Distribution and Characterization of Different Molecular Products of Pro-somatostatin in the Hypothalamus and Posterior Pituitary Lobe of the Mongolian Gerbil (*Meriones unguiculatus*)

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Antisera raised against various synthetic peptide fragments of the pro-somatostatin molecule were used to visualize immunohistochemically the distributions of different pro-somatostatin fragments in the hypothalamus and posterior pituitary of the Mongolian gerbil. To define the nature of the immunoreactive somatostatin-related molecular forms, gel chromatography combined with radioimmunoassays of hypothalamic and posterior pituitary extracts was performed. Within the hypothalamus, only trace amounts of somatostatin-28 and somatostatin-28(1-12) were present, whereas pro-somatostatin(1-76), pro-somatostatin(1-64), and somatostatin-14 peptides were present in equimolar amounts. In contrast, the posterior pituitary lobe contained equal amounts of somatostatin-14, somatostatin-28, and somatostatin-28(1-12) but no pro-somatostatin(1-76), indicating that pro-somatostatin is further processed during the axonal flow to posterior pituitary nerve terminals. The gel chromatographic data were further substantiated by immunohistochemical data. Thus, perikarya containing all of these five immunoreactivities were strictly confined to the periventricular area and parvocellular subset of the paraventricular nucleus. However, the number of somatostatin-28- and somatostatin-28(1-12)-immunoreactive perikarya was approximately 20% of the number of somatostatin-14- and pro-somatostatin(1-64)-immunoreactive cells. In other hypothalamic areas only somatostatin-14 and pro-somatostatin(1-64) immunoreactivities were detectable in cell bodies. These cell bodies were encountered in the organum vasculosum laminae terminalis; the suprachiasmatic, ventromedial, arcuate, perifornical, and posterior hypothalamic nuclei; and the median preoptic and retrochiasmatic areas. *In situ* hybridization histochemistry revealed that the cellular distribution of pro-somatostatin mRNA corresponds to that of somatostatin-14 and pro-somatostatin immunoreactivity, suggesting that the immunoreactive material observed within the cell bodies is synthesized there and that the differences in density of immunoreactivities may be explained by

intracellular processing of pro-somatostatin. Somatostatinergic nerve fibers and terminals in hypothalamic areas and the posterior pituitary lobe were immunoreactive to all of the employed antisera.

From the present results, obvious differences between intrahypothalamic and hypothalamo-pituitary somatostatinergic neurons emerge. Within hypothalamic neurons not projecting to the median eminence and the posterior pituitary lobe, pro-somatostatin is posttranslationally processed in the cell body predominantly into pro-somatostatin(1-64) and somatostatin-14. Otherwise, within periventricular neurons projecting to the median eminence and the posterior pituitary lobe, pro-somatostatin is posttranslationally processed during the axonal flow into pro-somatostatin(1-64), somatostatin-14, somatostatin-28, and somatostatin-28(1-12).

Originally, the tetradecapeptide somatostatin-14 (SS-14) was isolated from the porcine hypothalamus and shown to inhibit the release of growth hormone from the anterior pituitary lobe (Brazeau et al., 1973). Later, cDNAs coding for human and rat prepro-SS have been cloned and the peptide sequences deduced (Shen et al., 1982; Goodman et al., 1983). Gel filtration and sequence analysis of gastrointestinal and hypothalamic extracts from several species have revealed the following putative post-translational processing products of pro-SS: an N-terminal pro-somatostatin(1-64) [pro-SS(1-64)] molecule and a peptide consisting of the remaining 28 C-terminal amino acids of pro-SS, somatostatin-28 (SS-28) (Pradayrol et al., 1980; Schally et al., 1980; Goodman et al., 1983; Holst et al., 1988; Bersani et al., 1989). SS-28 contains a typical prohormone cleavage site and may be further processed into the C-terminal SS-14 and the N-terminal SS-28(1-12), both widely distributed throughout the brain (Benoit et al., 1982, 1984). The role of SS-14 and SS-28 as neurotransmitters/neurohormones is well established (Meyers et al., 1980; Lumpkin et al., 1981; Reichlin, 1983; Enjalbert et al., 1986; Brown et al., 1988; Meyer et al., 1989). Less is known about the physiological functions of SS-28(1-12), and some authors regard it as a fragment of no physiological importance (Vécsei and Widerlöv, 1990). In brain areas, the relative content of SS-14, SS-28, and SS-28(1-12) varies (Benoit et al., 1982, 1984; Gomez et al., 1983). Although prohormones have been considered to be less biologically active than their final products, SS-28 is, with regard to some peripheral actions, more potent than SS-14 (e.g., the inhibition of growth hormone release from the anterior pituitary lobe) (Tannenbaum et al., 1982). This is supported by the observation that SS-28 exhibits higher affinity to SS-14 receptors in the pituitary than SS-14,

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Table 1. Schematic presentation of the peptide fragments of the pro-SS molecule recognized by the antisera employed in this study

Fragment	Antisera			
	1654	1758	2098	4576
pro-SS(1-64)	—	—	+	—
pro-SS(1-76)	—	—	+	+
SS-28(1-12)	—	—	—	+
SS-14	—	+	—	—
SS-28	+	+	—	—

Positive response is depicted with a plus sign, while negative is depicted by a minus sign.

whereas in the brain, SS-28 exhibits a lower affinity than SS-14 for the receptor (Srikant and Patel, 1981; Leroux et al., 1985; Reubi and Maurer, 1985). In the gastrointestinal tract, the pro-SS(1-64) peptide has been shown to be a major product of processing of pro-SS (Holst et al., 1988; Bersani et al., 1989). Recently, a 7 kDa peptide fragment of the N-terminal part of the pro-SS molecule has been shown to be present in high amounts in the rat nervous system (Rabbani and Patel, 1990), and this peptide is suggested to be identical to pro-SS(1-63). However, the functional significance of pro-SS(1-64) or pro-SS(1-63) is at present unknown.

In the hypothalamus, somatostatinergic neurons are mainly localized in the periventricular cell group extending from the preoptic area to the median eminence (Hökfelt et al., 1975; Krisch, 1978; Dierickx and Vandesande, 1979). Lesion studies have shown that these cells are the main origin of the somatostatinergic nerve terminals in the perivascular spaces surrounding the portal vessels of the median eminence (Ibata et al., 1983; Beal et al., 1985). This projection is likely to influence the release of growth hormone from pituitary somatotrophs via a release of SS-14, SS-28, and SS-28(1-12) to the portal blood (Millar et al., 1983; Sheward et al., 1984; Pierotti et al., 1985). Apart from the periventricular area/median eminence-projecting neurons, somatostatinergic cell bodies and nerve fibers are present in several other hypothalamic areas as well as the posterior pituitary lobe (Hökfelt et al., 1975; Patel and Reichlin, 1978; Dierickx and Vandesande, 1979; Hoffman and Hayes, 1979). Thus, in addition to the somatostatinergic neuroendocrine system in the hypothalamus, a population of somatostatinergic neurons, which may also involve hypothalamic neurons, give rise to intracerebral projections. It is tempting to speculate that the pro-SS molecule undergoes differential post-translational processing dependent on whether the final product serves as a hypophysiotropic factor or an intracerebral transmitter substance.

The present study was undertaken to shed light on the anatomical distribution of pro-SS-derived peptide fragments in the hypothalamus and to identify the posttranslational processing products of pro-SS in different hypothalamic areas. By employing a wide variety of antisera recognizing different regions of the pro-SS molecule [pro-SS(20-36), SS-14, SS-28, and SS-28(1-12)], we have combined immunohistochemical visualization of

Figure 1. Diagrammatic representation of the pro-SS molecule. Binding regions of the various antisera employed in the present study are indicated. Antiserum 4576 recognizes the C-terminus of pro-SS(1-76) and SS-28(1-12) only when SS-14 is split off from pro-SS. Antiserum 1654 recognizes the full sequence of SS-28, but neither SS-14 nor SS-28(1-12).

neuronal elements with the gel chromatographic characterization of the immunoreactivities in hypothalamic extracts of the gerbil. This approach makes it possible to reveal the actual peptides present within cell bodies and their fate during axonal transport to nerve terminals (Lewis et al., 1986). To investigate that transcription of the prepro-SS gene is in fact further translated through peptide synthesis, we used *in situ* hybridization histochemistry to localize pro-SS mRNA in the hypothalamus. Furthermore, by comparing the data obtained by *in situ* hybridization histochemistry with the immunohistochemical data, it was possible to compare the regional synthetic activity with the content of the corresponding peptide.

Materials and Methods

Antisera. The antisera used in the present study were raised in rabbits as described in Holst and Åsted (1974). Antisera were raised against the following synthetic pro-SS fragments: 20-36 fragment of pro-SS (code 2098), carboxy-terminal part of SS-28(1-12) (code 4576), SS-28 (code 1654), and SS-14 (code 1758). The epitopes recognized by the various antisera are depicted in Figure 1 and Table 1. The antisera have previously been characterized in detail: The 2098 antiserum recognizes the 20-36 amino acid sequence of the pro-SS molecule and thus pro-SS-like immunoreactivity (pro-SS-LI) could represent the 20-36 fragment alone or extended versions thereof (Bersani et al., 1989). The 4576 antiserum recognizes the carboxy-terminal part of the SS-28(1-12) molecule but not C-terminally extended forms, and thus SS-28(1-12)-LI could represent SS-28(1-12) as well as amino-terminally extended forms of this part (Skak-Nielsen et al., 1987). Since the 1654 antiserum recognizes a part of the amino acid sequence linking the SS-28(1-12) and SS-14, it does not react with either SS-28(1-12) or SS-14 separately after these peptide fragments have been formed from the cleavage of SS-28 (Holst et al., 1988). Finally, the 1758 antiserum recognizes the carboxy-terminal part of the SS-14 molecule, and thus SS-14-LI could represent SS-14 as well as SS-28 (Baldissera et al., 1983). For immunohistochemistry, the antisera were diluted in potassium phosphate-buffered saline (KPBS; pH 7.4) containing 0.3% Triton X-100 (TX-100) and 1.0% BSA (KPBS-BT). The dilutions were as follows: codes 1758 and 2098, 1:1000; codes 4576 and 1654, 1:400.

Immunohistochemistry. Fifteen adult male Mongolian gerbils weighing 50–70 gm were anesthetized with tribromomethanol (250 mg/kg body weight, i.p.) and perfused via the vascular system with 0.05 M potassium phosphate-buffered saline (KPBS; pH, 7.4) containing heparin (15,000 IU/liter) for 3 min, followed by perfusion with 4% paraformaldehyde in 0.1 M phosphate buffer (pH, 7.4) for 15 min. The brains

Figure 2. Camera lucida drawings of coronal sections through the gerbil preoptic area and hypothalamus illustrating the distribution of immunoreactive nerve fibers (*left*) and cell bodies (*right*). The *left* column indicates the staining pattern seen after immunohistochemical visualization with antisera 1758 and 2098, whereas the *right* column indicates the distribution of immunoreactive elements after the use of antisera 1654 and 4576. Black dots indicate stained cell bodies, and short thin lines indicate labeled nerve fibers and terminals. For abbreviations, see Appendix.

1758 & 2098

a

1654 & 4576

b

c

d

Figure 3. Low-power photomicrograph showing the periventricular area of third ventricle at the level of the suprachiasmatic nucleus (SCN). A high number of immunoreactive cell bodies visualized with antiserum 2098 is seen in the dorsal two-thirds of the periventricular area. A few immunoreactive cell bodies are seen in the dorsomedial part of the suprachiasmatic nucleus (arrows). For other abbreviations, see Appendix. Scale bar, 200 μ m.

were cryoprotected in a 30% sucrose solution in KPBS (pH 7.4) for 2 d before either serial sagittal sections (20 μ m) or serial frontal sections (40 μ m) were cut on a cryostat. The sections were rinsed for 2 $\times$ 10 min in KPBS and then incubated at 4°C for 24 hr in primary antiserum. After this incubation, the sections were washed for 3 $\times$ 10 min in KPBS (pH 7.4) plus 0.1% TX-100 and 0.3% BSA (KPBS-BT) followed by incubation with biotinylated swine anti-rabbit IgG (E353, Dakopatts, Copenhagen, Denmark) diluted 1:200 in KPBS-BT at room temperature for 60 min. The sections were then washed for 3 $\times$ 10 min in KPBS-BT and finally incubated at room temperature for 60 min in avidin-biotin-conjugated horseradish peroxidase (K377, Dakopatts) diluted 1:1000 in KPBS-BT. After a wash for 10 min in KPBS-BT and for 2 $\times$ 10 min in 0.05 M Tris-HCl buffer (pH 7.6), the sections were reacted for peroxidatic activity by incubation in the latter buffer containing 0.025% 3,3'-diaminobenzidine (DAB) and 0.003% H_2O_2 for 20 min. After washing for 2 $\times$ 10 min in distilled water, sections were mounted on gelatin-subbed slides, dehydrated in a series of ethanol, and coverslipped. For topographical studies a similar series of sections was counterstained in thionine.

Immunohistochemical controls. In order to elucidate possible cross-reactivity with various antigens including the synthetic peptide the antiserum was raised against, antisera were preabsorbed for 24 hr with 2 μ M of each of the following four peptides: SS-14, SS-28, SS-28(1-12), and pro-SS(20-36).

In situ hybridization. The animals were fixed and cryoprotected as above. Brain sections (20 μ m) were cut in the frontal plane and placed on glass slides prerinsed in a 2% solution of RNase-removing agent AbSolve® (DuPont, Wilmington, DE). The sections were delipidated in a series of graded alcohol and rehydrated in diethylpyrocabonate (DEPC)-containing sterile water (DEPC- H_2O). The sections were then incubated in 1 mg/ml Proteinase K (Sigma) in 0.01 M Tris-HCl buffer (pH 7.4, with 0.05 mM $CaCl_2$) for 15 min at 37°C and rinsed for 30 min in 2 $\times$ SSC (300 mM sodium chloride, 30 mM sodium citrate, pH 7.0). The sections were thereafter preincubated in the hybridization buffer [1.0 gm dextran sulfate; 351 mg NaCl; 50 ml 2 M Tris-HCl, pH 7.4; 2 ml 50 $\times$ Denhardt's solution; 20 ml 0.5 M EDTA; 5 mg yeast tRNA (Sigma); 5 mg salmon testes DNA (Sigma); 5 ml deionized formamide, and 2.93 ml DEPC- H_2O] for 1 hr at 27°C. The hybridization was performed by adding the ^{35}S -labeled pro-somatostatin oligonucleotide probe (somatostatin antisense probe; DuPont, code NEP-203) to the hybridization buffer to a concentration of 0.67 pM (1.3×10^6 cpm/section) at 27°C and incubated overnight. After hybridization, the sections were rinsed for 30 min in 1 $\times$ SSC at 27°C followed by a rinse in 1 $\times$ SSC at room temperature. The slides were dried under stream of cold air and covered by Ilford K2 photographic emulsion diluted with distilled water 1:1 and placed in light-safe boxes for exposure at 4°C. After 7–14 d of exposure, the autoradiographs were developed for 3 min at 20°C in Kodak D-19, fixed in 30% thiosulfate, counterstained with cresyl violet, dehydrated in a series of alcohol, and coverslipped with Depex®. A separate set of sections was treated with RNase (1 μ g/ml, 1 hr at 37°C) before hybridization. A further control was carried out by omitting the oligonucleotide probe during hybridization, although all other conditions were maintained constant.

Cell counting. To quantify the immunohistochemically labeled sections and the labeled emulsion autoradiographs, the number of labeled cells was counted manually in selected areas. The counting was done twice and the procedure was performed bilaterally on corresponding sections from two different experiments. The recording of neurons was facilitated by positioning an ocular grid over the area and systematically counting the labeled neurons within each row of the grid.

Chromatographic analysis. Fifty adult male Mongolian gerbils were killed by decapitation, and the brains and pituitaries were rapidly removed. Acid ethanol extracts were prepared according to method II of Newgard and Holst (1981). Briefly, the frozen tissue was homogenized in 4 vol of acid ethanol at $-20^\circ C$ and centrifuged. Five volumes of ice-cold diethyl ether were added to the supernatant, and the aqueous proteinaceous phase was isolated at $-50^\circ C$. The resulting precipitate was dissolved in 1 M urea, buffered to pH 5.0, and applied to a 16 $\times$ 1000 mm (K16/100) column packed with Sephadex G50 fine grade (Pharmacia, Uppsala, Sweden), equilibrated, and eluted, at a flow rate of 300 ml/min at 4°C, with an albumin-containing phosphate assay buffer. Elution positions are referred to by their coefficient of distribution, $K_D = (V_e - V_0)/V_0$, where V_0 is the elution volume of a completely excluded substance; V_0 , the available inner volume, determined as the difference between the elution volumes of ^{125}I -labeled albumin (V_0) and $^{27}NaCl$, both added to the sample for internal calibration; and V_e , the elution volume of the substance in question. In separate experiments, gel filtrations were performed with synthetic somatostatin-14, synthetic somatostatin-28(1-12), synthetic somatostatin-28, and the natural porcine pro-somatostatin 1-64 (Bersani et al., 1989). Eluted fractions were assayed with radioimmunoassay based on antiserum codes 2098, 4576, and 1758 as described previously (Holst et al., 1988).

Results

Differences in the localization of stained neurons among the hypothalamic nuclei were observed. Therefore, hypothalamic immunoreactivities are referred to as prosomatostatin(20-36)-like immunoreactivity (pro-SS-LI), somatostatin-28(1-12)-like immunoreactivity [SS-28(1-12)-LI] somatostatin-28-like immunoreactivity (SS-28-LI), and somatostatin-14-like immunoreactivity (SS-14-LI), respectively. In those areas where no difference in distribution of various immunoreactivities was

Figure 4. High-power photomicrograph from the rostral periventricular area of the third ventricle (3V). Small ovoid, and medium-sized elongated immunoreactive cell bodies visualized with antiserum 1758 are seen in the periventricular area. Intraependymally situated nerve fibers endowed with boutons en passant are indicated with arrows. Scale bar, 50 μ m.

present, the immunoreactive elements are more conveniently referred to as somatostatin-like immunoreactivity (SRIF-LI).

Within the preoptic area and hypothalamus, immunoreactive cell bodies and nerve fibers were observed with all four antisera (Fig. 2). The number of immunoreactive cell bodies visualized with the various antisera differed significantly in certain areas (Table 2). Within the suprachiasmatic, the ventromedial, the dorsomedial, and the posterior hypothalamic nuclei, the retrochiasmatic area, the area of the tuber cinereum, and the caudal aspects of the lateral hypothalamic area, only pro-SS-LI and SS-14-LI cell bodies were present. However, in the periventricular area, the paraventricular nucleus (PVN), and arcuate nucleus, pro-SS-LI and SS-14-LI cell bodies as well as a small number of SS-28-LI and SS-28(1-12)-LI cell bodies were observed. Within the suprachiasmatic and ventromedial nuclei and the retro-

chiasmatic and the lateral hypothalamic areas, the density of pro-SS-LI and SS-14-LI fibers was higher than the density of SS-28-LI and SS-28(1-12)-LI fibers.

SS-immunoreactive neurons in the preoptic area and hypothalamus

Periventricular cell group. Small- to medium-sized immunoreactive perikarya were located periventricularly from the supraoptic to the mammillary recess (Figs. 2, 3; see also 7, 8). The cells were predominantly situated close to the ependyma of the third ventricle, and few immunoreactive cellular processes endowed with varicosities penetrated the ependyma (Fig. 4). Occasionally, an intraependymally situated immunoreactive cell body was observed (Figs. 5, 6). In the preoptic area, the immunoreactive cell bodies were restricted to the ventral aspect

Figure 5. High-power photomicrograph showing two SS-14-LI cell bodies in the periventricular area of the third ventricle. One cell body is apparently intraependymally situated (open arrow), whereas another is located in the neuropil of the periventricular strata (solid arrow). Scale bar, 25 μ m.

Figure 6. High-power photomicrograph of an intraependymally situated pro-SS-LI cell body. Scale bar, 25 μ m.

Figure 7. High-power photomicrographs of the periventricular subnucleus of the PVN. In *a*, a large number of SS-14-LI cell bodies is seen. A single intraependymally situated cell body can be seen (*open arrow*). The predominant type of immunoreactive cell body is small and ovoid (*solid arrows*), whereas only few medium-sized and elongated cell bodies are seen. In *b*, the *in situ* hybridization histochemical signal shows a high number of SS mRNA-containing cell bodies. A high grain density is seen over individually labeled cell bodies (*arrows*). 3V, third ventricle. Scale bars, 50 μ m.

of the periventricular area (Fig. 2*a*). At the rostral part of the suprachiasmatic nucleus, the immunoreactive periventricular cell bodies were observed in the full ventrodorsal extent of the periventricular area (Fig. 2*b*). Farther caudally, at the level of the paraventricular nucleus, the periventricular cells were encountered as distinct dorsal and ventral groups of perikarya (Fig. 2*c*).

The dorsal group was continuous with immunoreactive cell bodies in the periventricular and medial parvocellular subnuclei of the PVN. A very high density of immunoreactive perikarya was observed in the periventricular parvocellular subnucleus (Figs. 3, 7), whereas fewer were observed in the medial parvocellular subnucleus. Furthermore, only cells displaying pro-SS-LI and SS-14-LI were observed in the medial parvocellular subnucleus, whereas cells displaying SS-28-LI and SS-28(1-12)-

LI were strictly confined to the periventricular parvocellular subnucleus (Fig. 2*d,e*). Caudal to the PVN, the number of labeled cells in the dorsal periventricular cell group decreased significantly (Fig. 2*f*). The ventral periventricular group of perikarya was located medially to the anterior hypothalamic area and extended from the suprachiasmatic nucleus via the retrochiasmatic area into the arcuate nucleus.

Suprachiasmatic nucleus. In the suprachiasmatic nucleus, sparse and faintly labeled immunoreactive cells were encountered in the extreme dorsomedial portion of the nucleus (Fig. 3). However, occasionally a labeled cell body was observed in the most lateral aspects of the ventrolateral portion.

Tuber cinereum. Within the tuber cinereum, scattered immunoreactive cells were observed in the medial portion of the ventromedial hypothalamic nucleus, the dorsomedial hypota-

lamic nucleus, the retrochiasmatic and perifornical areas, and the area of the tuber cinereum. In the retrochiasmatic area, immunoreactive cells were scattered throughout the area from the optic chiasm to the arcuate nucleus (Fig. 9). This cell group merged with the periventricular cell group and laterally with the immunoreactive cells encountered in the area of the tuber cinereum (Fig. 10). The immunoreactive cell bodies encountered in lateral aspect of the area of the tuber cinereum were medium-sized and elongated (Fig. 11), whereas those encountered in the medial aspect were small and rounded (Fig. 13). Occasionally, pro-SS-LI and SS-14-LI cells were encountered in the rostral portion of the ventromedial nucleus, whereas the nucleus was devoid of immunoreactive cell bodies at more caudal levels. At the midlevel of the ventromedial nucleus, the area of the tuber cinereum contained labeled cells surrounding the nucleus laterally from the dorsolateral part to the ventrolateral part (Fig. 2f,g). In particular, the region intervening between the ventromedial and the dorsomedial nuclei contained a high number of immunoreactive cell bodies (Fig. 12).

Dorsal and lateral region (caudal parts). Within the dorsal hypothalamic area, a low number of scattered immunoreactive cells were located ventral to the mammillothalamic tract and ventrolateral to the thalamic reuniens nucleus (Fig. 2g). Within the lateral hypothalamic area, scattered immunoreactive cells were encountered in its ventromedial and caudal parts (Fig. 2h).

Arcuate nucleus. The arcuate nucleus contained a moderate number of immunoreactive cell bodies with a morphology different from the SRIF-LI cells located in the periventricular area. The small cell bodies of the arcuate nucleus were unevenly stained (Figs. 14–16). It should be emphasized that within the caudal part of the nucleus, only cell bodies displaying pro-SS-LI and SS-14-LI were observed. In the rostral part of the arcuate nucleus, the pro-SS-LI and SS-14-LI cell bodies were more numerous than the SS-28-LI and SS-28(1-12)-LI cell bodies and were continuous laterally with loosely arranged immunoreactive cells in the area of the tuber cinereum.

SS-immunoreactive nerve fibers in the preoptic area and hypothalamus

In addition to the areas containing immunoreactive perikarya, several areas contained immunoreactive nerve fibers. In general,

Figure 8. High-power photomicrograph from the periventricular area of the third ventricle. A high density of grains is seen overlaying individual cell bodies, showing *in situ* hybridization signal for SS mRNA. Scale bar, 10 μ m.

fibers displayed with all employed antisera were of fine caliber with a beaded appearance.

The periventricular area contained a dense plexus of immunoreactive fibers, some of which could be followed into the ependyma, but intraventricular nerve fibers were never found. In the preoptic area, extensive plexuses of fibers were observed in the organum vasculosum laminae terminalis (OVLt), the anterior ventral preoptic and the medial preoptic nuclei, and the medial preoptic area (Fig. 2a,b). In contrast, the lateral preoptic area contained a labeled fiber plexus of low to moderate density.

The anterior hypothalamic area was only sparsely innervated by immunoreactive fibers (Fig. 2c–e), whereas the supra-chiasmatic nucleus exhibited a high density of immunoreactive fibers (Fig. 3). The retrochiasmatic area contained a plexus of moderate density of immunoreactive fibers (Fig. 2d,e). The mag-

Table 2. Number of cell bodies in hypothalamic areas visualized by either immunohistochemistry or *in situ* hybridization

Visualization method	Paraventricular nucleus ^a	Retrochiasmatic area ^a	Arcuate nucleus ^b	Periventricular nucleus ^b	Suprachiasmatic nucleus ^b
1758	75.25 $\pm$ 13.7	108.2 $\pm$ 9.9	46.50 $\pm$ 9.1	9.75 $\pm$ 2.2	23.00 $\pm$ 2.4
2098	82.25 $\pm$ 10.4	99.00 $\pm$ 4.24	47.75 $\pm$ 2.9	10.25 $\pm$ 1.5	25.25 $\pm$ 3.9
4576	15.25 $\pm$ 3.2 ^c	0.25 $\pm$ 0.5 ^c	0.25 $\pm$ 0.5 ^c	0.75 $\pm$ 0.5 ^c	0.00 $\pm$ 0 ^c
1654	19.25 $\pm$ 3.0 ^d	0.50 $\pm$ 1.0 ^d	0.00 $\pm$ 0.0 ^f	2.00 $\pm$ 0.8 ^d	1.00 $\pm$ 1.2 ^d
<i>In situ</i>	78.25 $\pm$ 8.1	106.00 $\pm$ 7.0	66.75 $\pm$ 3.8 ^e	10.00 $\pm$ 1.4	22.75 $\pm$ 3.2

Values are given as means $\pm$ SEM; $n = 4$ in all cases.

^a $p < 0.05$ as determined by Friedman's test.

^b $p < 0.01$ as determined by Friedman's test.

^c $p < 0.05$ (1758, 2098, *in situ* vs. 4576) as determined by ANOVA.

^d $p < 0.05$ (1758, 2098, *in situ* vs. 1654) as determined by ANOVA.

^e $p < 0.01$ (1758, 2098 vs. 4576) as determined by ANOVA.

^f $p < 0.01$ (1758, 2098 vs. 1654) as determined by ANOVA.

^g $p < 0.05$ (1758, 2098 vs. *in situ*) as determined by ANOVA.

Figure 9. Low-power photomicrograph of the ventral part of the gerbil hypothalamus. A large number of pro-SS-LI cell bodies is seen in the retrochiasmatic area and the area of the tuber cinereum. 3V, third ventricle. Scale bar, 200 μ m.

nocellular PVN and supraoptic nucleus contained plexuses of immunoreactive fibers of low density (Fig. 2*b-d*).

In the ventromedial and the arcuate nuclei, the highest density of immunoreactive fibers in the hypothalamus proper was encountered. Very dense pro-SS-LI and SS-14-LI fiber plexuses were observed in the medial portion of the ventromedial nucleus (Figs. 10, 12). Within the arcuate nucleus, the fiber plexus became increasingly denser when followed in direction of the median eminence.

Throughout the lateral hypothalamic area, pro-SS-LI and SS-14-LI fibers were observed having a moderate to low density in the ventral aspects and a high density in the dorsal aspects close to the zona incerta, whereas only single SS-28(1-12)-LI and SS-28-LI fibers were seen in this area.

SS immunoreactivity in the median eminence and the posterior pituitary

In the rostral part of the median eminence, a very high density of SRIF-LI fibers terminating in the perivascular spaces was

observed. In the caudal part, the density of SRIF-LI fibers was lower (see Fig. 17*a-c*). Fibers originating from the rostral part of the median eminence coursed via the infundibular stalk into the posterior pituitary lobe. A very dense accumulation of SRIF-LI fibers could be seen in the external zone of the median eminence. The internal zone of the median eminence contained fewer SRIF-LI elements, and individually discernible nerve fibers could be followed in a direction more or less parallel to the ventral surface of the brain via the infundibular stalk into the posterior pituitary lobe. In the rostral part of the posterior pituitary lobe, the SRIF-LI fibers spread diffusely, often to terminate in close apposition to blood vessels (see Fig. 17*e*). In the central part of the posterior pituitary lobe, SRIF-LI fibers were sparsely distributed in close proximity to the intermediate lobe.

In situ hybridization

In order to investigate whether cellular labeling revealed with the immunohistochemical staining was accompanied by an in-

Figure 10. Low-power photomicrograph of the ventral part of the gerbil hypothalamus at a level 240 μ m caudal to that shown in Figure 9. A high density of pro-SS-LI fibers is seen in the medial part of the ventromedial hypothalamic nucleus (VMH). A population of scattered pro-SS-LI cell bodies is seen in the area of the tuber cinereum and the lateral hypothalamic area (arrows). The boxed area is shown at higher magnification in Figure 10. 3V, third ventricle. Scale bar, 200 μ m.

Figure 11. Higher magnification of the boxed area in Figure 10. A large triangular cell body is seen in the lateral hypothalamic area (arrow). Scale bar, 25 μ m.

tracellular content of pro-SS mRNA, we performed *in situ* hybridization histochemistry on gerbil brain sections. A clear correspondence between the distribution of perikarya containing SS hybridization signal and SS was detected. Hybridization signal produced with the oligonucleotide probe was found in perikarya in all areas throughout the preoptic area and hypothalamus that contained neuronal somata displaying pro-SS-LI and SS-14-LI. Generally, the number of cell bodies revealed by *in situ* hybridization corresponded to that revealed by immunohistochemistry (Table 2). The densest hybridization signal in the hypothalamus was found in the periventricular cell group (Figs. 7b, 8). A general correspondence between the number of grains

Figure 13. High-power photomicrograph of SS-14-LI cell bodies in the area of the tuber cinereum lying dorsal to the VMH. In some of the cell bodies, the immunoreactive material is apparently distributed throughout the cytoplasm (solid arrows). However, in other cell bodies, the immunoreactive material is confined to only a part of the cellular extent (open arrows). Scale bar, 25 μ m.

overlaying individual cell bodies and the intensity of the immunohistochemical labeling was observed (Fig. 7). However, within the arcuate nucleus, the number of pro-SS-LI and SS-14-LI cell bodies was about 80% of the number of cell bodies containing SS mRNA as revealed by *in situ* hybridization (Table 2). Furthermore, in the arcuate nucleus the individual neuronal somata were visualized with a very dense *in situ* hybridization

Figure 12. Low-power photomicrograph of the ventral part of the gerbil hypothalamus at a level 240 μ m caudal to that shown in Figure 10. A high density of pro-SS-LI cell bodies and nerve fibers is seen in the arcuate nucleus (Arc). Dorsal to the ventromedial hypothalamic nucleus (VMH), many pro-SS-LI cell bodies are seen in the area of the tuber cinereum (arrows). 3V, third ventricle. Scale bar, 200 μ m.

Figure 14. High-power photomicrograph of the arcuate nucleus. A moderate number of unevenly stained SS-14-LI cell bodies (arrows) and a high density of immunoreactive fibers is seen. 3V, third ventricle. Scale bar, 25 μ m.

Figure 15. High-power photomicrograph of the arcuate nucleus. A high density of grains is seen over cell bodies histochemically visualized by *in situ* hybridization to SS mRNA. 3V, third ventricle. Scale bar, 25 μ m.

signal (Figs. 14–16), whereas they displayed a faint pro-SS-LI and SS-14-LI labeling.

Gel filtration

The combined gel filtrations and radioimmunoassays of the tissue extract from gerbil hypothalami and posterior pituitary lobes clearly demonstrate the presence of two large and several smaller molecular forms of the pro-SS molecule. The chromatographic data are presented in Figure 18.

From the hypothalamus, equimolar amounts of peptides coeluting with genuine pro-SS (1–76), pro-SS(1–64), and synthetic SS-14 were extracted. Much lower quantities of peptides coelut-

ing with synthetic SS-28 and SS-28(1–12) were present in the hypothalamic extracts. The peak value of the hypothalamic SS-14 elution profile corresponds to 3000 pmol/gm wet weight tissue.

The immunoreactive elution profiles of extracts from posterior pituitary lobes differed from those of hypothalamic extracts. The predominant molecular species of the pro-SS molecule coeluted with synthetic SS-28(1–12), whereas the molecular species coeluting with genuine pro-SS(1–76) was absent. The majority of large molecular fragments recognized with the 2098 antiserum coeluted with genuine pro-SS(1–64). Equimolar amounts of peptides coeluting with synthetic SS-28 and SS-14, respectively, were apparently present in extracts from posterior pituitary lobes.

Discussion

The present study represents the first attempt to describe the anatomical distribution and molecular nature of pro-SS and its processed fragments pro-SS(1–64), pro-SS(1–76), SS-14, SS-28, and SS-28(1–12) in the preoptic area, hypothalamus, and pituitary in a mammalian species by means of immunohistochemistry and gel chromatography. Additionally, the localization of pro-SS mRNA was studied in the same areas.

The localization of somatostatinergic cell bodies in the gerbil hypothalamus is largely consistent with earlier immunohistochemical observations from other mammalian species performed with antisera against SS-14 (Krisch, 1978; Dierickx and Vandesande, 1979; Hoffman and Hayes, 1979; Bennett-Clarke et al., 1980; Papadopoulos et al., 1986). Furthermore, the distribution of somatostatinergic cell bodies revealed by *in situ* hybridization is largely consistent with that described in the rat (Fitzpatrick-McElligott et al., 1988).

The *in situ* hybridization histochemistry and the localization of pro-SS mRNA to individual cell bodies establish the neuronal sites of synthesis of pro-SS throughout distinct regions of the preoptic area and hypothalamus of the gerbil. Except for the

Figure 16. Medium-power photomicrograph of the arcuate nuclei of the gerbil hypothalamus. A high density of SS mRNA-containing cell bodies visualized by means of *in situ* hybridization histochemistry is seen in the nuclei. Individually labeled cell bodies are easily seen as a dense accumulation of grains (arrows). For abbreviations, see Appendix. Scale bar, 100 μ m.

Figure 17. A series of medium-power photomicrographs from the gerbil pituitary stalk (*a-c*). A dense plexus of SS-28-LI (*a*), SS-14-LI (*b*), and pro-SS-LI (*c*) nerve fibers is seen in the external zone of the median eminence, whereas a lower density of immunoreactive nerve fibers is seen in the internal zone. The immunoreactive fibers are observed to course to the posterior pituitary lobe via the infundibular stalk. In *d* a survey camera lucida drawing of the gerbil hypothalamo-pituitary complex is shown. Within the posterior pituitary (*e*), either thin immunoreactive fibers endowed with terminals en passant (*small solid arrows*) or large bulbous immunoreactive terminals (*open arrows*) are seen. For abbreviations, see Appendix. Scale bars: *a-c*, 100 μ m; *e*, 10 μ m.

Figure 18. Gel chromatographic profiles of extracts from hypothalamus (left column) and posterior pituitary lobe (right column). The amount of immunoreactivity is expressed as the total amount of applied immunoreactivity to the column. Elution positions of known fragments of the pro-SS molecule are depicted above each column.

arcuate nucleus, the densities of neurons containing pro-SS mRNA, pro-SS-LI, and SS-14-LI appear to be similar in the examined cell groups. This seems to suggest a similar sensitivity of these two independent techniques and to the equal access of their reagents to the brain sections. The somatostatinergic neuroendocrine neurons in the periventricular area displayed the densest *in situ* hybridization signal and most intense immunohistochemical labeling of all examined cell groups. Since these cells are of comparable size to those observed in other hypothalamic areas, such as the area of the tuber cinereum, the higher grain density does not simply reflect a larger perikaryal size in this area. The regional differences in the amount of grains encountered per cell could be the result of larger synthetic activity

of the periventricular cell group when compared to, for example, the area of the tuber cinereum as earlier suggested (Uhl and Sasek, 1986). The mismatch between a higher number of pro-SS mRNA-containing cells and the relative low number of pro-SS-LI and SS-14-LI cells observed for the arcuate nucleus could be caused by such diverse factors as small cytoplasmic storage capacity, fast axonal transport of newly synthesized pro-SS, and rapid cellular degradation of newly synthesized peptide. The mere presence of mRNA within a cell body does not always indicate that peptide synthesis takes place, as seen, for example, in the thalamocortical pathway where cholecystikinin mRNA was found (Burgunder and Young, 1988) but not cholecystikinin immunoreactivity (Innis et al., 1979). It may therefore be

that only a fragment of the pro-SS message is actually translated within arcuate neurons.

In the preoptic area and hypothalamus, a differential regional distribution of SS-14-LI, pro-SS-LI, SS-28-LI, and SS-28(1-12)-LI cell bodies and nerve fibers was observed. The observed differences were supported by the gel chromatographic analysis. The chromatographic data of hypothalamic extracts demonstrate the presence of two large and three smaller molecular species of the pro-SS molecule. About equimolar amounts of peptides coeluting with pro-SS(1-76), pro-SS(1-64), and SS-14 were present within the hypothalamus, whereas trace amounts of SS-28 and SS-28(1-12) were present. In general, this observation corresponds to the immunohistochemical observations. However, the presence of a pro-SS(1-76) fragment is surprisingly high compared to the immunohistochemical data inasmuch as only the presumably pituitary-projecting somata were properly stained with the 4576 antiserum. Abundant pro-SS(1-64)-like peptide has recently been reported to be present in rat hypothalamus (Rabbani and Patel, 1990), while the pro-SS(1-76) form was present in low amounts. This discrepancy could be either due to species difference or due to different extraction methods.

Earlier investigations have preferentially focused on the role of SS-14 and SS-28 in pituitary physiology (Rorstad et al., 1979; Tannenbaum et al., 1982; Reichlin, 1983), but the present data suggest that SS-28(1-12) could be a posterior pituitary neuroendocrine transmitter candidate. Within extracts from the posterior pituitary lobe, the large pro-SS(1-76) fragment was absent, indicating that it is further degraded during the axonal flow from hypothalamic cell bodies to the posterior pituitary terminals. This is not the case with pro-SS(1-64), which appears to be conserved during its course to the posterior pituitary lobe. We have recently reported a similar distribution of pro-SS-derived molecular species in the rat posterior pituitary lobe (Mikkelsen et al., 1991). In extracts from the posterior pituitary lobe, the SS-28(1-12) fragment was by far the most predominant, while equimolar amounts of SS-28 and SS-14 were present. Several physiological experiments have shown that SS-14, SS-28, and SS-28(1-12) all are released to the portal blood (Millar et al., 1983; Sheward et al., 1984; Pierotti et al., 1985). In the context of the posterior pituitary lobe being a distal extension of the median eminence, it is possible that a similar pattern of release takes place in the posterior pituitary lobe itself.

As shown in Table 2, the distributions of pro-SS-LI and SS-14-LI cell bodies were equivalent. However, SS-28-LI and SS-28(1-12)-LI cell bodies were restricted to hypothalamic areas known to send projections to blood-brain barrier-free areas communicating through the vascular system with the anterior pituitary and the systemic circulatory system. The presence of a very low number of immunohistochemically stainable SS-28-LI and SS-28(1-12)-LI perikarya compared to the number of SS-14 and pro-SS-LI perikarya was reflected by low amounts of extractable peptide. However, many areas contained SS-28-LI and SS-28(1-12)-LI nerve fibers that were labeled nearly as intensely and were similarly distributed as that of SS-14-LI and pro-SS-LI nerve fibers. Keeping in mind that immunohistochemistry is an unreliable method for quantitative studies, it should be stressed that the concentrations of various pro-SS fragments are not necessarily identical. These observations suggest that the posttranslational processing of the pro-SS molecule within the intrahypothalamic cell bodies leads to the formation of three major SS-14, pro-SS(1-76), and pro-SS(1-64) pools,

whereas the SS-28 and SS-28(1-12) fragments are further degraded. However, SS-28-LI and SS-28(1-12)-LI are present in nerve fibers, which could reflect either that these peptide fragments escape the posttranslational degradation within the cell bodies (not detected by immunohistochemistry) or that processing of the pro-SS molecule takes place within the nerve fibers, too. This is in agreement with earlier investigations, which have shown that within the hypothalamus, SS-14 is the most predominant peptide, whereas in the median eminence and the posterior pituitary SS-14 and SS-28 are present in equimolar amounts (Pierotti and Harnmar, 1985), supporting the view that two populations of SS-containing hypothalamic neurons with different processing enzyme activity exist.

The hypothalamic nuclei and areas, in which pro-SS-LI and SS-14-LI cells were encountered, harbor intracerebrally projecting neurons establishing synaptic contact with neurons in other brain areas. Within the brain, SS-28 exhibits three- and sixfold lower affinity than SS-14 to SS-14 receptors in the hypothalamus and cortex, respectively (Srikant and Patel, 1981; Leroux et al., 1985). On the contrary, the affinity of SS-28 to SS-14 receptors in the anterior pituitary lobe was 3.2-fold higher than that of SS-14 (Srikant and Patel, 1981). Based on the present results, it is tempting to speculate that SS-14 predominantly has neurotransmitter actions within the CNS, whereas SS-28 is more likely to exert a peripheral hormonal action mediated through a release to the portal and systemic blood circuit. SS-14 as well as SS-28 exhibit an inhibitory effect of growth hormone from cultured rat pituitary cells, with SS-28 exerting a longer-lasting action than SS-14 (Tannenbaum et al., 1982). Hypothalamic areas exerting an apparently higher density of pro-SS-LI and SS-14-LI compared to SS-28-LI and SS-28(1-12)-LI were the suprachiasmatic nucleus, the medial portion of the ventromedial nucleus, and the arcuate nucleus. This could mean that the former peptides are more actively engaged in the regulation of these areas and thus influence functions such as circadian rhythmicity, food intake, and neuroendocrine control of the pituitary. However, the roles of SS-28(1-12), pro-SS(1-63) and/or pro-SS(1-64), and pro-SS(1-76), if any, are at present unknown.

The presence of somatostatinergic fibers in the posterior pituitary is controversial. In a recent study, Kawano and Daikoku (1988) did not show SRIF-LI in the posterior pituitary lobe of the rat. However, other studies have immunohistochemically confirmed the presence of pro-SS-related molecular species in nerve fibers within the posterior pituitary lobe of the rat (Hökfelt et al., 1975; Mikkelsen et al., 1991) and the dog (Hoffman and Hayes, 1979). Apart from the well known projection from the magnocellular PVN to the posterior pituitary lobe, additional projections arise from parvocellular PVN subnuclei and most of the hypothalamic periventricular nucleus (Swanson and Kuypers, 1980; Ju et al., 1986; Larsen et al., 1991). The immunohistochemically identified cell bodies in the present study were parvocellular and predominantly located in the periventricular hypothalamic nucleus, suggesting that these neurons contribute to the somatostatinergic hypothalamo-pituitary projection. The role of SS in the physiology of the posterior pituitary lobe is at present only speculative.

Appendix

The following abbreviations are used in the figures:

3V,	third ventricle
ac,	anterior commissure

AHA,	anterior hypothalamic area
AMPO,	anterior medial preoptic nucleus
AP,	anterior pituitary lobe
Arc,	arcuate nucleus
BST,	bed nucleus of the stria terminalis
cp,	cerebral peduncle
DM,	dorsomedial hypothalamic nucleus
f,	fornix
fr,	fasciculus retroflexus
GP,	globus pallidus
HDB,	nucleus of the horizontal limb of the diagonal band
ic,	internal capsule
ImL,	intermediate pituitary lobe
LA,	lateroanterior hypothalamic nucleus
LH,	lateral hypothalamic area
LHb,	lateral habenular nucleus
LPO,	lateral preoptic area
ME,	median eminence
MHb,	medial habenular nucleus
ml,	medial lemniscus
MM,	medial mammillary nucleus
MPN,	medial preoptic nucleus
MPO,	medial preoptic area
mt,	mammillothalamic tract
opt,	optic tract
ox,	optic chiasm
pePVN,	periventricular parvicellular subnucleus
PF,	parafascicular thalamic nucleus
PH,	posterior hypothalamic area
pmPVN,	posterior magnocellular subnucleus
Pn,	pontine nuclei
PP,	posterior pituitary lobe
PV,	paraventricular thalamic nucleus
PVN,	paraventricular hypothalamic nucleus
RCh,	retrochiasmatic area
Re,	thalamic reuniens nucleus
SCN,	suprachiasmatic nucleus
sm,	stria medullaris
SO,	supraoptic nucleus
sox,	supraoptic decussation
SPF,	subparafascicular thalamic nucleus
st,	stria terminalis
TC,	area of the tuber cinereum
VMH,	ventromedial hypothalamic nucleus
ZI,	zona incerta

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