TEMPORAL PATTERNS OF SOMATOSTATIN IMMUNOREACTIVITY IN THE CEREBROSPINAL FLUID OF THE RHESUS MONKEY: EFFECT OF ENVIRONMENTAL LIGHTING¹

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Abstract

A rabbit antiserum to somatostatin was used to develop radioimmunoassay methods for measuring somatostatin in monkey cerebrospinal fluid (CSF). By gel permeation chromatography, at least five molecular weight forms of immunoreactive somatostatin (IRS) were identified in monkey CSF; two of these species co-migrated with either synthetic somatostatin-14 or somatostatin-28. The 24-hr profile of CSF somatostatin immunoreactivity was obtained from five monkeys during diurnal lighting, constant light, and constant darkness. During diurnal lighting, all five monkeys had a clear ultradian component in CSF IRS of 4 to 5 hr duration; this pattern was not affected significantly by constant light or dark. In addition, three of the five monkeys exposed to diurnal lighting showed a diurnal rhythm in CSF IRS, with higher hormone levels during darkness. In some animals, this diurnal rhythm also could be demonstrated during constant light or dark.

Somatostatin, a tetradecapeptide that inhibits growth hormone (GH) (Brazeau et al., 1973) and thyrotropin release from the pituitary (Vale et al., 1974), has a widespread distribution in the central nervous system as shown by immunohistochemistry and radioimmunoassay (RIA). The highest levels of somatostatin in the rat are found in the medial basal hypothalamus (Brownstein et al., 1975) although substantial levels also have been reported in the preoptic area (Kobayashi et al., 1977) and the circumventricular organs (Palkovits et al., 1979). Subcellular fractionation of brain tissue has indicated that 60 to 80% of the somatostatin is localized in the synaptosomal fraction (Epelbaum et al., 1977). Pharma-

cological studies with synthetic somatostatin have demonstrated a direct effect on neuronal activity (Renaud et al., 1975) and a modification of behavior in intact animals (Rorstad et al., 1980). In view of these findings, it has been postulated that somatostatin functions as a neurotransmitter or neuromodulator in addition to its role as a hypothalamic releasing hormone.

Somatostatin has been identified by radioimmunoassay (RIA) in human cerebrospinal fluid (CSF) (Patel et al., 1977; Kronheim et al., 1977), but little is known about the temporal patterns of this peptide in that system. The purpose of this study was to identify the somatostatin-like immunoreactivity in CSF from the rhesus monkey and to determine the temporal profiles of CSF somatostatin during different environmental lighting conditions.

Materials and Methods

Animals. Adult male rhesus monkeys were purchased from the National Institutes of Health primate colony and were adapted to chronic chair restraint as previously described (Reppert et al., 1979). The animals were exposed to 3 to 4 days of diurnal lighting (12 hr light, 12 hr dark), with lights on from 6 A.M. to 6 P.M., followed by 3 to 4 days of constant light and another 4 days of constant dark. CSF was withdrawn continuously (1 ml/hr) from an indwelling cisternal subarachnoid catheter

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using methods previously described (Reppert et al., 1979); 2-ml fractions were collected. The refrigerated samples were removed every 24 hr and stored at -70°C . Neither 24-hr refrigerator storage nor repeated (three times) freeze-thawing of pooled monkey CSF significantly affected the level of somatostatin measured by RIA.

Materials. The sources of the peptides used are as follows: somatostatin for immunization, dihydrosomatostatin, *N*-Tyr-somatostatin, [Tyr¹²]somatostatin, bradykinin triacetate, leucine enkephalin, methionine enkephalin, neurotensin, angiotensin, bombesin, adrenocorticotrophic hormone-1-24 (ACTH₁₋₂₄), oxytocin, thyrotropin-releasing hormone (TRH), and substance P, Bachem (Torrance, CA); somatostatin for RIA standard, [Tyr¹]somatostatin, luteinizing hormone-releasing hormone (LHRH), Beckman (Palo Alto, CA); vasoactive intestinal polypeptide (VIP), motilin, cholecystokinin (CCK)-octapeptide, and secretin, Peninsula (San Carlos, CA); porcine glucagon, α -melanocyte-stimulating hormone (α -MSH), and melatonin, Sigma (St. Louis, MO); Arg-vasotocin, Merck (West Point, PA); Arg-vasopressin, Ferring A/B (Malmö, Sweden); des[Ala¹, Gly², NH₂³, COOH¹⁴]somatostatin and des[NH₂³, COOH¹⁴]somatostatin, Ayerst (Montreal, Quebec, Canada); [Ser¹⁰]somatostatin, *N*³-acetyl des[Ala¹, Gly²]somatostatin, and bisacrylamide des[Ala¹, Gly²]somatostatin, Dr. A. Schonbrunn, Harvard School of Public Health (Boston, MA); the alanine-substituted somatostatin analogues in Table I, Dr. J. Rivier, Salk Institute (La Jolla, CA); somatostatin-28, Drs. P. Brazeau and N. C. Ling, Salk Institute; rat insulin, Dr. R. Chance, Eli Lilly Laboratories (Indianapolis, IN); and rat GH, rat prolactin (PRL), rat thyrotropin-stimulating hormone (TSH), human GH, and human PRL, National Institute of Arthritis, Metabolism, and Digestive Diseases (Bethesda, MD).

The sources of the following reagents are as follows: human serum albumin (HSA; Cohn fraction V) and horse heart cytochrome *c* (Type III), Sigma; Na[¹²⁵I], Amersham (Arlington Heights, IL); normal rabbit serum, Pel-Freez Biologicals (Rogers, AR); and goat anti-rabbit γ -globulin, Biotek (Shawnee Mission, KS).

RIA for somatostatin. Antiserum for somatostatin was generated in a New Zealand white rabbit using methods previously described (Arnold and Fernstrom, 1980). RIA was performed using a 1:150,000 final dilution of rabbit antiserum (batch M4) and approximately 10^4 cpm of ¹²⁵I-labeled [Tyr¹]somatostatin (Schonbrunn and Tashjian, 1978) in a final volume of 0.6 ml with an assay buffer of 0.1 M sodium phosphate, pH 7.4, containing 0.01 M EDTA, 0.05 M NaCl, 0.02% sodium azide, and 0.1% HSA. Assay tubes containing standards (1.25 to 320 pg of somatostatin) or samples of 0.2 ml of CSF were incubated at 4°C for 24 hr before the addition of 0.15 ml of appropriately diluted normal rabbit serum and goat anti-rabbit γ -globulin. After an additional 24-hr incubation, the assay tubes were centrifuged and the radioactivity in the pellet was determined.

Gel permeation chromatography. Pooled monkey CSF (30 ml) was lyophilized, reconstituted in 2 ml of 1 M acetic acid, and added to a 0.9×50 cm column of Bio-Gel P-10. The column was eluted with 1 M acetic acid at 4°C ; 1-ml fractions were collected. Fractions were lyoph-

ilized, reconstituted in 1 ml of RIA buffer, and assayed for somatostatin.

Data analysis. Periodicity in CSF somatostatin immunoreactivity was evaluated by power spectral analysis (Terman and Terman, 1980) for each animal in each of the test periods (light/dark, constant light, or constant dark). A computer program transformed the 2-hr values for immunoreactive somatostatin (IRS) to relative deviations from a least squares regression line fitted to the data sample. The correlation of the data with sine and cosine functions was determined independently for a succession of test periods between 2 and 30 hr in 0.05-hr increments. The magnitude of the best fitting sine function for each test period was defined as $M = \sqrt{r_{\sin}^2 + r_{\cos}^2}$, and the peak phase was defined by $\phi = \tan^{-1}(r_{\sin}/r_{\cos})$. The spectra were expressed graphically in terms of spectral power, M^2 . A one-tailed *t* test was used to evaluate the significance of the spectral components ($p < 0.01$ was chosen as the minimum level of significance).

Results

RIA of somatostatin. The displacement of labeled somatostatin by varying amounts of standard somatostatin or other peptides is shown in Figure 1. The only peptide tested clearly showing cross-reactivity was bradykinin triacetate; this peptide was less than 0.01% as potent as somatostatin at displacing ¹²⁵I-labeled [Tyr¹]somatostatin from rabbit antiserum. On the other hand, native bradykinin at concentrations up to 0.94 mol/liter $\times 10^{-6}$ showed no cross-reactivity.

The immunological cross-reactivity of a number of analogues of somatostatin is presented in Table I. The substitution of alanine for a single amino acid in the central portion of the somatostatin molecule (e.g., amino acids 6 to 12) results in substantially diminished cross-reactivity. Alterations of the NH₂-terminal portion of the somatostatin molecule (by substituting alanine for amino acids 2 or 4 or adding an NH₂-terminal tyrosine) did not affect its immunoreactivity greatly. Somatostatin-28 is equipotent with somatostatin-14 on a molar basis (Fig. 2).

The sensitivity of the RIA, defined as the concentration of somatostatin which resulted in the binding of labeled somatostatin 2 SD below mean binding in the absence of somatostatin, was 1.3 pg/tube. The ED₅₀ for somatostatin (defined as the concentration of somatostatin required to displace 50% of ¹²⁵I-labeled [Tyr¹]somatostatin specifically bound to antiserum) in the RIA was 3.5×10^{-11} mol/liter (34.4 pg/tube). The interassay coefficients of variation for samples containing means of 52, 25, and 12 pg/tube were 11.4, 12.7, and 10.6%, respectively. The intra-assay coefficient of variation was less than 10%.

The inhibition curve for serial dilutions of pooled monkey CSF was compared with a standard curve using synthetic somatostatin. The resultant log-logit slopes of the inhibition curves for the somatostatin standard (−1.22) and monkey CSF (−1.19) were not significantly different ($p < 0.05$).

Gel permeation chromatography. Chromatography of pooled monkey CSF on a Bio-Gel P-10 column revealed five major peaks of immunoreactivity (Fig. 3). *Peak IV*

Figure 1. Binding of ^{125}I -labeled $[\text{Tyr}^1]\text{somatostatin}$ to rabbit antiserum as a function of somatostatin or other peptide concentrations (moles per liter). B/B_0 , Ratio of ^{125}I -labeled $[\text{Tyr}^1]\text{somatostatin}$ bound in the presence of competing peptide to that bound in the absence of competing peptide; *h*, human; *r*, rat.

represents material that co-chromatographs with synthetic somatostatin, whereas *peak III* behaves similarly to somatostatin-28. *Peak I* migrates approximately with cytochrome *c* ($M_r = 12,300$); *peak II* migrates intermediate between cytochrome *c* and somatostatin-28. The remaining material (*peak V*) migrates after synthetic somatostatin. The proportion of somatostatin immunoreactivity measured in *peaks I* to *V* is 22, 24, 26, 13, and 15%, respectively.

CSF somatostatin. CSF samples obtained continuously from five monkeys over the three lighting schedules were assayed for somatostatin immunoreactivity (Figs. 4 and 5). IRS levels in CSF ranged from 7 to 117 pg/ml.

Periodicity in CSF IRS levels was evaluated by power spectral analysis for each animal during each of the three lighting conditions (Fig. 6). Ultradian components to CSF IRS periodicity were observed in all monkeys tested during each of the three lighting regimens (Fig. 5). These components ranged from 3.35 to 16.60 hr in duration and generated a pattern of periodicity which varied from animal to animal. However, during diurnal lighting, all five monkeys had a clear ultradian component of 4 to 5 hr in duration. This rhythm persisted during constant light in three of the monkeys (animals 615, 854, and 771) and in constant darkness in four monkeys (animals 585, 641, 854, and 771).

During diurnal lighting conditions, three of the five monkeys (animals 585, 615, and 771) had a significant ($p < 0.01$) daily component (between 23 and 24 hr) in their CSF IRS levels. In each of these three animals, higher levels of IRS generally were observed during the dark

period of the daily cycle. Of the remaining two monkeys, one (animal 641) had a significant rhythmic component of 20.95 hr, while the other (animal 854) did not have a significant daily component during diurnal lighting conditions. Exposure of the monkeys to constant conditions modified the daily periodicity of CSF somatostatin in some of the animals. During constant light, only one monkey (animal 615) had a significant rhythmic component of near 24 hr duration (22.70 hr). In a monkey (animal 585) which previously had a significant daily component (23.95 hr) during diurnal lighting conditions, constant light abolished a daily rhythmic component but generated a shorter component of 20.70 hr duration. During the subsequent exposure to constant darkness, two monkeys (animals 615 and 771) had a significant daily component in CSF somatostatin of 22.75 and 24.90 hr, respectively.

Discussion

This study provides evidence for the existence of multiple forms of somatostatin in monkey CSF. By gel permeation chromatography, five peaks of somatostatin immunoreactivity have been identified. *Peak IV* corresponds to the tetradecapeptide somatostatin, whereas *Peaks III* and *I* reflect higher molecular weight species of approximately 3,000 and 12,000 daltons, respectively. *Peak II* also reflects larger molecular weight material intermediate between 3,000 and 12,000 daltons. By its elution positions, *peak V* may represent a molecular species smaller than the native tetradecapeptide. In the literature, a host of studies have dealt with the identifi-

TABLE I
Immunoreactivity of somatostatin analogues

Analogue	ED ₅₀ ^a mol/liter × 10 ¹¹
Somatostatin	4.8
Dihydrosomatostatin	29
N-Tyr-somatostatin	4.2
[Tyr ¹]Somatostatin	4.2
[Ala ²]Somatostatin	6.0
[Ala ⁴]Somatostatin	6.0
[Ala ⁶]Somatostatin	170
[Ala ⁷]Somatostatin	8,000
[Ala ⁸]Somatostatin	24,000
[Ala ⁹]Somatostatin	17.5
[Ala ¹⁰]Somatostatin	50
[Ser ¹⁰]Somatostatin	6.5
[Ala ¹¹]Somatostatin	3,400
[Tyr ¹¹]Somatostatin	131
[Ala ¹²]Somatostatin	48
[Ala ¹³]Somatostatin	9.8
N ³ -Acetyl des[Ala ¹ , Gly ²]somatostatin	4.0
Bisacrylamide des[Ala ¹ , Gly ²]somatostatin	15.0
Des[Ala ¹ , Gly ² , NH ₂ ³ , COOH ¹⁴]somatostatin	21.5
$\begin{array}{c} \text{H} \text{C} \text{---} \text{S} \text{---} \text{S} \text{---} \text{C} \text{H}_2 \\ \qquad \qquad \qquad \\ \text{HC-CO-Ser-Thr-Phe-Thr-Lys-Trp-Phe-Phe-Asn-Lys-NH-CH}_2 \\ 1 \qquad 2 \qquad 3 \qquad 4 \qquad 5 \qquad 6 \qquad 7 \qquad 8 \qquad 9 \qquad 10 \qquad 11 \qquad 12 \end{array}$	
Retroenantio isomer of des[Ala ¹ , Gly ² , NH ₂ ³ , COOH ¹⁴]somatostatin	790,000
$\begin{array}{c} \text{H} \text{C} \text{---} \text{S} \text{---} \text{C} \text{H}_2 \\ \qquad \qquad \qquad \\ \text{HC-CO-Ser-Thr-Phe-Thr-Lys-Trp-Phe-Phe-Asn-Lys-Cys-Gly-NHEt} \\ 1 \qquad 2 \qquad 3 \qquad 4 \qquad 5 \qquad 6 \qquad 7 \qquad 8 \qquad 9 \qquad 10 \qquad 11 \qquad 12 \qquad 13 \qquad 14 \end{array}$	
Retroenantio isomer of des[NH ₂ ³ , COOH ¹⁴]somatostatin	>1,060,000

^a ED₅₀, concentration of somatostatin or analogue required to displace 50% of [¹²⁵I-Tyr¹]somatostatin specifically bound to antiserum.

Figure 2. Radioimmunoassay standard curves were run with synthetic somatostatin-14 (○) and somatostatin-28 (▲) using the primary antiserum at a dilution of 1:120,000. Straight lines were fitted by a weighted least squares regression of logit (B/B_0) on $\log_e$ (dose) (Davis et al., 1980). The 50% binding values were 19.84 ± 1.68 fmol/tube for somatostatin-14 and 21.57 ± 0.51 fmol/tube for somatostatin-28. The corresponding slopes were -1.044 ± 0.063 and -1.097 ± 0.020 , respectively.

Figure 3. Gel permeation chromatography of pooled monkey CSF on a Bio-Gel P-10 column. The vertical arrows indicate the elution volumes of Cyt c (horse heart cytochrome c; $M_r = 12,300$), SS 28 (somatostatin-28), and SRIF (somatostatin).

cation of multiple species of somatostatin in mammalian brain and gut extracts. In addition to isolating the 1,600-dalton somatostatin molecule, various workers have identified 12,000-dalton and 3,000- to 4,000-dalton species (Zyznar et al., 1979; Trent and Weir, 1981), an interne-

Figure 4. Patterns of CSF somatostatin from three monkeys (animals 585, 615, and 641) during periods of diurnal lighting, constant light, and constant dark. CSF was collected as 2-hr fractions.

diate species of approximately 6,000 daltons (Lauber et al., 1979; Sagar et al., 1982), and a species smaller than 1,600 daltons (Rorstad et al., 1979). Our finding of multiple forms of somatostatin in monkey CSF differs from a report identifying a single species of somatostatin (the tetradecapeptide) in human CSF (Kronheim et al., 1977). Whether this reflects a difference in experimental protocol or in species cannot be firmly established at this time. Most likely, the presence of multiple species of somatostatin in monkey CSF suggests that axon terminals that release somatostatin into CSF release many forms of the molecule. However, the chromatographic conditions used in this study cannot rule out the possibility that the immunoreactive material of higher apparent molecular weight than synthetic somatostatin consists either of somatostatin oligomers or of somatostatin bound to some other material.

With the use of a sensitive and specific assay for somatostatin, this study describes the temporal pattern of CSF somatostatin immunoreactivity in the rhesus monkey. IRS levels in CSF varied markedly over the period of observation, with a range of 7 to 117 pg/ml. The periodicity of CSF somatostatin immunoreactivity was evaluated by power spectral analysis. By this method, all monkeys in this study exhibited ultradian components in CSF IRS rhythms. Of particular interest is the 4- to 5-hr periodicity in all monkeys tested during

diurnal lighting conditions. This ultradian component by and large was well maintained during constant lighting conditions. Interestingly, Steiner et al. (1978) have described a serum GH rhythm with a periodicity of 4.1 hr in another Old World primate, the baboon. In a recent study, Quabbe et al. (1981) describe a similar pattern of GH secretion in the rhesus monkey. Whether CSF somatostatin has a physiological role in GH regulation has yet to be determined, although a recent study (Lumpkin et al., 1981) finds that the intraventricular injection of somatostatin paradoxically stimulates GH release. Indeed, it is conceivable that CSF provides a physiological medium for hormonal communication with peptides such as somatostatin between various brain neurons.

Three of the five animals had a daily (23- to 24-hr) component for CSF IRS during diurnal lighting conditions, with higher peptide levels during the dark periods. Exposure to constant light or constant dark resulted in a loss of daily periodicity in all but one or two animals.

The significance of both ultradian and daily rhythmicity in CSF IRS cannot be firmly established at this time. It has been suggested that the measurement of the CSF peptide level is probably reflective of the diffusion of molecules following synaptic release by brain neurons or the result of direct secretion by these cells into CSF (Jackson, 1980). In the frog, it has been demonstrated that the ependymal cells in the median eminence have

Figure 5. Patterns of CSF somatostatin from two monkeys (animals 854 and 771) during periods of diurnal lighting, constant light, and constant dark. CSF was collected as 2-hr fractions.

Figure 6. Power spectra for periodicity in CSF somatostatin in five monkeys (animals 585, 615, 641, 854, and 771) during each of three lighting conditions: LD (12 hr light, 12 hr dark), LL (constant light), and DD (constant dark). ●, spectral component significant at $p < 0.001$; ○, $p < 0.01$.

bidirectional transport activities for the transport of substances between hypophyseal portal blood and CSF (Nakai and Naito, 1974). It is unlikely, however, that CSF peptide levels are indicative of simple diffusion of these water-soluble molecules from blood across a blood-brain barrier into CSF (Jackson, 1980).

Interestingly, two recent reports document dramatic daily rhythms in CSF levels of the neuropeptides oxytocin and vasopressin in the monkey and the cat (Reppert et al., 1981; Perlow et al., 1981). Whether CSF rhythms are common to all neuropeptides is unknown at present, although subsequent investigation on this topic is already underway.

Note added in proof. After submission of this paper, a report by Berelowitz et al. (1981) appeared which also describes a nocturnal elevation in IRS in the rhesus monkey.

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