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A cholecystokinin-like peptide is present in 5-hydroxytryptamine neurons in the spinal cord of the lamprey

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The spinal cord of the lamprey contains a row of 5-hydroxytryptamine (5-HT) cell bodies in the intermediate plane, just ventral to the central canal, as well as numerous 5-HT containing fibres with the highest concentrations in the Müller-fibre plane in the ventromedial part of the spinal cord (Honma 1970, Baumgarten 1972, Van Dongen *et al.* 1985b). In previous studies, we have investigated whether or not these 5-HT neurons also contain a biologically active peptide, primarily focusing on substance P and TRH, which have been shown to occur in 5-HT neurons in several mammals (Hökfelt *et al.* 1984). However, coexistence of 5-HT and a hitherto not defined tachykinin could only be seen, at most, in some single neurons (Van Dongen *et al.* 1985b, and in press). In the present study, we have continued this analysis using an antiserum specific for the mid-sequence 17–23 of cholecystokinin-33 (CCK-33) and report here that the majority of 5-HT cell bodies in the lamprey spinal cord contain a peptide reacting with this antiserum.

The spinal cord of adult lampreys (river lamprey, *Lampetra fluviatilis* and silver lamprey, *Ichthyomyzon unicuspis*) were dissected out under anaesthesia and immersed in ice-cold 10% formalin with 0.2% picric acid added as described before (Van Dongen *et al.* 1985b). After fixation for 2 h the tissues were rinsed for at least 24 h in 10% sucrose in 0.1 M phosphate buffer. Transverse and horizontal serial sections (thickness 14 μm) were cut on a cryostat and processed according to the indirect immunofluorescence technique of Coons and co-workers (Coons 1958). Briefly, sections were incubated in a humid atmosphere at 4 °C for 24 h with CCK antiserum no. 1561 in a dilution of 1:400. This antiserum was raised in rabbits against porcine CCK-33 coupled by carbodiimide to bovine serum albumin (Rehfeld 1985). It recognizes approximately the 17–23 sequence of CCK-33. This sequence is thus outside the C-terminal octapeptide amide which is conventionally considered to be the biologically active part of CCK-33. Adjacent sections were incubated under the same conditions with anti-serum raised to 5-HT coupled to bovine serum albumin (1:400) (Steinbusch *et al.* 1978). After rinsing in phosphate buffered saline (PBS), the sections were incubated with fluorescein-isothiocyanate (FITC) conjugated swine anti-rabbit antibodies (Dakopatts, Copenhagen) for 30 min at 37 °C. After mounting in a mixture of glycerine and 0.1 M phosphate buffer with added *p*-phenylenediamine, the sections were analysed in a fluorescence microscope and photographed. After photography, sections incubated with CCK antiserum were reincubated with 5-HT antiserum as described above, and sections incubated with 5-HT antiserum were now processed with CCK antiserum. The sections were re-photographed and the micrographs compared with regard to distribution of 5-HT- and CCK-immunoreactive cells and fibres.

After incubation with 5-HT antiserum, many fluorescent cell bodies were seen in the midline immediately ventral to the central canal. These neurons gave rise to a dense plexus of varicose fibres in the ventromedial part of the spinal cord,

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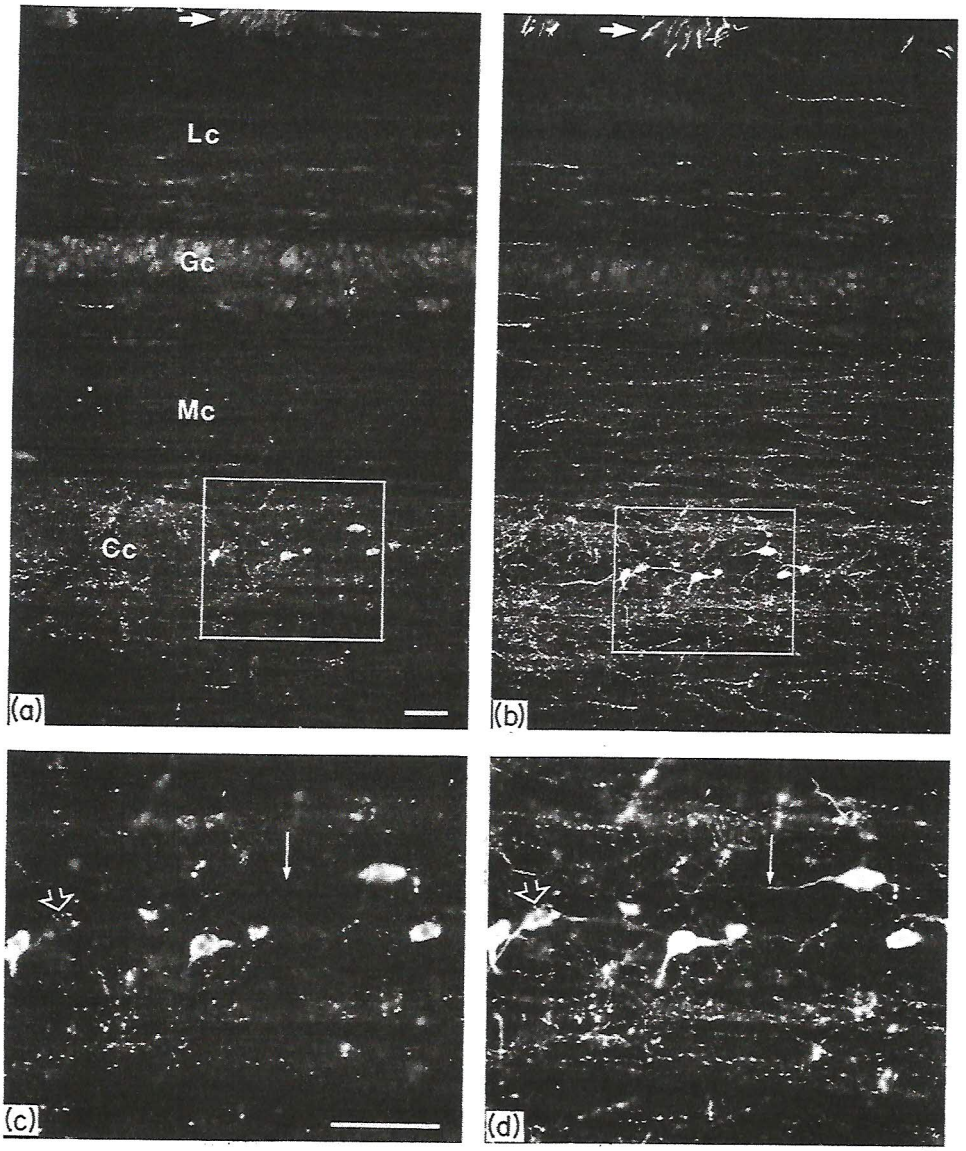

Fig. 1a–d. Immunofluorescence micrographs of horizontal sections of the lamprey spinal cord after incubation with antiserum to CCK-33 (a, c) and 5-HT (b, d). Areas (c) and (d) represent high magnifications of part of (a) and (b) respectively, as indicated by rectangles. Areas (a) and (b) show the same section, which has first been incubated with CCK antiserum and, after photography, with 5-HT antiserum. (a, c) Several CCK-immunoreactive cell bodies can be seen in the midline (central canal column, Cc) and they are surrounded by numerous processes. Note the lack of fibres in other layers with some single exceptions. (b, d) After reincubation of the section with 5-HT antiserum, only one cell body (open arrow) appears, which is probably not CCK-immunoreactive, or perhaps only weakly positive. There are, however, more 5-HT-positive fibres than CCK-immunoreactive ones in this area. Furthermore, many 'newly stained' 5-HT-positive longitudinal fibres can be seen in the medial (Mc), lateral gray (Gc) and lateral (Lc) columns, as well as in the roots (thick arrows). Note that processes from the CCK/5-HT-immunoreactive cells can be followed for much longer distances after 5-HT incubation (cf. thin arrows in Fig. 1c and d), suggesting that CCK-like immunoreactivity is difficult to demonstrate in non-terminal processes. Bars indicate 50 μm.

forming bilateral fibre plexus columns (van Dongen *et al.* 1985b). In addition, numerous 5-HT-positive fibres were seen in the dorsal, lateral and ventral spinal axon columns. Further 5-HT-positive fibres were also seen in dorsal and ventral roots. After incubation of adjacent sections with CCK antiserum, immunoreactive cell bodies with a similar distribution and in similar numbers were observed ventral to the central canal (Fig. 1a, c). Fibres which were CCK-immunoreactive were seen in the ventromedial aspects of the cord forming two rows of dense fibre networks closely overlapping with the 5-HT plexus in this area (Fig. 1a, c). Very few CCK-immunoreactive fibres were seen in the dorsal, lateral or ventral spinal axon columns and none in the dorsal or ventral roots (Fig. 1a). After reincubation of 5-HT stained sections with CCK antiserum, the distribution of immunoreactive structures was identical to the one seen after incubation with 5-HT antiserum alone, that is, no newly stained structures were observed. After reincubation of CCK stained sections with 5-HT antiserum, a few 5-HT-positive cell bodies were encountered, in addition to the CCK immunoreactive ones (Fig. 1b, d). In addition, numerous fluorescent 5-HT-positive, apparently CCK-negative fibres were seen in the areas described above (Fig. 1b).

The present results demonstrate that a majority of 5-HT cell bodies in the midline, ventral to the central canal contain a CCK-like peptide. This conclusion is based on the fact that (1) in many pairs of adjacent sections approximately the same number of CCK- and 5-HT-positive cells were seen with overlapping distribution, and (2) after reincubation of 5-HT stained sections with CCK antisera and of CCK stained sections with 5-HT antiserum, only very few *newly* stained cell bodies appeared. Furthermore, this peptide also seems to be present in the ventromedial 5-HT fibre plexus forming two fibre columns along the ventral surface of the spinal cord. There are, however, numerous 5-HT-positive fibres in the spinal cord, which seem to lack CCK-33-like immunoreactivity. Whether these fibres, in fact, lack the peptide, or whether the concentrations of the CCK-like peptide are too low to be detected with our immunohistochemical technique, remains to be elucidated. The latter possibility is favored to some extent by the fact that the double-staining experiments directly showed that often 5-HT-, but not CCK-like immunoreactivity could be seen in processes emanating from 5-HT/CCK cells. These findings agree with the recent demonstration of a CCK-like peptide in some 5-HT neurons in the rat (Mantyh & Hunt 1984).

The exact nature of the CCK-like immunoreactivity demonstrated in the present study is unknown. The antibody used, in contrast to most other CCK-antisera, reacts with the mid-portion of CCK-33 and does not recognize the C-terminal octapeptide. Incubation with antiserum raised against CCK-(1–8) does not stain the 5-HT neurons in the lamprey spinal cord, although strongly fluorescent fibres can be visualized in other areas (to be published). Thus, the CCK-like peptide seen in the 5-HT neurons in the lamprey spinal cord may represent a hitherto unknown compound, possibly produced by differential processing from the CCK precursor molecule. Attempts are now being made to obtain more information on the structure of the CCK-like immunoreactivity in the lamprey spinal cord.

The lamprey spinal cord represents a favourable model for physiological analysis of neuronal events, including the mechanism of action of neurotransmitters (Grillner 1985). Thus, electrophysiological analysis can be carried out on segments of the cord kept *in vitro* for several days, and intracellular recordings can be made from many types of identified neurons. Recently, we have been able to characterize in this preparation some of the actions of 5-HT on single neurons with intracellular recordings and have observed that 5-HT causes a reversible reduction of the late after-hyperpolarization after-action potentials (Van Dongen *et al.* 1985a). Thus, the lamprey 5-HT/CCK system may offer a model to analyse the mechanisms of interaction of two compounds contained in and perhaps released from the same neurons. Such information would be of interest in view of the fact that numerous neurons in the mammalian brain seem to contain both a classical transmitter and a peptide (Hökfelt *et al.* 1984). It should, however, be emphasized that it will be necessary to characterize chemically the CCK-like peptide in the lamprey spinal cord before such studies can be carried out.

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