Colocalization of Somatostatin with GABA or Glutamate in Distinct Afferent Terminals Presynaptic to the Mauthner Cell

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The presence of somatostatin in afferent fibers impinging on the goldfish Mauthner (M-) cell was determined using immunohistochemical methods, combined with confocal and electron microscopy, and the relationship of this peptide with inhibitory and excitatory terminals was studied. Somatostatin-reactive boutons were present only on the distal part of the M-cell's lateral dendrite. Somatostatin immunoreactivity was observed in typical large myelinated club endings (LMCEs) corresponding to mixed (electrical and chemical) eighth nerve primary afferent fibers. The axoplasm of these fibers contained dense-core vesicles (DCVs) dispersed among round vesicles. We have made a novel finding that the excitatory transmitter glutamate is present in LMCEs. Colocalization of this amino acid with somatostatin was detected in 75% of these endings using postembedding staining with gold particles of various sizes. The other structures labeled by somatostatin antibody were found to be small vesicle boutons (SVBs), which establish symmetrical synapses and contain a population of pleomorphic vesicles with DCVs scattered among them. Double labeling with antibodies against glutamic acid decarboxylase and GABA allowed the definition of three types of biochemically characterized terminals: [somatostatin-GABA], [GABA], and [somatostatin]. However, the occurrence of DCVs in SVBs stained for GABA alone suggests that neuropeptides other than somatostatin may also coexist with GABA in this class of boutons. The coexistence of somatostatin with both inhibitory and excitatory neurotransmitters acting on the same region of a postsynaptic cell is discussed in relation to the role postulated for this peptide in synaptic plasticity.

[Key words: somatostatin, GABA, glutamate, colocalization, dendrite, Mauthner cell, immunocytochemistry]

Various peptides are found in terminals of central neurons, where they are often stored in dense-core vesicles (DCVs) and colocalized with more classical neurotransmitters (Hökfelt, 1991). For example, it has been reported that GABA coexists with cholecystokinin, vasoactive intestinal peptide, neuropeptide Y, substance P, or somatostatin in the neurites of the rat (Jones and Hendry, 1986). In the course of an ultrastructural study of GABAergic innervation of the goldfish Mauthner cell, we observed the presence of numerous DCVs in small vesicle boutons (SVBs) as reported previously (Tuttle et al., 1987) and preferentially SVBs containing GABA. In an attempt to identify the related peptide, we focused our attention on somatostatin-14 for the following reasons. It is expressed in most representatives of the phylogeny (Argos et al., 1983), including fish (Buchanan et al., 1987; Christenson et al., 1991; Zupanc et al., 1991) in which a molecule identical to the mammalian somatostatin-14 has been identified (Epelbaum, 1986). Further, this cyclic neuropeptide may coexist with GABA in the lamprey spinal cord (Christenson et al., 1991).

The extremity of the M-cell lateral dendrite is richly innervated by glutamic acid decarboxylase (GAD)–immunoreactive boutons (Petrov et al., 1991) and the corresponding GABAergic boutons are adjacent to the large myelinated club endings (LMCEs) that comprise the main electrical and chemical excitatory inputs to this neuron (Furshpan, 1964; Korn and Faber, 1976; Faber and Korn, 1978), a spatial relationship between excitatory and inhibitory afferents comparable to that for distal dendrites and dendritic spines of hippocampal (Somogyi et al., 1984) and neostriatal (Takagi et al., 1983) neurons. In this article, we show that some of these inhibitory afferents contain both somatostatin and GABA. Surprisingly, we have also observed for the first time somatostatin immunoreactivity in excitatory terminals, that is, in LMCEs. Previous work has shown that long-term potentiation (LTP) occurs at these excitatory terminals (Yang et al., 1990) and it involves activation of NMDA receptors. Further, the excitatory amino acid antagonist γ-D-glutamylglycine abolishes the EPSPs at this synapse (Wolszon and Faber, 1988). Taken together, these results suggest that the transmitter for the chemical component of this mixed synapse is glutamate and, indeed, with postembedding immunocytochemistry we demonstrate now that glutamate is present in all the LMCEs where it is colocalized with the neuropeptide somatostatin-14 in about 75% of the endings studied.

Materials and Methods

Fifteen adult goldfish (Carassius auratus), 10-12 cm in length, were perfused through the heart with saline fixative after being anesthetized with 0.3% 3-aminobenzoic acid ethyl ester (MS222; Sandoz) and paralyzed with Flaxedil (Rhône Poulenc; 1 μg/kg body weight).

Fixation procedures. For fluorescence microscopy, animals were perfused for 20 min with fresh cold paraformaldehyde (4%) in 120 mM phosphate buffer (PB) at pH 7.4. After dissection, the brains were immersed overnight in paraformaldehyde and sucrose (40%) in phosphate-buffered saline (PBS), at 4°C. Brainstem were cut (30 μm) with a freezing microtome and slices were treated for 30 min with 0.25% ammonium chloride in PBS to block the free aldehyde groups. For peroxidase ex-
experiments, fish were treated as described above, except that a perfusion mixture of 2% paraformaldehyde and 2% glutaraldehyde was used in order to preserve somatostatin reactivity and the brain slices (30 μm) were cut with a vibratome without surroce treatment. For electron microscopy, four fish were perfused with a mixture of 2% paraformaldehyde and 2% glutaraldehyde in PBS at pH 7.4. The brains were sectioned with a vibratome and the M-cell-containing slices (80 μm) were kept in PBS.

Immunohistochemistry. For light microscopy, sheep anti-GAD and rabbit anti-somatostatin-14 (Amersham) antibodies were diluted in PBS at 1:200 and 1:500, respectively. The secondary antibody was incubated with somatostatin antiserum or a mixture of the two antibodies overnight, at room temperature, and in presence of 0.25% Triton X-100 and 0.12% gelatin for double labeling experiments. After extensive washing with PBS, GAD and somatostatin were revealed with a donkey anti-goat coupled to tetramethyl isothiocyanate (TRITC; Nordic Immunological Labs) (1:200 in PBS), respectively, for 3 hr. The sections were finally mounted with Mowiol ( Hoechst, Frankfurt, Germany) and examined either with a Zeiss microscope equipped for fluorescence or a confocal microscope (Molecular Dynamics, Sunnyvale, CA) coupled to a graphic station. Briefly, 15 successive optical sections (focus step size, 0.5 μm) were obtained at appropriate settings of the argon laser wavelengths for excitation (488 and 514 nm) and long-pass filters (515 and 530 nm) for FITC and TRITC, respectively. Data from the same histological sample were collected consecutively at the identical depth for GAD and somatostatin, and were stored in a host computer. The background noise was reduced and the contrast was enhanced using a Gaussian (3 × 3 × 3) filter. Final images were reconstructed by superposing the two sets of digitized optical sections.

For peroxidase staining, slices were incubated overnight in the primary anti-somatostatin-14 polyclonal serum (1:500), for further assessment with an avidin–biotin–peroxidase complex (ABC) (Vector Laboratories, Burlingame, CA). For this purpose, the floating sections were dipped sequentially for 1 hr in secondary biotinylated antibody (donkey anti-rabbit, 1:200; Amersham) and (2) 2 hr in ABC (1: 100). The bound horseradish peroxidase (HRP) was visualized by using diaminobenzidine tetrahydrochloride (DAB) as the chromogen, by incubating preparation for 15 min in 0.03% DAB in 0.1 M Tris-HCl, pH 7.4, and then adding 0.03% H2O2 to the solution. The reaction was controlled visually and stopped by extensive washing with Tris-HCl. Sections were then dehydrated in graded alcohol, in order to enhance the contrast, and mounted on slide with Eukitt.

Postembedding technique. Vibratome sections (80 μm) were incubated in osmium tetroxide (2%, 20 min), dehydrated in graded ethanol and embedded flat in Araldite. In one experiment, osmium tetroxide treatment was omitted and the dehydrated preparation was reacted with 1% ethanol phosphotungstic acid (EPTA) for 2 hr at 60°C. Serial ultrathin sections were cut with a diamond knife and placed on Formvar-coated nickel grids and treated as follows: (1) 1% H2O2 in H2O (7 min); (2) somatostatin-14 (1:500) or GABA (1:150 to 1:250) or glutamate (1:5000) antiserum in 0.03 M Tris buffer (pH 7.4) containing 0.9% NaCl, 0.1% Triton X-100 overnight at room temperature; (3) 0.5% polyethylene glycol (10 min) in Tris NaCl buffer; (4) goat anti-rabbit antibody coupled to 15 nm (Amersham) or 5 nm (Biocell) gold particles or goat anti-mouse antibody coupled to 5 nm gold particles (Amersham) diluted 1:50 in the buffer used in the previous step for 2 hr; (5) 2% uranyl acetate (15 min). Some serial sections were processed only with 2% uranyl acetate (15 min) followed by 1% lead citrate (2–5 min) in order to better visualize ultrastructure. For colocalization studies of GABA and somatostatin, alternate sections were reacted with each antibody. The results were analyzed with STATGRAPHICS software. In the case of LMCEs, we arbitrarily counted gold particles inside the area included between the point where these terminals lose their myelinated sheath and the presynaptic membrane. In the histograms, results from three animals, processed with the same immunohistochemical procedure, were pooled.

Results

Distribution of somatostatin-containing inputs

The single staining experiments with somatostatin-14 antiserum showed that somatostatin-containing afferents are segregated to a restricted region of the M-cell (Fig. 1). No immunoreactivity for the peptide was detected on the soma, including the axon cap (Fig. 1A), a specialized neuropil that surrounds the axon initial segment. At this brainstem level, a few varicose fibers (not shown), with a discontinuous staining, were observed close to the small ventral dendrites that originate from the soma, but never in direct contact with them. Numerous positive boutons were observed beyond the bifurcation of the lateral dendrite, with both fluorescent (not shown) and peroxidase (Fig. 1B) staining. The latter technique, which allows a glutaraldehyde fixation, gave a more intense labeling and therefore better visualization of immunoreactive boutons. The proportion of stained profiles was already decreased at the level of the bifurcation of the dendrite.

Ultrastructural analysis of somatostatin-immunoreactive boutons

Postembedding immunogold labeling for somatostatin was used to determine the fine morphological features of stained terminals. This method, which is quite sensitive to the antibodies, indicated that there is a low amount of somatostatin in individual terminals, and in order to differentiate labeled endings from unlabeled ones, the density of gold particles was computed in all SVBs and LMCEs. The background noise in the M-cell cytosol facing the selected boutons was determined within the same method. The raw data are shown as frequency histograms for the M-cell cytosol (Fig. 2A), SVBs (Fig. 2B), and LMCEs (Fig. 2C). These skewed histograms (Fig. 2B, C) indicate that all SVBs and LMCEs do not contain the same amount of somatostatin immunoreactivity. To differentiate the stained processes from the unstained ones, a threshold of 2.21 gold particles/μm2 was set. It corresponds to the average density plus SD (1.31 ± 0.98 gold particles/μm2, n = 62) measured in the M-cell which is free from peptide. Profiles with a density above this limit were considered as labeled (Fig. 2D). The number of gold particles in the latter was 3.65 ± 1.89 (n = 103) in SVBs and 1.97 ± 0.84 (n = 45) in LMCEs after subtraction from density in the M-cell cytosol. The values in these endings are significantly different from the background noise (Student’s t test, p < 0.001). On the distal dendrite, 65% of the SVBs (103 positive for 159 analyzed) and 75% of the LMCEs (45 positive for 60 analyzed) contained somatostatin immunoreactivity. The labeled SVBs had common ultrastructural features; namely, they (1) establish sym-
Figure 1. Preferential location of somatostatin labeled boutons in specific regions of the M-cell synaptic bed. A, Micrograph obtained with Nomarski optics showing the lack of immunoreactivity inside the axon cap, on the soma (S), and on the small ventral dendrites (arrowheads) issuing from it. B, Positively stained boutons (arrows) and presumed club endings (arrowheads) distal to the bifurcation of the lateral dendrite (LD). Scale bars: A, 30 μm; B, 15 μm.

metrical synaptic contacts with the target cell (Fig. 3A, A); (2)
are filled with a pleiomorphic population of small vesicles, and
large and numerous mitochondria usually situated away from
the presynaptic membrane (Fig. 3A, B); and (3) also contain
DCVs, which are better visualized in semithin sections stained
with EPTA (Fig. 3C). Occasionally, accumulation of two or
three 15 nm gold particles was observed over DCVs (see also
Localization of somatostatin in nerve endings, below), but la-
beling was also always present over axoplasm and fewer particles
were found over some mitochondria (Fig. 3A). These DCVs
were not usually associated with the presynaptic active zone,
and were observed anywhere in the bouton.

Colocalization of GAD, GABA, and somatostatin
The occurrence of symmetrical synapses and pleiomorphic ves-
icules in somatostatin-immunoreactive SVBs suggested that SVBs
contain inhibitory neurotransmitters (ref. in Kom et al., 1989)
such as GABA. To test for this possible colocalization we con-
ducted double labeling experiments for GAD and somatostatin.
As shown in Figure 4, peptide immunoreactivity is present in
GAD-positive terminals (Fig. 4A, A). This colocalization is
maximal on the distal part of the lateral dendrite whereas at its
medial portion, only GAD staining was observed (not shown).
The weak labeling, especially for somatostatin, led us to study
regional distributions with confocal microscopy. Just proximal
to the dendritic bifurcation, the majority of the boutons were
only GAD immunoreactive and they were intermingled with a
few [somatostatin-GAD]-positive terminals (Fig. 4B, B). At the
most distal part of the dendrite we could distinguish three cat-
egories of afferents: [somatostatin-GAD], and [GAD] or [so-
matostatin] alone (Fig. 4C, C). The latter impinged exclusively
on the extremity of the dendrite (Fig. 4C). All stained terminals
had a mean diameter of 1.97 ± 0.43 μm (n = 22), in agreement
with previous electron microscopic data for the size of SVBs
(Nakajima, 1974). Electron microscopic studies with GABA and
somatostatin antisera on serial ultrathin sections confirmed the
existence of these three populations of SVBs (Fig. 5). The [GABA-
somatostatin] population corresponds to SVBs that are enriched
in DCVs (mean diameter ± SD = 109 ± 14 nm; n = 40)
termingled with pleiomorphic small vesicles (Fig. 5A, A), and
carries symmetrical synapses (Fig. 5A, B). In this area, only few
boutons had somatostatin immunoreactivity alone (Fig. 5B, B); they contained a mixed complement of small vesicles and of
fewer DCVs. Some GABA-positive SVBs that did not contain
somatostatin (Fig. 5C, C) displayed the same basic ultrastra-
tural characteristics, including DCVs (104 ± 13 nm; n = 43),
suggestive of another peptide.

Coexistence of glutamate and somatostatin
An unexpected finding with postembedding staining with the
anti-somatostatin antiserum was the appearance of this peptide
in the LMCEs (Fig. 6A). The ultrastructure of LMCEs was an-
alyzed in serial ultrathin sections (Fig. 6B). In addition to gap
junctions spanning the membrane apposed to the dendrite and
attachment plate, there were asymmetrical synapses close to
terminal borders with pre- and postsynaptic differentiation of
the same length. The DCVs (105 ± 18 nm; n = 30) were usually
observed associated with numerous round, small vesicles. Dou-
ble staining experiments were conducted with anti-glutamate
monoclonal and anti-somatostatin polyclonal antibodies in or-
der to identify the excitatory neurotransmitter and its coexis-
tence with somatostatin. This colocalization was observed in
many LMCEs (Fig. 6A, C) that also displayed, as expected, DCVs
(Fig. 6A) and glutamate immunoreactivity, a high density of
gold particles being observed over mitochondria (Fig. 6C) and
over small round vesicles. A quantitative analysis of the density
of gold particles associated with glutamate immunoreactivity
was conducted to ascertain that the concentration of glutamate
was higher in these endings than in other terminals, particularly
those containing GABA. The density measurements were per-
formed as mentioned above for somatostatin. All afferent bou-
tons (i.e., SVBs and LMCEs) impinging on the distal portion of
the lateral dendrite were measured, since glutamate is used as
a precursor of GABA or for metabolic purposes. As shown in
Figure 7, there was indeed a high density of gold particles in LMCEs (46.35 ± 13.11 gold particles/μm²; n = 19). Further, two populations of SVBs were distinguished on the basis of this criterion, namely, SVB1 and SVB2 with 13.26 ± 4.27 gold particles/μm² (n = 23) and 30.17 ± 5.76 gold particles/μm² (n = 27), respectively. These values were obtained after correcting for the M-cell cytosol density (9.86 ± 3.09; n = 15) and they are significantly different from each other (Student’s t test; p < 0.001).

The detection of somatostatin in LMCEs with electron microscopy contrasts with the fact that confocal microscopy of immunofluorescent preparations did not provide evidence for the presence of this molecule in their parent axons. This discrepancy could be due to (1) the lower concentration of somatostatin in LMCEs as compared to SVBs, (2) the difficulty at the level of terminal endings to recognize them among other boutons with fluorescence, and finally (3) the use of paraformaldehyde for fluorescence. This aldehyde is not necessarily the best fixative for revealing binding sites of an antibody prepared against a glutaraldehyde coupled antigen (see Materials and Methods). Thus, we used an HRP method with glutaraldehyde fixation for a better correlation of light and electron microscopic observations. Under these conditions, a few LMCEs were somatostatin positive (Fig. 8A, A). At the extremity of the lateral dendrite, staining was seen in both en face (Fig. 8A, A) and transversal sections (Fig. 8A, B) of LMCEs surrounded by numerous negative profiles. Some large-diameter (5–16 μm) labeled fibers (Fig. 8B, C) were also found in the vestibular nerve in agreement with the evenly distributed immunogold staining over club endings.

Localization of somatostatin in nerve endings

As shown above, postembedding immunocytochemistry with 15 nm gold particles showed somatostatin immunoreactivity in different SVBs and LMCEs axoplasm compartments (Figs. 3, 5, 6) and sometimes over DCVs, which are considered to be the site of the storage of peptides. However, since such large gold particles (15 nm) can reduce the accurate localization of the antigen, we performed another set of experiments, where the peptide was visualized with smaller (5 nm) gold particles (Fig. 9). With this marker, a preferential accumulation of gold particles over DCVs was generally observed in positive SVBs (Fig. 9A, D) and LMCEs (Fig. 9B, C) and was more conspicuous, given the larger number of (1) included particles, and (2) labeled DCVs. This result agrees with previous reports that peptides may be stored in DCVs (Takagi et al., 1983; De Biasi and Rustioni, 1988; Hokfelt, 1991). These stained terminals also displayed DCVs with fewer (Fig. 9C) or no (Fig. 9A) gold particles. Yet, as already noticed with 15 nm particles, we also observed the presence of 5 nm particles over vesicular compartments (Fig. 9D) and some mitochondria (Fig. 9B).

Somatostatin immunoreactivity in the vestibular nucleus

The observation of positive fibers in the eighth nerve, and the fact that the lateral dendrite of the M-cell lies in the vestibular nucleus, led us to analyze the distribution of somatostatin im-
Figure 3. Ultrastructural features of somatostatin-positive boutons revealed with gold particles on the distal part of the M-cell lateral dendrite. A1 and A2. Ultrathin sections of the same ending showing an SVB (A1) stained for somatostatin (densities: 5.41 gold particles/μm² for SVB; 0.88 gold particle/μm² in the M-cell cytosol facing the terminal) and the lack of gold particles in the absence of the first antibody (A2). This SVB had DCVs (arrowheads) and symmetrical synapses (arrows). Note its location between two LMCEs (CE1, CE2). B. Two labeled terminals (T1, 4.7 gold particles/μm²; T2, 5.64 gold particles/μm²) with fewer DCVs (arrowheads) located near a negative bouton (asterisk; 1.5 gold particles/μm²). Here, the background noise estimated over a 19 μm² surface area is equivalent to 1.4 gold particles/μm². C. Semithin (0.5 μm) EPTA-treated section with numerous DCVs (arrowheads) and active zones (arrows) inside a somatostatin-positive terminal (9.54 gold particles/μm² for SVB; 1.37 gold particles/μm² in the M-cell cytosol). Scale bars: A1, A2, B, 1 μm; C 0.5 μm.

Figure 4. Relative distribution of GAD (left column) and somatostatin (right column) in double-labeled afferent inputs. A1 and A2. Presence of numerous GAD-positive boutons at the extremity of the dendrite (arrowheads); some are also stained for somatostatin (arrows); the pictures were obtained at the same focus. B1–C1. Confocal microscopic images of other preparations demonstrating that reactive profiles are positive for GAD, GAD-somatostatin, and somatostatin. Proximal to the bifurcation of the dendrite (B1, B2), an abundance of GAD-positive boutons (arrowheads) is in contrast with few double-stained terminals (arrows). Beyond the bifurcation (C1, C2), there are GAD-positive (arrowheads), and GAD-somatostatin–positive (arrowheads) profiles with a progressively increasing number of somatostatin-positive (crossed arrows) boutons. A1–C1 and A2–C2, TRITC and FITC filters for GAD and somatostatin, respectively. Scale bars: A1 and A2, 10 μm; B1–C2, 20 μm.
munocoreactivity in this region. Immunoreactive profiles were observed in apposition to vestibular neurons located close to the tip of the M-cell lateral dendrite (not shown). However, numerous vestibular neurons did not receive detectable somatostatin input.

Double labeling experiments also revealed the presence of numerous GAD-positive boutons, in contact with vestibular neurons as well as double stained [somatostatin-GAD] profiles on a few of them (not shown). As mentioned above, the peroxidase reaction is more sensitive and it showed some somatostatin-positive vestibular neurons (not shown) lying close to unstained ones. In these cells, the somatostatin immunoreactivity was present in the whole cytoplasm without clear compartmentalization.

**Discussion**

In this study, we have found, in addition to the already reported (Somogyi et al., 1984; Jones and Hendry, 1986; Sloviter and Nilaver, 1987) coexistence of somatostatin with GABA, the presence of this peptide in glutamatergic excitatory endings. Several subtypes of afferents have been identified with respect to this and other transmitters, particularly at the extremity of the M-cell lateral dendrite, as summarized in Figure 10.

**Specificity of the somatostatin-14 antiserum**

Several lines of evidence support the notion that the somatostatin antiserum is as specific in fish as in other vertebrates. The pattern of staining, which includes neuronal cell bodies and varicose and thick fibers, corresponds to similar descriptions in rat (Finley et al., 1981; Takagi et al., 1983; Johansson et al., 1984; Sloviter and Nilaver, 1987), cat (Somogyi et al., 1984), and lamprey (Buchanan et al., 1987; Christenson et al., 1991). Furthermore, the restriction of the staining to the periphery of the brainstem, the reticular formation, its lack in the cerebellum (not shown) and in the vestibular nucleus have been also reported in comparable areas of the rat using both immunocytochemistry (Finley et al., 1981; Takatsuki et al., 1981; Johansson et al., 1984) and biochemical measurements (Douglas and Palkovits, 1982). Furthermore, for a given structure that was labeled, others of the same type were negative, even though they had the same morphological features. Accordingly, gold particle densities in different profiles indicate that only a fraction of the SVBs (65%) and LMCEs (75%) are enriched in somatostatin immunoreactivity, in comparison with the M-cell background noise and with data from the tissue-free resin (1.1 ± 0.9 gold particles/μm²; n = 23). Both somatostatin-positive SVBs and LMCEs contained DCVs (105-109 nm) that exhibited 20 ± 7 gold particles/μm²; somatostatin, 8.95 gold particles/μm²; n = 23). Both somatostatin-positive SVBs and LMCEs contained DCVs (105-109 nm) that exhibited accumulation of small gold particles. This is in agreement with reports that in the rat hypothalamic periventricular nucleus (Horvath et al., 1989) and neostriatum (Takagi et al., 1983) somatostatin immunoreactivity is present in large (100-160 nm) neurosecretory granules and in large DCVs (128 nm), respectively. The observation of unstained DCVs in these positive terminals can be related to the presence of another peptide such as neuropeptide Y, which is known to coexist with somatostatin (Jones and Hendry, 1986), but may not share the same DCVs. Alternatively, this could be due to the postembedding staining used in this study, where only one side of the section was available for the reaction. On the other hand, in agreement with an earlier report of Takagi et al. (1983), we also found somatostatin immunoreactivity in axoplasm and associated with some mitochondria and with vesicular compartments in SVBs and LMCEs.

**Segregation of somatostatin-positive boutons on the M-cell surface**

Somatostatin was found to be present on the distal portion of the lateral dendrite in SVBs and LMCEs. The latter type of excitatory endings are only present in this area (Korn et al., 1989). Concerning the inhibitory SVBs, the restricted and stereotyped distribution of somatostatin terminals on the distal part of the lateral dendrite of the M-cell contrasts with the broader distribution of GABA and glycine. Although GABA-containing afferents are more numerous at the extremity of the lateral dendrite, they were found everywhere except in the axon cap (Petrov et al., 1991). Indeed, iontophoretic applications of GABA produce inhibitory responses in the M-cell (Faber and Korn, 1980) although glycinergic transmission is more widely distributed at the M-cell surface (ref. in Korn et al., 1989). The distribution of the somatostatin-containing fibers on the M-cell is similar to that in rat cortex, where they synapse with distal dendrites of pyramidal neurons (Takagi et al., 1983, Jones and Hendry, 1986; Sloviter and Nilaver, 1987), and in lamprey spinal cord, where the largest responses to somatostatin can be recorded on the dendritic shaft of the stretch receptor neuron (Christenson et al., 1991). This phylogenetic consistency suggests that somatostatin intervenes in the regulation of the dendritic properties. At the ultrastructural level, the majority of somatostatin-positive boutons contacting the M-cell correspond to SVBs (Nakajima, 1974).

The somatostatin-immunoreactive SVBs have structural features similar to terminals described by Tuttle et al. (1987), who recognized three types of SVBs, and more specifically, to their type A. Their small vesicles are more oval and round, and they contain more DCVs, mitochondria, and glycogen particles than type C. In the latter type, synaptic vesicles vary from flattened to round, whereas in SVB type B, most of them are flat.

**Colocalization of GABA and somatostatin**

The extremity of the M-cell lateral dendrite is innervated by GABAAergic fibers (Petrov et al., 1991). Double labeling with GAD and somatostatin antisera revealed the coexistence of both markers in terminals distributed mainly beyond the bifurcation. 

*Figure 5* Ultrastructural characteristics of the three populations of biochemically different SVBs revealed by postembedding staining for GABA (left) and somatostatin (right). A, and A,, Coexistence of GABA (dilution, 1:250) and somatostatin immunoreactivity (GABA, 12.63 gold particles/μm²; somatostatin, 8.95 gold particles/μm²) in the same bouton near a double-unstained terminal (asterisks). Note the widespread distribution of DCVs (arrowheads) and the presence of a synaptic contact (arrow) in the stained bouton. B, and B,, Example of a terminal positive for somatostatin (6.45 gold particles/μm²) but not for GABA (0.53 gold particle/μm²). This bouton bearing active zones (arrow) does not display any DCVs, in contrast to the nearby double-stained ending (asterisks; GABA, 9.20 gold particles/μm²; somatostatin, 6.54 gold particles/μm²). C, and C,, A GABA- (dilution, 1:130) labeled bouton (45.43 gold particles/μm²) that contained DCVs (arrowheads) and was not immunoreactive for somatostatin (0.00 gold particles/μm²). In all these pictures somatostatin background levels in the M-cell cytosol ranged from 1.3 to 2.1 gold particles/μm² as measured from surfaces larger than 9 μm². Scale bars, 0.5 μm.
of the dendrite. Ultrastructural analysis of GABA and somatostatin with immunogold postembedding techniques revealed at least three populations of SVBs, a result that was also suspected by confocal microscopy analysis. The double-labeled SVBs, which represent one of these subpopulations, display numerous DCVs and mitochondria, therefore fulfilling some SVB type A features (see above). Since boutons containing GABA alone had the same characteristics, including DCVs, these organelles are not necessarily associated with somatostatin and they could be associated with other neuropeptides such as cholecystokinin, vasoactive intestinal peptide, or substance P as in the rat neocortex (reviewed in Jones and Hendry, 1980). The assumption that both [GABA-somatostatin] and [GABA] SVBs are of type A implies that their axons are unmyelinated and therefore probably originate from local interneurons (Tuttle et al., 1987). This notion is strengthened by observations in rat neostriatum, where all somatostatin-positive axons of medium-size aspiny neurons lack myelin (Takagi et al., 1983). The third, [somatostatin], population of SVBs has few DCVs and mitochondria, like SVBs of the C type; however, their scarcity did not allow for an accurate classification. This observation raises the problem of whether another fast-acting neurotransmitter is colocalized with somatostatin. Glycine would be a possible candidate since SVBs are generally inhibitory (ref. in Korn et al., 1989) and glycine receptors are present in this part of the cell (Seitanidou et al., 1988; Triller et al., 1990). In addition, somatostatin is associated with a high-affinity glycine uptake in the Xenopus retina (Smiley and Basinger, 1988).

Coexistence of glutamate and somatostatin
Glutamate immunoreactivity was found in LMCEs and in two groups of SVBs (1 and 2). The latter, which contain a pleomorphic population of vesicles, are known to be inhibitory. The SVB1 are probably glycinergic boutons since the density of gold particles mainly found on mitochondria was only slightly above the background level. The SVB2 had a higher content in glutamate and DCVs were present in 85% of them. Hence, they correspond to GABAergic SVB type A (see above). The presence of glutamate in these endings accounts for its role as a substrate of GAD, the synthetic enzyme for GABA. The density of gold particles was the highest in LMCEs. These endings transit both electrically and chemically (Furshpan, 1964; Korn and Faber, 1976; Lin and Faber, 1988) in agreement with electron microscopic (Robertson et al., 1963; Nakajima, 1974) and freeze-fracture (Tuttle et al., 1986) data. Previous iontophoretic (Diamond and Roper, 1973) and pharmacological (Wolzson and Faber, 1988) studies suggest that the chemical component of LMCEs is mediated by glutamate. This notion is strengthened by the demonstration of an NMDA-dependent LTP (Yang et al., 1990) and is confirmed here with immunocytochemistry. However, the presence of another excitatory amino acid such as aspartate can not be excluded. Indeed, the demonstration by Tracey et al. (1991) of the coexistence of both glutamate and aspartate in primary afferent neurons raises the possibility that these two neurotransmitters could be released simultaneously to activate NMDA receptors.

Although, somatostatin is commonly colocalized with inhibitory neurotransmitters, we have also found it in primary excitatory afferents. Coexistence of substance P, in primary glutamatergic fibers, was reported in the superficial laminae of rat spinal cord (De Biasi and Rustioni, 1988) and has been suggested for the adrenocorticotropic hormone in the pigeon vestibular ganglion (Güntürkün and Deviche, 1992). In addition, the presence of DCVs in the LMCEs, away from the presynaptic grid, suggests that somatostatin could be released independently from glutamate at nonspecialized areas as suggested for another system (Zhu et al., 1986), and given this disposition, it could act on adjacent synapses as shown for glycine (Faber and Korn, 1988).

Functional implication
The coexistence of the same peptide in both excitatory and inhibitory endings raises the question of its function(s). In any case, the presence of somatostatinergic neurons in the vestibular nucleus, adjacent to the lateral dendrite, supports the hypothesis that these second-order interneurons synapse on the distal half of the M-cell lateral dendrite and that they probably are inhibitory. On the distal part of the M-cell lateral dendrite, somato-

Figure 7. Glutamate immunoreactivity in different terminal profiles contacting the distal part of the lateral dendrite. The values (mean number of gold particles/μm² ± SEM) are above that in the M-cell cytosol (dashed line; 9.86 ± 0.9; n = 15). Note that the density of the label is the highest in LMCEs (56.21 ± 3.00; n = 19). Two populations of SVBs are distinct with respect to glutamate content: SVB1 (22.48 ± 1.04; n = 24) and SVB2 (40.03 ± 1.10; n = 27). Double asterisks indicate that values are significantly different when tested two by two (p < 0.001, Student’s t test).
Somatostatin released by these cells could act postsynaptically to complement GABA action by hyperpolarizing the M-cell through activation of a K+ conductance as described in lamprey (Christenson et al., 1991). A presynaptic effect of somatostatin, which reduces GABA release, could also occur, as reported in the rabbit cerebral cortex at concentrations below those that elicit postsynaptic effects (Scharfman and Schwartzkroin, 1989). Such a disinhibition by somatostatin could also be produced by its release at the edges of excitatory afferents, thereby reinforcing excitation. This sequence could contribute to the induction of LTP on the lateral dendrite, by high-frequency stimulation that is known to increase somatostatin release (Bonanno et al., 1988) in other structures. Somatostatin could also potentiate electrotone coupling via gap junctions by inhibiting the entry of calcium through voltage-dependent Ca2+ channels (Wang et al., 1990). Facilitatory effects of somatostatin in the production of LTP have been implicated in guinea pig hippocampus where this peptide significantly enhances potentiation in cysteamine (a somatostatin depletor) treated animals (Matsuoka et al., 1991).

In addition, a recent report of Wang et al. (1993) demonstrates that somatostatin specifically enhances glutamate responses mediated by NMDA receptors.

In conclusion, we have shown that somatostatin was colocalized with both glutamate and GABA neurotransmitters, suggesting a dual role for this peptide in the modulation of synaptic activity.

Figure 8. Eighth nerve inputs immunostained with somatostatin antiserum and their relationships with the distal lateral dendrite of the M-cell (DLD). A, and A', Micrographs obtained from the same section, at different levels of focus with Nomarski optics. Tangential view (A', arrow) and transverse sections (A', arrows) of immunoreactive club endings close to unstained profiles (arrowheads). B and C, Somatostatin-containing afferents (arrows) within the eighth nerve (VIII N) and intermingled with negative profiles (arrowheads). The dashed line indicates the edge of the brainstem. Fixation with 4% paraformaldehyde for fluorescence (B) and 2% paraformaldehyde, 2% glutaraldehyde for peroxidase reactions (A, C). Scale bars, 25 μm.

Figure 9. Localization of somatostatin in presynaptic endings synapsing on the lateral dendrite. A, A small vesicles bouton exhibiting preferential accumulation of somatostatin immunoreactivity (5 nm gold particles) over (solid arrowheads) DCVs. Note the presence of unstained DCVs (open arrowheads) and immunogold labeling over other axoplastic compartments exemplified by arrows. B, General view of an LMCE displaying DCVs filled with (solid arrowheads) or devoid of (open arrowhead) somatostatin. Note the presence of sparse gold particles in the axoplasm and over (arrow) a mitochondrion (Mi). C, Higher magnification of region I of the LMCE shown in B. The labeled DCVs display gold particles associated with their rim (arrowheads). D, Another example from an SVB showing the preferential accumulation of gold particles on the rim of DCV (arrowhead). MC, Mauthner cell; A, Attachment plate; *, chemical excitatory synapse. Scale bars: A, 0.2 μm; B, 0.5 μm; C and D, 0.1 μm.
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Figure 10. Summary diagram showing the relative distribution of excitatory glutamatergic (GLU) LMCEs and inhibitory GABAAergic (GABA) SVBs containing somatostatin (SOM) on distal parts of the lateral dendrite (LD). At this level (see box on the M-cell drawing), the relatively few SVBs containing only somatostatin also contain a second transmitter that, as with "empty profiles" elsewhere, may be glycine (see Colocalization of GAGA and somatostatin). Toward the medial part of the dendrite, there are only GABAergic and glycergic terminals intermingled with large vesicle boutons (LVBS) and small myelinated club endings (SMCEs). AC, S, VD, and VIII N, axon cap, soma, ventral dendrite, and eighth nerve, respectively.
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