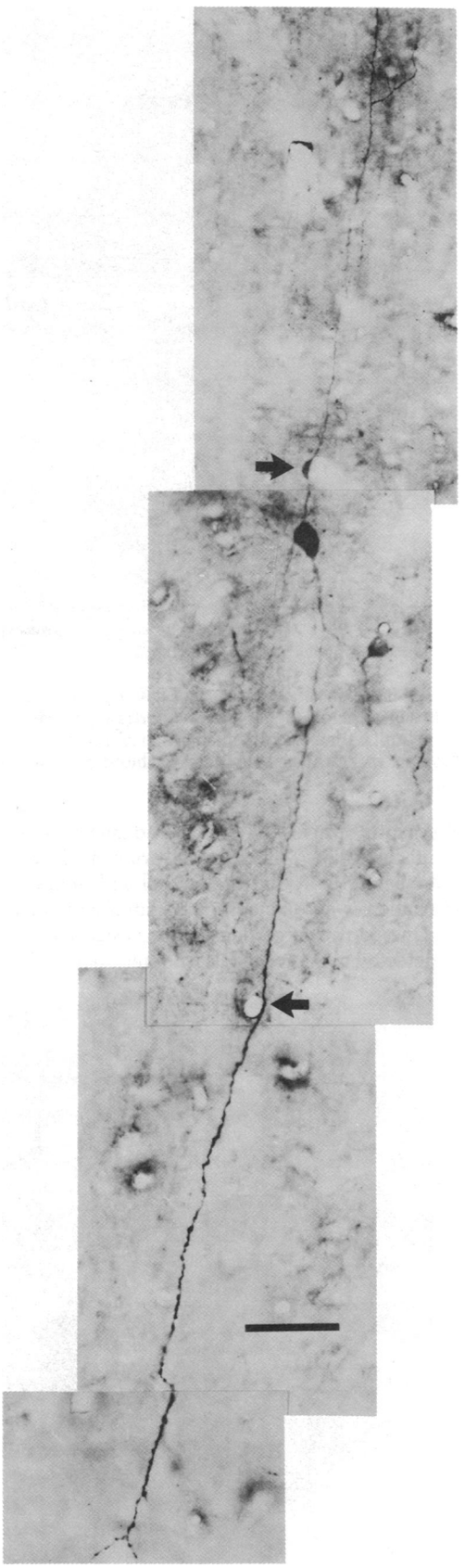

FIG. 2. Photomicrograph of a CCK-immunoreactive neuron in layers II and III of rat cerebral cortex. Long processes originate from the upper and lower poles of the small soma and branch infrequently. The processes are in proximity to blood vessels at two points (arrows). (Bar = 40 μm.)

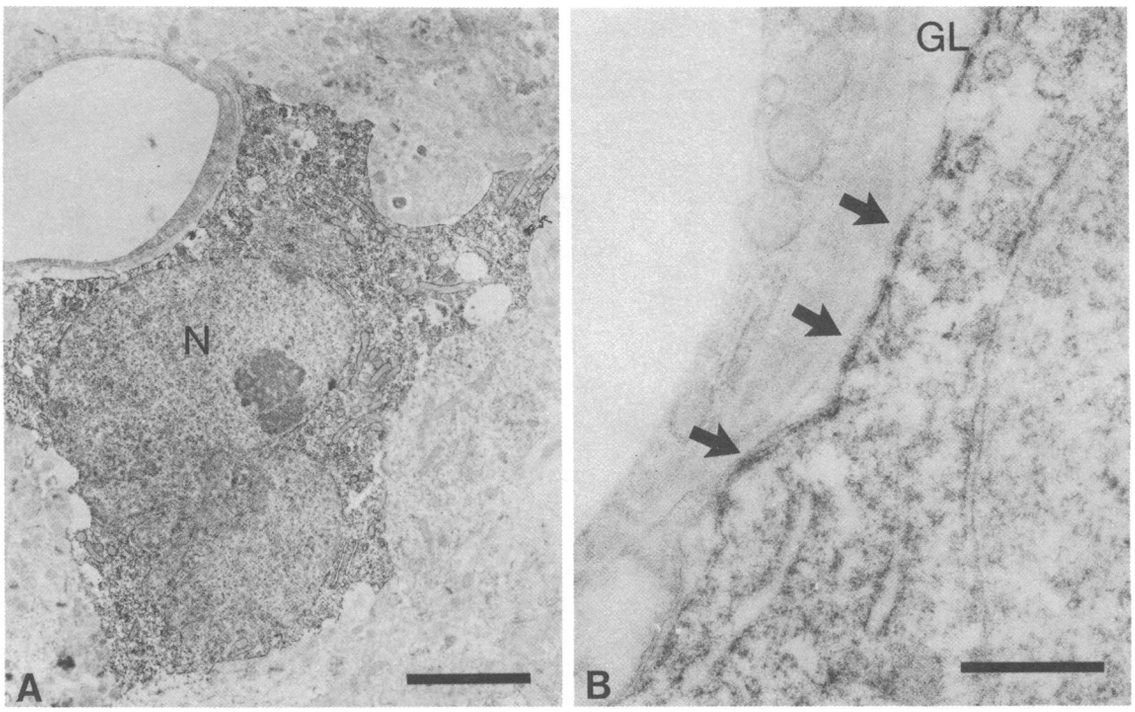

FIG. 3. (*A*) Electron micrograph of a CCK-immunoreactive neuron in layer II of monkey motor cortex. The soma of the neuron lies in close proximity to a blood vessel (upper left) and partially enfolds part of another neuron (upper right). The nucleus (N) is deeply infolded. (Bar = 4 μm.) (*B*) Higher magnification electron micrograph of a section serial to the one shown in *A*. The soma of the CCK-immunoreactive neuron establishes intimate contact with the basal lamina of the blood vessel at several points (arrows); at other places, a glial lamella (GL) more typically intervenes. (Bar = 1 μm.)

spherical synaptic vesicles 40–60 nm in diameter and usually a single mitochondrion. The reaction product of immunocytochemical labeling is free in the cytosol and under our conditions of fixation is not obviously associated with a particular organelle. One or two large dense core vesicles are only occasionally detected in the terminals whether examined in single or in serial sections. Relatively few axon terminals in the cortex display CCK-like immunoreactivity. The majority synapse on the somata and proximal dendrites of both pyramidal and nonpyramidal cells. At these sites, the terminals appear to form exclusively symmetric synapses. Other immunoreactive terminals synapse on small dendrites and, though they usually form

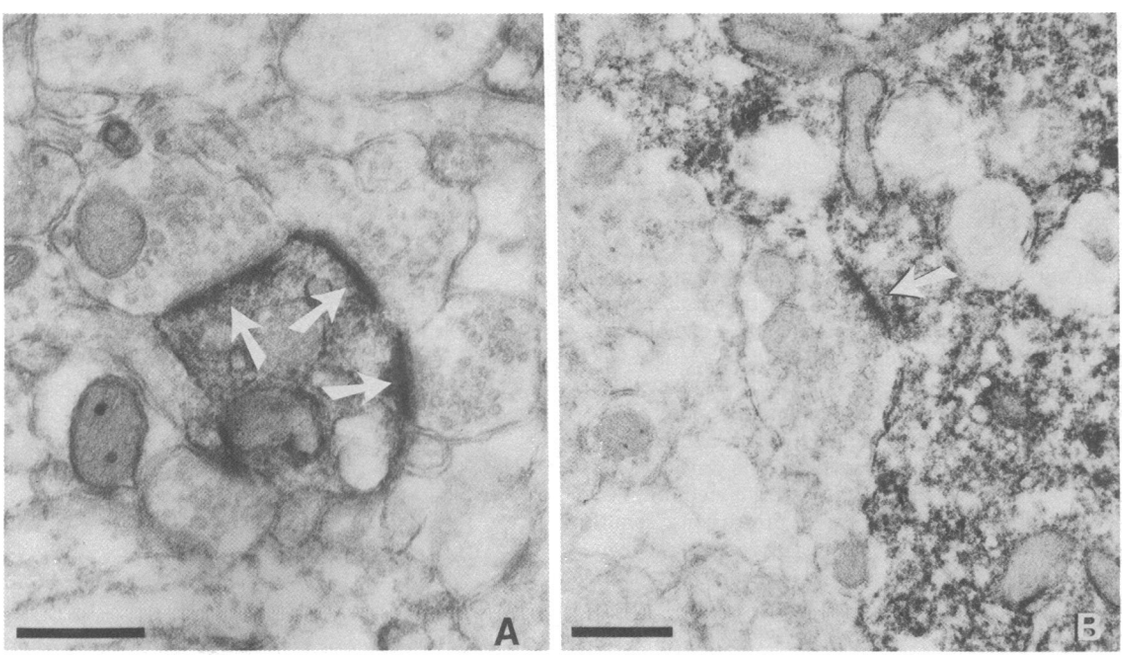

FIG. 4. Electron micrographs of synapses onto CCK-immunoreactive neurons. (*A*) Rat parietal cortex. Three terminals form asymmetric synapses (arrows) with a small labeled process. (*B*) Monkey somatic sensory cortex. A terminal forms an asymmetric synapse (arrow) with the soma of a CCK-immunoreactive neuron. Labeled somata receive very small numbers of symmetric and asymmetric synapses. (Bars = 1 μm.)

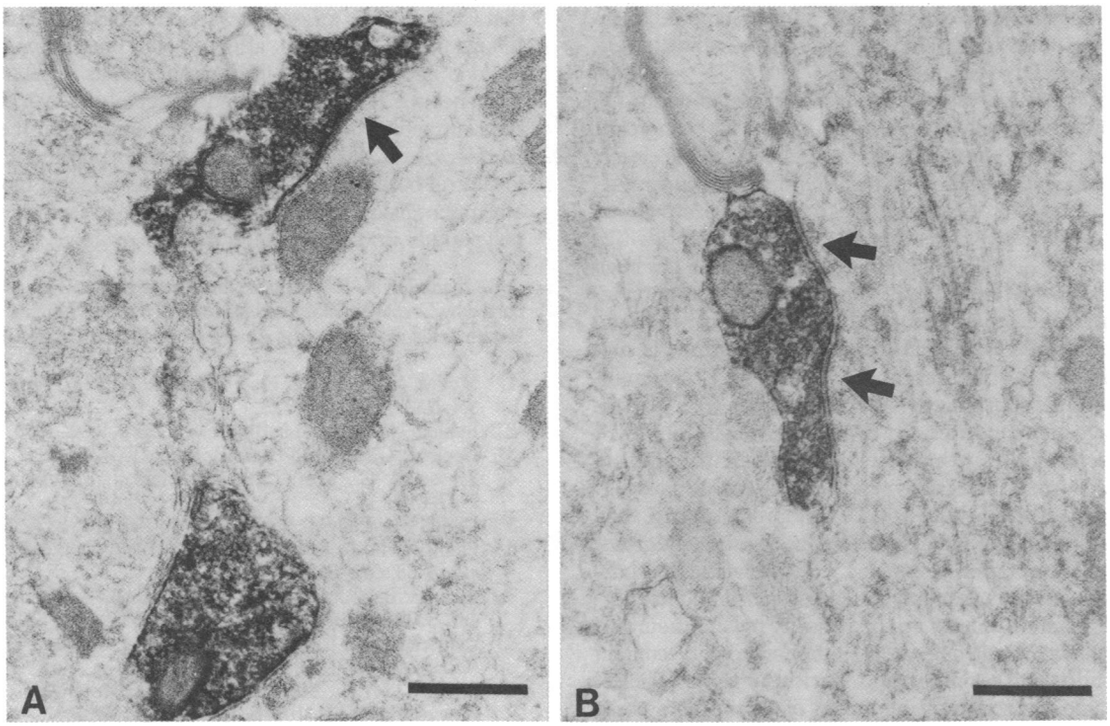

FIG. 5. Electron micrographs of synapses formed by axon terminals displaying CCK-like immunoreactivity. The terminals form symmetric synapses (arrows) with a large isolated dendrite in layer II of rat cerebral cortex (*A*) and with the apical dendrite of a layer IIIB pyramidal cell in monkey somatic sensory cortex (*B*). The reaction product is in the cytosol of the terminal and not in the small spherical synaptic vesicles. Large dense core vesicles are not present in the majority of the CCK-immunoreactive terminals. (Bars = 1 μm.)

symmetric synapses, they are sometimes associated with synaptic thickenings that are more asymmetric.

In some cases, the somata and processes of CCK neurons are closely associated with other unlabeled neurons or with the walls of capillaries or other blood vessels presumed to be venules or arterioles from the presence of smooth muscle cells in their walls. The somata and processes of a CCK neuron can virtually surround the soma of an unlabeled cell (Fig. 6) or a small blood vessel (Fig. 3). In no case are membrane specializations seen at the contacts between the CCK neurons and the unlabeled somata or blood vessels, but the CCK cells can be in direct contact with another neuron or with the basal lamina of a vessel without any intervening astroglial processes (Fig. 3). Similar associations of unlabeled cells are not evident.

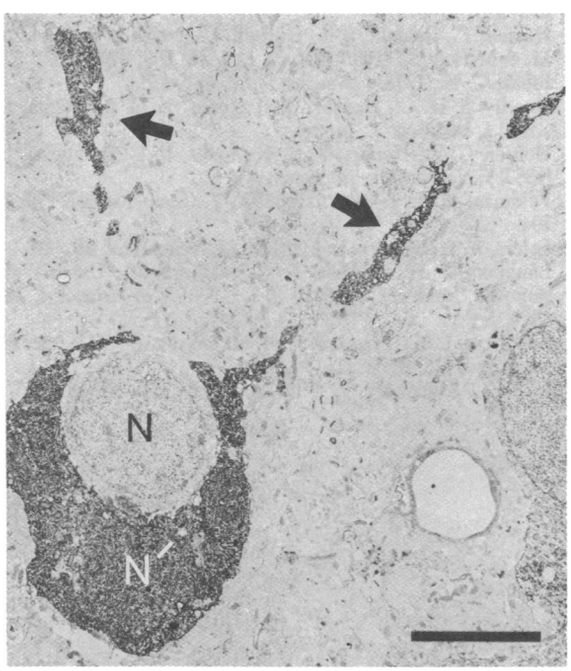

FIG. 6. Electron micrograph of a CCK-immunoreactive neuron in layer II of monkey somatic sensory cortex. The soma and dendrites of the labeled neuron virtually surround the soma of a small unlabeled neuron. The nuclei of the unlabeled (N) and labeled (N') cells are seen. No membrane specializations were seen between the cells. (Bar = 8 μm.)

DISCUSSION

Based on their dendritic morphology, neurons in rat and monkey cerebral cortex displaying CCK-like immunoreactivity are clearly nonpyramidal cells, mainly of the bipolar type. Several studies have previously indicated the presence of CCK and other peptide transmitter candidates in bipolar neurons of the cerebral cortex (10–12). In the present study, we were able to demonstrate the fine structure of CCK neurons in rat and monkey cerebral cortex and the nature of the synapses they make with other neurons. The presence of both symmetric and asymmetric synapses on their somata is typical of small nonpyramidal cells generally (13). The symmetric synapses formed by terminals presumably arising from axons of intrinsic CCK neurons and the close nonsynaptic associations these neurons have with other neurons and with small blood vessels raise several points for discussion.

Iontophoretic application of CCK onto cortical (6) neurons produces a marked increase in spontaneous activity, as would be expected from an excitatory neurotransmitter. Evidence from the present study shows that CCK-like immunoreactivity occurs predominately in cortical terminals forming symmetric synapses. These data together seem to represent an exception to the general principle of an association between excitatory

synapses and asymmetric contacts (14), unless some other inhibitory transmitter is coexistent with and co-released with CCK at the immunoreactive terminals.

Immunocytochemical studies have localized somatostatin (10), vasoactive intestinal polypeptide (11), and avian pancreatic polypeptide (12) in neurons of the cerebral cortex that closely resemble CCK neurons. In neurons of both the rat cortex (11) and human cortex (15), somatostatin-like immunoreactivity has been reported to coexist with avian pancreatic polypeptide-like immunoreactivity. CCK-like immunoreactivity, itself, has been found to coexist with dopamine in neurons of the substantia nigra and ventral tegmental area (16). Coexistence of CCK and γ-aminobutyric acid (GABA) in some cortical neurons is suggested by findings in the present study showing CCK-immunoreactive terminals forming symmetric synapses with pyramidal cell somata and dendrites. GABAergic terminals identified immunocytochemically with antiserum against glutamic acid decarboxylase form symmetric synapses at these same sites (17, 18). However, several features of CCK neurons suggest they are distinct from glutamic acid decarboxylase-positive neurons: (*i*) no convincing example of bipolar glutamic acid decarboxylase-positive neurons was found in monkey sensory motor cortex (19) while such cells make up the majority of CCK neurons; (*ii*) axon terminals displaying CCK immunoreactivity contain few mitochondria and mainly small spherical synaptic vesicles. When the same methods of fixation and electron microscopic preparation were used, glutamic acid decarboxylase-positive terminals consistently contain numerous mitochondria and flattened vesicles (18); (*iii*) the very close association between many CCK neurons and either blood vessels or other neurons is not shared by the glutamic acid decarboxylase-positive neurons. Final determination of a lack of CCK and glutamic acid decarboxylase coexistence in cortical neurons must, however, await double immunohistochemical labeling studies.

Golgi-impregnated bipolar neurons that closely resemble the CCK neurons have been identified in the rat visual cortex (20) but, unlike CCK neurons, their axons form exclusively asymmetric synapses (21). Hence, two or more populations of intrinsic cortical neurons with bipolar morphologies probably exist but the transmitter associated with the cells forming asymmetric synapses remains to be identified.

The close association between CCK neurons and small blood vessels and unlabeled neurons suggests the possibility of a nonsynaptic function for CCK in the cerebral cortex. It may be that the CCK cell serves to monitor circulating blood factors and factors released by other neurons that could affect its own functional state. An alternative possibility is suggested by the fact that release of vasoactive intestinal polypeptide from autonomic nerve terminals in sweat glands and in the submandibular gland causes vasodilation (22, 23) and thus may promote secretion induced by co-release of acetylcholine. No evidence that CCK also has a vasoactive function has yet been reported, though it does, of course, affect smooth muscle tone in the gall bladder (24). Diffusion of CCK away from its release sites might influence the excitability of neurons enfolded by a CCK cell or the contractile state of arterioles or venules that it surrounds. In this way, CCK and possibly the other peptides could serve to control both local neuronal function and local blood flow in a manner analogous to that suggested for vasoactive intestinal polypeptide in the peripheral nervous system.

We thank Dr. K. L. Valentino for her assistance. This work was supported by Grants NS10526 and NS18335 from the National Institutes of Health and a grant from the American Parkinson Disease Foundation.

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