Substance P and Cholecystokinin-Like Peptides in *Helix* Neurons and Cholecystokinin and Serotonin in a Giant Neuron

Abstract. *Immunohistochemical procedures revealed that the serotonin-containing cell situated in each metacerebral ganglion of the snail* Helix *also contains a cholecystokinin-like peptide, but is devoid of substance P. In radioimmunoassays the cholecystokinin-like material showed similarities to the carboxy terminal regions of mammalian cholecystokinin and gastrin. Since much is known about the morphology and synaptic connections of this serotonin neuron, the role of the cholecystokinin-like peptides can now be investigated by neurophysiological methods, thus opening the way to discovering whether a neuron can use more than one transmitter.*

In the metacerebral ganglia of pulmonate and opisthobranch mollusks (*1*, *2*) is a giant serotonin-containing neuron (GSC) measuring 180 μm across its major axis and capable of being dissected from the living animal (*1*, *3*). Evidence in favor of the idea that the serotonin is used as a neurotransmitter by the cells (*1*, *2*) is impressive. The suggestion that the GSC's are both cholinergic and serotonergic neurons (*2*, *4*) has, because of a lack of conclusive data, come under criticism (*5*, *6*). The proposal that neurons may use more than one transmitter substance has been supported by immunohistochemical studies showing that certain peptides—for example, substance P and cholecystokinin octapeptide (CCK8)—are associated with some vertebrate neurons that may contain a classical transmitter of their own (*7*, *8*). These peptides may also be transmitters in their own right (*9*, *10*).

The purpose of this report is to demonstrate that specific invertebrate neurons also contain peptides resembling substance P and CCK and that the CCK-like peptides coexist with serotonin in the GSC's. To our knowledge, this is the first report that a transmitter-like peptide coexists with a classical transmitter molecule in a specifically identifiable neuron with known characteristics. Further studies on the GSC may now make it possible to designate a role for the peptide at a molecular level.

The garden snail *Helix aspersa* was used in this study. Circumesophageal ganglia were dissected and fixed in 4 percent paraformaldehyde with 0.1*M* phosphate buffer (*p*H 7.4) for at least 1 hour. Sections 10 μm thick were cut on a cryostat and processed for immunohistochemistry according to Coon's indirect procedure (*11*). Alternate sections were incubated with antiserum (L112) to synthetic COOH-terminal tetrapeptide of CCK (*12*), with monoclonal antibody to substance P (NC 1/34 HL) (*13*), or with monoclonal antibody to serotonin (*14*). After being rinsed, the sections were incubated with fluorescein isothiocyanate (FITC)–conjugated goat antibodies to rabbit serum, rinsed, mounted, and examined in a fluorescence microscope (Zeiss). In control preparations, at least 200 μg of synthetic unsulfated COOH-terminal CCK8, substance P, or serotonin was added per milliliter of antiserum or monoclonal antibody. In sections taken throughout the central ganglionic

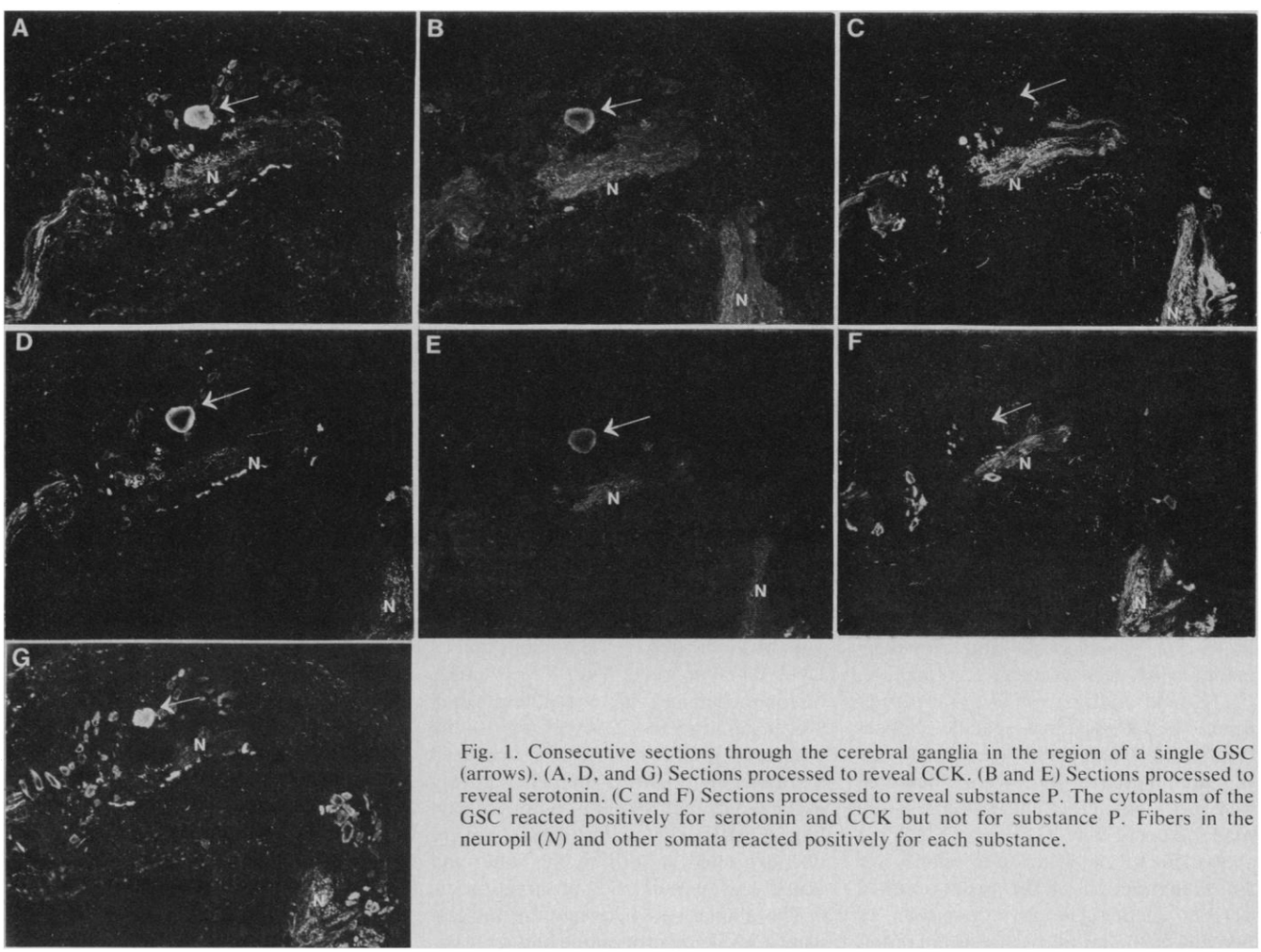

Fig. 1. Consecutive sections through the cerebral ganglia in the region of a single GSC (arrows). (A, D, and G) Sections processed to reveal CCK. (B and E) Sections processed to reveal serotonin. (C and F) Sections processed to reveal substance P. The cytoplasm of the GSC reacted positively for serotonin and CCK but not for substance P. Fibers in the neuropil (*N*) and other somata reacted positively for each substance.

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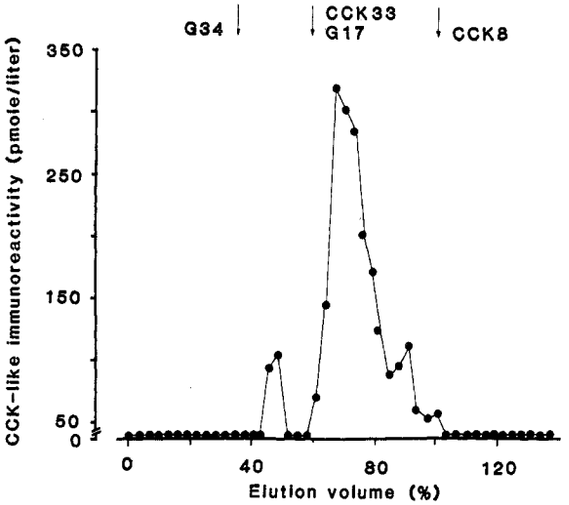

Fig. 2. Separation on Sephadex G-50 (superfine; 1 by 100 cm in 0.05*M* ammonium bicarbonate, 4°C) of a boiling-water extract of snail ganglia. A sample equivalent to 0.08 g of *Helix* supraesophageal ganglion was applied to the column together with bovine serum albumin and $Na^{125}I$ to mark the void volume (detected by absorption at 280 nm) and salt region, respectively. The columns were calibrated with natural human gastrin (34 residues; *G34*), 17-residue gastrin (*G17*), natural porcine CCK (33 residues; *CCK33*) and its synthetic COOH-terminal sulfated octapeptide (*CCK8*). Recovery of immunoreactivity and of standard peptides in tissue extracts was more than 75 percent of the total applied. Elution volumes are expressed as a percentage from void to ^{125}I.

mass, somata and processes demonstrated immunoreactivity to CCK, substance P, or serotonin. Control sections showed a dark background devoid of any obvious fluorescent structures. A specific examination of the GSC's revealed that the cells reacted positively to the serotonin monoclonal antibody (Fig. 1). Serotonin immunoreactive material was associated with the cytoplasm alone and not with the nucleus. The GSC's cytoplasm also reacted positively to the COOH-terminal–specific CCK antiserum but not to substance P antibody (Fig. 1).

The demonstration of serotonin immunoreactivity in the GSC's is consistent with previous findings that have revealed by a variety of biochemical methods that the neuron is serotonergic (*1, 2*).

The CCK-like immunoreactive material in *Helix* ganglia was extracted by boiling tissues in water (0.1 g/ml) for 5 minutes, homogenizing, and centrifuging (2000*g*) for 10 minutes. In radioimmunoassays with antiserum L112, the concentrations of immunoreactive material, relative to CCK8 standard, were 72 ± 5 pmole/g. Antiserum L112 reacts about equally with human heptadecapeptide gastrin (G17) and CCK8 and with their common COOH-terminal tetrapeptide, G4 (*12*). In contrast, in assays with another COOH-terminal–specific antiserum (L48) (*15*), only trace amounts of immunoreactivity were detected (0.4 ± 0.2 pmole/g). The L48 antiserum reacts equally with G17 and CCK8 but shows 50- to 100-fold lower affinity for G4. Reextraction of the pellets with 3 percent acetic acid gave less than 10 percent of the initial CCK-like material.

Separation of ganglia extracts by gel filtration on Sephadex G-50 revealed a major peak of activity eluting between G17 and CCK8 (Fig. 2), which indicates a molecule of a size intermediate between these peptides.

In addition, minor peaks of activity eluted earlier and later than the main one. The minor forms probably correspond to large and small molecular variants, respectively, and may well represent biosynthetic precursors or side products.

The combined immunochemical and chromatographic data indicate that the molluscan immunoreactive material is distinguishable from the main forms of mammalian gastrin and CCK, and also from the gastrin-like activity in molluscan gastrointestinal tract which was reported to resemble mammalian gastrin in immunochemical and chromatographic properties (*16*). The material demonstrated by L112 is also distinguishable from the tetrapeptide Phe-Met-Arg-Phe-NH_2 (FMRFamide) isolated from *Macrocallista* ganglia (*17*), which slightly resembles the COOH-terminal tetrapeptide (Trp-Met-Asp-Phe-NH_2) of CCK. This was indicated by the findings that (i) L112 failed to react with FMRFamide (immunochemical potency less than 0.0001 relative to CCK8), and (ii) the molluscan CCK-like material was well extracted in boiling water, but poorly extracted in acid, whereas factors resembling FMRFamide in *Helix* ganglia were well extracted in both boiling water and acetic acid (unpublished observations).

The similarities between the molluscan CCK-like neuropeptide and its mammalian counterparts in their COOH-terminal regions are compatible with the idea that the COOH-terminal regions of these peptides are conserved because they include the minimum fragment needed for biological activity (*18*). Isolation and full chemical characterization of the *Helix* CCK-like activity are now needed, however, to establish the nature of the structural similarities. The availability of pure *Helix* CCK-like material is also essential for physiological studies in mollusks, as well as for providing radioimmunoassay standards to estimate true concentrations in tissue extracts.

Our studies indicate that CCK-like peptides exist not only in vertebrate neurons (*19*), but also in specific neurons of invertebrates.

To our knowledge, this is the first time that the coexistence of CCK immunoreactivity and serotonin in the same neuron has been established, although certain vertebrate neurons have been shown to contain both dopamine and CCK-like material (*20*). Substance P–like immunoreactivity has been demonstrated in certain invertebrates (*21*); therefore its occurrence in specific neurons in *Helix* is of significance. The lack of substance P–like immunoreactivity in the GSC's is interesting in view of the finding that certain vertebrate neurons contain both serotonin and substance P–like material (*22, 23*). There is already evidence that CCK is a neurotransmitter in the vertebrate brain (*9*); its presence in GSC's now makes it possible to study the peptide's role at the cellular level. Many morphological, electrophysiological, pharmacological, and biochemical data about the GSC are now available (*1, 2*); the way is now open for a detailed study of the physiology of neurons that use more than one neurotransmitter.

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Entorhinal Cortex Lesions Induce a Decreased Calcium Transport in Hippocampal Mitochondria

Abstract. *Lesions to the entorhinal afferent of the hippocampus in rats caused marked changes in calcium transport into mitochondria. Pyruvate-supported calcium transport into mitochondria from the denervated hippocampus was decreased to a larger extent than succinate-supported transport, and adenosine triphosphate–supported transport was not significantly modified. Although cytochrome oxidase and succinate dehydrogenase activities were not significantly changed by entorhinal lesions, pyruvate flux through pyruvate dehydrogenase was significantly decreased, and this effect was correlated with changes in pyruvate-supported calcium transport. The active portion of pyruvate dehydrogenase decreased, whereas total pyruvate dehydrogenase was not modified. These data suggest that denervation might initiate dendritic atrophy and subsequent growth responses by modifying calcium regulation through a change in the phosphorylation of pyruvate dehydrogenase.*

Partial denervation of the hippocampus sets in motion a series of anatomical changes, including dendritic atrophy (*1*), glial hypertrophy and atrophy (*2*), and a growth response in undamaged fibers ("sprouting") and denervated dendrites (*3*). Because these phenomena are a clear manifestation of anatomical plasticity of mature neuronal circuits and because they may be involved in the behavioral and physiological sequelae of brain damage, it is important to identify the biochemical processes that promote and regulate them. Studies of plasticity resulting from electrical stimulation of hippocampal afferents have suggested the existence of a cellular mechanism that could mediate the degenerative and growth responses to denervation. High-frequency activation of certain hippocampal pathways modifies the endogenous phosphorylation of a protein (*4*) that has been identified as the alpha subunit of the mitochondrial enzyme pyruvate dehydrogenase (PDH) (*5*). Pyruvate dehydrogenase activity, which is regulated by the phosphorylation and dephosphorylation of its alpha subunit (the enzyme being inactive in its phosphorylated form), is, in turn, tightly coupled to calcium sequestration by mitochondria (*6*). These results prompted the hypothesis that the regulation of cytosol calcium in the pre- and postsynaptic elements of neuronal connections is strongly influenced by synaptic activity (*7*). Increases in cytosol calcium levels have been linked both to growth (*8*) and degeneration (*9*) in neurons. Therefore the possibility exists that denervation initiates dendritic atrophy and subsequent growth responses (formation of new spines) by removing presynaptic regulation of the calcium "buffering" mechanism provided by mitochondria in the denervated cells. We therefore measured calcium transport and PDH activity in hippocampal mitochondria of rats after producing lesions of the entorhinal cortex.

One week after unilateral lesioning of the entorhinal cortex, the pyruvate-supported calcium accumulation by the crude mitochondrial fraction was markedly reduced (−41 percent) in the ipsilateral, denervated hippocampus as compared with the contralateral, control hippocampus (Table 1). The values found in the contralateral hippocampus were not significantly different from those in the hippocampi of control, unlesioned rats (*10*). The succinate-supported calcium accumulation was affected to a lesser extent (−25 percent), and the adenosine triphosphate (ATP)–supported calcium accumulation was not significantly altered (Table 1). The ratios of pyruvate to succinate and of pyruvate to ATP were thus markedly reduced on the lesioned side (Table 1). In the same group of animals, metabolic flux through PDH was also significantly reduced (−25 percent) (Table 1). At various times after lesioning, the changes in pyruvate decarboxylation correlated well with the changes in pyruvate-supported calcium transport (Fig. 1); the slope of the curve was about 0.54 and the coefficient of correlation was .83 ($P < .01$). However, there was no correlation between the changes in pyruvate-supported calcium transport and the changes in succinate dehydrogenase (SDH) activity ($r = .19$). Only the active portion of PDH was decreased on the lesioned side (−22 percent), the total PDH activity being unmodified (Table 2). The ratio of active to total PDH activity, which is generally considered an index of the phosphorylated state of PDH (*11*), was therefore also significantly decreased (−20 percent). The decrease in pyruvate-supported calcium transport was present as early as 24 hours after production of the lesion, reached a maximum about 5 days after lesioning, and was still present and of a similar magnitude as long as 6 months

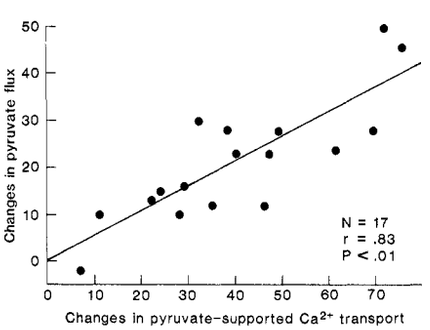

Fig. 1. Correlation between changes in pyruvate-supported calcium transport and changes in pyruvate flux at various times after lesioning of the entorhinal cortex. At various times (1 day to 6 months) after production of a unilateral entorhinal cortex lesion, calcium transport supported by pyruvate and pyruvate flux through pyruvate dehydrogenase were determined in mitochondrial fractions prepared from hippocampus (legend to Table 1). The changes in both parameters represent the percent decrease on the lesioned side in comparison with the control side.

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