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Distribution of the sst_1 and sst_2 Receptor mRNAs in the Hypothalamus and Uptake of Somatostatin by Neurons in the Brain of the Adult Rat

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To M. Davis, K. Jarrett, & H.J. Heinz
Thanks for all your help

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ABSTRACT

The neuropeptide somatostatin is both a hormone and a neuromodulator; It is the major physiological inhibitor of growth hormone secretion. With the aim of identifying the receptor subtypes through which this neuropeptide may be exerting its neuroendocrine actions in the brain, we have examined by *in situ* hybridization the distribution of the mRNA for the sst_1 and sst_2 receptors in the hypothalamus of adult male and female rats. Both receptor subtypes were highly expressed in the medial preoptic area, suprachiasmatic nucleus and arcuate nucleus. High sst_1, but low sst_2 receptor expression was evident in the para- and periventricular nuclei as well as in the ventral premammillary nucleus. Conversely, moderate to high sst_2, but low sst_1 receptor mRNA levels were detected in the anterior hypothalamic nucleus, ventromedial and dorsomedial nuclei and medial tuberal nucleus. These distributional patterns conform to those of somatostatin binding sites as visualised by *in vitro* radioautography. The distribution of sst_1 receptor-expressing cells within the periventricular, paraventricular and suprachiasmatic nuclei was similar to that of neurons previously reported to contain and/or express somatostatin in the brain suggesting that some of the sst_1 receptors may correspond to autoreceptors. Within the arcuate nucleus, the distributions of both sst_1 and sst_2 receptor mRNA-expressing cells were comparable to that of neurons previously found to selectively bind somatostatin-14 within this area. Given that over one third of these cells also contain and express growth hormone-releasing factor, the present findings suggest that both of these receptor subtypes are involved in the central regulation of growth hormone-releasing factor secretion by somatostatin.

Several reports focusing on peripheral cells have indicated that somatostatin can be internalized after binding to membrane associated somatostatin receptors. Moreover, it has recently been reported that the neuropeptides neurotensin and substance P, both of which interact with G protein coupled receptors, can be internalized into central neurons by receptor- mediated endocytosis. In light of these reports, we investigated what role the G protein coupled sst_1 or sst_2 receptors might play in mediating the internalization of somatostatin. When expressed on the surface of COS 7 cells transfected with the appropriate cDNA, the sst_1 receptor internalized between 20 and 25% of specifically bound

^{125}I-Tyr^{0}[DTrp^{8}]somatostatin while the sst_2A receptor internalized up to 75% of specifically bound. Furthermore, we were able to visualize internalized ^{125}I-Tyr^{0}[DTrp^{8}]somatostatin in COS 7 cells *in vitro* using a combined biochemical and morphological technique. In slices of rat brain processed *ex vivo* for the detection of radioligand uptake, we found that neurons in the arcuate and periventricular nuclei, zona incerta, medial habenula, hippocampus, and retrosplenial and frontal cortices sequestered radioactivity intracellularly. The distribution of some of these cells, namely in the zona incerta, arcuate and periventricular nuclei of the hypothalamus was similar to the distribution of dopaminergic cells previously documented in those regions. Furthermore, uptake of the radiolabeled ligand by these cells was abolished by nomifensin, a pharmacological inhibitor of the dopamine transporter, suggesting that the dopamine transporter might have mediated the uptake of radioactivity, presumably in the form of free ^{125}I-Tyr, by neurons in these regions. By contrast, uptake of the iodinated ligand in the retrosplenial and frontal cortices, hippocampus, and medial habenula, areas that do not contain cells known to express the dopamine transporter, might involve receptor- mediated internalization, as the distribution of labeling in these areas was highly reminiscent of the distribution of sst_2 receptor mRNA in the same regions. In summary, the present results indicate that while the dopamine transporter may mediate the uptake iodinated metabolite(s) of ^{125}I-Tyr^{0}[DTrp^{8}]somatostatin by neurons in some regions of the adult rat brain, uptake of ^{125}I-Tyr^{0}[DTrp^{8}]somatostatin in some other regions of the rat brain might be the result of sst_2 receptor- mediated internalization.

RÉSUMÉ

La somatostatine est un neuropeptide qui joue le rôle à la fois d'une hormone et d'un neurotransmetteur. Son rôle le mieux documenté est l'inhibition physiologique de la sécrétion de l'hormone de croissance. Dans le but d'identifier les sous types de récepteurs centraux responsables des effets neuroendocrines de la somatostatine, nous avons étudié par hybridation in situ la distribution des ARNm codant pour les récepteurs sst1 et sst2 dans l'hypothalamus de rats adultes males ou femelles. Les deux sous types de récepteurs sont fortement exprimés dans l'aire préoptique médiane, le noyau suprachiasmatique et le noyau arqué. Les récepteurs sst1 sont aussi exprimés dans le noyau para et periventriculaire où il n'y a qu'une faible expression de sst2. Au contraire, des taux modérés à importants de récepteurs sst2 sont observés dans le noyau hypothalamique antérieur, les noyaux ventro- et dorsomédian et dans le noyau tubéro-médian alors que peu de récepteurs sst1 y sont exprimés. Cette distribution est en accord avec la répartition des sites de liaison de la somatostatine visualisés par autoradiographie in vitro. La distribution des cellules exprimant le récepteur sst1 au sein des noyaux périventriculaire, paraventriculaire et suprachiasmatique est comparable à celle des neurones décrits comme contenant et/ou exprimant la somatostatine dans le cerveau suggérant que certains de ces récepteurs pourraient être des autorécepteurs. Au sein du noyau arqué, la distribution des ARNm codant pour les récepteurs sst1 et sst2 correspond à celle des neurones qui y lient la somatostatine (1-14). Considérant que plus du tiers de ces cellules contiennent et expriment le facteur de libération de l'hormone de croissance, nos résultats suggèrent que les deux types de récepteurs sont impliqués dans la régulation de la sécrétion de cette hormone par la somatostatine.

De nombreuses études au niveau de cellules périphériques ont montré que la somatostatine peut être internalisée après liaison à son récepteur membranaire. De plus, il a été décrit récemment que deux neuropeptides, la neurotensine et la substance P, tous deux interagissant avec des récepteurs couplés à une protéine G, peuvent être internalisés dans des neurones du système nerveux central par un mécanisme d'endocytose impliquant son récepteur. Au vu de ces résultats nous avons d'abord cherché à déterminer le rôle que les récepteurs sst1 et sst2 pouvaient jouer dans les mécanismes d'internalisation de la somatostatine. Les cellules COS-7 transfectées avec le cDNA codant pour le récepteur sst1 internalisent 20 à 25% de la ^{125}I-Tyr0[DTrp 8] somatostatine liée spécifiquement à ces cellules, alors que les cellules exprimant le récepteur sst2 en internalisent jusqu'à 75%. L'examen radioautographique de ces dernières confirme que l'internalisation est

confinée aux cellules efficacement transfectées. Nous avons ensuite visualisé la ^{125}I-Tyr0[DTrp 8] somatostatine internalisée dans des tranches de cerveau de rat. L'examen radioautographique des tranches superfusées in vitro avec 1.5 nM de ^{125}I-Tyr0[DTrp 8] somatostatine montre un marquage sélectif de neurones dans le noyau arqué, le noyau périventriculaire, de la zona incerta, l'hipoccampe et les cortex rétrosplénial et frontal. La distribution de certaines de ces cellules dans la zona incerta, les noyaux arqué et periventriculaire est la même que celle décrite pour les cellules dopaminergiques. De plus, la capture de somatostatine par ces cellules est inhibée par la nomifensine, un inhibiteur pharmacologique du transporteur de la dopamine, suggérant que le transporteur de la dopamine peut être responsable de la capture de radioactivité dans ces régions, probablement sous la forme de ^{125}Tyr libre. Par contre, la capture de somatostatine dans le cortex frontal et rétrosplénial, l'hipoccampe et l'habénula médiane, régions qui ne contiennent pas de cellules qui expriment le transporteur de la dopamine, semble être le résultat de l'internalisation de la somatostatine par un mécanisme dépendant du récepteur, ce qui est corroboré par la distribution de ce marquage identique à celle décrite pour les ARNm codant pour le récepteur sst2. En conclusion, nos résultats montrent que le transporteur de la dopamine peut être responsable de la capture de la radioactivité sous la forme de métabolites iodés de la somatostatine dans certaines régions cérébrales, alors que le récepteur sst2 peut être responsable de la capture de ^{125}I-Tyr0[DTrp 8] somatostatine dans d'autres régions.

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LIST OF ABBREVIATIONS

cDNA:	Complimentary Deoxyribonucleic Acid
CNS:	Central Nervous System
GH:	Growth Hormone
G Protein:	Guanine Nucleotide Binding Protein
GRF:	Growth Hormone- Releasing Hormone
^{125}I-SRIF:	^{125}I-Tyr^{0}[DTrp^{8}]somatostatin14
mRNA:	Messenger Ribonucleic Acid
SRIF:	Somatostatin
sst_1:	Somatostatin Receptor Subtype 1
sst_2:	Somatostatin Receptor Subtype 2
sst_2A:	Unspliced Variant of the sst_2 Receptor
sst_2B:	Splice Variant of the sst_2 Receptor

* SSTR1 and SSTR2 are used in the Manuscript section of the thesis as abbreviations for the somatostatin receptor subtype 1 and the somatostatin receptor subtype 2 respectively.

GENERAL INTRODUCTION

The existence of growth hormone- release inhibitory factors was first reported in 1968 by Krulich *et al.* who were attempting to demonstrate growth hormone (GH) releasing activity in rat hypothalamic extracts (Krulich 1968). In 1973, Brazeau and colleagues reported the chemical structure of this hypothalamic growth hormone- release inhibitory substance that they termed somatostatin (SRIF) (Brazeau, 1973). It is now known that there are two forms of bioactive SRIF derived from the same precursor molecule; the tetradecapeptide SRIF-14, and its N terminally extended form somatostatin-28 (SRIF-28) (Epelbaum, 1992). Both of them suppress GH release, as well as thyroid stimulating hormone and prolactin release from the anterior pituitary (Epelbaum, 1992). In addition to its role as a neuroendocrine regulator of pituitary GH secretion, SRIF (which refers heretofore to both SRIF-14 and SRIF-28) has been implicated in a variety of other functions both within and outside the central nervous system (Epelbaum, 1994; Reichlin, 1983).

Somatostatin, a hydrophobic protein, acts as both a hormone and a neuromodulator, and does so by interacting with high affinity cell surface receptors that transduce signals that evoke biological responses. Molecular biological studies have demonstrated the existence of five distinct SRIF receptor subtypes that have been termed sst_1, sst_2, sst_3, sst_4, and sst_5 according to the order in which they were cloned. The five known SRIF receptor subtypes are seven transmembrane domain spanning guanine nucleotide binding protein (G protein)- coupled receptors (Bell, 1993; Epelbaum, 1994).

The binding of peptide ligands to G protein-coupled cell surface receptors is frequently followed by internalization of receptor-ligand complexes (Morel, 1994). Internalization commonly occurs by rapid receptor- mediated endocytosis, and is often proceeded by lysosomal degradation of the peptide ligand (Gorden, 1982; O'Connor, 1983; Pastan, 1981). In the nervous system, internalization has previously been observed only for large polypeptide ligands such as growth factors (Bernd, 1983; Walicke, 1991),

however, recent *in vivo* data indicate that neurotransmitters may be internalized by neurons following interaction with their respective receptors (Beaudet, 1994).

In vivo Synthesis of Somatostatin

In mammals, SRIF is synthesized from a 116 amino acid prepro-somatostatin peptide that is derived from a single gene (Epelbaum, 1994). The prepro peptide is enzymatically cleaved to the 92 amino acid pro-somatostatin that contains the SRIF moiety at its carboxy-terminal; the propeptide is further cleaved to yield SRIF-14 and SRIF-28 as well as three other cleavage products that lack known biological activity (Epelbaum, 1994; Patel, 1995; Rabbani, 1990). Pro-somatostatin is proteolytically cleaved at a dibasic Arg-Lys site to produce SRIF-14, and at a monobasic Arg residue to produce the mature SRIF-28 peptide (Rabbani, 1990). Although little is known about the role of pro-hormone converting enzymes and the subcellular compartmentalization of the processing events, it appears that furin, PC1, and PC2, all members of the subtilisin-related serine convertase family, play a role in the maturation of SRIF from its prepro-hormone (Patel, 1995). The active site on both SRIF-14 and SRIF-28 is localized to amino acid positions 7-10 (Epelbaum, 1992). The distribution of the two biologically active molecular forms of SRIF varies from one tissue to the next; in the brain SRIF-14 accounts for 70%-80% of SRIF-like immunoreactivity (Patel, 1981).

Localization and Function of Somatostatin

Although SRIF was originally isolated from hypothalamic extracts, the peptide was later found to be distributed widely throughout the body and to subserve a multitude of functions.

Periphery

SRIF is found in secretory cells of epithelial origin throughout the gastrointestinal tract, where it inhibits, via autocrine and paracrine mechanisms, the secretion of gastrin, pepsin, and hydrochloric acid in the stomach, as well as the secretion of bile, and the release of secretin and vasoactive intestinal peptide (Patel, 1992). The secretion of SRIF by D cells, located in mucosal glands, into the lumen of the digestive tract influences the contractility of the gastrointestinal tract and the absorption of fluids (Patel, 1992). Furthermore, SRIF inhibits both exocrine and endocrine pancreatic function; aside from an inhibitory effect on pancreatic fluid, bicarbonate, and enzyme production, SRIF produced in the δ cells of the islets of Langerhans influences carbohydrate metabolism via paracrine inhibitory actions on the release of glucagon and insulin from α and β cells respectively (Patel, 1992). SRIF is also found in other organs throughout the body including the adrenal medulla where it inhibits angiotensin-II-simulated aldosterone secretion and acetylcholine-stimulated catecholamine secretion, in the thyroid where it inhibits thyroid stimulating hormone-stimulated T3 and T4 release, and in the kidneys where it inhibits both hypovolemicaly induced renin secretion and antidiuretic hormone induced water absorption (Patel, 1992; Reichlin, 1983).

Somatostatinergic cells are also found in the peripheral nervous system. The renal hilar arteries are innervated by SRIF containing varicosities (Reinecke, 1988). A proportion of the small primary sensory neurons in the dorsal root ganglia involved in nociception use SRIF as a neurotransmitter (Kai, 1989). SRIF is also synthesized in the perikarya of the sensory ganglia of the vagus and sciatic nerves and transported bidirectionally toward the central nervous system and the sites of sensory innervation (MacLean, 1988).

Central Nervous System

Somatostatinergic nerve cell bodies are found throughout the neuroaxis, save for the cerebellum; the vast majority of SRIF immunopositive neurons are localized within the diencephalon and are particularly concentrated within the hypothalamus (Patel,

1992). SRIF- containing neurons that project to the median eminence, and are thereby directly responsible for the regulation of growth hormone secretion by the anterior pituitary gland, encompass approximately 80% of SRIF immunopositive cells in the hypothalamus (Critchlow, 1981). These cells lie within the anterior periventricular nucleus (Finley, 1981; Johansson, 1984) just beside the lateral margin of the third ventricle in an ovoid area comprising three to four layers (Finley, 1981). Axons projecting from these cells course laterally from the periventricular nucleus and turn to run caudally toward the median eminence where they terminate at the *zona externa* (Epelbaum, 1992). In addition to projecting to the median eminence, SRIF neurons within the periventricular nucleus project to the arcuate nucleus (Epelbaum, 1992) and to various structures within the limbic system (Kirsch, 1979). The vast majority of the remainder of hypothalamic SRIF immunopositive neurons are localized in the arcuate nucleus, the parvocellular portion of the paraventricular, and in the ventrolateral part of the ventromedial nucleus (Finley, 1981; Johansson, 1984). The neurons in these other hypothalamic nuclei do not, however, project significantly to the median eminence. Nonetheless, they could be involved in the central modulation of growth hormone secretion as well as thyroid stimulating hormone and prolactin secretion.

Elsewhere in the diencephalon, SRIF like immunoreactivity has been observed in the epithalamus and subthalamus; the thalamus, however, is largely devoid of SRIF immunopositive structures (Johansson, 1984; Finley, 1981).

High numbers of SRIF-positive cell bodies are found in the zona incerta (Johansson, 1984). High concentrations of SRIF immunoreactive perikarya are also observed in the central nucleus amygdala (Finley, 1981). In the cerebral cortex, somatostatinergic immunoreactivity is found in cell bodies in layers II-VI (Finley, 1981); the greatest aggregation of immunopositive cells was, however, found in layers V and VI (Finley, 19981; Patel, 1992).

The hippocampus contains numerous SRIF immunoreactive cell bodies at all rostral to caudal levels. Specifically, the majority of SRIF-positive cells are observed in the outer part of the stratum oriens; fewer SRIF immunopositive cells are found within the pyramidal cell layer and the stratum radiatum (Johansson, 1984). The hilus of the

dentate gyrus also contains large numbers of immunoreactive perikarya (Johansson, 1984).

In the rhombencephalon, the cranial nerve nuclei, including the nucleus tractus solatarious, nucleus gracilis, nucleus cuneatus, the trigeninal nucleus, and the facial nucleus generally contain only low numbers of SRIF immunoreactive cell bodies (Johansson, 1984). A notable exception is the dorsal cochlear nucleus that contains high numbers of somatostatinergic cell bodies (Johansson, 1984).

Several regions also contain somatostatinergic fibers. Fibrous networks strongly immunoreactive for SRIF are found in the arcuate, ventromedial, and medial preoptic hypothalamic nuclei (Johansson, 1984; Finley, 1981). The zona incerta contains low to moderately dense SRIF-positive fibrous networks (Johansson, 1984).

A dense patchy network of SRIF fibers is found within the lateral habenula, whereas only a weak network of fibers is observed within the medial habenula; neither region contains immunopositive cells (Johansson, 1984). The somatostatinergic projection to the habenula arises from the preoptic part of the periventricular nucleus (Epelbaum, 1994).

SRIF-positive fibers also form a dense network in the outer part of the molecular layer of the dentate gyrus of the hippocampus with decreasing intensity toward the middle part (Johansson, 1984). A high density of immunoreacive fibers is also found within the stratum lacunosum (Johansson, 1984). Somatostatinergic innervation of the hippocampus is via projections from the periventricular cell group that course in the superior fornix (Epelbaum, 1994).

In the mesencephalon, the substantia nigra contains low numbers of thick somatostatinergic fibers in the pars compacta and single fibers in the pars reticulata (Johansson, 1984).

Besides its neuroendocrine function, and in keeping with its widespread distribution in the central nervous system structures, SRIF plays a role in a number of central functions in the mammal. These effects are heterogeneous and often contradictory.

At the cellular level, it appears that SRIF has both stimulatory and inhibitory effects on the electrophysiological activity of neurons (Epelbaum, 1986). In a detailed

study of cortical neurons, Delfs and Dichter demonstrated that some neurons were directly inhibited by SRIF and that others were stimulated by the peptide (Delfs, 1983). Recently, SRIF has been shown to hyperpolarize CA1 pyramidal hippocampal neurons by a mechanism involving the production of an arachadonic acid metabolite (Schweitzer, 1993). It has been suggested that SRIF exerts its central stimulatory effects by disinhibiting other neurotransmitter defined systems such as gamma-aminobutyric acid (Robbins, 1985).

At the biochemical level, SRIF has been shown to influence various neurotransmitter systems. It inhibits noradrenalin and potassium induced thyrotropin-releasing hormone release (Epelbaum, 1986). Dopaminergic turnover in the limbic system and the striatum is increased following intracerebroventricular administration of SRIF (Chesselet, 1983; Epelbaum, 1986). Acetylcholine turnover in the diencephalon and the hippocampus is also increased following intracerebroventricular administration of SRIF (Epelbaum 1986).

A number of reports indicate that SRIF can have a multitude of often contradictory behavioural effects. SRIF has been reported to both decrease (Segal, 1974) and increase (Cohn, 1975; Havlicek, 1976) locomotor behaviour in a number of different paradigms. The peptide has also been reported to induce analgesia (Epelbaum, 1992) and sleep (Brown, 1975). Finally, given the peptide's localization in the neocortical areas and within the limbic system, it is not unexpected that SRIF has been implicated in learning and memory (Bell, 1995).

In addition to its role in the normal adult central nervous system, a role for SRIF as a trophic factor during neurodevelopment has been suggested (Epelbaum, 1992).

Abnormal SRIF function has been implicated as being pathognomonic in a number of central nervous system disorders including Alzheimer's disease, Huntington's disease, temporal lobe epilepsy, and depression (Patel, 1992).

Somatostatin Receptors

The multitude of biological responses evoked by SRIF are mediated by membrane bound, ligand specific receptors on responsive cells. In 1978 Schonbrunn and Tashjian described high affinity functional binding sites for SRIF in cultured GH_4C_1 rat anterior pituitary cells using ^{125}I-Tyr^{11}-SRIF (Schonbrunn, 1978). It is now well established that the binding sites for SRIF, originally described using radioactive SRIF analogues, correspond to functional SRIF receptors.

Classification and Pharmacology

The SRIF receptors can be grouped into two subclasses based upon their respective affinities for various SRIF analogues in radioligand binding studies. Modifications to the ring structure of SRIF have produced several analogues that have helped to define and classify the two subclasses of SRIF receptors (Tran, 1985). These analogues comprise the biologically active central ring of SRIF but, unlike the endogenous peptide, have relatively stable amino acid sequences (Epelbaum, 1992). The SRIF analogues are not only less susceptible to peptidase activity, and therefore have longer half lives, but also have distinct affinities for the different SRIF receptor subtypes (Epelbaum, 1992). In the brain, shorter synthetic SRIF analogues such as octreotide competed with ^{125}I-SRIF for SRIF binding sites in a biphasic manner, whereas in other tissues, including the pituitary gland, the displacement of ^{125}I-SRIF from SRIF binding sites by octreotide binding curves was monophasic (Reubi, 1984; Tran, 1982). While ^{125}I-SRIF binds with the same affinity in all tissues, the use of octreotide in binding studies demonstrated that, in the brain, there are at least two pharmacological subclasses of SRIF receptors, one with a nanomolar (type A or 1 subfamily) and the other with a micromolar (type B or 2 subfamily) affinity for octreotide (Reubi, 1984). Although SRIF-28 has been shown in transfected cell systems to have a slightly greater affinity than SRIF-14 for the SRIF receptors (Raynor, 1993), both peptides at nanomolar concentrations are full agonists of both subclasses (Moyse, 1989).

Results from functional assays also argue in favour of the concept of receptor heterogeneity. The SRIF analogues octreotide, MK678, and RC 160 inhibit growth hormone and glucagon release much more potently than they inhibit insulin release, while SRIF-28 seems to be more potent than SRIF -14 at inhibiting insulin release from pancreatic β cells (Srikant, 1987). In addition, various halogenated SRIF analogues were equipotent to SRIF-14 with respect to inhibiting gastric acid secretion, but were found to be more potent than SRIF-14 at inhibiting GH secretion (Meyers, 1978).

Mechanisms of Action

SRIF receptors are known to exert biological activity via transmembrane signal transduction pathways (Patel, 1992). The type 1 subclass of receptors is negatively coupled, via G_i, to the membrane bound enzyme adenylate cyclase, and thus, ligand binding inhibits the formation of cyclic adenosine monophosphate from adenosine triphosphate (Bell, 1993). Furthermore, because SRIF can block the secretion of hormones (other than growth hormone) that use a cyclic adenosine monophosphate independent secretory pathway, it is believed that guanine triphosphate insensitive- SRIF receptors may be coupled to potassium channels by G_k (Patel, 1992) and/or to calcium channels by other yet unclassified G proteins (Yajima, 1986). Accordingly, they are able to hyperpolarize cellular membranes by increasing the potassium current, and reduce, either directly or indirectly, the concentration of intracellular calcium needed for granule exocytosis (Yajima, 1986). Finally, SRIF receptors may be positively coupled to a membrane associated tyrosine phosphatases (Patel, 1990).

Molecular Biology of SRIF Receptors

Attempts to isolate and purify SRIF receptors from central and peripheral sources produced inconclusive results. It is, however, generally accepted that SRIF receptors are heavily glycosylated proteins that lack disulphide bridges (Epelbaum, 1992) and belong to the G protein-coupled seven transmembrane domain receptor superfamily (Reisine,

1995). Five SRIF receptor subtypes have recently been identified and shown to couple to guanine nucleotide binding proteins (Reisine, 1995) and to multiple effector systems including adenylyl cyclase, ion channels, and tyrosine phosphatases (Bruns, 1995; Delesque, 1995; Kleuss, 1995). It is believed that the carbohydrate component of the receptors may be involved in mediating high affinity ligand binding (Rens-domiano, 1992).

Recent molecular biological studies have demonstrated the existence of five SRIF receptor subtypes with distinct regional distributions (Bruno, 1993; Epelbaum, 1994). These cloned receptors have been termed sst_1 (Yamada, 1992), sst_2 (Yamada, 1992), sst_3 (Yasuda, 1992), sst_4 (Bruno, 1992), and sst_5 (O'Carrol, 1992) according to the order in which they were isolated. The sst_2 receptor has two isoforms generated from the same gene; sst_2A, the unspliced form, and sst_2B, a 23 amino acid shorter splice variant (Epelbaum, 1994; Kong, 1994; Reisine, 1994). Sequence and hydropathy analyses have confirmed that the SRIF receptors contain seven putative transmembrane domains characteristic of other G protein-coupled receptors (Bell, 1993; Epelbaum, 1994); they are most closely related to the opioid receptor family, with whom they have 30% sequence homology (Yasuda, 1992). The cloned SRIF receptors have a high degree of amino acid and structural homology amongst themselves, however, it is believed that they may represent a unique neurosecretagogue receptor subfamily as their sequences differ from any other known receptors (Bell, 1993). It should be noted that interspecies differences in SRIF receptors exist. However, in general, there is greater than 90% amino acid sequence homology between the same receptor subtype in different species and between 35% and 60% identity between different receptor subtypes in the same species suggesting genetic conservation (Epelbaum, 1994).

On the basis of earlier studies described above, SRIF receptors were subdivided into two pharmacological subclasses. More recent pharmacological studies in transfected cells have demonstrated that the sst_2A/B, sst_3, and sst_5 receptors correspond to the type 1 subclass in that they display a relatively high affinity for the biologically stable synthetic SRIF agonists MK678 and octreotide and are sensitive to GTP and divalent cations (Epelbaum, 1994). The sst_1 and sst_4 receptors comprise the type 2 subclass that displays

relatively lower affinity, and lacks selectivity, for MK678 and octreotide and is unlikely to be coupled to adenylate cyclase (Epelbaum, 1994). The heterogeneity in SRIF receptor subtypes and the differential expression of these subtypes in various tissues allows SRIF to potentiate a plethora of diverse biological actions

SRIF Binding in the CNS

The regional and subregional distributions of SRIF binding sites in the rat brain have been reported using film and emulsion radioautography after incubation with various iodinated or tritiated SRIF analogues (Krantic, 1992). There is by and large concordance between the endogenous peptide localization and concentration and the distribution of binding sites (Epelbaum, 1993). The distribution of binding sites in the telencephalon and diencephalon of the rat is described summarily below.

The localization of SRIF binding sites in the hypothalamus of the adult rat varies both between and within the different nuclei. In the anterior hypothalamus, moderate concentrations of SRIF binding sites have been reported in the preoptic area (Uhl, 1985). The anterior hypothalamic area, according to many studies, does not comprise SRIF binding sites (Leroux, 1985; Reubi, 1985; Uhl, 1985). The periventricular (Uhl, 1985), paraventricular (Leroux, 1985), and supraoptic (Reubi, 1985; Uhl, 1985) nuclei are moderately enriched with SRIF binding sites.

In the mediobasal hypothalamus, SRIF binding sites are found in the retrochiasmatic area where they form a small oval cluster between the base of the third ventricle, and the caudal fibers of the optic chiasm (Epelbaum, 1989). Within the arcuate nucleus, labeled foci are mainly clustered within the ventral portion of the nucleus with a small number of binding sites lying more laterally along the base of the brain (Epelbaum, 1989). The density of binding sites within the arcuate nucleus intensifies along the rostral-caudal extent of these structures (Epelbaum, 1989). Interestingly, the distribution of SRIF binding sites in the arcuate nucleus conforms closely with the distribution of GRF- positive neurons in this nucleus (Epelbaum, 1989). Simultaneously, McCarthy *et al.* (McCarthy, 1992), and Bertherat *et al.* (Bertherat, 1992) demonstrated that growth

hormone-releasing hormone neurons are a subpopulation of SRIF receptor- expressing cells in the arcuate nucleus of the rat. Approximately one third of growth hormone-releasing hormone immunopositive neurons in the arcuate nucleus were reported to bind ^{125}I-SRIF (McCarthy, 1992). The ventromedial nucleus and the median eminence do not contain significant numbers of binding sites (Reubi, 1985; Uhl, 1985).

Within the borders of the posterior hypothalamus, the lateral hypothalamic area was found to contain low (Uhl, 1985) to moderate (Leroux, 1985) densities of SRIF binding sites. The mammillary nucleus was found to contain a low density of binding sites in a single study (Uhl, 1985).

Moderate levels of binding of SRIF have been described in the caudate-putamen and the thalami nuclei (Epelbaum, 1993). In addition to the hippocampus, other limbic structures, including the basolateral amygdaloid nucleus, the medial habenula, the interpeduncular nucleus, the septum, and the bed nucleus of the stria terminalis, also contain dense accumulations of SRIF binding sites (Reubi, 1985; Epelbaum, 1993). The lateral habenula, by contrast, contained very few binding sites (Katayama, 1990).

The pattern of labeling observed across the subregions of the hippocampal formation is also heterogeneous. Whereas the molecular and granular cell layers of the dentate gyrus, the subiculum, and all areas but the pyramidal layer of the CA1 field of Ammon's horn of the hippocampus, contain high concentrations of SRIF binding sites, the CA3 region is virtually devoid of SRIF binding sites (Reubi, 1985; Epelbaum, 1993). While a high density of binding sites was observed in the stratum oriens, moderate binding intensity was observed in both the lacunosum molecular and radiatum layers (Katayama, 1990).

A distinct heterogeneous pattern of binding is observed in the cerebral cortex. The deeper cortical layers (V and VI) (Epelbaum, 1993) are highly enriched with SRIF receptors, whereas the more superficial layers contain only a modicum of SRIF binding sites (Reubi, 1985).

Somatostatin binding sites are generally distributed uniformly between neuronal perikarya and the surrounding neuropil, thereby making it difficult to differentiate between somatodendritic and axonal labeling (Epelbaum, 1993). However, in a few areas,

including the arcuate nucleus of the hypothalamus, SRIF binding is selectively localized over neuronal perikarya (Epelbaum, 1993).

Tissue Distribution of the Somatostatin Receptor Subtypes

To determine the potential role that each SRIF receptor subtype plays in mediating the numerous biological effects of SRIF, it is necessary to understand not only the signal transduction pathways utilized by the different receptors and the distribution of SRIF binding sites, but also to have an accurate understanding of the anatomical pattern of expression of each receptor subtype. To this end, a number of studies focusing on the central effects of SRIF have examined the regional distributions of the SRIF receptor subtypes in the central nervous system and pituitary gland.

Pituitary

The pituitary gland has been found, using *in situ* hybridization histochemistry, to express both the sst_2 and sst_3 receptor mRNAs, but not sst_1 mRNA (Senaris, 1994). It is believed, based upon pharmacological data from studies using MK-678, and octreotide, that the inhibition of growth hormone secretion, at the level of the anterior pituitary, is governed by the sst_2 and/or sst_5 receptors (Hoyer, 1994).

Central Nervous System

Using solution hybridization/ nuclease protection analysis with sequence specific cRNA probes, Bruno and colleagues recently described the tissue distribution of the mRNA encoding the five known receptor subtypes in the rat brain. (Bruno, 1993). They reported that sst_1 and sst_2 receptor mRNAs were widely distributed within the central nervous system with the highest levels of expression in the hippocampus, hypothalamus, cortex, and amygdala (Bruno, 1993). mRNA corresponding to the sst_3 receptor was found throughout the central nervous system with the notable exception of the cerebellum that was devoid of sst_3 receptor hybridization signal (Bruno, 1993). Other authors have,

however, based upon *in situ* hybridization histochemistry using oligoprobes, reported that sst_3 mRNA is heavily concentrated in the granule cell layer of the cerebellum; it is believed that these receptors lie on inhibitory interneurons that modulate the activity of the Purkinje neurons (Perez, 1994). sst_4 receptor mRNA exhibited a widespread distribution with the highest levels of expression in the hippocampus and the cortex (Bruno, 1993), however, it is also found in high concentrations in the lateral habenula (Bell, 1995). The sst_5 receptor was unique in that its mRNA was highly localized within the hypothalamus and preoptic area (Bruno, 1993).

Kong and colleagues (Kong, 1994) determined the tissue distribution of the sst_1 sst_2A/B and sst_3 receptor mRNAs by RNA blotting using specific cDNA probes. They reported that the highest concentrations of sst_1 mRNA were detected in the cerebral cortex, hippocampus, and hypothalamus (Kong, 1994). All tissues found to express sst_2 contained both forms (A and B) of this receptor subtype; sst_2B was, however, by comparison to sst_2A, expressed at much higher levels in the hypothalamus (Kong, 1994). sst_3 mRNA was detected in moderate to high concentrations in the cerebral cortex, cerebellum, hippocampus, midbrain, and hypothalamus by these authors (Kong, 1994). Kong *et al.* found, by *in situ* hybridization histochemistry, that the sst_2 mRNA detected in the hypothalamus was localized within the ventromedial and dorsomedial hypothalamic nuclei (Kong, 1994). They did not, however, report the detection of the sst_1 or sst_2 receptor mRNAs in the hypothalamus by *in situ* hybridization histochemistry.

The localization of the sst_1, sst_2, and sst_3 receptor mRNAs within the hypothalamus of the rat, as detected by *in situ* hybridization using oligoprobes, were described, in brief, by Perez *et al.* (Perez, 1994). These authors found that the anterior hypothalamus, the median eminence, the suprachiasmatic, arcuate, and ventromedial nuclei expressed sst_3 receptor mRNA (Perez, 1994). sst_1 receptor mRNA was expressed in the supraoptic, ventromedial, and arcuate nuclei, as well as in the median eminence. sst_2 receptor mRNA was reported to be localized within the ventromedial and arcuate nuclei (Perez, 1994). Perez *et al.* reported that the periventricular hypothalamic nucleus did not contain sst_1, sst_2, or sst_3 receptor mRNA (Perez, 1994).

The mRNA encoding the various SRIF receptor subtypes thus showed both a neuroanatomically specific and overlapping pattern of expression. In light of the disagreement between various reports on the distribution of the sst_1 and sst_2 probes in the hypothalamus (Breder, 1992; Bruno, 1993; Kong, 1994; Perez, 1994) and the lack of a detailed description of the regional and cellular distributions of these receptor subtypes within the hypothalamus, the present study was undertaken to elucidate the potential physiological role of the individual receptor subtypes in the transduction of SRIF's neuroendocrine actions in the brain.

G Protein-Coupled Receptors

A plethora of transmembrane spanning receptors for hormones and neurotransmitters are coupled to guanine nucleotide binding proteins. Upon receptor-ligand interaction, these receptors stimulate or inhibit various second messenger effector systems. The superfamily of G protein-coupled receptors is characterized by a seven transmembrane domain spanning structure, and includes the adrenergic, muscarinic, dopaminergic, most serotoninergic, and all known peptide receptors (Mantyh, 1995; Strosberg, 1991).

Binding of a ligand to its G protein-coupled receptor induces a conformational change in the receptor and catalyzes the exchange of a bound guanine diphosphate for a guanine triphosphate (Darnel, 1991; Strosberg, 1991). Once activated by the exchange, the three subunits of the G protein, $G\alpha$, $G\beta$, and $G\gamma$ dissociate (Darnel, 1991); the $G\alpha$ subunit goes on to modulate effector function (i.e. activation or inhibition of adenylate cyclase) (Strosberg, 1991). Finally, the cycle is completed when the bound guanine triphosphate is hydrolyzed to guanine diphosphate and the three subunits of the protein complex reassociate (Darnel, 1991).

G Protein-Coupled Receptor Agonist Internalization

Data from studies on cultured cells expressing various G protein-coupled receptors indicate that numerous G protein-coupled receptors undergo phosphorlyation, endosomal internalization, ligand dissociation/degradation, dephosphorlyation, and finally receptor recycling to the plasma membrane (Caron, 1993; Kobilka, 1992; Lefkowitz, 1993; Senogles, 1990). Moreover, several studies over the last decade in cell culture systems and in tissue preparations have demonstrated, both biochemically and morphologically, that various ligands interacting with G protein-coupled receptors are internalized. Muscarinic cholinergic agonists (Strosberg, 1991), β–adrenergic agonists (von Zastrow, 1992), glucagon (Amherdt, 1989), substance P (Mantyh, 1995), thyrotropin-releasing hormone (Ashworth, 1995), and neurotensin (Mazella, 1991) have all been shown to be internalized following agonist occupation of the ligand binding site on their respective G protein-coupled receptors.

The precise mechanisms involved in G protein-coupled receptor endocytosis and ligand internalization are still largely unknown. It appears that some receptors may be internalized via a mechanism involving clathrin coated pits, whereas other receptors appear to be internalized by a mechanism that does not involve coated pits. For example, in human epidermoid carcinoma A-431 cells, β_2-adrenergic receptors undergo internalization in non-clathrin coated vesicles (Raposo, 1989), whereas thyrotropin-releasing hormone appears to be internalized with its receptor via a mechanism involving clathrin coated pits in GH-Y rat pituitary cells (Ashworth, 1995). Just what causes such fundamental differences in the mechanism of internalization utilized by structurally homologous receptors remains unclear.

The internalization process is believed to subserve a number of different biological functions. Internalization of receptor- ligand complexes renders these complexes inaccessible to membrane impermient ligands; they are therefore sequestered following internalization (Ashworth, 1995). Sequestration may be important for desensitization of receptors and to facilitate the reactivation of the receptor following

ligand dissociation/degradation (Ashworth, 1995). Internalization may also represent a mechanism for clearing ligand from the extracellular space, for degrading the ligand, and thus, for terminating the action of the ligand (Posner, 1986). Internalization of receptor agonists may represent the initial step involved in targeting ligands to the nucleus. Nuclear binding sites for G protein-coupled receptor agonists have been described for gonadotropin-releasing hormone (Millar, 1983), angiotensin-II (Re, 1984), and SRIF (Todisco, 1994). It is believed that these and other peptides may act directly at the nuclear level, like steroid hormones, to alter gene expression (Morel, 1994).

Internalization of G Protein-Coupled Receptor Agonists in the Brain

Recent evidence suggests that peptide neurotransmitters that act via G protein-coupled receptors may be internalized following interaction with their respective neural receptors. To date, two such neurotransmitters have been identified in the central nervous system. Neurotensin has been found to be internalized and mobilized in neurons both *in vitro* (Mazella, 1991) and *in vivo* (Beaudet, 1994; Castel, 1994; Faure, 1995a), and Mantyh and colleagues reported, just a few months ago, that substance P evokes internalization of its receptor- ligand complex in the striatum *in vivo* (Mantyh, 1995).

Neurotensin

That neurotensin is internalized in neurons was suggested by several lines of evidence. Firstly, neurotensin receptor agonists induce a decrease in the number of cell surface receptors expressed in cultured rat neurons (Vanisberg, 1991). In addition, intrastriatal administration of iodinated neurotensin leads to an accumulation of radiolabeled peptide in the ipsilateral substantia nigra pars compacta (Castel, 1990). This phenomenon was shown to be receptor- mediated, as it was abolished by co-injection of an excess of non-radioactive peptide, and microtubule-dependant, as it was ablated by the intracerebroventricular administration of colchicine (Castel, 1990).

In 1991, Mazella *et al.* demonstrated that neurotensin binds and is internalized by embryonic neurons from mouse brain in culture (Mazella, 1991). Subsequently, it was found that neurotensin is also internalized by a hybrid cell line (SN17) derived from septal cholinergic cells (Faure, 1992). That neurotensin is internalized along with its G protein-coupled receptor was demonstrated in the SF9 insect line transfected with the cDNA encoding the high affinity neurotensin receptor (Faure, 1995a). Cross-linking of a nitro-azido derivative of neurotensin to cell surface receptors at 4°C and raising the temperature to 37°C did not prevent ligand from entering the cells as would be predicted if the ligand was internalized without it receptor (Faure, 1995a).

The cellular mechanisms responsible for the internalization of neurotensin in neurons have begun to be elucidated. (Beaudet, 94). Beaudet *et al.* (Beaudet, 1994) demonstrated, both biochemically (using acid-washes) and anatomically (by confocal microscopy) that, like for other ligands that function via G protein coupled receptors and are internalized in peripheral tissue, neurotensin is internalized in neurons both *in vitro* and *in vivo*. Neurotensin is internalized in a time- and temperature- dependent fashion; maximal internalization occurs at a temperature of 37°C and an incubation duration of between 40 and 60 minutes in SN17 cells (Beaudet, 1994). The internalization process is believed to involve endocytosis as it is inhibited by phenylarsine oxide (Faure, 1995b). Moreover, internalization was found to be receptor- mediated as it: (1) was found to be concentration dependent (Faure, 1995b); (2) to reach a half maximal effect at a ligand concentration of 0.3 nM which corresponds to the K_D of ^{125}I-neurotensin binding to neurons in the presence of phenylarsine oxide (Beaudet, 1994); (3) is prevented by co-injection of cold neurotensin (Castel, 1990); (4) occurs selectively in neurons known to express high affinity neurotensin binding sites (Beaudet, 1994); and (5) is abolished by mutations in the amino acid sequence of the C terminus of the receptor (Chabry, 1995) .

Confocal microscopic analysis of serial optical sections of neurons in the substantia nigra pars compacta of animals injected with fluoresceinyl-thiocarbamy-[Glu^1]neurotensin in the caudate- putamen suggest that, once internalized, receptor-ligand complexes are sequestered within granular compartments (Beaudet, 1994). Electron microscopy revealed that these granular compartments may correspond to endosomal and

lysosomal elements (Beaudet, 1994). This observation is consistent with the report by Ashworth indicating that thyrotropin-releasing hormone is similarly sequestered following internalization (Ashworth, 1995). Likewise, these data (Beaudet, 1994) are concordant with contemporary models of receptor- mediated endocytosis in which ligands are sequestered in endosomal compartments where they dissociate from their receptors; ligands may then be recycled via the endoplasmic reticulum-Golgi apparatus pathway or targeted to lysosomes for degradation (Shepherd, 1989).

Neurotensin was found to be mobilized following internalization. Cellular migration of internalized neurotensin from distal processes to neuronal cell bodies, and from the periphery to the perikaryon was observed in the substantia nigra (Beaudet, 1994; Castel, 1994). Whereas confocal microscopic studies indicated that internalized neurotensin clusters around the nuclear membrane without penetrating it, electron microscopic investigations indicated that internalized neurotensin can be mobilized into the nucleus (Beaudet, 1994), a discrepancy that needs clarification. The possibility that neurotensin, like angiotensin-II (Re, 1984), may be internalized by its G protein- coupled receptor and subsequently alter the transcriptional activity of the cell cannot therefore be excluded.

Substance P

Substance P evoked internalization of its G protein-coupled receptor in the rat striatum occurs within one minute of a striatal injection of substance P (Mantyh, 1995). Approximately 60% of substance P receptor immunoreactive neurons at the site of the injection internalized the injected neuropeptide (Mantyh, 1995). In dendrites, substance P is mobilized from the plasma membrane into endosomes (Mantyh, 1995). Internalization of substance P is receptor- mediated as: (1) the quantity of peptide internalized is dependent upon the concentration of peptide injected (Mantyh, 1995), and (2) internalization is blocked by co-injection of the neurokinin 1 receptor antagonist RP-67,580 (Mantyh, 1995).

Internalization of somatostatin

Whether or not SRIF is internalized following ligand-receptor interaction is controversial. Several reports indicate that SRIF is internalized in pancreatic (Amherdt, 1989; Viguerie, 1987), hypophyseal (Draznin, 1986; Morel, 1986), and human carcinoid tumor (Lamberts, 1995) cells following application of exogenous SRIF ligands. Other reports, to the contrary, indicate that SRIF is not internalized to an appreciable extent in either GH_4C_1 pituitary cells (Presky, 1986) or in RINm5F insulinoma cells (Sullivan, 1986). Nevertheless, *in vivo* peptide scintigraphic data (Breeman, 1995) argue in favour of internalization.

Draznin *et al.* initially demonstrated that SRIF can be internalized (Draznin,1985) after its interaction with G protein-coupled receptors on anterior pituitary cells in primary culture. Using gold conjugated SRIF (G-SRIF), these authors demonstrated that the internalization process was both time- and temperature- dependent (Draznin, 1985). After two minutes of incubation with G-SRIF at 37°C, the ligand was found, by electron microscopy, to be distributed over the plasma membrane; there was virtually no internalization (<2.8% of total cell- associated radioautographic grains found intracellularly) at this time (Draznin, 1985). After twenty minutes of incubation with G-SRIF at 37°C, 12% of total cell- associated SRIF was found localized intracellularly and to be associated with coated vesicles, intermediate sized vesicles, lysosomes, and the Golgi apparatus (Draznin, 1985). When incubations were carried out at 4°C, G-SRIF was not found to enter the hypophyseal cells (Draznin, 1985). Moreover, the process was shown to be receptor- mediated as it could be reduced with an excess of non- radiolabeled ligand (Draznin, 1985).

Subsequently, Morel and colleagues demonstrated that adrenocorticotropin secreting mouse tumor AtT-20 cells, which are known to express the SRIF receptors, internalize radiolabeled SRIF-28 (Morel, 1986). When AtT-20 cells are exposed to SRIF for prolonged periods of time, there is a marked diminution in receptor density (Morel, 1986). It was found that this ligand- induced receptor down regulation occurred

concomitantly with internalization of SRIF (Morel, 1986). Presumably SRIF was internalized as a receptor- ligand complex as internalization was inhibited by 80% when cells were coincubated with the radiolabeled peptide an excess of non- radiolabeled SRIF (Morel, 1986) and because other reports indicate that the SRIF-14 membrane receptor density is decreased by 40% following an extended exposure to SRIF (Heisler, 1985; Srikant, 1985). Consistent with the report by Draznin *et al.* (Draznin, 1985), Morel *et al.* (Morel, 1986) found that time- dependent labeling patterns were observed for the plasma membrane, secretory granules, lysosomes, the Golgi apparatus, and the nuclear membrane. Whereas plasma membrane labeling was maximal after only 1 hour of ligand exposure, lysosomal labeling was maximal after 18 hours of exposure (Morel, 1986). The finding that the nuclear membrane can be labeled with a SRIF analogue (Morel, 1986) suggests that SRIF, like angiotensin-II (Re, 1984) may be internalized by its G protein-coupled receptor in order to effect the transcriptional activity of the cell.

Somatostatin binding sites have been reported to be present on the surface of pancreatic acinar cells (Esteve, 1984). SRIF binds to these sites with a high affinity (24±1.1 fmol/mg protein) and is rapidly internalized following an initial 5 minute lag period (Viguerie, 1987). A plateau, corresponding to internalization of 20.4±1.3% of total cell-associated specific radioactivity, was achieved at 45 minutes in this assay (Viguerie, 1987) that employed the widely accepted acid-washing protocol of Haigler (Haigler, 1980). As was the case with the previous reports of SRIF internalization, the process of internalization was diminished when an excess of unlabeled SRIF was added to the incubation medium containing the radiolabeled peptide (Viguerie, 1987). Internalization was also shown to be saturable with a half maximal concentration for internalization equal to 0.4 nM (Viguerie, 1987). Finally, internalization was found to be energy dependent as the amount of radioactivity internalized was reduced to 4 ± 1.5% of cell-associated specific radioactivity when the temperature of the incubation was lowered to 5°C (Viguerie, 1987).

Amherdt and colleagues also demonstrated that SRIF is internalized by neonatal rat islet cells (Amherdt, 1989). Following binding to membrane-associated SRIF

receptors, radiolabeled SRIF-14 is internalized in a time- and temperature- dependent fashion in β and α cells (γ cells internalized SRIF poorly) with maximal internalization observed at 37°C and 60 minutes (Amherdt, 1989). Consistent with earlier reports (Draznin, 1985; Morel, 1986), endocytosed SRIF was found to be processed intracellularly as indicated by the time dependent pattern of labeling observed over various subcellular compartments including endocytotic vesicles, lysosomes, secretory granules, and the Golgi apparatus (Amherdt, 1989). A notable exception to the concordance between the three studies is the nuclear membrane labeling reported by Morel *et al.* (Morel, 1986) which was not reported by either Draznin (Draznin, 1985) or Amherdt (Amherdt, 1989). Interestingly, the report by Amherdt was the first to demonstrate that a peptide hormone can be internalized by a cell that normally secretes it (Amherdt, 1989).

By contrast, no significant internalization of SRIF was observed with GH_4C_1 cells, a clonal strain of rat pituitary tumor cells that possesses high affinity SRIF receptors (as well as epidermal growth factor (EGF) receptors) (Presky, 1986). At temperatures ranging from 4°C to 37°C and times from 1 to 5 hours, 86 ± 4% of specifically bound [^{125}I-Tyr^{1}]SRIF was acid extractable (i.e. associated with the cell surface) (Presky, 1986). These data, as well as data indicating that thyrotropin-releasing hormone is internalized by GH_4C_1 cells (Hinkle, 1982) suggest that while GH_4C_1 cells possess the capacity to internalize some peptide hormones, they do not appreciably internalize SRIF.

Similarly, RINm5F insulinoma cells fail to copiously internalize SRIF (Sullivan, 1986). Greater than 80% of saturably bound radiolabeled SRIF could be displaced from RINm5F cells independent of the time and temperature of the incubation by washing them with an acidic buffer (Sullivan, 1986).

Why some SRIF receptors internalize relatively large quantities of SRIF and others do not is unclear. Differences in the amino acid sequences of the receptors, particularly in their C termini, might contribute to the observed differences as mutations to the C terminus of the neurotensin receptor have been reported to alter the receptor's potential to internalize neurotensin (Chabry, 1995). Similarly, C terminal amino acid

sequences have been reported to be critical for ligand internalization mediated by the thyrotropin- releasing hormone (Nussenzveig, 1993), gastrin- releasing peptide (Benya, 1993), and the β2-adrenergic (Barak, 1994) receptors. The various SRIF receptors are known to couple to different G proteins (Markstein, 1989). Differential coupling to distinct G proteins by different receptor subtypes, or by the same receptor subtype in different cell types, might similarly affect the efficiency of internalization.

SPECIFIC OBJECTIVES

Although all five SRIF receptor subtypes are known to be expressed to variable extents in the rat hypothalamus, little is known about the sub-regional and cellular localization of each subtype. In hopes of elucidating the potential physiological role of the individual receptor subtypes in the transduction of SRIF's neuroendocrine actions in the brain, we investigated the distributions of the mRNA for the sst_1 and sst_2 receptors in the hypothalamus of the adult rat by *in situ* hybridization histochemistry.

In addition, given that internalization of G protein-coupled receptors has recently been demonstrated in the brain, we investigated whether or not SRIF is internalized by cells in the rat brain. To this end, an *ex vivo* system that could provide both biochemical and anatomical data respecting the internalization of SRIF was developed.

In the first set of experiments, film and slide autoradiograms previously processed for the *in situ* hybridization detection of the mRNAs encoding the sst_1 and sst_2 receptor subtypes in the rat brain were analyzed to determine the regional and cellular distributions of these two receptors. Light macroscopic analysis of film autoradiograms permitted us to map the regional distributions of the two receptor subtypes within the nuclei of the hypothalamus and to semi-quantitatively assess the regional density of expression of each subtype. Light microscopic analysis of slide autoradiograms allowed us to define the cellular distribution of the sst_1 and sst_2 receptors in the nuclei of the rat hypothalamus involved in the neuroendocrine regulation of GH secretion. The findings of these experiments have now been published (Beaudet, 1995).

In the second set of experiments, coronal sections of the rat brain from the level of the optic chiasm, rostrally, to the substantia nigra, caudally, were incubated, *ex vivo*, with ^{125}I-SRIF to attempt to determine indeed if SRIF is internalized by cells in the central nervous system. These experiments employed the biochemical acid washing protocol originally developed by Haigler (Haigler, 1980), and which is now widely used to assay for internalization of ligands into intracellular compartments. After *ex vivo* processing,

the tissue was coated with nuclear emulsion. Autoradiograms were analyzed by a computer- aided image analysis system, and the distribution of internalized radioactivity was assessed.

CHAPTER I

Patterns of Expression of sst_1 and sst_2 Receptors in the Hypothalamus of the Adult Rat: Relationship to Neuroendocrine Function

MANUSCRIPT

PATTERNS OF EXPRESSION OF SSTR1 AND SSTR2 SOMATOSTATIN RECEPTOR SUBTYPES IN THE HYPOTHALAMUS OF THE ADULT RAT: RELATIONSHIP TO NEUROENDOCRINE FUNCTION§

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Abbreviations: III, third ventricle; AMPO, anterior medial preoptic nucleus; Arc, arcuate; DTT, dithiothreitol; GH, growth hormone; GRF, growth hormone-releasing factor; ME, median eminence; MPO, medial preoptic nucleus; Pa, paraventricular nucleus; Pe, periventricular nucleus; PBS, phosphate-buffered saline; RCh, retrochiasmatic nucleus; SRIF, somatostatin; TE, Tris-EDTA; TMC, tuberal magnocellular nucleus.

ABSTRACT

The neuropeptide SRIF is the major physiological inhibitor of growth hormone secretion. With the aim of identifying the receptor subtypes through which this neuropeptide may be èxerting its neuroendocrine actions in the brain, we have examined by *in situ* hybridization the distribution of the mRNA for SSTR1 and SSTR2 isoforms in the hypothalamus of adult male and female rats. Both receptor subtypes were highly expressed in the medial preoptic area, suprachiasmatic nucleus and arcuate nucleus. High SSTR1, but low SSTR2, expression was evident in the para- and periventricular nuclei as well as in the ventral premammillary nucleus. Conversely, moderate to high SSTR2, but low SSTR1, mRNA levels were detected in the anterior hypothalamic nucleus, ventromedial and dorsomedial nuclei and medial tuberal nucleus. Taken together, these distributional patterns conform to those of SRIF binding sites as visualized by *in vitro* autoradiography, suggesting that an important proportion of SSTR1 and SSTR2 receptors in the hypothalamus are associated with the perikarya and dendrites of intrinsic neurons. The distribution of SSTR1-expressing cells within the periventricular, paraventricular and suprachiasmatic nuclei was similar to that of neurons previously reported to contain and/or express SRIF in the brain suggesting that some of the SSTR1 receptors may correspond to autoreceptors. Within the arcuate nucleus, the distribution of SSTR1 and SSTR2 mRNA-expressing cells was comparable to that of neurons previously found to selectively bind somatostatin-14 within this area. Given that over one third of these cells also contain and express growth hormone-releasing factor, the present findings suggest that both of these receptor subtypes are involved in the central regulation of growth hormone-releasing factor secretion by SRIF. Taken together, the present results suggest that SSTR1 and SSTR2 SRIF receptor mRNAs are heavily expressed in those neurons containing SRIF and/or growth hormone-releasing factor and thereby imply a role for both SSTR1 and SSTR2 SRIF receptor subtypes in neuroendocrine regulation of growth hormone secretion in both sexes of this species.

INTRODUCTION

Somatostatin (SRIF), a tetradecapeptide originally isolated from ovine hypothalamus on the basis of its ability to inhibit growth hormone (GH) release from cultured rat pituitary cells[3], has subsequently been shown to be the major physiological inhibitor of GH secretion (see[39], for a review). The NH_2-terminally extended form of the tetradecapeptide, SRIF-28, which is a naturally occurring hypothalamic peptide[36] also exerts potent, long-acting inhibition of spontaneous GH release[40]. Both SRIF-14 and SRIF-28 induce their GH-inhibitory actions at the level of the pituitary by binding to high-affinity membrane receptors located on pituitary cells[34,37,38]. However, a growing body of evidence indicates that SRIF also regulates GH secretion through its action within the central nervous system[7,11,13,19,20,41]. In keeping with this interpretation is the demonstration, by radioligand binding studies, of moderate to high concentrations of high affinity SRIF binding sites throughout the mediobasal hypothalamus[16,18,43]. Furthermore, double labelling studies have recently provided anatomical evidence for the association of SRIF receptors with a subpopulation of neurons that both contain[22] and express[2] GH-releasing factor (GRF) within the arcuate nucleus of the hypothalamus, thereby providing strong support for the concept of a direct influence of SRIF on the GRF hypothalamo-hypophyseal system.

The existence of multiple SRIF receptor subtypes in mammalian brain was originally postulated on the basis of both biological and pharmacological studies[30,33,38,42]. Two general types of SRIF receptors, SRIF-1 and SRIF-2, emerged as a result of these studies. More recently, molecular biological studies have demonstrated the existence of at least five distinct SRIF receptor subtypes, designated SSTR1 to SSTR5, all of which are widely expressed in brain[5,23,46,47]. Based on their structure and pharmacological profile, these different subtypes may be divided into two main classes of receptors: the pair SSTR1/SSTR4 on the one hand and the SSTR2/SSTR3/SSTR5 group on the other hand. All five isoforms exhibit high affinity for the endogenous ligands SRIF-14 and SRIF-28,

and SSTR1 and SSTR2 appear to be pharmacologically and functionally equivalent to the SRIF-2 and SRIF-1 classes of receptors, respectively[10,32].

While all of these receptors were found to be expressed to a variable extent in both human and rodent hypothalamus, there is marked disagreement between recent reports regarding the pattern of expression of SRIF receptor mRNA within this region of the brain[4,15,27]. Furthermore, little is known on the sub-regional and cellular localization of each receptor type within this structure, particularly with regards to neuronal systems involved in the control of GH secretion. With the aim of elucidating the potential physiological role of the individual SRIF receptor subtypes in the transduction of SRIF's neuroendocrine actions in brain, we have examined the distribution of the mRNA for SSTR1 and SSTR2 in the hypothalamus of the adult rat using *in situ* hybridization histochemistry. In view of the striking sexual dimorphism in pattern of GH secretion in the rat[24], both sexes of this species were studied.

EXPERIMENTAL PROCEDURES

Animals and Tissue Preparation

Adult Sprague-Dawley rats (150-200g) were purchased from Charles River Canada (St. Constant, Quebec), maintained on a 12-hour light/12-hour dark cycle (lights on: 0600 h) and given free access to Purina rat chow and tap water. Animals (5 males and 4 females) were sacrificed under sodium pentobarbitol anesthesia (50 mg/kg ip) by transaortic perfusion with 0.17 M phosphate-buffered saline (PBS) followed by a 4% paraformaldehyde solution in 0.1 M PO_4 buffer, pH 7.4, at room temperature. Brains were then dissected out of the skull, post-fixed by immersion in the same fixative for 2 additional hours at room temperature and cryoprotected overnight by immersion in a 30% sucrose solution in 0.2 M PO_4 buffer at 4°C. Brains were subsequently snap-frozen in isopentane at -40°C and stored at -80°C until used.

Coronal sections, 20 mm-thick, were cut on a cryostat from the nucleus of the diagonal band, rostrally, to the mammillary bodies, caudally, and thaw-mounted on polylysine (50 mg/ml)-coated microscope slides. The sections were then dehydrated in graded ethanols (70%, 95% and 100%; 3 min/bath), air-dried and stored at -80°C until hybridization histochemistry was performed.

Probe Preparation

The original plasmids mSSTR1 and mSSTR2 were generously provided by Dr. Graeme Bell, Howard Hughes Medical Institute, University of Chicago. The mouse SSTR1 probe is a 413 bp *Ban* II-*Acc* I fragment of the gene which encodes amino acids 214-352, and the mouse SSTR2 probe is a 458 bp *BstE* II-*Xba* I fragment of the cDNA which encodes amino acids 254-369, the stop codon and 107 nucleotides of the 3'-flanking/untranslated region. The DNA fragments described above were sub-cloned into the plasmid vector pGEM-3Z (Promega Biotec, Madison, WI). To obtain antisense probes, the plasmids were linearized with *Eco*RI and transcribed with the Gemini II system (Promega Biotec, Madison, WI) using SP6 RNA polymerase and [^{35}S]-uridine 5'-[a-

thio]triphosphate (DuPont-NEN, Boston, MA). Sense probes were prepared from *Hind* III-linearized plasmid DNA using T7 RNA polymerase. Aliquots were stored at -70°C. Prior to use for *in situ* hybridization, the identity and integrity of the transcripts were verified by PAGE against known standards. The final probe specific activity was approximately 2.74 x 10^9 dpm/mg.

In Situ Hybridization

The frozen sections were thawed for 30-45 min at room temperature, rehydrated in graded ethanols (100%, 95%, 70%; 3 min/bath) and washed 3 min in H_2O. To increase the accessibility of the probe to intracellular mRNA strands, sections were then immersed in 0.1% proteinase K in Tris-EDTA (TE) buffer (0.1M Tris HCl, 50 mM EDTA, pH 8) for 30 min at 37°C followed by a rapid rinse in H_2O. To reduce non-specific hybridization, sections were acetylated by incubation for 10 min under agitation in 0.1M triethanolamine, pH 8, containing 0.25% acetic anhydride, incubated at room temperature in 0.1M Tris-glycine pH 8, rinsed 3x5 min in a double concentration of standard saline citrate (2xSSC) and pre-hybridized by immersion for 1 h at room temperature in 4xSSC containing 1xDenhart's and 10mM mercaptoethanol. The sections were then dehydrated in sequence in 70%, 95% and 100% ethanol (5 min/bath) and covered with hybridizing solution (80 ml/3 sections) consisting of the same buffer as used for pre-hybridization, supplemented with 1% sarcosyl, 50% formamide, 10% dextran sulfate, 10mM dithiothreitol (DTT), and $2x10^7$ cpm/ml of the appropriate ^{35}S-labelled probe. They were then coverslipped and incubated overnight at 60°C in humid chambers. Following hybridization, the slides were immersed in 4xSSC to remove the coverslips, rinsed 3x10 min in 4xSSC, incubated 30 min at 37°C in 4xSSC containing 20 mg/ml RNase-A and washed in decreasing concentrations of SSC (x2, x0.5 and x0.1) containing 0.25% DTT (10 min bath at room temperature). They were then rinsed in 0.1xSSC containing 0.25% DTT at 60°C for 30 min, dehydrated in 70% ethanol containing 0.1xSSC and 0.25% DTT and then successively in 95% and 100% ethanol (3 min/bath) and finally dried under a cool stream of air.

To control for non-specific hybridization, additional sections were incubated in parallel with equivalent concentrations of ^{35}S-labelled sense instead of antisense probe. To

control for cross-reactivity, a hundredfold excess of cold SSTR2 or SSTR1 antisense probes were added to the ^{35}S-labelled SSTR2 and SSTR1 probes, respectively.

Autoradiography and Analysis

For film autoradiography, sections were apposed to Beta Max tritium-sensitive film (Amersham) in X-ray cassettes for 1 to 3 weeks of autoradiographic exposure. The films were developed in GBX (Eastman, Kodak, Rochester, NY). The slides were subsequently coated with NTB2 nuclear emulsion (Kodak) diluted 1:1 with distilled water, exposed for a further 4 to 6 weeks in light-proof boxes and developed in freshly prepared D-19 for 4 min at 18°C. They were then stained with toluidine blue, coverslipped with Permount and examined under a Leitz Aristoplan microscope.

RESULTS

Macroscopic examination of film autoradiograms of coronal sections of the rat brain taken from the magnocellular preoptic area, rostrally, to the mammillary bodies, caudally, revealed different topographic patterns of distribution for SSTR1 and SSTR2 mRNAs (Figs. 1a,b). Thus, although intense hybridization signal was evident for both probes throughout the cerebral cortex, SSTR1 mRNA was selectively distributed over the outermost and innermost cortical layers, sparing layer 4, whereas SSTR2 mRNA was confined to deep layers in the retrosplenial and frontal cortices but was more homogeneously distributed in parietal, insular and piriform cortex (Figs. 1a,b). Conversely, intense SSTR2 hybridization signal was observed in the medial habenular nucleus as well as in the pyramidal cell layer of Ammon's horn and the granule cell layer of the dentate gyrus, whilst SSTR1 mRNA signal was not detected in the medial habenular nucleus and was very low in the hippocampus and dentate gyrus (Figs. 1a,b). Finally, a moderate hybridization signal for SSTR2 was detected in the mediodorsal thalamic nucleus, however, labelling of SSTR1 mRNA was not apparent in this region (Figs. 1a,b). Control sections prepared with a sense probe for either SSTR1 (Fig. 1c) or SSTR2 (not shown) did not comprise specific labelling in any region of the rat brain. Furthermore, the addition of an excess of a dissimilar cold probe did not displace hybridization of either the SSTR1 or SSTR2 probes. Finally, no obvious qualitative or quantitative differences were observed between male and female rats for either probe.

Hypothalamic Distribution of SSTR1 mRNA

In sections from the rostral hypothalamus, moderate to high concentrations of SSTR1 mRNA were detected throughout the medial preoptic area (Fig. 2a; Table I). By high resolution autoradiography, approximately 60% of neurons in this region were found to be labelled with a markedly higher proportion of cells labelled in the anterior medial preoptic nucleus (AMPO) and medial preoptic nucleus (MPO) than in the remainder of the area (Fig. 3b). By contrast, the lateral preoptic area and anterior hypothalamic nucleus were

only sparsely labelled (Table I). More caudally, moderate to high SSTR1 hybridization was apparent in the para- and periventricular nuclei dorsally and in the suprachiasmatic nucleus ventrally (Fig. 2b; Table I). In the paraventricular nucleus, cells were both more numerous and more intensely labelled in the magnocellular division of the nucleus than in its parvocellular segment (Fig. 3a). In the periventricular nucleus, the labelling was found in liquid emulsion-processed sections to correspond to scattered, intensely positive neurons distributed beneath the ependymal border of the 3rd ventricle (Fig. 3a). Within the suprachiasmatic nucleus, labelled cells were confined to the less densely cellular ventrolateral sub-division of the nucleus.

Among the highest concentrations of SSTR1 mRNA detected in the hypothalamus were within the arcuate nucleus (Figs. 2c,d; Table I). Labelling in this structure was not homogenous as the caudal pole exhibited a more intense hybridization signal than the rostral pole (Table I). At the cellular level, SSTR1 mRNA was evident in approximately 30% of neurons rostrally and 60% of neurons caudally (Figs. 3c; 4a,c). Low levels of SSTR1 mRNA were also detected in neurons of the dorsomedial portion of the ventromedial hypothalamic nucleus and in a modicum of neurons in the ventrolateral portion of this same nucleus (Fig. 2c; Table I).

More caudally, weak SSTR1 hybridization signal was detected in the dorsomedial and medial tuberal nuclei (Fig. 2d; Table I). The tuberal magnocellular nucleus also hybridized the SSTR1 probe, but more intensely (Fig. 3c). Within the mammillary bodies, SSTR1 mRNA was detected in moderate concentrations in the ventral premammillary nucleus (Table I). Hybridization signal was only occasionally observed in the dorsal premammillary nucleus. The lateral mammillary nucleus showed little SSTR1 hybridization signal, whereas the supramammillary nucleus was virtually label free. Finally, SSTR1 positive neurons were scattered throughout the lateral hypothalamus.

Hypothalamic Distribution of SSTR2 mRNA

SSTR2 mRNA was detected within essentially the same hypothalamic areas as SSTR1 mRNA. Its distribution, however, was much less discrete than that of the SSTR1 probe (Fig. 2). Furthermore, it was proportionally much more heavily concentrated than

SSTR1 mRNA in a number of hypothalamic nuclei including the anterior hypothalamic nucleus, the dorsal- and ventromedial nuclei and the medial tuberal nucleus (Fig. 2, Table I). However, the signal to noise ratio in liquid emulsion-processed material was not as high as with the SSTR1 probe (compare Figs. 4a,b).

Within the medial preoptic area, a moderate to high SSTR2 hybridization signal, detectable over approximately 30% of local neurons, was detected in the AMPO and MPO. A weaker signal, corresponding to approximately 15% of neurons, was evident in the remainder of the structure (Fig. 2e). The concentration of SSTR2 mRNA detected in the paraventricular nucleus was low by comparison with that detected for SSTR1 (Table I). The SSTR2 probe labelled only scattered neurons in the parvocellular division and approximately 10% of the neurons in the lateral magnocellular division of the nucleus. In the periventricular nucleus, only a few scattered SSTR2 mRNA-positive neurons were observed. The suprachiasmatic nucleus comprised moderate SSTR2 hybridization signal which was confined, like the SSTR1 signal, to the ventrolateral segment of the nucleus (Fig. 2f).

SSTR2 hybridization pattern within the arcuate nucleus was comparable to that of SSTR1 in that the caudal pole exhibited a more intense concentration of SSTR2 mRNA than the rostral pole (Figs. 2g,h; Table I). However, at the cellular level, it was observed that whereas SSTR1-labelled cells were mainly concentrated medially, next to the borders of the 3rd ventricle (Fig. 4a), SSTR2 mRNA was more heterogeneously distributed throughout the entire mediolateral extent of the nucleus (Fig. 4b). Approximately 20 and 40% of neurons in the arcuate nucleus were labelled with the SSTR2 probe in the rostral and caudal poles, respectively.

In contrast to SSTR1, which was barely apparent in the dorsomedial portion of the ventromedial hypothalamic nucleus (Fig. 2c), SSTR2 mRNA was heavily concentrated in this region (Fig. 2g; Table I). In fact, nearly all neurons showed some degree of hybridization signal in this segment of the nucleus. A weak SSTR2 hybridization signal was also detected in the ventrolateral portion of the ventromedial hypothalamic nucleus.

Intense SSTR2 hybridization signal was detected in the medial tuberal nucleus, along the basolateral edges of the hypothalamus (Fig. 2h; Table I). In contrast to SSTR1

mRNA, which was observed in moderate to high concentrations in the ventral premammillary nucleus and in low concentrations in the dorsal premammillary nucleus, SSTR2 mRNA was rarely observed in either of these two nuclei. Both the lateral mammillary and supramammillary nuclei were found to comprise low concentrations of SSTR2 mRNA.

Figure 1. Film autoradiograms of 20 μm-thick coronal sections labelled with SSTR1 (a) and SSTR2 (b) antisense riboprobes. Control section in (c) was incubated with a SSTR1 sense probe. Note the difference in the labelling patterns produced by the two antisense probes and the absence of specific hybridization of the sense probe. Abbreviations: Arc: arcuate nucleus; Cx: cerebral cortex; DG: dentate gyrus; Hi: hippocampus; MD: mediodorsal thalamic nucleus; MHb: medial habenular nucleus. Scale bar = 1.5 mm.

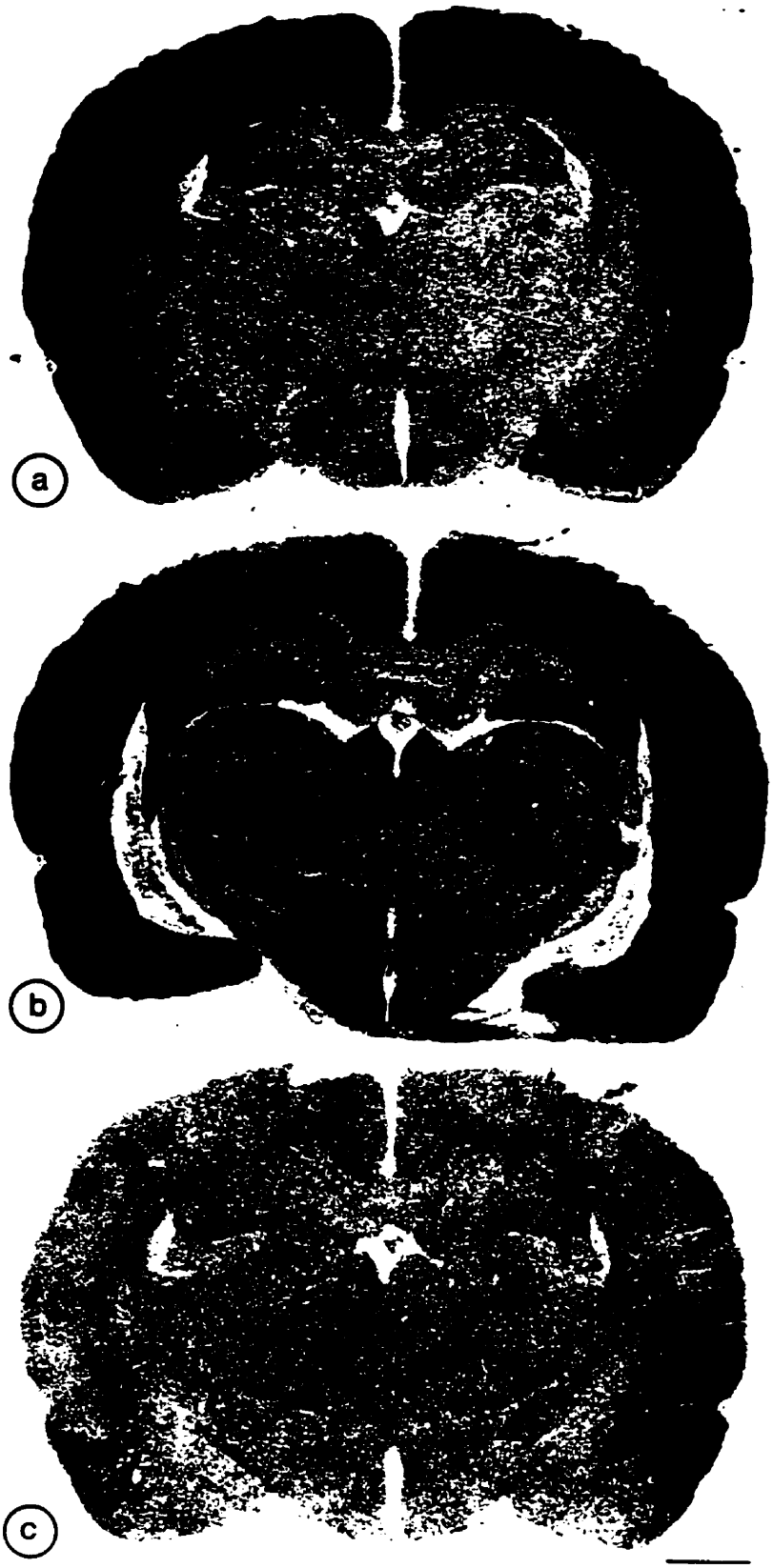
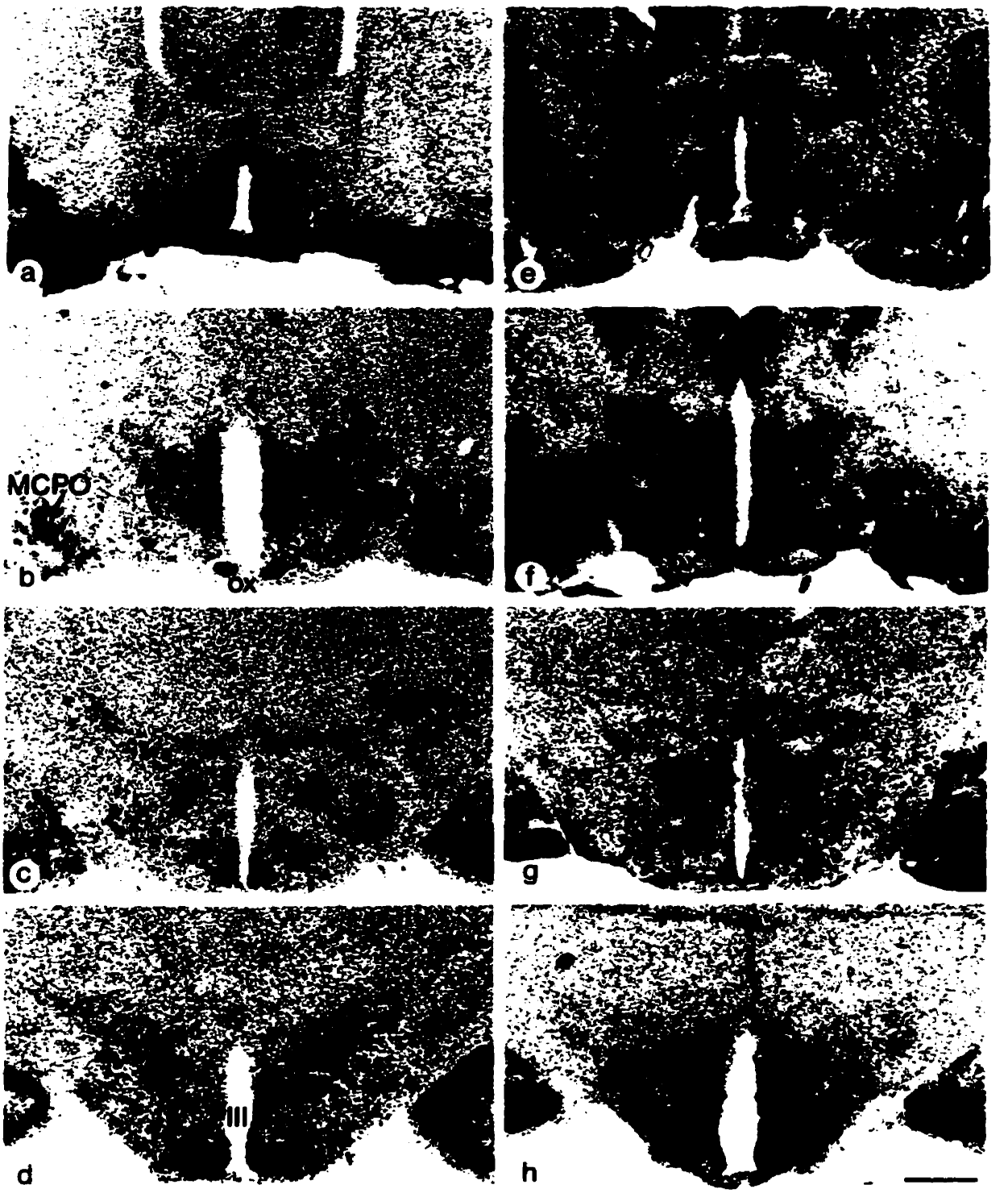

a
b
c

Figure 2. Regional distribution of SSTR1 (a-d) and SSTR2 (e-h) mRNA as detected by *in situ* hybridization across four different rostrocaudal levels (from a, e, rostrally to d, h, caudally) of the rat hypothalamus. Film autoradiograms. Two weeks of exposure. Abbreviations: ac: anterior commissure; Arc: arcuate nucleus; DM: dorsomedial hypothalamic nucleus; MCPO: magnocellular preoptic nucleus; MPA: medial preoptic area; MTn: medial tuberal nucleus; ox: optic chiasm; Pe: periventricular nucleus; SCh: suprachiasmatic nucleus; VM: ventromedial nucleus; III: third ventricle. Scale bar = 1.0 mm.


a
e
MCPO
b
ox
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g
III
d
h

Figure 3. Cellular distribution of SSTR1 mRNA in the hypothalamus of adult rat as detected by *in situ* hybridization using liquid emulsion autoradiographic processing. Darkfield. In (a), SSTR1 mRNA hybridizing cells are evident in the parvocellular division of the paraventricular nucleus (Pa) as well as along the border of the third ventricle (III), within the periventricular nucleus (Pe), and in the retrochiasmatic area (RCh). In (b), a dense cluster of labelled cells is detected in the medial preoptic nucleus (MPO); ox: optic chiasm. A few scattered cells are also visible in the remainder of the preoptic area. In (c), dense SSTR1 hybridization signal is apparent over most of the cells comprising the arcuate (Arc) and tuberal magnocellular nucleus (TMC); ME: median eminence. Scale bar = 75 μm.

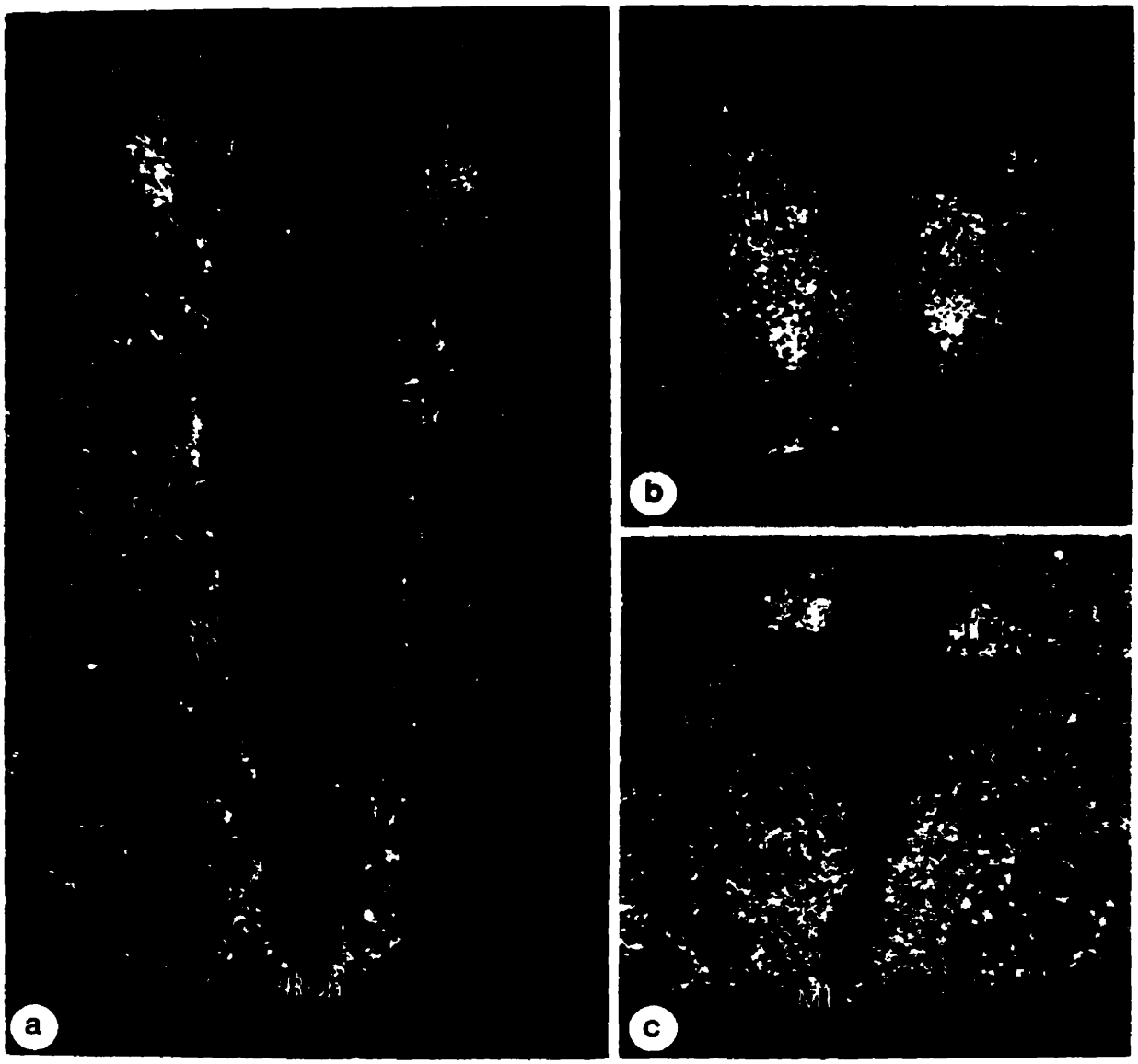
a
b
c

Figure 4. Comparative distribution of SSTR1 (a) and SSTR2 (b) mRNA in the arcuate (Arc) nucleus of the hypothalamus of an adult rat. Note that the signal in (a) is both more intense and more restricted than in (b). Also, note the absence of hybridization signal in the median eminence (ME). Scale bar = 150 μm. The brightfield view in (c) demonstrates that the hybridization signal (here SSTR1) originates from a subset of neuronal perikarya. Scale bar = 20 μm.

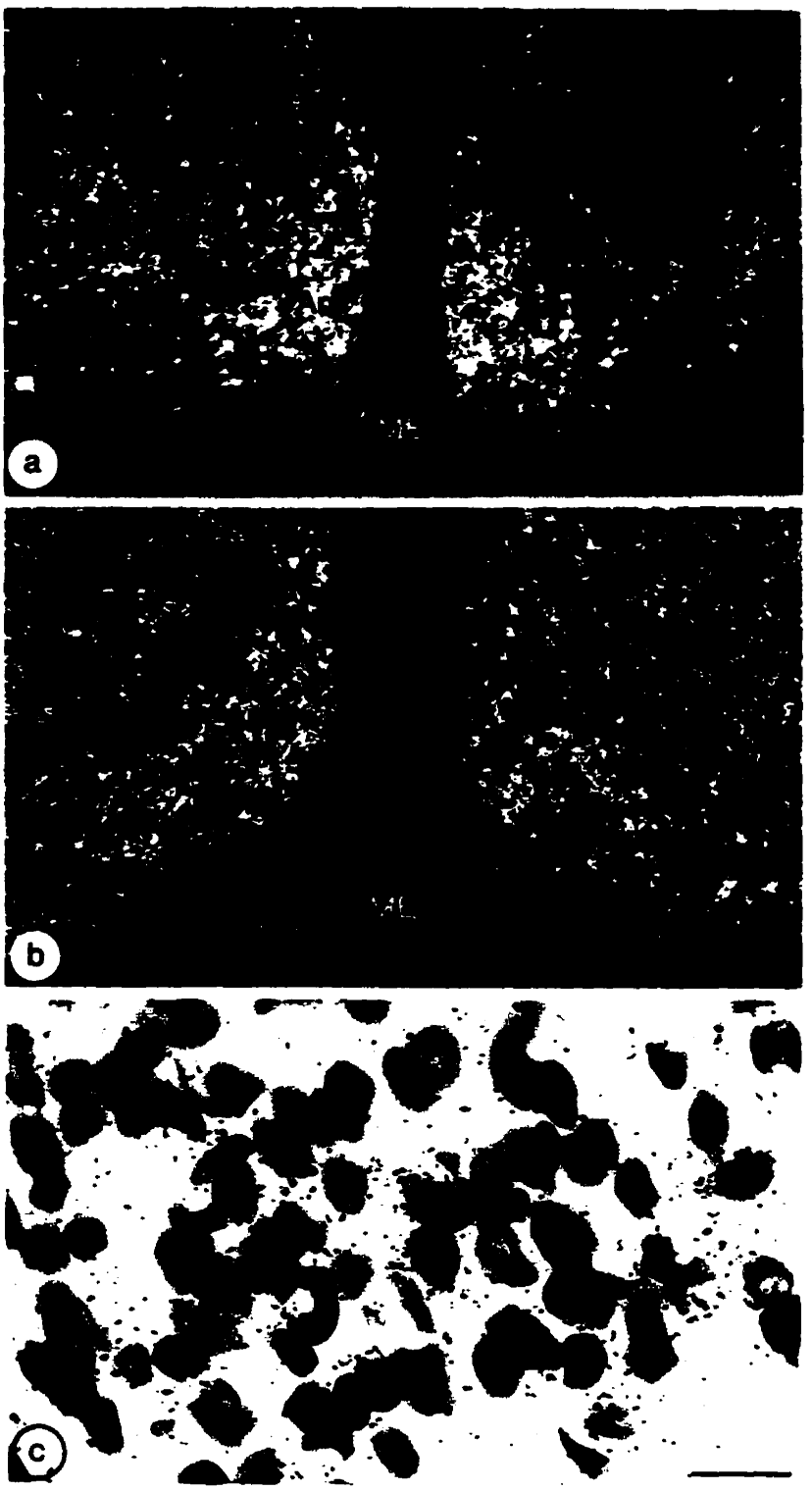
a
b
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Table I. Distribution of sst_1 and sst_2 receptor mRNA in hypothalamus of adult rat
*Relative values established separately for each probe. Data based on joint film and light microscopic observations.

REGION	LABELLING DENSITY*	
	SSTR1 mRNA	SSTR2 mRNA
Medial preoptic area	+++	+++
Lateral preoptic area	+	+
Anterior hypothalamic nucleus	+	++
Paraventricular nucleus	+++	+
Periventricular nucleus	++	+
Suprachiasmatic nucleus	++++	++
Arcuate nucleus (rostral pole)	+++	++
Arcuate nucleus (caudal pole)	++++	+++
Ventromedial nucleus	+	+++
Dorsomedial nucleus	+	++
Medial tuberal nucleus	+	++++
Ventral premammillary nucleus	++	+
Lateral mammillary nucleus	+	+

DISCUSSION

The present report provides a comprehensive description of both the regional and cellular distribution of SSTR1 and SSTR2 mRNA throughout the hypothalamus of the adult rat. Several lines of evidence indicate that the labelling patterns yielded by each of the two probes utilized in our study reflect specific hybridization to SSTR1 and SSTR2 mRNA: 1) the finding that no hybridization was detected with sense riboprobes; 2) the observation that the addition of an excess of a dissimilar cold antisense probe did not displace hybridization of either SSTR1 or SSTR2 probes; and 3) the concordance of hybridization patterns with previously reported distributions of SSTR1 and SSTR2 mRNA in extrahypothalamic areas of mouse and rat brain[4,15,27]. Indeed, the laminar patterns of SSTR1 and SSTR2 expression described here in sensory motor, retrosplenial, insular and pyriform cortices were comparable to those previously observed by *in situ* hybridization in corresponding regions of both mouse[4] and rat[15,27] cerebral cortex. Similarly, the presence of strong SSTR2 and the absence of SSTR1 hybridization signal over the medial habenular nucleus as well as the detection of abundant SSTR2 mRNA within the pyramidal cell layer of Ammon's horn and the granular cell layer of the dentate gyrus conformed to earlier *in situ* hybridization data gathered in either mouse[4] or rat[15,27] brain. In general, the patterns of expression of SSTR1 and SSTR2 were similar in male and female rats.

The distribution of SSTR1 mRNA described here in the rat hypothalamus closely corresponds to that previously reported in mouse hypothalamus using the same riboprobe[4] but differs on several accounts from that recently reported for the rat based on the use of oligoprobes[27]. In particular, moderate to high levels of SSTR1 mRNA were detected here in the anterior periventricular, paraventricular, suprachiasmatic, premammillary and mammillary nuclei, whereas Pérez *et al.*.[27] reported SSTR1 mRNA levels as being undetectable within the same regions of the rat brain, a discrepancy which may be most likely accounted for by the notoriously lower sensitivity of oligoprobes as compared to riboprobes.

The intra-hypothalamic distribution of SSTR2 mRNA observed in the present study was also more extensive than that previously observed by Pérez *et al.*.[27] in the rat. Thus, we found significant expression of SSTR2 mRNA in the medial preoptic area as well as in the periventricular, suprachiasmatic, dorsomedial, medial tuberal and mammillary hypothalamic nuclei, whereas Pérez *et al.*.[27] did not. We also found considerably more widespread expression of SSTR2 mRNA in the rat than Breder *et al.*.[4] did in the mouse. Indeed, these authors reported the retrochiasmatic area and paraventricular and arcuate nuclei as the only sources of SSTR2 mRNA in this species. This discrepancy probably reflects true species differences since we used the same riboprobe and followed a similar *in situ* hybridization protocol as these authors and since our distributional patterns in extra-hypothalamic areas were consistent with theirs. Furthermore, the results of RNA blotting experiments have shown high levels of SSTR2 mRNA expression in adult rat hypothalamus[6,15] in conformity with the present findings. In fact, recent evidence suggests that the rat-mouse differences in expression of SSTR2 mRNA are not confined to the hypothalamus but are also present in the striatum, midbrain, pons, medulla and cerebellum[6].

Two splice variants of SSTR2 mRNA referred to as SSTR2A (unspliced) and SSTR2B (spliced) have been identified in mouse and rat tissues[25,44]. These two receptors exhibit similar affinities for a number of SSTR2-selective ligands including MK-678[31]. However, the hypothalamus was shown to express much higher levels of SSTR2B than SSTR2A mRNA[15]. Thus, although the probes used in the present study did not distinguish between the two forms of the SSTR2 mRNA, it may be assumed that the bulk of SSTR2 mRNA detected here corresponds to the spliced form.

The widespread distribution of both SSTR1 and SSTR2 mRNA observed here in the rat hypothalamus conforms with the presence of specific [Tyr^{11}]SRIF-14, [Leu^{8},D-Trp^{22},Tyr^{25}]SRIF-28 and [Tyr^{0},D-Trp^{8}]-SRIF-14 binding sites documented throughout this area by *in vitro* autoradiography[16,18,43]. Indeed, all of these ligands have been shown in transfected cells to bind with high affinity to both SSTR1 and SSTR2 receptor subtypes[29]. Furthermore, the preferential expression of SSTR2 over SSTR1 mRNA in structures such as the dorsomedial and ventromedial nuclei correlates well with the earlier demonstration of preferential binding of the SSTR2-selective ligand MK 678[29] within these two nuclei[21].

Conversely, the preferential expression of SSTR1 over SSTR2 mRNA in the paraventricular nucleus might account for the detection of relatively high levels of SRIF-28, . but not of SRIF-14, binding in the paraventricular nucleus since studies in transfected cells have shown SRIF-28 to bind with greater affinity to the SSTR1 than to the SSTR2 subtype[29].

Taken together, the regional distribution of SSTR1 and SSTR2 mRNA unraveled in the present study conforms remarkably closely to that of SRIF binding sites revealed by autoradiography using a variety of radioactive ligands. Thus, among the highest concentrations of SSTR1 and/or SSTR2 mRNA were found here in the medial preoptic area, anterior hypothalamic nucleus, para- and periventricular nuclei, dorso- and ventromedial nuclei and arcuate nucleus all of which have been shown to contain moderate to high levels of SRIF binding sites in radioligand binding studies[9,16,18,21] (for a review, see[17]). One can surmise from this comparison that an important proportion of SSTR1 and SSTR2 receptors in the hypothalamus are "post-synaptic", i.e., are associated with the perikarya and/or the dendrites of neurons by which they are expressed. Indeed, our own high resolution autoradiographic studies had already demonstrated a clearcut somato/dendritic association of SRIF binding sites with neurons of the arcuate and periventricular nuclei[9]. The congruence between the present results and those of earlier receptor autoradiographic studies also suggests that a significant contingent of hypothalamic SRIF binding sites correspond to either the SSTR1 or the SSTR2 subtype of the receptor. This is obviously not to say that other SRIF receptor subtype(s) may not also share a similar topographic or even cellular distribution as these two since SSTR3, SSTR4 and SSTR5 mRNAs have also been detected in adult rat hypothalamus.

The distributional pattern of SSTR1 and, to a lesser extent, SSTR2 receptor subtypes in the peri- and paraventricular nuclei, visualized here using liquid emulsion coating procedures, was highly reminiscent of that of SRIF-containing neurons detected by immunohistochemistry within the same region[1,8,12,45] (compare Fig. 3a with Figs. 34a and b in[12]). Furthermore, high numbers of SSTR1- and SSTR2-expressing cells were detected in the suprachiasmatic and arcuate nuclei which have been shown to comprise neurons that both contain[8,12,45] and express[14] endogenous SRIF. Earlier reports have demonstrated a

direct inhibitory action of SRIF on SRIF release in cultured hypothalamic cells[28,35] suggesting that SRIF may autoregulate its own secretion and, as a consequence, possibly modulate GH release[20]. Although double labelling studies will obviously be needed to determine whether either or both of SSTR1 and SSTR2 receptor subtypes are indeed expressed by SRIF-containing cells, the present observations strongly suggest that a proportion of SSTR1 and perhaps also of SSTR2 receptors may correspond to SRIF autoreceptors.

Among the highest concentrations of hypothalamic SSTR1 and SSTR2 mRNA were detected in the arcuate nucleus. Interestingly, the neuronal populations expressing each of these receptor subtypes showed considerable overlap suggesting that SSTR1 and SSTR2 receptor subtypes may be co-expressed by a subpopulation of arcuate neurons. This would be in keeping with the recent demonstration by Patel *et al.*.[26] that the same cell can express multiple SRIF receptor genes. Within the arcuate nucleus, the distribution of SSTR1- and SSTR2-hybridizing cells was remarkably similar to that of neurons previously found to bind [^{125}I]D-Trp^{8}-SRIF-14[9]. Moreover, previous studies from our[22] and Epelbaum's[9] laboratories have demonstrated that one third of these neurons possessing SRIF binding sites both contain and express GRF. The present results therefore imply that the receptors present on the surface of arcuate GRF neurons may express either or both SSTR1 and/or SSTR2 receptor subtypes. Although this interpretation will obviously need confirmation from double labelling experiments, our results already strongly suggest that both SSTR1 and SSTR2 receptor subtypes are likely to be involved in the central regulation of GRF secretion, and hence of GH release, by SRIF.

In summary, the results reported here demonstrate that: (1) in contrast to previous reports, patterns of SSTR1 and SSTR2 hybridization show considerable overlap within the hypothalamic-hypophysiotropic axis of the adult rat; and (2) SSTR1 and SSTR2 SRIF receptor mRNAs are heavily expressed in those hypothalamic neurons that contain and express SRIF and/or GRF. Taken together, these findings imply a role for both SSTR1 and SSTR2 SRIF receptor subtypes in neuroendocrine regulation of GH secretion in both sexes of this species.

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CHAPTER II

Internalization of Somatostatin

MATERIALS AND METHODS

Animals

Adult male Sprague-Dawley rats (body weight 180-200g were purchased from Charles River Canada (St Constant, Quebec), maintained on a twelve hour light/ twelve hour dark cycle (lights on: 0600 h), and given free access to Purina rat chow and tap water. All studies were conducted in accordance with the guidelines of the Canadian Council on Animal Care.

Competition for SRIF Binding Sites on Cortical Homogenates by [DTrp^8]Somatostatin and Iodinated Somatostatin

To verify the pharmacological properties of ^{125}I-Tyr^0[DTrp^8]SRIF and [DTrp^8]SRIF, the ability of [DTrp^8]SRIF to displace ^{125}I-SRIF binding to cortical homogenates was assessed. Rats (n=2) were killed by decapitation and their brains rapidly dissected from the skull. The cortex was dissected and placed in a 10 x volume of 5 mM Tris-HCl containing 2 mM EDTA, homogenized for 20 sec, and then centrifuged at 15,000g for 15 min at 4°C. The supernatant was aspirated, and the pellet resuspended in the same volume of buffer and centrifuged at 15,000g for 15 min at 4°C. Following centrifugation, the supernatant was aspirated, and the pellet frozen at -20°C until use.

At the time of the binding assay, the frozen pellet was resuspended in 50 mM Tris-HCl buffer containing 0.2% bovine serum albumin and 0.8 mM 1,10 phenantroline (pH 7.5). The concentration of protein used in the assay was 0.5 µg/ml membrane. Cortical homogenates (50µg) were incubated with ^{125}I-Tyr^0[DTrp^8]SRIF (0.5 nM, 2000 Ci/mmol), (Kindly provided by Georges Gaudriault) in the presence of increasing concentrations of [DTrp^8]SRIF (Peninsula) (10^{-12} M, 10^{-11} M, 10^{-10} M... 10^{-6} M) for 30 minutes at room temperature. Binding was terminated by the addition of 2 ml ice-cold 50 mM Tris-HCl buffer (pH 7.5). The binding cocktail was filtered through glass microfiber

filters (GF/C, Whatman) that were presoaked in 0.3% polyethylenimine. The filters were washed 2x with ice-cold 50 mM Tris-HCl buffer (pH 7.5). The radioactivity bound to membranes was retained on the filters and counted in a gamma detector. Non-specific binding was measured in the presence of 1 μM unlabeled [Dtrp^8]SRIF.

Cell Culture

COS 7, monkey kidney, cells were grown in Dubelco's minimum essential medium (MEM) containing glutamine and supplemented with 44 mM $NaHCO_3$, 10% fetal calf serum, and 50 mg/l gentamicin. Transfections were done with either the cDNA recombinant plasmid encoding either the sst_1 receptor or the sst_2 receptor (generously provided by Terry Reisine, University of Pennsylvania, School of Medicine). The cDNA encoding the sst_1 receptor was subcloned into the eukaryotic expression vector pCMV-6b that contains the cytomegalovirus promoter. The cDNA encoding the sst_2A receptor was subcloned into the eukaryotic expression vector pcDNA I, that contains the cytomegalovirus promoter. Transient transfections were performed with 1μg of recombinant plasmid by the DEAE-dextran precipitation method (Cullen, 1987) onto semi-confluent COS 7 cells grown in 100-mm cell culture dishes. Briefly, 1μg of plasmid was diluted in 50 μl of water to which 950 μl of Tris buffered saline/ DEAE-Dextran buffer (pH 7.5) was added. 1 x 10^6 cells were plated in 100 mm^2 culture dishes and incubated for 30 min with the plasmid containing cocktail at 37°C, incubated for 3 hours at 37°C with Dubelco's MEM containing glutamine and supplemented with 44 mM $NaHCO_3$, 10% fetal calf serum, 50 mg/l gentamicin, and 100 μM chloroquine. The cells were washed with Tris buffered saline (pH 7.4) and incubated with Dubelco's MEM containing glutamine and supplemented with 44 mM $NaHCO_3$, 10% fetal calf serum, and 50 mg/l gentamicin for approximately 55 hours at 37°C. All experiments using COS 7 cells were conducted approximately 55 hours after the time of transfection.

Internalization Kinetics

In order to evaluate the kinetics of internalization of SRIF mediated by the sst_1 and sst_2A (splice variant of the sst_2 receptor) receptor subtypes, biochemical assays were done with COS 7 cells transfected with the appropriate cDNA. The assay employed a classical binding methodology in conjunction with the acid-wash protocol of Haigler (Haigler, 1980).

2×10^5 cells were plated in 12 mm diameter cell culture dishes and covered with 250µl of culture medium at 37°C. The culture medium was aspirated, and cells were equilibrated for 10 min at 37°C in Earle's-Tris-HEPES buffer (pH 7.4) supplemented with 0.01% glucose and 1% bovine serum albumin. The equilibration buffer was subsequently aspirated and replaced with 250 µl of binding buffer containing ^{125}I-Tyr^0[DTrp^8]SRIF (0.5 nM, 2000 Ci/mmol) in the presence of 1 mM of the enzyme inhibitor 1,10-phenantroline; cells were incubated for 3, 5, 10, 20, 40, 60 min at 37°C in the presence or absence of 10 µm phenylarsine oxide, an internalization blocker. At the end of the incubation, cells were washed for 2 min with either 1 ml Earle's-Tris-HEPES buffer (internalized plus extracellular radioactivity) or 1 ml of Earle's-Tris-HEPES buffer containing 0.5 M NaCl (pH 4) to dissociate surface bound radioactivity (internalized radioactivity alone). The COS 7 cells were then harvested by addition of 1 ml of 0.1 M NaOH and filtered through GF/C glass filters (Millipore). The filters were washed 2X with 5 ml of ice cold Earle's-Tris-HEPES buffer and counted in a gamma counter. In all cases, parallel incubations were performed in the presence of 1 µM unlabeled [Dtrp^8]SRIF to determine non- specific binding. The fraction of radioactivity resistant to the acid-wash was considered to have been sequestered intracellularly. Subtraction of data points obtained in the absence of phenylarsine oxide after acid- washing from data points obtained without an acid- wash step provided a quantitative measure of the amount of radioactivity sequestered intracellularly. The efficiency of internalization was calculated to be the percent of ^{125}I-Tyr^0[DTrp^8]SRIF specific binding determined in the presence of phenylarsine oxide, that, in the absence of phenylarsine oxide at each time point, was resistant to the acid-wash step.

Radioautography with COS 7 Cells in Culture

With the aim of visualizing internalized SRIF, radioautograms of COS 7 cells transfected with the sst_2A receptor cDNA were prepared. 2 x 10^5 cells were plated in 35 mm diameter cell culture dishes and covered with 3 ml of culture medium at 37°C. The culture medium was aspirated, and cells were equilibrated for 10 min at 37°C in Earle's buffer (pH 7.4) supplemented with 0.01% glucose and 1% bovine serum albumin. The equilibration buffer was removed, and cells were then incubated in either: (1) 2 ml of Earle's binding buffer (pH 7.4) containing ^{125}I-Tyr^0[DTrp^8]SRIF (0.35 nM, 2000 Ci/mmol) in the presence of 1 mM 1,10-phenantroline to prevent peptide degradation; or (2) the same buffer plus 1 x 10^{-5} M [DTrp^8]SRIF (Peninsula) to determine the non-specific binding. The cells were incubated for 30 min at 37°C. At the end of the incubation, cells were washed 3x with Earle's buffer (pH 7.4), washed for 2 min at room temperature in the same buffer containing 0.5 M NaCl (pH 4.0) to dissociate surface-bound ligand, and subsequently fixed with 0.2 M PO_4 buffered glutaraldehyde solution (pH 7.4) containing 0.18 M NaCl, to preserve cellular morphology, for 30 min at 4°C. After fixation, cells were dehydrated in graded ethanols (70%, 95%, 100%; 3 min/concentration). The outer walls of the culture dishes were removed, and the cells were coated with NTB2 liquid nuclear emulsion (Kodak). The emulsion was exposed to the radioactivity for two weeks in light tight boxes, and then developed for 2.5 min in D19 developer (Kodak) diluted 1:1 with water at 18°C. The cells were allowed to dry and were then counterstained with Toluidine blue. The proportion of labeled cells was determined by counting the number of labeled and unlabeled cells in a field of view at 40x magnification (average of 3 randomly selected fields of view). Controls consisted of non-transfected cells incubated in parallel with transfected cells.

Uptake of ^{125}I-SRIF in Rat Brain Slices Ex Vivo

The ability of SRIF to be internalized by brain cells was assayed *ex vivo.* Adult male rats were killed by decapitation. The brain was rapidly dissected from the cranium and immersed in an ice-cold oxygenated (95% O_2, 5% CO_2) Ringer's solution containing 130 mM NaCl, 20 mM $NaHCO_3$, 1.25 mM KH_2PO_4, 1.3 mM $MgSO_4$, 5 mM KCl, 10

mM dextrose anhydrate, and 2.4 mM $CaCl_2$. 350 μm- thick sections were cut on a vibrating microtome (Vibratome) from the level of the optic chiasm, rostrally, to the level of the substantia nigra, caudally. The slices were then equilibrated for 20 min in oxygenated Ringer's containing 0.2% BSA at room temperature, and then equilibrated an additional 20 minutes at 37°C in the same buffer.

Following equilibration, slices were incubated for 15 (n=2 animals) or 45 (n=2 animals) minutes at 37°C in oxygenated Ringer's containing ^{125}I-Tyr^0[DTrp^8]SRIF (1.5 nM; 2000 Ci/mmol) in the presence of 0.2% BSA and 0.01% bacitracin to inhibit peptide degradation.

Control sections were processed by co-incubating slices with 1.5 nM ^{125}I-Tyr^0[DTrp^8]SRIF (2000 Ci/mmol) together with 1 x 10^{-5}M [Dtrp^8]SRIF for 15 minutes (n=2 animals) or 45 minutes (n=2 animals) at 37°C.

Moreover, in order to determine the mechanism(s) by which exogenous SRIF is able to gain access to the intracellular compartment, the effects of various drugs were tested. After equilibration, slices were incubated for 45 min at 37°C in oxygenated Ringer's containing ^{125}I-Tyr^0[DTrp^8]SRIF (1.5 nM; 2000 Ci/mmol), 0.2% BSA, and 0.01% bacitracin supplemented with: (1) octreotide (Sandoz) (10 μM) (n=2 animals); or (2) nomifensin (Sigma), an inhibitor of the dopamine transporter (10 μM) (n=1 animal).

To assess the energy dependency of the uptake process, slices (n=3 animals) were equilibrated for 40 min at 4°C in oxygenated Ringer's, and then incubated for 45 min at 4°C in oxygenated Ringer's containing 1.5 nM ^{125}I-Tyr^0[DTrp^8]SRIF (2000 Ci/mmol) in the presence of 0.2% BSA and 0.01% bacitracin.

After incubation with the radiolabeled peptide, slices were washed 5 x 8 min in 20 ml of oxygenated Ringer's at 37°C (or 4°C for slices radiolabeled at 4°C) and then washed for 3.5 min in Earle's buffer containing 0.18 M NaCl (pH 4.0). Subsequently, slices were fixed for 30 min in a 0.2 M PO_4 buffered glutaraldehyde solution (pH 7.4) containing 0.18 M NaCl to preserve cellular morphology at room temperature. Brain slices were then cryoprotected overnight by immersion in a 30% sucrose solution in 0.2 M PO_4 buffer at 4°C.

Brain slices were then mounted on blocks of OCT mounting medium at -20°C and re-cut into 20 μm- thick sections. Sections were thaw mounted onto 3-aminopropyltriethoxyailane-coated slides (2%) warmed to 45°C and then incubated overnight at 37°C. The sections were then dehydrated in graded ethanols (70%, 95%, 100%; 5 min bath), defatted in BDH xylene substitute (2 x 10 min), and rehydrated in graded ethanols (100%, 95%, 70%; 5 min/bath) and water (3 x 30 sec). Sections were allowed to dry overnight and were then coated with NTB2 liquid nuclear emulsion (Kodak). The emulsion was exposed to the radiolabeled peptide for 14 days in light tight boxes and then developed (2.5 min at 18°C) in D19 developer (Kodak) diluted 1:1 with water. Finally, sections were counter stained with Toluidine blue.

Light microscopic observations were made using a Leitz Aristoplan microscope. Sections were analyzed under both dark and bright field illumination. Labeled cells were identified as having higher than background levels of radioautographic grains concentrated over their cell bodies. The neuroanatomical distribution of labeled cells were mapped in dark field on select sections using a computer aided (HISTO software) image analysis system (BIOCOM, Les Ulis, France) coupled to a Leitz Diaplan microscope. Semi-schematic diagrams were constructed from maps prepared on the image analysis system.

RESULTS

Competition for SRIF Binding Sites on Cortical Homogenates by [DTrp^8]Somatostatin and Iodinated Somatostatin

In order to characterize the pharmacological properties of ^{125}I-Tyr^0[DTrp^8]SRIF and [Dtrp^8]SRIF, the ability of [Dtrp^8]SRIF to displace ^{125}I-SRIF binding was assayed using rat brain cortical homogenates. Increasing concentrations of non-radiolabeled [Dtrp^8]SRIF monophasically displaced the binding of ^{125}I-Tyr^0[DTrp^8]SRIF to cortical homogenates (Fig. 1). The degree of displacement of ^{125}I-SRIF binding by [Dtrp^8]SRIF was concentration-dependent. The IC_{50} of [Dtrp^8]SRIF on ^{125}I-SRIF binding was 0.3 nM.

Internalization Kinetics on COS 7 Cells Transfected with sst_1 or sst_2A Receptor cDNA:

The kinetics of internalization of ^{125}I-Tyr^0[DTrp^8] mediated by the sst_1 and sst_2A receptors were characterized using whole COS 7 cells transfected with the appropriate cDNA (Fig. 2). Figure 2 shows the amount of ^{125}I-SRIF that was resistant to acid-washing (i.e. sequestered intracellularly) as a percent of total specific (i.e. surface plus intracellular radioactivity) at each time interval by COS 7 cells transfected with cDNA encoding either the sst_1 receptor (open circles) or the sst_2A receptor (closed circles). When cells were incubated with ^{125}I-SRIF in the presence of phenylarsine oxide and an excess of non- radiolabeled SRIF and subsequently washed with an acidic buffer, uptake of radioactivity was abolished. Mean ± standard error of the mean of three experiments.

Cells transfected with the sst_2A receptor cDNA recombinant plasmid rapidly internalized ^{125}I-SRIF with a $t_{1/2}$ = 5-6 min. The $t_{1/2}$ is the time at which 38% (half maximal internalization efficiency) of specifically bound SRIF was acid-wash resistant (i.e. had been sequestered intracellularly). The initial increase in internalization efficiency between 0 and 10 minutes was exponential and a maximal plateau was achieved at 10 min.

By comparison to cells transfected with sst_2A receptor cDNA, cells transfected with the sst_1 receptor cDNA did not internalize ^{125}I-SRIF with as high a capacity as cells transfected with the sst2A receptor cDNA. Only 20-25% of specifically bound radioactivity was internalized by COS 7 cells expressing the sst_1 receptor.

Radioautography with COS 7 Cells in Culture

Approximately 22% of COS 7 cells in the series transfected with the sst_2A receptor cDNA exhibited dense accumulations of radioautographic grains over their entire cytoplasm (Fig. 3a). By comparison, the remainder of cells showed radioautographic grain densities that were hardly above background (Fig. 3a).

When transfected cells were co-incubated with both ^{125}I-SRIF and an excess of unlabeled SRIF, there were no silver grain accumulations over cell bodies (Fig 3b). Similarly, when non-transfected COS 7 cells were incubated with ^{125}I-SRIF, accumulations of silver radioautographic grains were not observed over cell bodies.

Uptake of ^{125}I-SRIF in Rat Brain Slices Ex Vivo

Microscopic examination of rat brain slices incubated *ex vivo* with ^{125}I-SRIF and subsequently washed with a hyperosmolar NaCl buffer (pH 4) to remove surface bound radioactivity revealed accumulations of silver radioautographic grains over populations of nerve cell bodies as well as over the neuropil in discrete neuroanatomical regions (Table I). Neurons in some regions, particularly within the diencephalon, were intensely labeled, whereas neurons in other regions of the brain were only weakly to moderately labeled. The patterns of distribution observed in sections incubated for either 15 or 45 min were indistinguishable. The regional and cellular patterns of distribution are described below.

Intensely labeled cells were detected in the diencephalon (Fig. 4), particularly in the mediobasal hypothalamus. The most intensely labeled cells in the hypothalamus were localized within the arcuate and periventricular nuclei (Fig. 5). Within the arcuate nucleus, intensely labeled cells tended to cluster dorsally and medially near the border of the third ventricle. In the rostral pole of the arcuate nucleus (Fig. 5a), approximately 50% of neurons were intensely labeled, and approximately 30% were moderately to intensely

labeled, whereas in the caudal pole of the nucleus (Fig. 5b), approximately 8% of neurons were intensely labeled, and 18% moderately to intensely labeled. The median eminence was unlabeled in all cases (Fig. 5b). Within the periventricular nucleus, intensely labeled cell bodies were found just beside the ependymal cell border of the third ventricle (Fig. 5a). The greatest number of labeled cells in this nucleus was observed at the level of the junction between the retrochiasmatic area and the rostral pole of the arcuate nucleus. Approximately 8-10 intensely labeled neurons per 20 μm- thick section were found unilaterally at the level of the optic chiasm, 15-20 at the level of the retrochiasmatic area, and approximately 5 labeled neurons at the level of the rat brain just caudal to the xiphoid thalamic nucleus (Paxinos, 1986).

The most intensely labeled cells in this paradigm were found in the medial part of the zona incerta (Fig. 6b). The greatest aggregate of labeled cells in this region was at the level of the extreme rostral pole of the arcuate nucleus, just caudal to the level of the retrochiasmatic area, where approximately 70% of neurons were intensely labeled. Caudally, the proportion of labeled neurons dropped off sharply and approximated 5% of all neurons in this region at the level of the rat brain just caudal to the level of the xiphoid thalamic nucleus (Paxinos, 1986).

Intensely labeled cells were also detected more caudally, at the level of the mesencephalon, in the substantia nigra pars compacta (Fig. 6a).

Weakly to moderately labeled cells were detected in several diencephalic nuclei. Four to seven lightly to moderately labeled neurons were detected in the dorsal-medial aspect of the paraventricular nucleus just dorsal and lateral to the apex of the third ventricle, and in the ventral-lateral aspect along the inferior border of the nucleus. Scattered moderately labeled neurons were also detected within the confines of the anterior hypothalamic area (Fig. 7d), the lateral hypothalamus, and in the region of the tuber cinereum.

Within the telencephalon, a number of different structures contained weakly to moderately labeled cells. A few faintly labeled neurons occupied the medial aspect of the paraventricular nucleus of the thalamus. A subpopulation of neurons in the dorsal lateral

aspect of the medial habenula was weakly to moderately labeled (Fig. 8c). Rostrally, approximately 30% of cells in the structure were moderately labeled, however, caudally, the number of labeled cells approached only 5% and the intensity of signal overlying these cells was considerably lower. The lateral habenula, on the contrary, did not embody labeled cells.

A few moderately labeled cells were observed in the hilus of the hippocampus (Fig. 7c). Furthermore, labeled cell bodies, ranging in number from 3 to 12 per 20μm-thick section, were found unilaterally along the pyramidal cell layer of the CA3 region of Ammon's horn. The dorsal medial blade of the subiculum contained a few weakly labeled cells. The CA1 and CA2 fields of Ammon's horn together generally contained not more than five moderately labeled cells unilaterally per 20 μm- thick section.

The retrosplenial region of the cingulate cortex contained a modicum of weakly to moderately labeled cells in layer V (Figs. 7a,b). Approximately 30% of neurons in these deeper cell layers were labeled. Labeled neurons were found primarily within the retrosplenial cortex, however, a small number of labeled neurons were also found in frontal cortical areas 1 and 2.

In addition to containing labeled cell bodies, some regions of the brain contained accumulations of radioautographic grains over the neuropil. Light to moderate densities of radioautographic grains were diffusely localized over the neuropil at all rostral to caudal levels within the dorsal lateral aspect of the medial habenula (Fig. 8c). The diffuse labeling of the neuropil was uniform across the rostral to caudal extent of the medial habenula.

The highest density of silver grains found in the hippocampus was in the stratum lacunosum moleculare (Fig. 8a). The radioautographic signal in this region was diffuse, and coincided with the anatomical position of the perforant pathway. A diffuse array of radioautographic grains was also observed over the neuropil in the medial blade of the subiculum of the hippocampus and along the interior aspect of the dentate granule cell layer (Fig. 7c).

4°C Conditions

Slices of rat brain incubated with ^{125}I-SRIF at 4°C exhibited only background levels of radioautographic signal. Labeled cells or fibers were not observed in any regions of the brain in these sections (Figs. 8b;9b)

Displacement of Uptake with [DTrp^8]Somatostatin

Slices incubated with ^{125}I-Tyr^0[DTrp^8]SRIF in the presence of more than a thousand fold higher concentration of non-radiolabeled [DTrp^8]SRIF (Figs. 8d;9c) contained accumulations of radioautographic grains in the same regions as slides incubated with the radiolabeled peptide alone (Figs. 8c;9a). Although the patterns of distribution of labeled cells in the two experimental conditions were homologous, the intensity of the radioautographic signal in sections incubated in the presence of an excess of non- radiolabeled SRIF, was, particularly in regions characterized by light to moderate labeling intensities, attenuated (Fig. 8d). The number of labeled cells in any region was not, however, appreciably diminished by incubation with ^{125}I-SRIF in the presence of an excess of non- radiolabeled peptide.

Octreotide

Slices incubated with ^{125}I-Tyr^0[DTrp^8]SRIF (1.5 nM) in the presence of octreotide (10μm) (Fig. 9d) had patterns of distribution of labeled cells and diffuse neuropil labeling homologous to slices incubated with ^{125}I-Tyr^0[DTrp^8]SRIF alone, and could not be distinguished from slices incubated with ^{125}I-SRIF in the presence of an excess of [DTrp^8]SRIF.

Nomifensin

Slices of rat brain diencephalic and mesencephalic tissue incubated with ^{125}I-Tyr^0[DTrp^8]SRIF (1.5 nM) in the presence of nomifensin (10 μM) (Fig. 10b) did not contain labeled cells in any region of the brain observed to contain intensely labeled cells when incubated with ^{125}I-Tyr^0[DTrp^8]SRIF alone.

Figure 1. Displacement of ^{125}I-SRIF binding to cortical rat brain homogenates by increasing concentrations of non-radiolabeled DTrp^{8}-SRIF. K_D = 0.3 nM. Representative graph from one experiment.

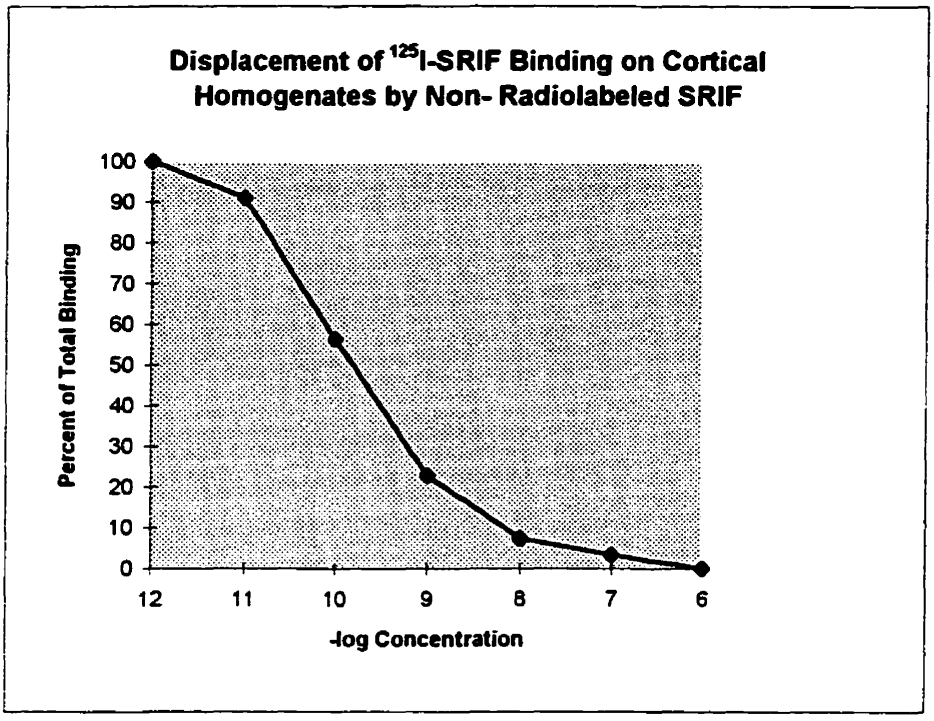
Displacement of 125I-SRIF Binding on Cortical Homogenates by Non- Radiolabeled SRIF
Percent of Total Binding
100
90
80
70
60
50
40
30
20
10
0
12
11
10
9
8
7
6
-log Concentration

Figure 2. Internalization kinetics of ^{125}I-SRIF mediated by sst_1 (open circles) and sst_2A (closed circles) receptors expressed on the surface of COS 7 cells. The efficiency of internalization at each time point was calculated to be the percent of specific ^{125}I-SRIF binding that, at 37°C in the absence of phenylarsine oxide, remained resistant to a NaCl acid- wash (pH, 4.0). Mean ± standard error of the mean of three experiments.

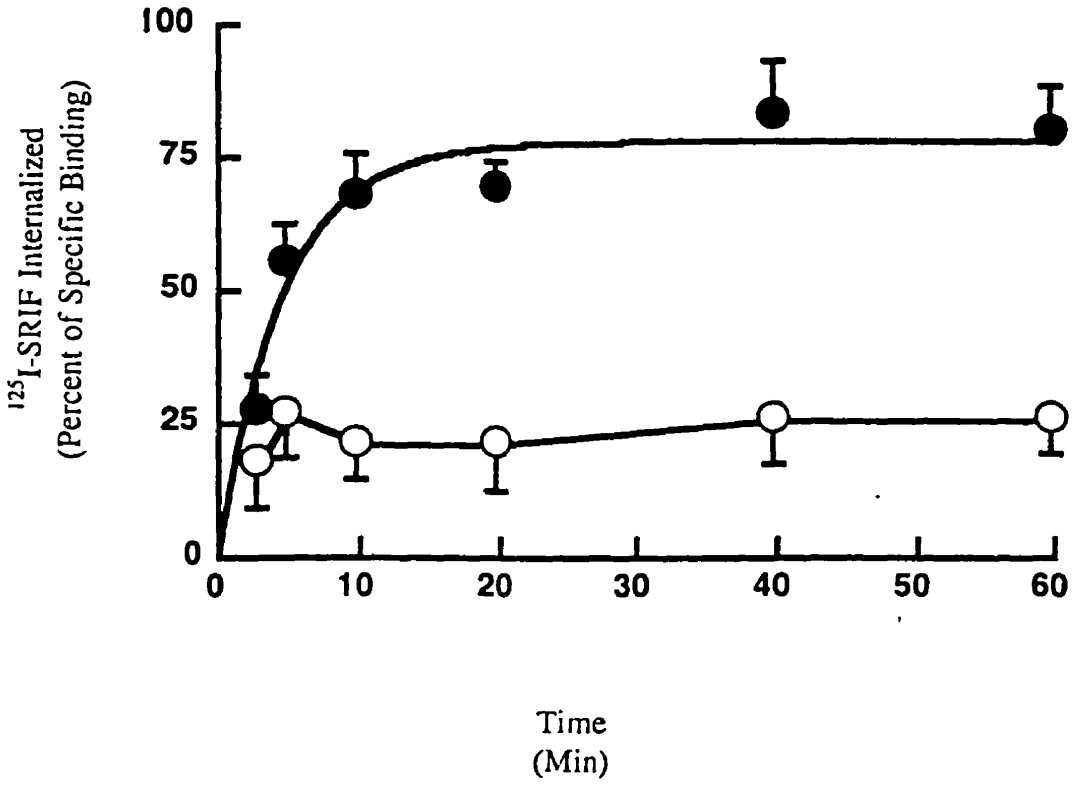
100
75
50
25
0
0
10
20
30
40
50
60
^{125}I-SRIF Internalized
(Percent of Specific Binding)
Time
(Min)

Figure 3. Autoradiograms of COS 7 cells transfected with the cDNA encoding the sst_2A receptor and incubated with 0.35 nM ^{125}I-SRIF in the absence (a), or in the presence (b), of 1 x 10^{-5} M unlabeled DTrp^8-SRIF for 30 min at 37°C and subsequently washed with a NaCl buffer (pH 4) to dissociate surface bound radioactivity. In (a), labeled cells (arrows) are visible over approximately 22% of cells, whereas only background levels of radioautographic signal are detected over cell bodies in (b). That only a proportion of cells in (a) are labeled reflects the yield of the cDNA transfection. (Magnification: x200)

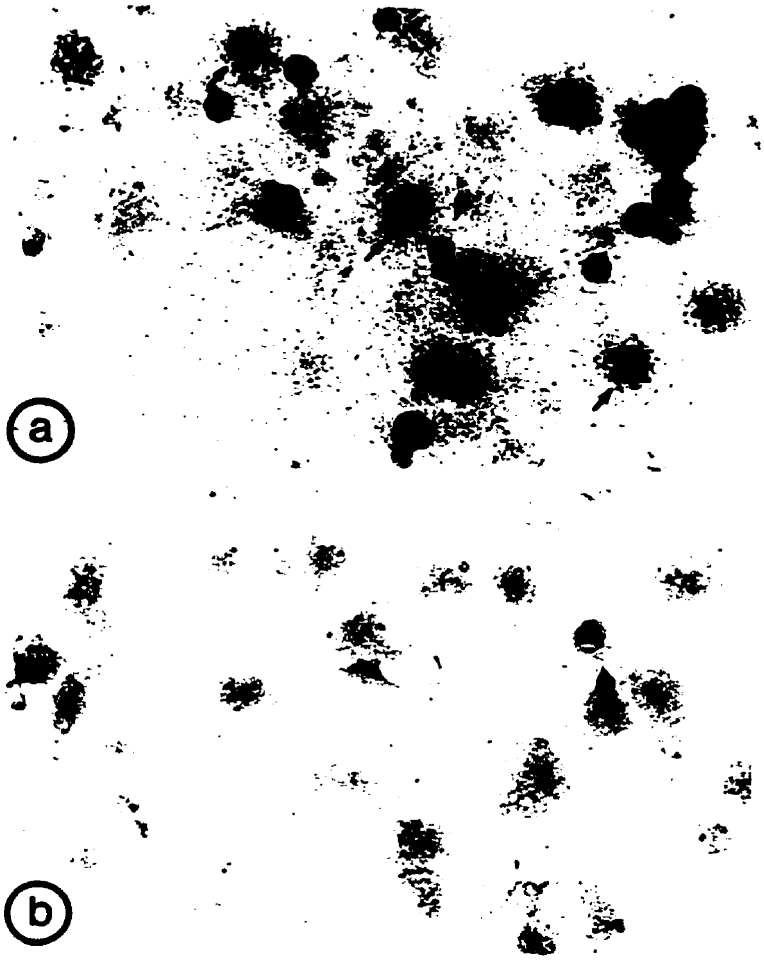
a
b

Figure 4. Representative semi-schematic diagrams of the diencephalon along the rostral (a) to caudal (c) axis of the rat brain illustrating the distribution of intensely labeled cells in sections of rat brain incubated for 45 min at 37°C with ^{125}I- SRIF and washed with a hyperosmolar NaCl buffer (pH 4) to dissociate surface bound radioactivity. •'s represent one labeled cell; x's represent clusters of three labeled cells. Note that the number of labeled cells in the arcuate and periventricular hypothalamic nuclei decreases along the rostral to caudal axis of the brain. The greatest aggregate of labeled cells in the medial part of the zona incerta is at the level of the rostral pole of the arcuate nucleus (b). AHA, anterior hypothalamic area; Arc, arcuate nucleus; f, fornix; LH, lateral hypothalamic area,; MCPO, magnocellular preoptic nucleus; ME, median eminence; opt, optic tract; Pa, paraventricular hypothalamic nucleus; Pe, periventricular hypothalamic nucleus; RCh, retrochiasmatic area; Rh, rhomboid thalamic nucleus; so, supraoptic nucleus; sox, supraoptic decussation; vmh, ventromedial hypothalamic nucleus; zi, zona incerta.

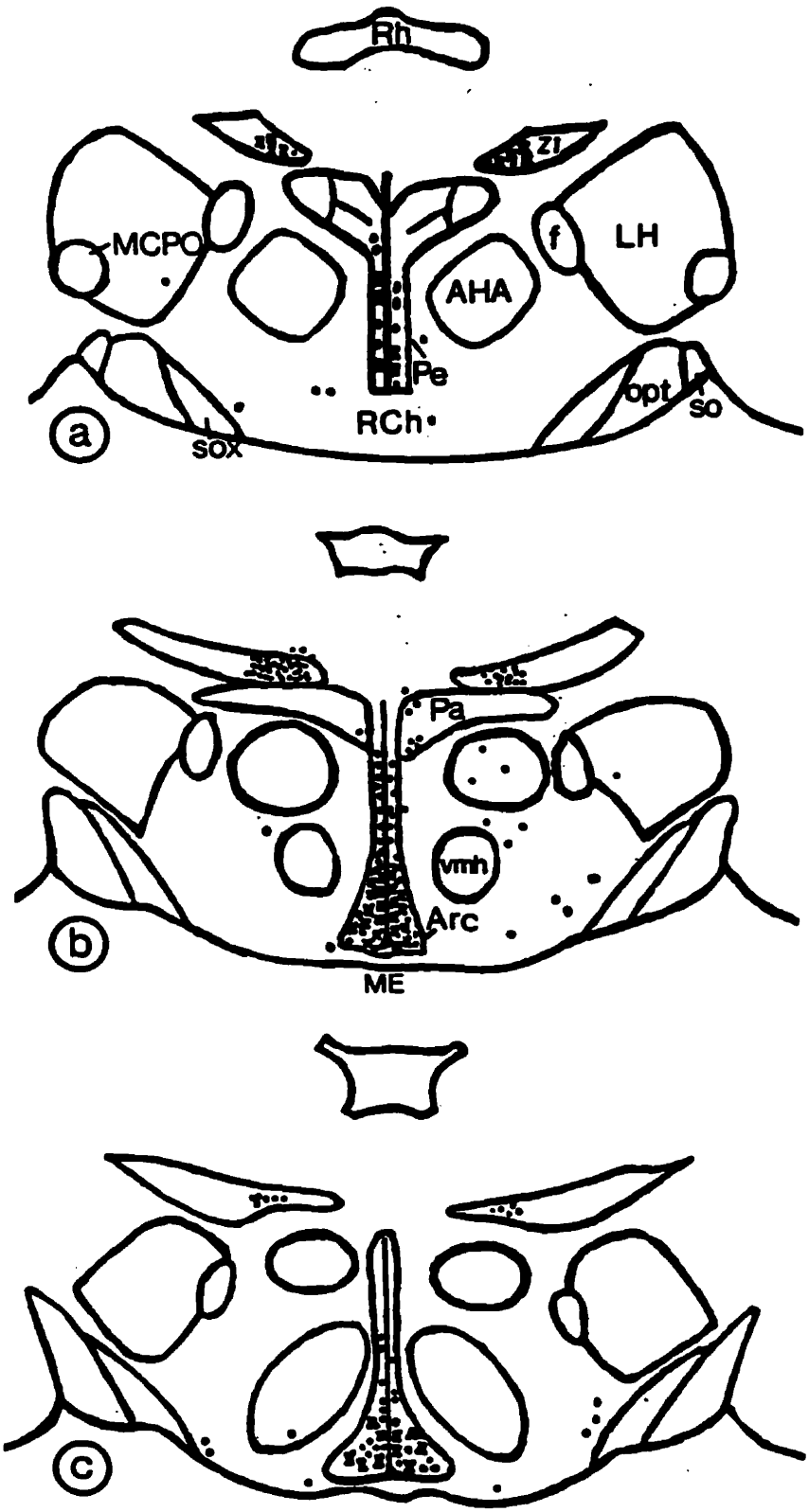
Rh
sfx
zi
MCPO
f
LH
AHA
Pe
opt
so
RCh
sox
a
Pa
vmh
Arc
b
ME
c

Figure 5. Autoradiographic distribution of iodinated ligand uptake in coronal sections of the rat brain taken at the level of the rostral pole (a) and caudal pole (b) of the arcuate nucleus. Sections were incubated with 1.5 nM ^{125}I-SRIF for 45 min at 37°C and subsequently washed with a NaCl buffer (pH 4) to dissociate surface bound radioactivity. Labeled cells are detected in the form of small silver grain foci prominent against a relatively high background labeling. In (a), labeled cells are localized throughout the arcuate nucleus as well as in the periventricular nucleus, just beside the border of the third ventricle. In (b), labeled cells are clustered dorsomedially in the arcuate nucleus and are less numerous in the periventricular nucleus as compared to (a). Arc, arcuate nucleus; ME, median eminence; Pe, periventricular nucleus; III, third ventricle. (Magnification: x100)

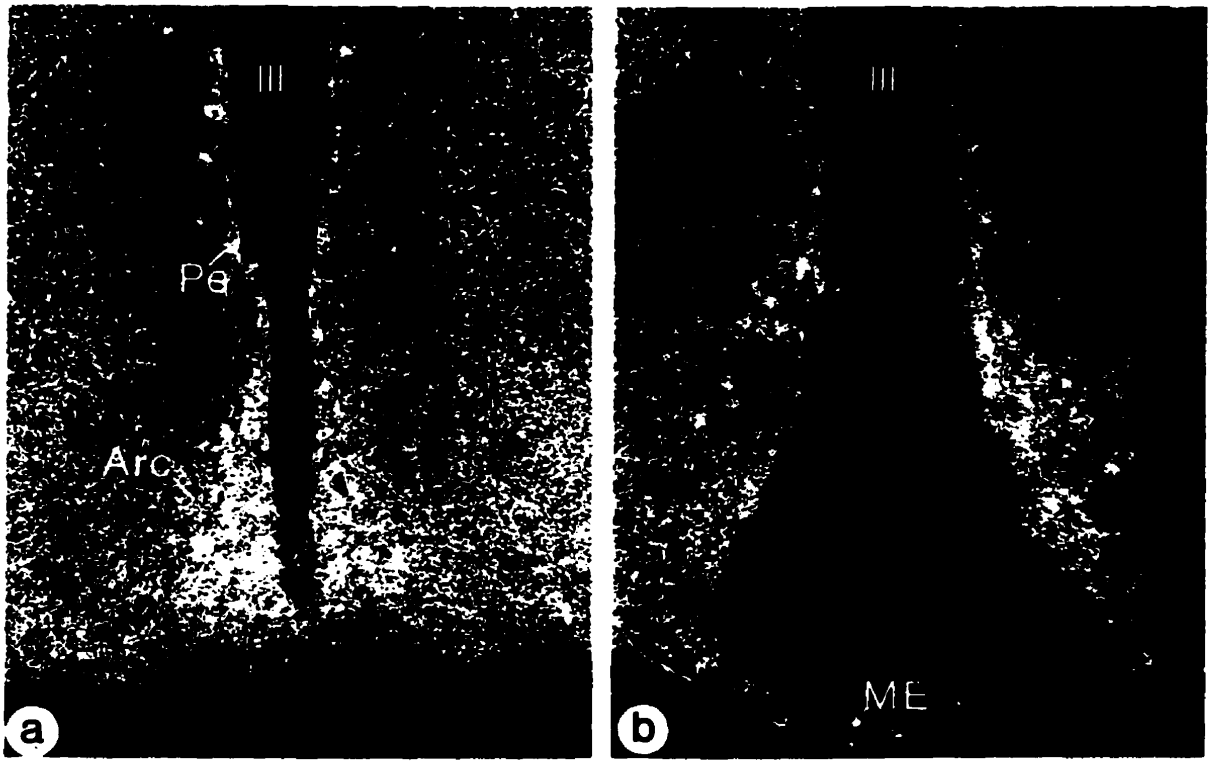
III
Pe
Arc
a
III
ME
b

Figure 6. Autoradiograms from sections of the rat brain incubated with 1.5 nM ^{125}I-SRIF for 45 min at 37°C and washed in a hyperosmolar NaCl buffer (pH 4) to dissociate surface bound radioactivity illustrating the distribution of intensely labeled cells in the substantia nigra (a) and the medial part of the zona incerta (b). In (a), labeled cells, viewed in darkfield, are confined to the pars compacta in the region of the A9 dopaminergic cell group. In (b), labeled cells are localized in the region of the A13 dopaminergic cell group. (Magnification: (a) x^{125}; (b) x313)

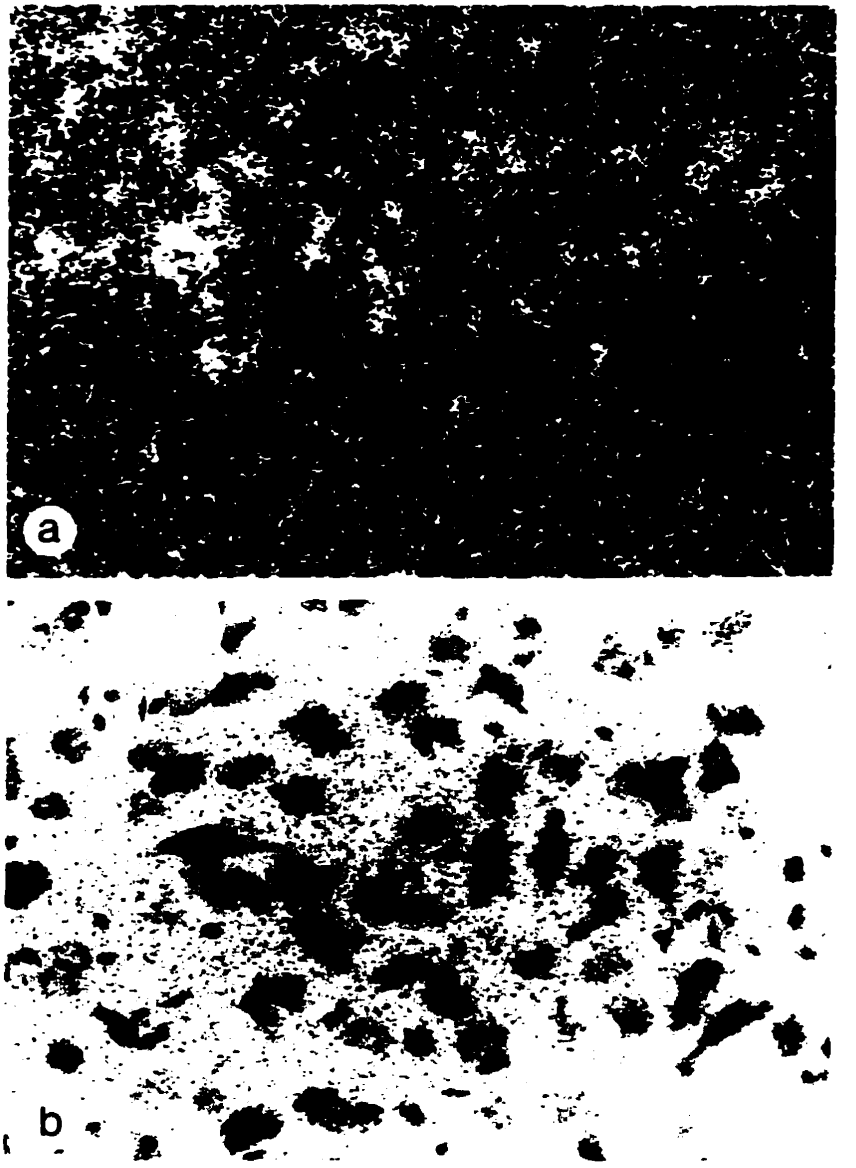
a
b

Figure 7. Darkfield (a, c) and brightfield (b, d) autoradiograms illustrating the distribution of weakly labeled cells in the retrosplenial cortex (a, b), hilus of the hippocampus (c), and anterior hypothalamic area (d) of sections of the rat brain incubated with 1.5 nM ^{125}I-SRIF for 45 min at 37°C and subsequently washed with a hyperosmolar NaCl buffer (pH 4) to dissociate surface bound radioactivity. In (a), numerous moderately labeled cells (arrows) are visible in layer V of the retrosplenial cortex. (b) confirms that the signal in (a) originates from radioautographic grains overlying pyramidal cells (arrows) in the deeper layers of the retrosplenial cortex. In (c), two moderately labeled cells are visible in the hilus (arrows) and a weak diffuse labeling is seen as a white band along the interior verge of the granule cell layer of the dentate gyrus (curved arrows). In (d), two moderately labeled cells (arrows) in the anterior hypothalamic area are visible amongst unlabeled perikarya. GC, granule cell layer; Hi, hilus; Mol, molecular layer. (Magnification: (a) x60; (b) x175; (c) x80; (d) x313)

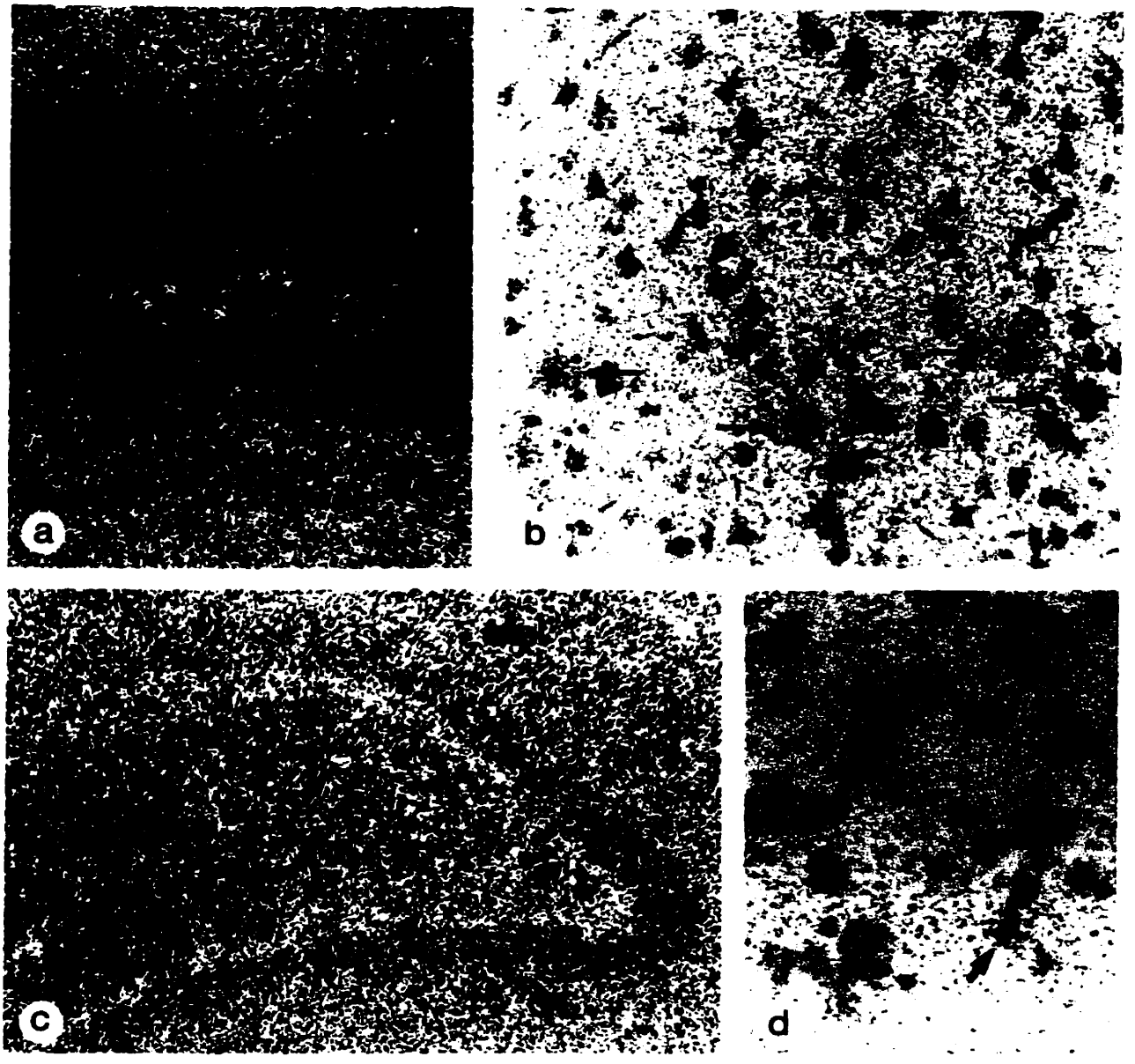
a
b
c
d

Figure 8. Distribution of radioautographic labeling in the hippocampus (a, b) and habenula (c, d) of coronal rat brain sections incubated with 1.5 nM ^{125}I-SRIF for 45 min at 37°C and subsequently washed in a NaCl buffer (pH 4) to dissociate surface bound radioactivity. Colour- enhanced images produced with the BIOCOM image analysis system. A diffuse radioautographic signal of moderate intensity is seen in the stratum lacunosum moleculare in slices incubated with ^{125}I-SRIF at 37°C (a), but not in slices incubated with ^{125}I-SRIF at 4°C (b). The radioautographic signal in the medial habenula (c) is attenuated when the slices are incubated with ^{125}I-SRIF in the presence of an excess of unlabeled DTrp^{8}-SRIF (d). GC, granule cell layer; Hi, hilus; LHab, lateral habenula; LMol, stratum lacunosum moleculare; MHab, medial habenula; Or, stratum oriens; Py, pyramidal cell layer; Rad, stratum radiatum.

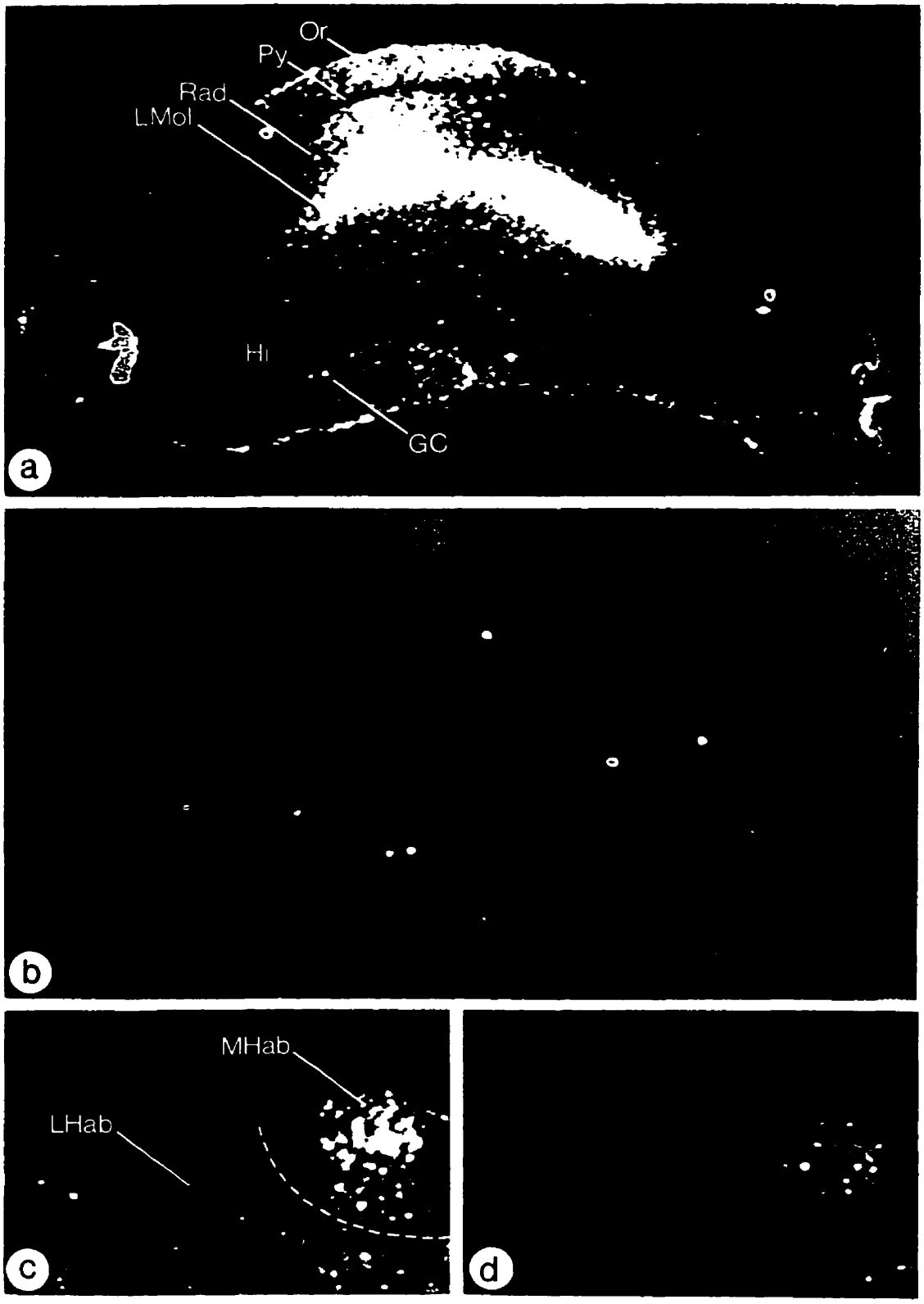
Or
Py
Rad
LMol
Hi
GC
a
b
MHab
LHab
c
d

Figure 9. Darkfield autoradiograms of coronal sections of the rat arcuate nucleus incubated with 1.5 nM ^{125}I-SRIF for 45 min at 37°C (a,c,d) or 4°C (b) in the presence of an excess of non-radiolabeled Dtrp^{8}-SRIF (c) or Octreotide (d). Sections were subsequently washed with a NaCl buffer (pH 4) to dissociate surface bound radioligand. In (a,c,d), intensely labeled cells were detected in the arcuate nucleus, particularly in its dorsomedial aspect. In (b), labeled cells were not observed in the arcuate nucleus. Arc, arcuate nucleus; ME, median eminence; III, third ventricle. (Magnification: x100)

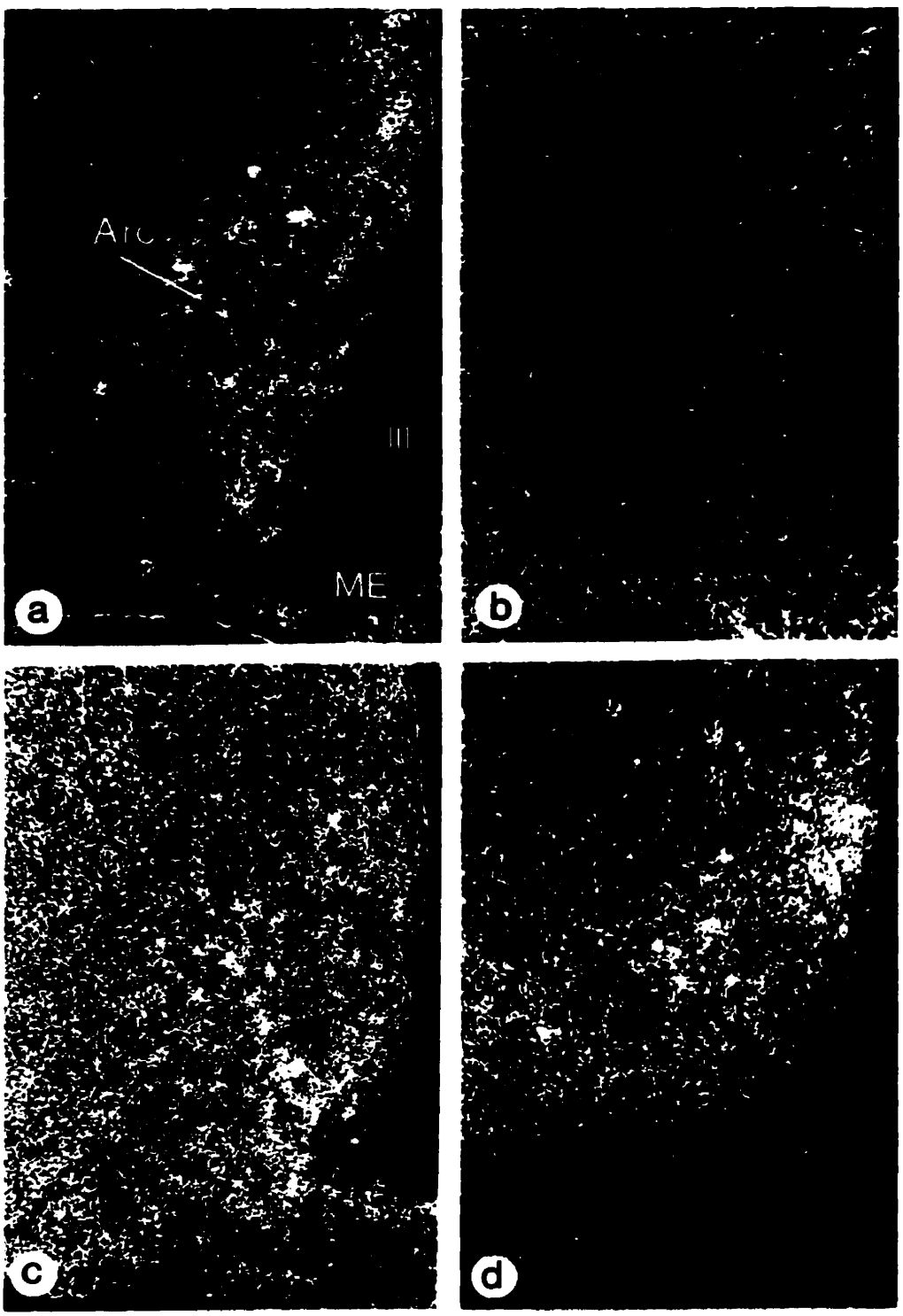
Arc
III
ME
a
b
c
d

Figure 10. Light microscopic autoradiograms of 20 μm- thick sections of the periventricular nucleus from rat brain slices incubated with 1.5 nM ^{125}I-SRIF in the absence (a), or in the presence (b), of 10 μM nomifensin, a pharmacological inhibitor of the dopamine transporter. A subset of cell bodies in (a), but not (b), are intensely labeled (arrows) beside the ependymal cell border of the third ventricle. III, third ventricle. (Magnification: x225)

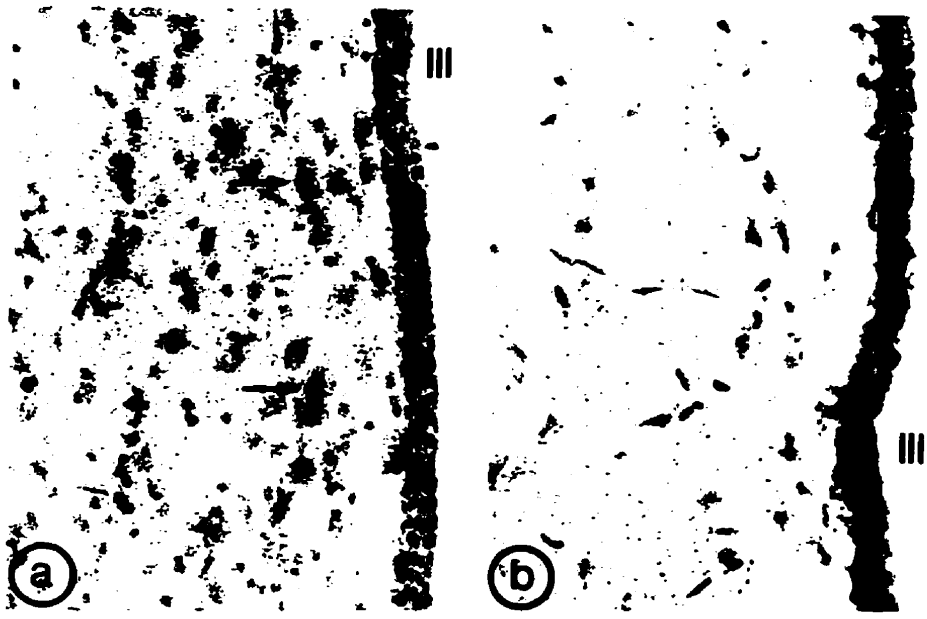
III
a
b
III

Figure 11. Comparative distribution of ^{125}I-SRIF binding sites (a) and sst_2A receptor immunoreactivity (b) in the rat brain. (a) Autoradiogram of ^{125}I-Tyr^0-DTrp^8-SRIF binding on a 20 μm- thick coronal rat midbrain section. Note that in both types of preparation, the labeling predominates in the deep layers of the cerebral cortex, the medial habenula, the hippocampal formation, and the amygdaloid complex. am, amygdaloid complex; cx, cerebral cortex; hi, hippocampal formation; mh, medial habenula. (Magnification: x7) (Figure kindly provided by Dr. Pascal Dournaud)


a
b

Table I. Distribution of Intracellularly Sequestered ^{125}I-Tyr^{0}[DTrp^{8}]Somatostatin in the Brain of the Adult Rat

‡Cell Number values indicate the relative proportion of cells labeled in each region; Intensity of Labeling values indicate the relative intensity of radioautographic grain density as compared to the density of grains over cell bodies in the zona incerta.

REGION	CELL NUMBER	INTENSITY OF LABELING
Periventricular Hypothalamic Nucleus-		
• Rostral	+++	***
• Caudal	+	***
Arcuate Nucleus (Rostral)		
• Dorsal	++++	***
• Ventral	+++	**
Arcuate Nucleus (Caudal)		
• Dorsal	+	***
• Ventral	++	**
Paraventricular Hypothalamic Nucleus	+	**
Anterior Hypothalamic Area	±	**
Tuber Cinereum	+	**
Lateral Hypothalamus	±	*
Zona Incerta-		
• Rostral	++++	***
• Mid	+	***
• Caudal	-	-
Paraventricular Thalamic Nucleus	+	*
Medial Habenula (Rostral)	++	** Diffuse **
Medial Habenula (Caudal)	+	* Diffuse **
Hippocampus		
• Stratum Lacunosum Moleculare		Diffuse ***
• CA3 Pyramidal Cells	+	**
• Hilus	+	**
Retrosplenial Cortex	+++	**
Frontal Cortex	+	*
Substantia Nigra Pars Compacts	++++	***

DISCUSSION

Several reports have indicated that SRIF is internalized into cells expressing functional SRIF binding sites (Amherdt, 89; Draznin, 85; Morel, 86; Viguerie, 87). Furthermore, Nouel *et al.* (Nouel, 94) recently reported, using a fluorescent analogue of SRIF, α-bodipy-[D-Trp^8]-SRIF and confocal microscopy, that SRIF is internalized into endosome-like vesicles in Chinese hamster ovary cells transfected with the cDNA encoding either the sst_1 or sst_2 receptor. To confirm these observations biochemically, we studied the kinetics of internalization of SRIF mediated by these two receptor subtypes using ^{125}I-Tyr^0[DTrp^8]SRIF as a ligand.

^{125}I-Tyr^0[DTrp^8]SRIF was selected for use in the present study as: (1) this ligand could be iodinated on the terminal Tyr residue; (2) DTrp^8 residues are known to enhance the stability of radioligands (Kitabgi, 1985); (3) this ligand has previously been used to study SRIF binding sites *in vitro* by quantitative (Bertherat, 1991), and non-quantitative (Epelbaum, 1989; McCarthy, 1992), radioautography; and (4) this ligand has been shown to bind to both sst_1 and sst_2 receptors expressed on transfected cells (Raynor, 1993).

The binding properties of ^{125}I-Tyr^0[DTrp^8]SRIF were assessed by binding to cortical homogenates. The K_D of 0.3 nM obtained here using ^{125}I-Tyr^0[DTrp^8]SRIF was consonant with the value of 0.28 nM previously reported for this ligand by Bertherat *et al.* (Bertherat, 1991). Taken together, the displacement data obtained here, and the previous use of this ligand in radioautographic binding studies (Bertherat, 1991; Epelbaum, 1989; McCarthy, 1992) indicate that ^{125}I-Tyr^0[DTrp^8]SRIF is an appropriate ligand for use here to study functional SRIF binding sites.

COS 7 Cells: Kinetics

To study the kinetics of SRIF internalization, we employed a variation of the acid extraction protocol initially developed by Haigler (Haigler, 1980) that has previously been used to define the kinetics of internalization of neurotensin (Chabry, 1995). This indirect biochemical method mimics the pH dependent physiological process that

dissociates peptides from their receptors within acidic prelysosomal vesicles (Dautry, 1983; Tycko, 1982). As such, this method enables the differentiation of internalized (acid-resistant) iodinated peptides from surface bound (acid-extractable) iodinated peptides.

When whole COS 7 transfected cells were incubated with ^{125}I-Tyr^0[DTrp^8]SRIF in the absence of phenylarsine oxide, a fraction of specifically bound ^{125}I-Tyr^0[DTrp^8]SRIF at times between 3 and 60 minutes remained resistant to the acid-wash step indicating that it had been sequestered intracellularly. That an excess of non-radiolabeled SRIF inhibited uptake of SRIF by transfected COS 7 cells suggests that uptake of ^{125}I-SRIF by transfected COS 7 cells occurred by a receptor- mediated mechanism. That phenylarsine oxide inhibited the uptake process in these same cells suggests that the transmembrane mobilization process involved endocytosis. Subtraction of data points obtained after acid- washing from data points obtained without an acid-wash step provided a quantitative measure of the amount of radioactivity sequestered intracellularly and allowed us to differentiate the kinetics of internalization from the kinetics of binding.

Notwithstanding that both SRIF receptor subtypes investigated internalized SRIF, the kinetics of internalization were distinct for each receptor subtype. Whereas cells transfected with the sst_1 receptor internalized between 20 and 25% of specifically bound SRIF by a low efficiency mechanism, cells transfected with the sst_2A receptor internalized up to 75% of specifically bound SRIF by a high efficiency ligand- induced mechanism.

Our results with COS 7 cells transfected with the sst_2A receptor cDNA are consistent with reports from several other groups that indicate that SRIF can be rapidly and perceptibly internalized into cells after binding to its receptor. Our data are concordant with those of Amherdt and colleagues who reported that when pancreatic islet α and β cells were incubated with [^{125}I-Tyr]SRIF-14 for 60 min at 37°C, approximately 60% of the total number of cell associated radioautographic grains were localized intracellularly (Amherdt, 1989). These authors reported that this process was both time- and temperature- dependent (Amherdt, 1989). Furthermore, light and electron

microscopic morphological observations indicate that a significant quantity (75.5%) of total cell associated SRIF is internalized into AtT-20 cells where it is found in association with the Golgi apparatus, lysosomes, cytoplasmic matrix, mitochondria, rough endoplasmic reticulum, secretory granules, nuclear membrane, and nucleus (Morel, 1986).

Our results with COS 7 cells transfected with the sst_1 receptor cDNA demonstrated a low efficiency of SRIF internalization reminiscent of that which was observed with RINm5F insulinoma cells (Sullivan, 1986) and GH_4C_1 pituitary cells (Presky, 1986) in culture. Iodinated SRIF analogues were reported not to be appreciably internalized into these cells (Presky, 1986; Sullivan, 1986). Approximately 75% of iodinated SRIF bound to RINm5F cells after 90 min of incubation at 37°C (Sullivan, 1986), and approximately 80% of iodinated SRIF bound to GH_4C_1 cells after 60 min of incubation at 37°C (Presky, 1986), could be removed by acid treatment. It is, therefore, conceivable that RINm5F insulinoma cells (Sullivan, 1986), and GH_4C_1 pituitary cells (Presky, 1986), had, in fact, internalized small quantities of SRIF via a low efficiency mechanism akin to that demonstrated here for the sst_1 receptor when expressed on COS 7 cells.

The exponential increase in the internalization rate observed between 0 and 10 min, and the half time of internalization equal to 5-6 min for cells transfected with sst_2A receptor cDNA were similar to those reported for the neurotensin receptor expressed on the surface of COS 7 cells (Chabry, 1995). Although our data do not indicate whether the sst_1 and sst_2A receptors were internalized along with the iodinated ligand, SRIF may be internalized as part of a receptor-ligand complex as suggested by the fact that both neurotensin (Faure, 1995a; Faure, 1995b) and substance P (Mantyh, 1995), neuropeptides that interact with G protein- coupled receptors, are internalized as receptor- ligand complexes.

The precise difference in the amino acid sequence between the sst_1 and sst_2A receptors that underlies the different kinetics of ligand internalization is unknown. The difference may be imparted by variations in amino acid sequences or quaternary structures of the two receptors, particularly in their C termini, as they differ in their

respective sequences by more than 51%. The former hypothesis would be in keeping with data indicating that point mutations to the C terminal tail of the neurotensin receptor can affect the efficiency of internalization (Chabry, 1995). Likewise, C terminal amino acid sequences have been reported to be critical for ligand internalization mediated by the thyrotropin- releasing hormone (Nussenzveig, 1993), gastrin- releasing peptide (Benya, 1993), and the β2-adrenergic (Barak, 1994) receptors. Further biochemical, molecular biological, and pharmacological studies are needed to substantiate this hypothesis.

The present study provides the first example of two receptor subtypes that internalize the same agonist differently. The kinetic profiles of internalization suggest that while internalization of SRIF mediated by the sst_2A receptor is ligand- induced, internalization of SRIF mediated by the sst_1 receptor may be ligand- independent. The differences in the kinetic profiles of SRIF internalization exhibited by the sst_1 and sst_2A receptors suggest that the physiological role of internalization of SRIF by each receptor may be distinct. Low efficiency internalization of SRIF mediated by the sst_1 receptor may be important for receptor recycling as is the case for the β2- adrenergic receptor (von Zastrow, 1992). On the other hand, high efficiency internalization of SRIF mediated by the sst_2A receptor may serve to promote peptide degradation as suggested by Viguerie *et al.* (Viguerie, 1987), or, in keeping with the recent demonstration of nuclear binding sites for SRIF (Todisco, 1994), may potentiate nuclear signal transduction by SRIF in cells expressing this receptor subtype.

COS 7 Cells: Radioautography

To investigate ligand- induced internalization of SRIF in the rat brain, we sought to develop a methodology to enable the visualization of intracellularly sequestered ^{125}I-SRIF. To this end, we combined classical biochemical (acid washing) and morphological (radioautography) techniques. Glutaraldehyde was used as a fixative in this paradigm as this dialdehyde rapidly stabilizes structures by cross-linking before there is any opportunity for extraction by the buffer (Glauert, 1981). In order to validate the methodology, we first examined internalization of ^{125}I-SRIF by COS 7 cells *in vitro*.

Using COS 7 cells transfected with the cDNA encoding the sst_2A receptor, we found that our assay permitted the visualization of ^{125}I-Tyr^0[DTrp^8]SRIF that had been sequestered intracellularly. Uptake of SRIF by COS 7 cells was receptor- mediated as: (1) uptake of ^{125}I-SRIF could be could be prevented by an excess of non- radiolabeled SRIF; (2) non-transfected cells did not take up ^{125}I-SRIF; and (3) only a proportion of cells in the transfected series were labeled. This proportion of labeled cells (approximately 22%) having successfully incorporated the plasmid encoding the receptor into their genome was congruent with the expected yield of transfection using the present technique in this cell line (Pollard, 1984).

Rat Brain Slices: Ex Vivo *Radioautography*

Once we had validated our methodology for visualizing internalized SRIF using COS 7 transfected cells *in vitro*, we applied an *ex vivo* variation of our technique to slices of rat brain tissue to determine if receptor- mediated internalization of SRIF occurred in the brain.

Subsequent to incubation of rat brain slices with ^{125}I-SRIF for 45 min at 37°C and acid- wash treatment, the intensity of labeling over cell bodies was not, however, equally intense in all regions. There were two types of labeling observed in slices of rat brain processed in our *ex vivo* paradigm. One type of labeling was intense and strictly perikaryal; the other was of weak to moderate strength and found over both perikarya and neuropil. The most intensely labeled cells were localized in the medial aspect of the zona incerta, the rostral and caudal poles of the arcuate nucleus, the periventricular nucleus, and the substantia nigra pars compacta. Less intensely labeled cell bodies were found in the deep layers of the retrosplenial and frontal cortices, the dorsal lateral aspect of the medial habenula, the hilus of the hippocampus, and in the anterior hypothalamic area/ tuber cinereum. Furthermore, diffuse extracellular labeling of weak to moderate intensity was observed in both the stratum lacunosum moleculare of the hippocampus, and in the medial habenula.

Slices of rat brain tissue incubated with ^{125}I-SRIF for 45 min at 4°C did not have silver grain accumulations over nerve cell bodies or neuropil in any region indicating that

the putative mechanism(s) by which the radioactivity gained access to the intracellular compartment was/were energy dependent.

To determine if the process(es) by which the iodinated compound was taken up into the intracellular compartment was/were saturable, slices of rat brain were co-incubated at a physiological temperature with ^{125}I-Tyr^0[DTrp^8]SRIF and an approximately 7000 fold higher concentration of unlabeled [Dtrp^8]SRIF and subsequently washed with an acidic buffer to dissociate surface bound radioactivity. The patterns of distribution of radiographic grains in slices of rat brain radiolabeled in the presence of an excess of unlabeled peptide were similar to the patterns observed in slices incubated with the radiolabeled peptide alone. The intensity of the radioautographic signal, as observed light microscopically, was, however, attenuated, particularly in those regions with weak to moderate intensities of labeling.

The regional pattern of distribution of neurons intensely labeled in our study did not conform to the regional pattern of distribution of SRIF binding sites previously defined using autoradiography with various iodinated SRIF analogues (Krantic, 1992) or to the patterns of expression of the sst_1 and sst_2 receptors described here, or in other studies (Bruno, 1993; Kong, 1994; Perez, 1994) using *in situ* hybridization histochemistry. For example, the most intensely labeled cells in this study were found in the zona incerta, a nucleus in which neither sst_1 nor sst_2 receptor mRNA was appreciably detected using *in situ* hybridization histochemistry (present study), and in which low to undetectable levels of SRIF binding sites were detected by autoradiography with iodinated SRIF analogues (Gulya 1985; Leroux, 1985; Leroux 1988; Krantic, 1989; Krantic, 1990; Reubi, 1985; Whitford 1985).

Furthermore, within the arcuate nucleus, the distribution of labeled cells did not correspond with the anatomical dispersion of SRIF binding found to be associated with growth hormone-releasing hormone neurons (Bertherat, 1991; Bertherat, 1992; Epelbaum, 1989; McCarthy, 1992), and which have been hypothesized to be directly regulated by SRIF (Tannenbaum, 1990). Whereas binding sites labeled with [^{125}I]Tyr^0-DTrp^8-SRIF14 in the arcuate nucleus are more numerous caudally than rostrally, and tend to spread laterally within the nucleus (Epelbaum, 1991; McCarthy, 1992), the cells in the

arcuate nucleus that were intensely labeled in our *ex vivo* assay were more numerous rostrally than caudally, and tended to be clustered dorsally and medially.

The distribution of intensely labeled neurons within the zona incerta, arcuate nucleus, periventricular nucleus, and substantia nigra pars compacta in our paradigm paralleled that of the dopaminergic neurons. We noted a striking similarity between the neuroanatomical distribution of intensely labeled cells in our study and that previously reported for dopaminergic cells as defined by fluorescence histochemistry and tyrosine hydroxylase immunocytochemistry (Björklund, 1984).

Within the diencephalon, the dopaminergic cells can be divided into four cell subgroups termed A11, A12, A13, and A14. The tuberal cell group, A12, occupies the arcuate nucleus, particularly its dorsal pole, and the periventricular nucleus rostral to the arcuate nucleus (Björklund, 1984). This cell group projects to the posterior and intermediate lobes of the pituitary (Björklund, 1984). The major aggregate of the caudal diencephalic cell group, A11, is found in the periventricular grey of the caudal thalamus, and is believed to project locally within the diencephalon and to give rise to dopaminergic spinal chord afferents (Björklund, 1984). The cells of the dorsal hypothalamic group, A13, are found clustered in the medial aspect of the zona incerta just ventromedial to the mammillothalamic tract (Björklund, 1984). Cells comprising the A14 subgroup are found within the periventricular hypothalamic nucleus from the level of the anterior commissure to the rostral border of the median eminence (Björklund, 1984).

Within the ventral tegmental area of the mesencephalon, the principal aggregate of dopamine containing neurons are found within the substantia nigra pars compacta; they are termed the A9 subgroup (Björklund, 1984).

The most striking correlation between the intensely labeled cells in our paradigm and the dopaminergic cells was with the A9, A12, and A13 cell groups (compare Fig. 4 with Fig. 1, Björklund, 1984). Even more strikingly, the intensity and distribution of labeling observed over strongly labeled neurons in our assay paralleled the intensity and distribution of neurons expressing the highest concentrations of dopamine transporter mRNA as detected by *in situ* hybridization histochemistry (Lorang, 1994). Indeed, not all dopaminergic cells express the dopamine transporter to the same extent (Lorang, 1994).

For example, the highest proportion of intensely labeled cells in our paradigm was found in the rostral pole of the medial aspect of the zona incerta. This same region contains amongst the highest levels of expression of the mRNA encoding the dopamine transporter (Lorang, 1994). On the other hand, amongst the lowest levels of both dopamine transporter mRNA and cellular labeling in our paradigm were observed in the region of the A14 dopaminergic cell group. These observations prompted us to investigate the possibility that radioactivity might have been taken up from the extracellular milieu into dopaminergic cells by the dopamine transporter.

The dopamine transporter uses the energy provided by the Na^+ gradient generated by the Na^+/K^+ ATPase to potentiate the rapid uptake of dopamine soon after its release into the synaptic cleft (Giros, 1993). In order to investigate whether the dopamine transporter, known to be expressed on dopaminergic neurons (Lorang, 1994), might have played a role in the uptake of SRIF, we incubated slices of the rat brain for 45 min at 37°C with ^{125}I-Tyr^0[DTrp^8]SRIF in the presence of nomifensin, a pharmacological inhibitor of the dopamine transporter with a K_i for the cloned rat dopamine transporter approximately 100 fold lower than that of dopamine (Giros, 1993). To our surprise, addition of nomifensin to the incubation medium completely abolished nerve cell body labeling in both the diencephalon and the substantia nigra. It is thus tempting to speculate that the intracellular sequestration of radioactivity by the intensely labeled neurons, as assayed here, may have been mediated by the dopamine transporter. However, given that n=1 for the nomifensin experiment, this interpretation is purely supposititious.

It is unlikely that ^{125}I-Tyr^0[DTrp^8]SRIF serves as a substrate for the dopamine transporter. The dopamine transporter, however, could recognize the iodinated tyrosine fragment separated from the SRIF moiety. Indeed, Sullivan and Schonbrunn reported that a putative extracellular metalloendoprotease cleaves ^{125}I-Tyr from ^{125}I-Tyr^{11}SRIF (Sullivan, 1986). Although bacitracin, a noncompetitive inhibitor of papain and subtilisin, was included in our incubation buffer, this compound is not particularly effective against metalloproteases (Sullivan, 1986), and therefore, we cannot exclude the possibility that free ^{125}I-Tyr may have been produced in the process of peptide degradation, and subsequently taken up by the dopamine transporter. Uptake of tyrosine into dopaminergic

cells would be consistent with the requirement of these neurons for tyrosine, the precursor in the synthesis of dopamine (Cooper, 1991). The present results would also be in keeping with positron emission tomographic studies in rhesus macaque monkeys indicating that radiolabeled tyrosine analogues such as ^{18}F-6-Fluoro-beta-fluoromethylene-m-tyrosineaccumulate accumulate in dopamine- rich neuroanatomical regions such as the caudate-putamen (DeJesus, 1992). It is possible that the uptake of radioactivity in our paradigm involved the interspecific uptake of iodinated tyrosine by the nomifensin sensitive- dopamine transporter, as it has already been shown that the dopamine transporter interspecifically takes up epinephrine and norepinephrine (Meriergerd, 1994; Giros, 1992).

In our paradigm, weakly to moderately labeled cells were found in the deeper layers of the retrosplenial and frontal cortices, medial habenula, and hilus of the hippocampus. Labeling of weak to moderate intensity was also observed over the neuropil in both the medial habenula and the stratum lacunosum moleculare. By contrast to what was observed for intensely labeled cells, the pattern of distribution of weakly to moderately labeled cells in the present study was markedly similar to the distribution of regions found to contain amongst the highest concentrations of sst_2 receptor mRNA in the rat brain (present study). For example, the medial habenula contained a modicum of moderately labeled cells and a diffuse array of radioautographic grains over the neuropil in its dorsal lateral aspect when rat brain slices were incubated with ^{125}I-SRIF at 37°C. Similarly, weakly to moderately labeled cells were observed in the deeper layers of the retorsplenial and frontal cortices, regions that contain considerable sst_2 receptor mRNA hybridization signal (present results). Furthermore, none of these regions, including the hippocampus, medial habenula, and the retrosplenial and frontal cortices, have been shown to contain dopaminergic cells or receive a *substantial* dopaminergic innervation (Björklund, 1984).

Although uptake of the radiolabeled peptide was not prevented by an excess of unlabeled [Dtrp^8]SRIF in the incubation medium, the extent of ^{125}I-SRIF uptake, particularly in regions with weakly to moderately labeled cells, was, albeit incompletely, attenuated. However, given the impressive similarity between the distribution of weakly

to moderately labeled cells and neuropil in our paradigm, and the distributions of both sst_2 mRNA reported here and of sst_2A receptor protein (Fig. 11, courtesy of Dr. Pascal Dournaud; unpublished data) in the rat brain, internalization of SRIF mediated by the sst_2A receptor should not be ruled out. sst_2A receptor protein was detected immunohistochemically over the large pyramidal cells in the deeper layers of the retrosplenial and frontal cortices, and over dendrites in the medial habenula, the stratum lacunosum moleculare, and in the hilus of the hippocampus. Indeed, whereas *in situ* hybridization histochemistry localized the sst_2 receptor mRNA to the pyramidal cell layer of Ammon's horn, the immunohistochemical data (Dournaud, unpublished) indicate that the sst_2A receptor protein is addressed to the apical dendrites of the pyramidal cells. Furthermore, *in vitro* radioautographic studies have demonstrated that iodinated SRIF analogues bind with moderate intensity in the stratum lacunosum moleculare but not in the CA1 and CA3 pyramidal cell layers (Katayama, 1990). The discordance between receptor message and protein localization may account for the moderate neuropil labeling observed in the present study in the region of the stratum lacunosum moleculare, and the lack of labeling over the pyramidal cell layer of the hippocampus. The distribution of weakly to moderately labeled cells in this report was notably similar to the pattern of distribution of sst_2A receptor protein and SRIF binding in the hippocampus, medial habenula, and retrosplenial cortex suggesting a possible role for the sst_2 receptor in the mediation of ^{125}I-SRIF uptake in these regions of the rat brain.

The hypothesis that weakly to moderately labeled cells might have internalized SRIF by a receptor- mediated mechanism would be in keeping with the demonstration of receptor- mediated internalization of ^{125}I-Tyr^0[DTrp^8]SRIF by neurons in primary culture (Dournaud, unpublished). Precisely why both [Dtrp^8]SRIF and octreotide attenuated, but did not inhibit the labeling of weak to moderately labeled cells is unclear. Further studies will undoubtedly be needed to clarify the mechanism by which weakly to moderately labeled cells sequestered radioactivity intracellularly.

In our paradigm, we detected non- saturable high efficiency uptake of ^{125}I-Tyr metabolite(s) of ^{125}I-Tyr^0[DTrp^8]SRIF, possibly mediated by the dopamine transporter, in the zona incerta, periventricular hypothalamic nucleus, the arcuate nucleus, and in the

substantia nigra pars compacta. We might have also detected receptor- mediated internalization of ^{125}I-Tyr^{0}[DTrp^{8}]SRIF in other regions of the rat brain including the medial habenula, the hippocampus, and the retrosplenial cortex, however, further studies are needed to confirm whether uptake of ^{125}I-Tyr^{0}[DTrp^{8}]SRIF by weakly to moderately labeled cells and neuropil was receptor- mediated. Indeed, the present study is in no way exhaustive, and methodological improvements should permit the detection and definition of all cells in the rat brain that internalize SRIF.

GENERAL CONCLUSIONS

In summary, the results reported here demonstrate that: (1) the patterns of sst_1 and sst_2 hybridization show considerable overlap within the hypothalamic-hypophysiotropic axis of the adult rat; (2) sst_1 and sst_2 receptor mRNAs are heavily expressed in those hypothalamic neurons that contain and express SRIF and/or GRF, and therefore imply a role for both the sst_1 and sst_2 receptors in the neuroendocrine regulation of GH secretion in both sexes of this species; (3) the sst_1 and sst_2A receptors are able to mediate internalization of SRIF when expressed on the surface of COS 7 cells, albeit to different degrees; and (4) while the dopamine transporter may mediate the uptake iodinated metabolite(s) of ^{125}I-Tyr^0[DTrp^8]SRIF by neurons in some regions of the adult rat brain, uptake of ^{125}I-Tyr^0[DTrp^8] SRIF in some other regions of the rat brain might be the result of internalization mediated by the sst_2 receptor.

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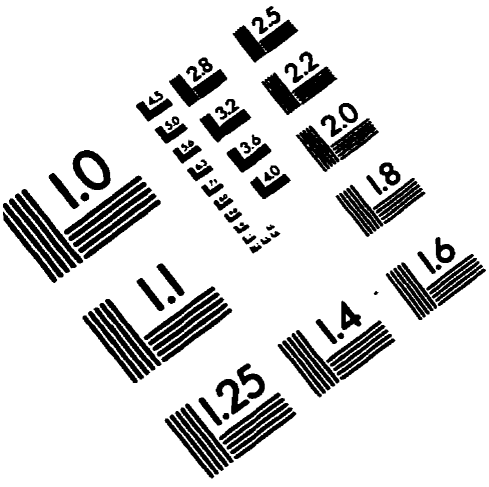
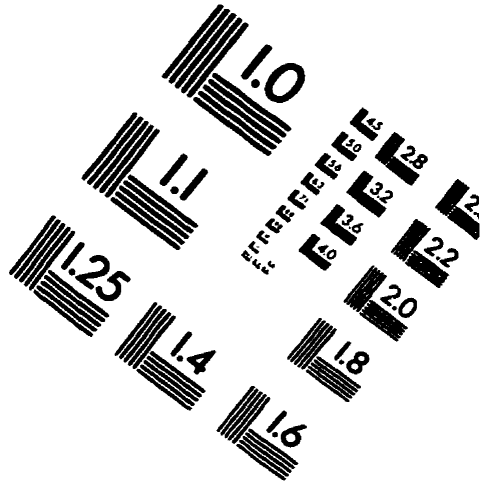
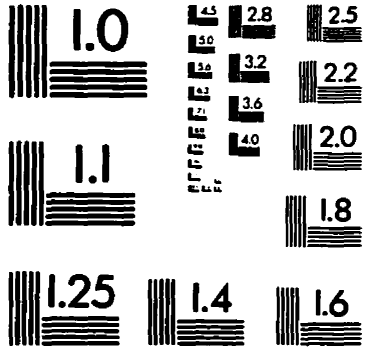
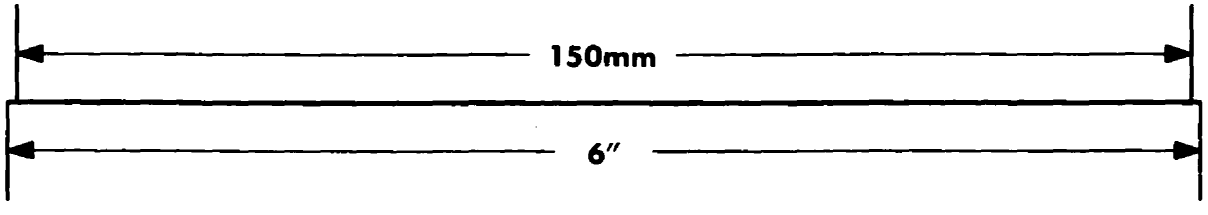
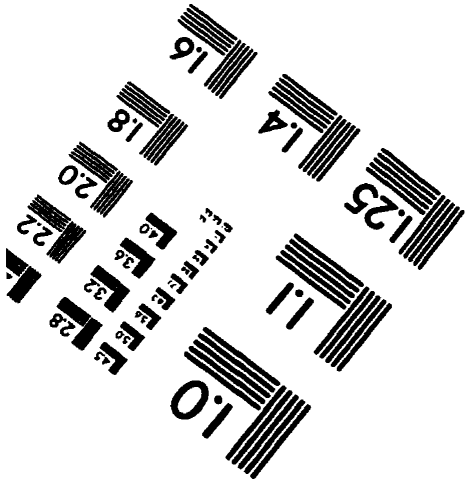
IMAGE EVALUATION TEST TARGET (QA-3)

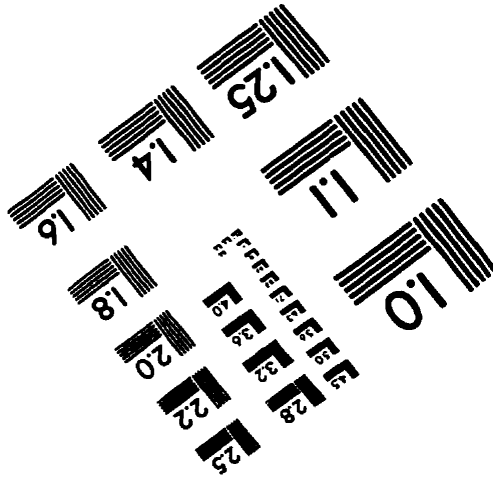












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