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Serotonin and substance P coexist in neurons of the rat's central nervous system

(autoradiography/fluorescence histochemistry/microspectrofluorimetry/immunofluorescence/unlabeled antibody–peroxidase antiperoxidase)

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ABSTRACT 5-Hydroxytryptamine (serotonin)-containing neurons in the rat's medullary raphe and interfascicularis hypoglossi cell groups were identified by means of autoradiography following prolonged intraventricular administration of 5-hydroxy[^{3}H]tryptamine, fluorescence histochemistry for the demonstration of endogenous 5-hydroxytryptamine, and microspectrofluorimetric analysis of excitation and emission spectra. Immunocytochemical methods (the unlabeled primary antibody–peroxidase antiperoxidase and indirect immunofluorescence methods) were applied with antisera to substance P in order to localize immunoreactivity in these medullary neurons. It was demonstrated that the raphe nuclei and the interfascicularis hypoglossi nucleus are heterogeneous cell groups that contain: (*i*) Neurons that display both an uptake–storage capacity for 5-hydroxy[^{3}H]tryptamine and a formaldehyde-induced fluorescence with spectral characteristics identical to those of the 5-hydroxytryptamine fluorophor. These cells exhibit high to low fluorescence intensities without detectable substance P-like immunoreactivity. (*ii*) Neurons with various 5-hydroxytryptamine fluorescence intensities and intense to low degrees of substance P-like immunoreactivity. (*iii*) Neurons with various degrees of substance P-like immunoreactivity without detectable 5-hydroxytryptamine fluorescence or 5-hydroxy[^{3}H]tryptamine uptake and storage capacity. These results indicate that some neurons contain high or low levels of only 5-hydroxytryptamine or substance P, whereas other neurons contain both 5-hydroxytryptamine and substance P in various proportions. The present findings demonstrate the presence of two putative transmitters, a biogenic amine and a polypeptide, within the same neuron in the mammalian central nervous system.

The gastrointestinal tract contains cells that store both small peptide hormones and a biogenic amine (1–3). Certain neurons in gastropod molluscs (4) and flatworms (5) contain both dopamine and 5-hydroxytryptamine (5HT, serotonin). The giant cerebral neuron in *Helix* has been demonstrated to release both acetylcholine and 5HT (6). Single, identified neurons of *Aplysia* have been shown by radioenzymatic micromethods to contain several putative transmitters, including 5HT, octopamine, and acetylcholine (7) and glutamate and glutamine (8). There is a growing body of data suggesting that while many nerves have only one transmitter, others in some species, during development or during hormone-dependent cycles, employ multiple transmitters (9). Somatostatin-like immunoreactivity has been reported (10) in some noradrenergic neurons of the peripheral sympathetic nervous system of guinea pigs. The present investigation provides data from a number of experimental approaches that two substances—one a biogenic amine, 5HT, and the other a polypeptide, substance P (SP)—that have been claimed as established or putative transmitters (9, 11, 12) can coexist in greater or lesser amounts in neurons of the mammalian central nervous system.

MATERIALS AND METHODS

Adult male Sprague–Dawley rats (200 g body weight) of American and Swedish origin were used. The animals were fed standard food pellets and water ad lib. and were maintained under controlled temperature and dark–light diurnal schedules.

Intraventricular Infusions of [^{3}H]5HT and Autoradiography. The animals, pretreated with a monoamine oxidase inhibitor (Clorgyline, 15 mg/kg body weight) 2 hr before the infusion, were anesthetized with 35% chloral hydrate (0.1 ml/100 g body weight), placed in stereotaxic headframes, and supplied with a low flow of 5% oxygen/95% carbon dioxide. Two separate sets of experiments were performed: (*i*) intraventricular infusions for 3 hr with 500 μl of 1 μM [^{3}H]5HT containing 10 μM unlabeled norepinephrine, and (*ii*) infusions for 3 hr with 500 μl of 1 μM or 10 μM [^{3}H]5HT. Following the infusion, the animals were perfused through the vascular tree with 1% (wt/vol) glutaraldehyde and 1% (wt/vol) formaldehyde in 0.12 M potassium phosphate buffer (pH 7.4). Tissues were stored for 48 hr in 30% sucrose in phosphate buffer and then sectioned at 30 μm while frozen. Alternate sections were prepared for light microscope autoradiography using NTB-2 emulsion, exposed for 30 days, developed in D19, and lightly counterstained with thionin. In addition, selected tissue slabs of the brain stem were taken after perfusion with aldehyde mixtures, postfixed in 2% osmium tetroxide, and embedded in epoxy resin. Semithin 1- to 4-μm sections were prepared from these slabs for high resolution autoradiography (for details of these methods see refs. 13–16).

SP Immunocytochemistry Using the Peroxidase Antiperoxidase (PAP) Method. Normal untreated rats, anesthetized with 35% chloral hydrate (0.1 ml/100 g body weight), were perfused with 1 liter of cold 4% formaldehyde in 0.12 M phosphate buffer (pH 7.3) for 0.5–4 hr. The dissected brains were soaked overnight in 30% sucrose in phosphate buffer. Cryostat or frozen sections 10 μm thick were cut and mounted serially on gelatin-coated slides and were kept frozen prior to use. Immunohistochemical reactions were performed by a modified unlabeled SP antibody–PAP technique previously reported (17, 18). Control sections were carried in parallel through the same sequence except that the first incubations were in SP antiserum that had been adsorbed with SP in excess. The preparations were observed with dark-field or bright-field optics.

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Abbreviations: 5HT, 5-hydroxytryptamine, serotonin; SP, substance P; PAP, peroxidase antiperoxidase antibody.

5HT Fluorescence Histochemistry. The Falck–Hillarp procedure was used for the histochemical demonstration of 5HT (19, 20). Tissue specimens from the medulla were frozen in liquid propane, cooled by liquid nitrogen, and then freeze-dried. Thereafter, specimens were exposed to gaseous formaldehyde of optimum humidity (90–97.5%) at +80° for 1–2 hr (21). After the specimens had been embedded in paraffin, the blocks were serially sectioned 10 μm thick and every fifth or tenth section was taken for analysis. For study under the fluorescence microscope or for recording of emission spectra, the sections were mounted on glass slides with Entellan under a coverslip. Excitation spectra were recorded from sections placed on spectral grade glass coverslips. Material used for the combined studies of 5HT fluorescence, microspectrofluorimetry, and SP immunofluorescence was treated with special care. Single sections, carefully placed on coverslips, were stretched gently for about 30 sec at 50–60° and mounted in xylene under a second, smaller coverslip for the 5HT microscopic and spectrofluorimetric analysis. Thereafter the second coverslip was carefully removed and the sections were subjected to staining for SP immunofluorescence. Each section was carefully surveyed in the fluorescence microscope (Zeiss, Standard Junior). Photographs were taken of all groups of 5HT-fluorescent perikarya present. Approximately 300 sections from at least six animals were subjected to these procedures. Photographs of 5HT-fluorescent perikarya were recorded on Scopix RP1 film and developed in Refinal (Agfa-Gevaert). This was followed by registration of excitation and emission spectra of 50 selected cells.

Microspectrofluorimetric Analysis. A modified Leitz fluorescence microspectrograph equipped with an MPV system was used. For emission spectra, mounted sections were excited with the 405-nm mercury line obtained by means of an interference filter (TAL 405, Schott and Gen) from a Philips CS 100 W-2 lamp. Epi-illumination (dichroic mirror no. 2) through oil immersion objectives (×25, numerical aperture 0.75) was used. A barrier filter with 50% transmission at 430 nm was inserted to minimize light scatter. For excitation spectra transillumination was used. The unmounted section was placed upside down against the dark-field condenser. Paraffin oil (Merck, spectral quality) served as the immersion medium. Care was taken to adjust the area measured (maximum slit width 3 μm) to the size of the fluorescent structure analyzed, and the area of section illuminated with excitation light was kept as small as possible in relation to the measuring area (22, 23). The time required for emission spectra registration was approximately 0.1 sec and for excitation spectra, 30 sec. Spectral changes due to decomposition were controlled for. Blank spectra were recorded in all cases from adjacent structures with no detectable formaldehyde-induced fluorescence and subtracted from the reading obtained from the test cell. Control spectra were obtained from a protein model prepared from 2% (wt/vol) bovine serum albumin solution containing 0.5 nM 5HT and treated with formaldehyde gas (+80°, 1 hr). All spectra were corrected for instrument factors leading to spectral distortion and expressed as relative quanta versus wavelength (22).

SP Immunofluorescence. The same sections used for 5HT fluorescence were recovered and used for SP immunofluorescence. Following 5HT fluorescence microscopy and spectrofluorimetric analyses, the upper coverslip was removed, the section was rinsed with xylene, and efforts were made to retain the tissue intact on the retaining coverslip. The identity of each section was noted along with the photographic records of the 5HT-fluorescent perikarya. The sections were processed for the indirect immunofluorescence method (24). After three brief rinses in 0.1 M phosphate-buffered saline, pH 7.4, the sections were incubated with either (*i*) antibody to SP [concentration 1:40 (18, 25)] or (*ii*) SP antibody pretreated with excess SP in order to serve as an antibody-adsorbed control (concentration 1:40). The incubation was carried out at 37° for 30 min in a humid environment. After a brief rinse with phosphate-buffered saline, the sections were reincubated under the same conditions with fluorescein isothiocyanate-conjugated sheep antiserum to rabbit IgG (diluted 1:4). The sections were rinsed well with phosphate-buffered saline, mounted in glycerol/phosphate-buffered saline (3:1, vol/vol), and examined in a Zeiss fluorescence microscope (26). All regions with SP immunofluorescent perikarya were photographed and the location and specimen number of each negative were recorded. Subsequently, the photographic records from 5HT fluorescence studies and the SP immunofluorescence studies were correlated with respect to their specimens of origin. Cell groups with perikarya displaying both 5HT fluorescence and SP immunofluorescence were examined for their precise correlations. The recovery of sections after both 5HT and SP procedures was very low (less than 5% of the original sample), and the recovery of matching photographs after both procedures was 1% of the original sample. The main reason for the low recovery was the fragility of sections from freeze-dried material that were subjected to the numerous agitations and solution changes.

Drugs and Substances Used. Norepinephrine hydrochloride (Sigma); [^{3}H]5HT creatinine sulfate (specific activity 16–24 Ci/mmol; Amersham Corp.); Clorgyline (courtesy May and Baker); substance P (Sigma); antibody to substance P (courtesy S. Leeman); PAP antibody (courtesy L. Sternberger); NTB-2 emulsion (Kodak); Entellan (Merck); fluorescein isothiocyanate-conjugated sheep antiserum to rabbit IgG (Miles).

RESULTS

5HT was detected by endogenous fluorescence and microspectrofluorimetry and by autoradiography after [^{3}H]5HT uptake in a number of locations in the brain, comparable to those previously reported (15, 27). Two of these 5HT-containing cell groups in the ventral medulla, the midline neurons in the nuclei raphe pallidus and raphe magnus and the neurons of the nucleus interfascicularis hypoglossi, were selected for further study with immunocytochemical procedures with SP antisera. Neurons with immunoreactivity to SP by the fluorescence and PAP methods are present in both sites. The results of this series of experiments are summarized in detail for the neurons of the interfascicularis hypoglossi (see Figs. 1 and 2). Similar results were obtained with cell bodies in the raphe magnus and pallidus.

The nucleus interfascicularis hypoglossi (28) consists of (*i*) medium-sized to large nerve cells (~30–60 μm in diameter), scattered individually along the descending rootlet fibers of the hypoglossal nerve, and (*ii*) small to medium-sized nerve cells (~20–40 μm in diameter), clumped together at the exit of the hypoglossal nerve rootlets from the brain. Processes of both these cell groups can be followed with some difficulty in Nissl-stained sections of the normal brain (Fig. 1*A*). However, with autoradiography after administration of [^{3}H]5HT and unlabeled norepinephrine (Fig. 1*B*) or with [^{3}H]5HT alone (Fig. 1*C*) neurons of both cell groups were labeled, thus exhibiting uptake–storage systems for the labeled transmitters. Unlabeled norepinephrine was used to prevent uptake of [^{3}H]5HT by probable catecholamine neurons. Fluorescence histochemical reactions display equivalent populations of neuronal cell bodies, better in the groups among the exiting rootlets of the hypoglossal

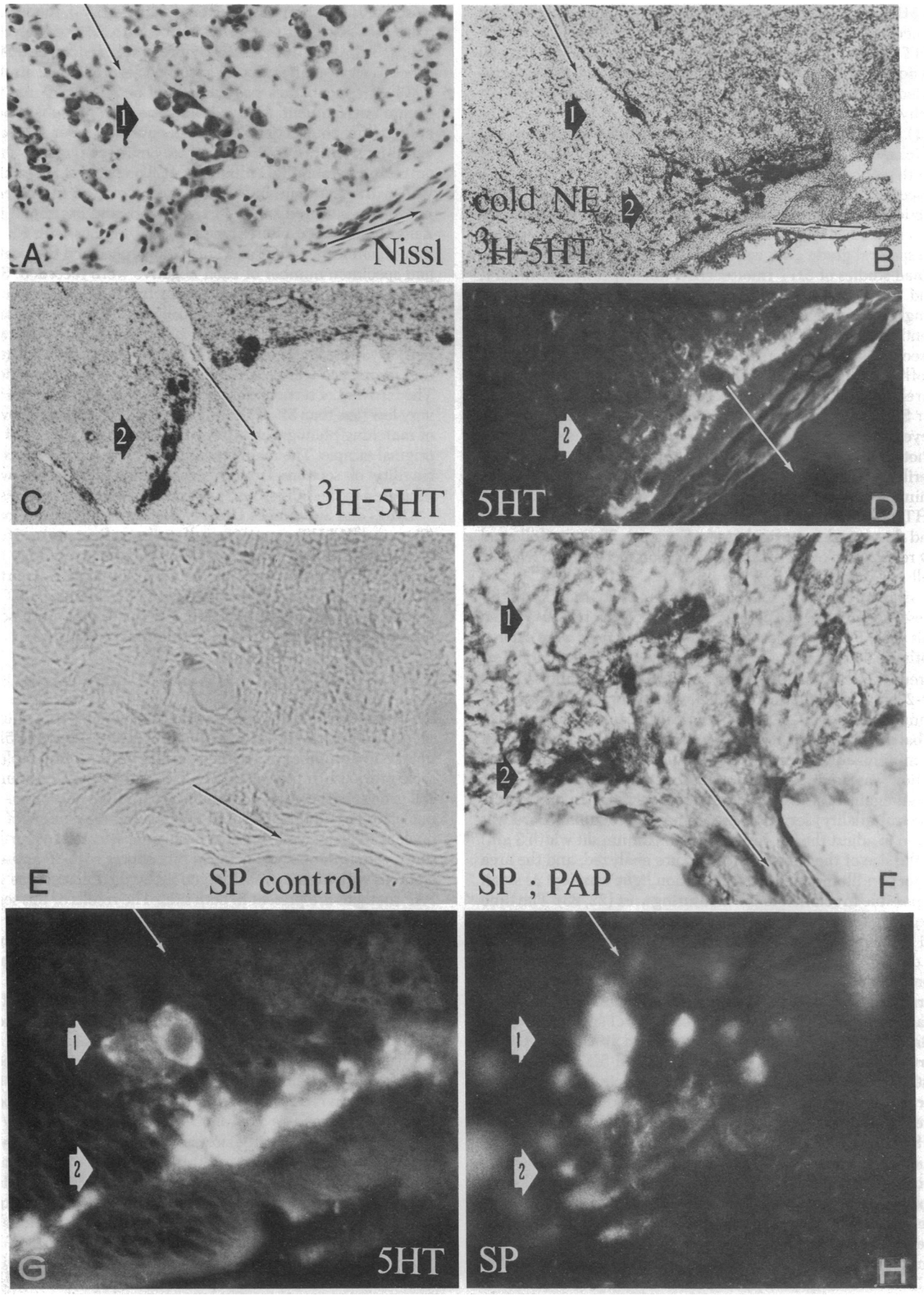

FIG. 1. *(Legend appears at bottom of the next page.)*

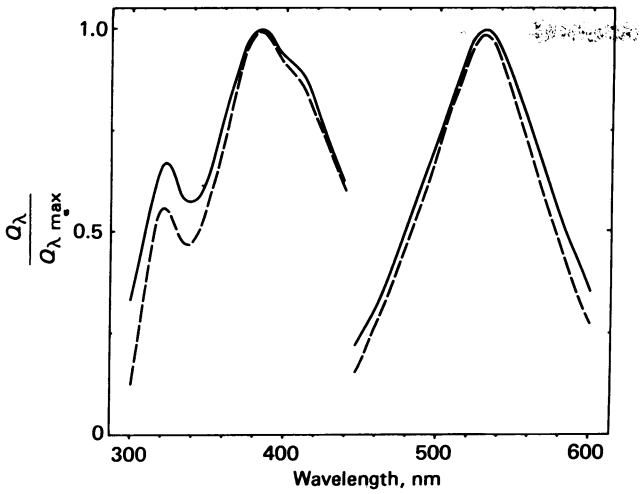

FIG. 2. Comparison of excitation (left) and emission (right) spectra recorded from a fluorescent 5HT cell (solid line) of the interfascicularis hypoglossi compared to a control protein model containing 0.5 mM 5HT (broken line) allowed to react with formaldehyde gas.

nerve than in those in the interfascicular portion. There is an intense, yellow fluorescence (Fig. 1*D*) that rapidly fades with UV illumination. Microspectrofluorimetric analyses demonstrate that the fluorescence of almost all these neuronal perikarya has spectral characteristics identical with those of the 5HT fluorophor: that is, peaks of excitation at 320–385 nm (shoulder at about 415 nm) and maximum emission at 525–530 nm (see Fig. 2). However, in a few neurons that were weakly fluorescent, the emission spectra showed, in addition to the peak at 525–530 nm, a small shoulder around 500 nm. These observations indicate the presence of another indole compound, possibly tryptamine, which is involved in a minor pathway in tryptophan metabolism (23).

Immunocytochemical studies with SP antiserum using the unlabeled primary antibody–PAP technique demonstrated a number of large immunoreactive neuronal cell bodies along the course of the hypoglossal nerve within the brain, as well as in clusters around the exit of the nerve (Fig. 1*F*). No immunoreactivity was found in adjacent sections incubated with SP antibody preadsorbed with SP in excess (Fig. 1*E*).

Experiments were conducted to demonstrate endogenous 5HT by fluorescence microscopy and SP immunoreactivity by the indirect Coons method (24) on cells of the interfascicularis hypoglossi in the same section (Fig. 1 *G* and *H*). The clusters of small and medium-sized fusiform neurons at the exit of the hypoglossal nerve are intensely fluorescent with endogenous 5HT, whereas the larger neurons along the course of the nerve are less intensely fluorescent (Fig. 1*G*). A reverse relationship holds for the results of SP immunofluorescence. Intense immunoreactivity was found in the large neurons along the course of the nerve and a fluorescence that was less intense, but significantly above background, was found in the neurons at the exit of the nerve. In a careful examination of these cells by juxtaposing photographic transparencies, it was found that approximately 70% of the neurons at the exit of the nerve showed high 5HT fluorescence and low SP immunoreactivity; the remaining 30% of the cells showed only intense 5HT fluorescence. Along the course of the hypoglossal nerve, about 50% of neurons with low 5HT fluorescence had intense SP immunoreactivity and vice versa. This suggests that whereas some neurons contain only one of the two transmitters, others contain both in reciprocal amounts, and the remaining cells contain neither one. However, because of the limitations in sensitivity of the techniques employed, it cannot be stated that cells showing 5HT fluorescence without detectable SP-like immunoreactivity are completely devoid of SP-like compounds, or vice versa.

DISCUSSION

These findings indicate that some central nervous system 5HT neurons can also contain an SP-like polypeptide, possibly in amounts inversely proportional to those of 5HT: neurons with high 5HT levels have small amounts of SP and vice versa. The mammalian central nervous system is supplied by numerous 5HT neurons and their processes. These were demonstrated histochemically in the brain stem of rat by utilizing formaldehyde-induced fluorescence (27). Subsequent studies (29–32) demonstrated the pathways and axon terminals of serotonin neurons. Since then, the observations have been expanded to include other species (33–35). The localization of 5HT systems has also been studied by means of intraventricular administration of [^{3}H]5HT (13, 14, 36–38), and the locations of neurons and axons with selective uptake of this chemical have been charted in a recent detailed study (15). The possibility that 5HT neurons might contain another putative transmitter was indicated in previous studies of neuronal perikarya in the raphe nuclei of rat and monkey (14, 39). It was discovered that individual neurons differ in their uptake of [^{3}H]5HT in a manner that appears to be independent of their distance from the ventricular surface and independent of the diffusion gradient of [^{3}H]5HT. These observations allowed the speculation that (*i*) 5HT may be a major transmitter in some neurons with greater uptake for [^{3}H]5HT, and (*ii*) in raphe neurons with low uptake for exogenous [^{3}H]5HT, other transmitter molecules may also be involved. SP, the hypotensive peptide originally detected in equine brain and gut (40) and characterized (41) as an undecapeptide, occurs in the central nervous system as well as in the peripheral nervous system (18, 26, 42–46). The evidence suggesting that SP, either in the intact state or as a precursor to smaller molecules, is a transmitter continues to grow (44–50). The action of SP has been reported to be calcium

FIG. 1 (*on preceding page*). Photomicrographs of the nucleus interfascicularis hypoglossi in the rat. A common labeling code is used in all eight panels taken from various preparative techniques. The course of the hypoglossal nerve within the medulla and after its exit from the brain is indicated by small arrows. The large arrows enclosing the numeral 1 indicate the group of larger neurons along the medullary course of the hypoglossal nerve rootlets. The large arrows enclosing the numeral 2 indicate the group of smaller neurons clustered at the exit of the hypoglossal nerve rootlets. (*A*) Nissl preparation of the normal adult brain. (×200.) (*B*) Autoradiogram after prolonged intraventricular administration of unlabeled ("cold") norepinephrine and [^{3}H]5HT. (×200.) (*C*) Autoradiogram after prolonged intraventricular administration of [^{3}H]5HT. (×200.) (*D*) Fluorescence histochemical demonstration of endogenous 5HT cells. (×200.) (*E*) Control preparation incubated with SP antiserum preadsorbed with SP in excess. No SP immunoreactivity is present. (×400.) (*F*) SP immunoreactive cells and fibers are demonstrated by the unlabeled SP antibody–PAP technique. (×400.) (*G*) Section prepared for the Falck–Hillarp technique demonstrates endogenous 5HT at various levels of intensity in individual cells of the interfascicularis hypoglossi. (×400.) (*H*) The same section has been washed well and reincubated with SP antiserum and allowed to react according to the indirect immunofluorescence method. Intense SP-like immunofluorescence is present in neurons along the course of the hypoglossal nerve and low SP-like immunofluorescence is present in neurons at the exit of the nerve. (×400.)

dependent (45) with an excitatory (46, 47) or inhibitory (49, 50) effect, depending on location.

These data contribute evidence in support of the hypothesis that neuronal perikarya can synthesize and store multiple neurotransmitters, and suggest that the concept that one nerve cell makes and releases only one transmitter (51) needs to be modified with respect to the mammalian central nervous system as well. Further investigations are needed for the localization of multiple transmitters in nerve terminals at the ultrastructural level.

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