INTERLEUKIN-1β EFFECTS ON SOMATOSTATIN RELEASE in vitro

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by

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Abstract

Interleukin-1ß (an acute phase immune response cytokine) affects central nervous system and hypothalamic control of many neuroendocrine functions. Injections of endotoxins, the cell wall components of gram negative bacteria, have been used experimentally to induce the secretion of interleukin-1ß (IL-1ß) and other cytokines in mammals. Long term endotoxin treatment in rats causes a decrease in the rate of somatic growth, accompanied by an initial suppression of growth hormone (GH) release parameters (the mass/concentration, peak frequency and baseline levels of secreted GH during a specified time interval) followed by a rebound. The hypothalamic peptide somatostatin (SS) is the primary inhibitor of GH release. IL-1ß has been shown to acutely ($\leq$ 30 min) and chronically (> 24 hr) stimulate SS biosynthesis, but no studies have observed IL-1ß effects on SS secretion during the intermediate time periods (in hours). The suppression of somatic growth and the initial inhibition of GH release parameters suggests that at some intermediate time point during exposure, endotoxins may adversely regulate somatostatin release through direct mechanisms or via IL-1ß mediation. This study used organotypic slice cultures of neonatal rat hypothalamic explants to test the hypothesis that IL-1ß induces an inhibition of somatostatin release when cultures are exposed to the cytokine for durations of several hours. This hypothesis may explain how the removal of the negative regulator of GH release would result in the rebound in GH secretion observed in rats treated with endotoxins. In this study, the 2 hr and 12 hr IL-1ß incubations resulted in an inhibition of somatostatin release below

basal secretion levels. These data indicate that an IL-1ß inhibition of SS release may mediate the rebound in GH secretion observed in rats treated chronically with endotoxins. The 24 hr incubation with IL-1ß had no significant effect on somatostatin release, suggesting that the chronic effects of IL-1ß on growth and GH secretion may be sustained through other hypothalamic mechanisms. This and other data in the literature also suggest that IL-1ß plays a complex role in somatostatin regulation having the ability to stimulate (minutes), inhibit (hours) or not alter (days) somatostatin release.

Certain prostaglandins are known to mediate IL-1ß actions. In order to clarify the mechanisms by which IL-1ß may inhibit somatostatin secretion, the synthesis of prostaglandin was inhibited. 12 hr incubations of indomethacin alone caused a significant decrease in somatostatin secretion from the cultures derived from the first, and most rostral slices only. Indomethacin combined with IL-1ß did not significantly alter somatostatin release compared to basal levels suggesting that in this culture system, under the conditions described, that IL-1ß exhibits its effects on somatostatin secretion through a prostaglandin dependent mechanism. Further study will be needed to determine the precise mechanisms and time course by which IL-1β affects somatostatin secretion.

Table of Contents

List of Figures

List of Abbreviations

ACTH = adrenocorticotropic hormone
Arc = arcuate nucleus
B_{max} = maximum binding capacity
cAMP = cyclic adenosine 3', 5'-monophosphate
CNS = central nervous system
CRE = cAMP responsive element
CREB = CRE-binding protein
CRH = corticotrophin-releasing hormone
DAB = 3,3'-diaminobenzidine
DIV = day *in vitro*
DMEM/F-12 = dulbecco's modified eagle media: nutrient mixture F-12
DNA = deoxyribonucleic acid
ED = embryonic day
GABA = gamma-aminobutyric
GH = growth hormone
GHRH = growth hormone-releasing hormone
ICC = immunocytochemistry
icv = intracerebroventricular
IL-1ß = interleukin-1ß
INF I = interferon I
ip = intraperitoneal
ir = immunoreactive
iv = intravenous
K_d = dissociation constant
kDa = kilodalton
LPS = bacterial lipopolysacharide
ME = median eminence
mRNA = messenger ribonucleic acid
NMDA = N-methyl-D-aspartate
NSB = mon-specific binding tubes
OVLT = organum vasculosum lamina terminalis
PAP = peroxidase-antiperoxidase
PBS = phosphate buffered saline
PeVN = periventricular nucleus
PGE_2 = prostaglandin E_2
PMA = phorbol 12-myristate 13-acetate
PND = post natal day
preproSS = preprosomatostatin
proSS = prosomatostatin
PVN = paraventricular nucleus
REF = reference tubes
RIA = radioimmunoassay
RM = releasing medium
SCM = serum-containing medium
SFM = serum free medium
SS = somatostatin
TC = total count tubes
TNF = tumor necrosis factor
TRH = thyrotropin-releasing hormone
VMN = ventromedial nucleus

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Dedication

As acknowledgment and thanks for all their love, support and encouragement throughout all my years, I would like to dedicate this thesis to my family and especially my mother, Yvonne Georgina Tittle, who died on May 12, 1997 while this thesis was being completed.

I INTRODUCTION

The hypothalamus is the center of many physiological systems that regulate many bodily functions including growth, reproduction, fluid balance, and stress responses. Chemical messengers of the immune system have been shown to affect hypothalamic systems and other central nervous system-mediated events. This paper is concerned with specific immune system effects on somatostatin, a hypothalamic regulator of growth.

Interleukin-1ß is an immunological chemical messenger that is transiently expressed when an immune reaction is triggered and helps coordinate general host defense mechanisms. This thesis tests the hypothesis that interleukin-1ß will inhibit hypothalamic somatostatin secretion when exposure occurs for the time period of hours, as part of a compensatory mechanism initiated to counteract the negative effects of the cytokine on growth. This study used organotypic slice cultures of neonatal rat hypothalamic explants to observe the effects of Interleukin-1ß on SS secretion.

Included in this introduction will be information on SS isolation, the role of SS in the regulation of growth hormone, a description of the SS gene and finally a description of biosynthesis and distribution of the SS peptide. As neonatal rats are used in the experimental model individual subsections in the introduction, examining age-related changes in the growth hormone system and the ontogeny of the SS gene and peptide will be presented to address potential differences between neonatal and adult rats. Information describing physiological SS release will be followed by a subsection discussing the regulatory factors involved in SS secretion which may or may not be present due to the inherent loss of afferent

innervation in *in vitro* models. Later in the introduction, an overview of relevant information on IL-1ß will also include some recent speculation on the transfer of IL-1ß past the blood-brain barrier. Current knowledge on the influence of the immune system on the growth hormone system and SS is provided and the introduction ends with the purpose and hypothesis of the thesis.

1. SOMATOSTATIN

1.1. Somatostatin Isolation

The hypothalamus regulates the function of the anterior pituitary gland by releasing various neuropeptides that diffuse from the intercellular space into the portal vascular system (see reviews: Halasz, 1994; Gomez-Pan and Rodriguez-Arnao, 1983). The hypothalamic neuropeptides are carried to the anterior pituitary gland and influence the synthesis and secretion of adenohypophyseal hormones (Gomez-Pan and Rodriguez-Arnao, 1983). Several hypothalamic hormone including, somatostatin were isolated and characterized functionally.

Geoffery Harris, labelled by some as 'the Father of Neuroendocrinology' along with his colleagues demonstrated the vascular link between the hypothalamus and the anterior pituitary (Harris, 1937), identified the portal vessel system (Green and Harris, 1947) and established the pituitary's functional dependence on its link with the hypothalamus (Harris and Jacobsohn, 1952). Subsequently Scharrer and Scharrer (1954) coined the term 'neurosecretion' from their observations that neurons in the hypothalamus secrete neuropeptides Following these studies, several groups began to attempt to extract and

experiment with specific hypothalamic peptides. Krulich et al. (1968) first extracted a hypothalamic factor capable of inhibiting growth hormone (GH) secretion. The GH release inhibiting factor was later isolated and characterized from ovine hypothalamus by Brazeau et al. (1973) while the group was searching for a stimulatory substance. This cyclic tetradecapeptide was termed growth hormone-release inhibiting hormone (GH-RIH), somatotropin-release inhibiting factor (SRIF) or somatostatin (SS) (Brazeau et al., 1973; Rivier, 1974). Somatostatin was subsequently isolated and characterized from porcine hypothalami by Andrew Schally and his colleagues (Schally et al., 1976).

Somatostatin is found in other organs, and has many other known functions. Koerker et al. (1974) identified the functional significance of somatostatin in the regulation of insulin and glucagon secretion from the pancreas. Dubois (1975) demonstrated the storage of SS in the pancreatic delta cells and Hökfelt et al. (1975) supported a peripheral mechanism for insulin and glucagon regulation by demonstrating the localization of SS immunoreactive cells near glucagon-containing cells and SS immunoreactivity in epithelial cells of large and small pancreatic ducts. Somatostatin is located in the antral mucosa of the stomach, and in lesser densities is distributed in the duodenum, jejunum and the exocrine pancreas (Guillemin and Gerich, 1976; Reichlin, 1983). Gastrointestinal SS inhibits the secretion of gastrin, gut glucagon, secretin and cholecystokinin. SS is also present in salivary glands where it has been shown to decrease salivary flow (Reichlin, 1983). SS is localized in the thyroid gland (Kronheim et al., 1976; Reichlin, 1983) and is postulated to be involved in the autocrine regulation of calcitonin (Reichlin, 1983).

1.2. Growth Hormone Axis Physiology

GH plays a major role in the control of metabolism and is a key regulator of growth in children and adults (see review by Arimura, 1994). The episodic secretion of GH in male rats, humans, and most mammalian species is controlled by the interplay of two hypothalamic peptides, somatostatin (SS) and growth hormone-releasing hormone (GHRH) (Tannenbaum, 1984; Robinson, 1991; Thorner et al., 1990; Frohman et al., 1990; Guillemen and Gerich, 1976). Somatomedins are a group of peptides under GH control that exhibit growth-promoting and insulin-like activities on several tissues (Tannenbaum, 1984). Both GH and somatomedins have been shown to regulate GH secretion through hypothalamic interactions (Becker et al., 1995; Berelowitz et al., 1981). The pattern of GH secretion is sexually dimorphic as female rats have a more continuous pattern of secretion and exhibit differential responses to exogenous GHRH and SS, compared to male rats (Tannenbaum, 1991; Robinson, 1991: Robinson et al., 1990; Jansson et al., 1984). GH secretion is also different during pregnancy in humans; pulsatile pituitary GH secretions decrease dramatically while nonpulsatile GH secretions from the placenta increase (Caufriez et al., 1994). Placental GH appears to functionally replace the pituitary isoform as it stimulates the secretion of maternal insulin-like growth factors but is not detected in the fetal circulation (Evain-Brion, 1994). Maternal insulin-like growth factor secretions are correlated with placental weight after delivery and baby birth weight (Caufriez et al., 1994). Otherwise fetal GH ontogeny appears to be independent of GH secreted into maternal plasma (Evain-Brion, 1994).

In rats, pituitary GH is measurable by at least embryonic day 18 (Nogami and Tuchibana, 1993; Rieutort, 1974) and plasma GH levels are first detected around embryonic day 19 (Rieutort, 1974; Khorram and McCann, 1984). Pituitary GH content reaches adult levels at postnatal day (PND) 20 (Rieutort, 1974), whereas plasma GH concentrations peaks around birth and falls to adult ranges between PND 5 and 10 (Rieutort, 1974; Khorram and McCann, 1984). Rieutort (1974) hypothesized that the peak in plasma GH release during early postnatal life corresponded to an event or stimulus closely related to the general stress of birth. Spencer et al. (1994) have shown that hypothalamic peptides are important in the control of gestational GH release as somatostatin antiserum administered to pregnant rats increased the mean birth weight of the offspring. Exogenous GH injections mimicked the effects of SS antiserum and increased the mean birth weight of the newborn rats (Spencer et al., 1994). SS is also an important regulator of GH secretion in the adult (see review by Arimura, 1994).

1.2.1. Growth Hormone Axis

Through the interplay of the inhibitory actions of SS and the stimulatory effects of GHRH, GH release in male rats is characterized by an ultradian or less than 24 hr rhythm with high amplitude GH secretory bursts (> 200 ng/ml) occurring at 3-4 hr intervals throughout a 24 hr period (Tannenbaum and Martin, 1976; Tannenbaum et al., 1990; Robinson, 1991; Donaghue et al., 1990; Kin-Chuen et al., 1995). The GH secretory episodes often show a complex double or triple peak, characterized by a sudden rise followed by a fall in plasma GH levels (Tannenbaum and Martin, 1976) which is consistent with the known

half-life of GH in the rat (Tannenbaum, 1984). During the intervening trough periods, basal plasma GH levels fall below the sensitivity of the assays (Tannenbaum and Martin, 1976; Donaghue et al., 1990; Kin-Chuen et al., 1995). The GH secretory rhythm is not affected by changes in the light dark-cycle (Tannenbaum and Martin, 1976) and is probably governed by an endogenous central pulse generator which is sensitive to feedback control by GH, as well as to nutritional, environmental, psychological and other endocrine cues (Robinson, 1991) The GH secretory pattern is also modified by sleep as GH levels increase during slow-wave sleep (Thorner et al., 1990). In humans the number of peaks per 24 hours is 6.7 and 11 for men and women respectively (Thorner et al., 1990). GH secretion is episodic in other species but the rhythm is not as obviously regular as in the male rat (Robinson, 1991; Tannenbaum, 1991).

The role of the episodic release of hypothalamic GHRH and SS in regulating the pattern of GH release has been established by many studies. GHRH has been verified as the hypothalamic peptide responsible for maintaining the peaks in GH release (see review by Arimura, 1994; Tannenbaum, 1991; Robinson, 1991; Tannenbaum, 1984). Passive immunoneutralization of endogenous GHRH through the intracerebroventricular (icv) administration of specific GHRH antisera, virtually abolished the spontaneous peak in GH release from the anterior pituitary (Arimura, 1994; Tannenbaum, 1991) although basal GH release was not apparently effected (Arimura, 1994). Administration of the GHRH antagonists, (N-Ac-Tyr^1, D-Arg^2) GRF-(1-29)-NH_2, also show negative effects on GH release similar to those obtained by passive GHRH immunoneutralization (Lumpkin et al., 1989).

Immunoneutralization techniques have also been used to determine the physiological significance of SS in the control of GH secretion (Tannenbaum, 1991; Robinson, 1991; Tannenbaum, 1984). Basal GH levels are elevated by passive SS immunoneutralization and GH secretory pulses are not abolished (Tannenbaum, 1991). SS immunoneutralization does not alter GH peak amplitude, GH peak frequency or mean 6 hr plasma GH levels (Arimura, 1994). Tannenbaum (1994) observed that SS immunoneutralization during the trough periods caused a rapid surge of GH secretion and significant elevation of subsequent GH trough levels. GH trough values did not reach undetectable levels throughout a 6 hr sampling period in SS anti-serum treated animals compared to values in control animals.

In male rats, GHRH-induced GH release is time-dependent with the GH secretory rise being significantly greater when exogenous GHRH was administered during the peak period as compared to a trough period of GH secretion (Arimura, 1994; Tannenbaum, 1991). Administration of anti-SS blocked the weak GHRH-induced GH release observed during trough periods of GH secretion, indicating that the weak GHRH-induced response is due to an antagonism by endogenous SS released in larger amounts during the nadir in GH secretion (Tannenbaum, 1991; Tannenbaum, 1984).

Studies that have simultaneously altered the normal secretion of SS and GHRH have shown that the coordinated actions of the two neuropeptides are necessary to produce the GH release pattern. Continuous infusions or serial GHRH injections decrease the GH pulse amplitude (Tannenbaum, 1991) but the GH secretory profile is not affected by exogenous neuropeptide administration (Tannenbaum, 1991; Robinson, 1991). The GH pulse rhythm

was entirely extinguished when continuous GHRH administration was coupled with immunoneutralization of endogenous SS (Tannenbaum, 1991; Robinson, 1991). These studies demonstrate that GH rhythmicity is preserved by the episodic release of endogenous SS from the hypothalamus during continuous GHRH infusion (Tannenbaum, 1991). Robinson (1991) postulated that GHRH pulses that did not release GH may be necessary to build up pituitary stores of GH that are not released while SS tone is high. The stored GH can be discharged in a large burst by the first pulse of GHRH arriving after SS secretion has waned. Growth hormone levels characteristically rebounded above preinfusion levels upon the termination of continuous intravenous SS infusion (Guillemin and Gerich, 1976). Clarke et al. (1988) observed that the magnitude of the post SS GH rebound was attenuated by the administration of anti-GHRH, leading them to speculate that GHRH plays a role in the rebound in GH secretion observed after SS withdrawal.

Tannenbaum (1991) surmised from the passive immunoneutralization and continuous infusion results (described above) that in male rats, SS and GHRH are released from the ME in reciprocal 3-4 hr cycles (about 180° out of phase) to generate the ultradian rhythm of GH secretion observed in peripheral blood. Fukuhara et al. (1993) used an enzyme immunoassay to measure SS peptide levels in the supernatant of homogenated suprachiasmatic tissue. They observed that the cellular content of immunoreactive SS in the suprachiasmatic nucleus peaked once per 24 hr and may contribute to the endogenous SS circadian rhythm. SS mRNA was higher during GH pulses, while the GHRH genetic transcript was greater during trough periods, demonstrating that the mRNA signals for the two neuropeptides were also

involved in an ultradian rhythm 180° out of phase with the presumed secretion of the respective peptides (Tannenbaum, 1991).

Actual measurements of portal plasma SS and GHRH have shown that the secretions of both peptides are indeed pulsatile. Plotsky and Vale (1987) measured SS and GHRH in portal plasma samples collected from the cannulated infundibular stalks of male rats anesthetized with a ketamine:xylazine mixture. Mean portal immunoreactive GHRH levels rose from <160 pg/ml to a mean peak concentration of 890 ± 65 pg/ml (n=7) during one 20 min collection period. The mean immunoreactive SS concentration was 112 ± 9 pg/ml during periods of trough GH secretion. This level declined by 37 ± 5 percent in collections coinciding with periods of expected GH pulses before returning to initial levels. However, the correlation between SS and GH secretion is largely inconsistent (Plotsky and Vale, 1987).

Anesthetics have been shown to have negative effects on GH secretion (Robinson, 1991) therefore Frohman et al. (1990) developed a experimental model that used conscious animals. GHRH-like peptide immunoreactivity is undetectable in portal blood samples from rats anesthetized with urethane or pentobarbital (Plotsky and Vale, 1987) and the characteristic GH secretion in rats is also abolished by these anesthetics (Robinson, 1991). The findings in anesthetized animals may not necessarily reflect a physiological neuropeptide release profile, whereas the hypophyseal portal blood collected from an artificial sinus placed anterior to the pituitary gland, can be collected from conscious animals (Arimura, 1994). Frohman et al. (1990) used such a model to measure hypophyseal SS and GHRH in oophorectomized sheep. The secretions of both GHRH and SS were pulsatile with peak

values of 25 - 40 pg/ml (GHRH) and 65 - 160 pg/ml (SS), and mean (± SD) values of 21 ± 7 pg/ml (GHRH) and 72 ± 33 pg/ml (SS). Assessment of pulse frequency indicated a mean interpulse interval of 71 min and 53 min for GHRH and SS, respectively (Frohman et al., 1990).

Frohman's group examined the interrelationship between the secretory patterns of GHRH, SS and GH. Their analysis indicated a significant concordance of GHRH peaks with those of GH ($p < 0.05$) and with the onset of the rise in GH secretion preceding the actual peak ($p < 0.02$). There was, however, no significant association found between GHRH secretory peaks and SS secretory troughs, or vice versa, or between troughs in SS release and peaks in GH secretion.

1.2.2. Age-Related Differences

GH secretion is also related to age. Older rats have decreased GH pulse amplitude although trough levels remain unchanged (Morimoto et al., 1988). Morimoto et al. (1988) used immunocytochemistry (ICC) to show that GHRH immunoreactivity (ir) was greater in the ME of younger rats (three months) than old rats (24 months) whereas SSir intensity was largely unaffected. Morimoto's group demonstrated that the age-related changes in GH secretion were not due to a change in the pituitary response to GHRH, but possibly due to a dysfunction or hypofunction in the synthesis, transport, and/or release of GHRH. The numbers of GHRH neurons in the arcuate nucleus (Arc) and periventricular area or the amount of SS-containing neurons in the preoptic area and anterior nucleus did not change with age. Morimoto et al. (1988) speculated that the age-related decline in GHRH may be

caused by a decrease in catecholamine concentrations with age. This hypothesis is supported by the finding that exogenous L-Dopa administration has been shown to restore the GH pulse in old rats (Sonntag et al., 1990).

Ge et al. (1989) used an *in vitro* hypothalamic rat model, in order to examine the difference in GHRH and SS release from cultures derived from male and female rats at 10, 30, 75 days and 14 months of age. Their studies demonstrated that the release of GHRH from the hypothalami of rats was related to sex, whereas the release of SS is related principally to age. Ge and colleagues observed that the hypothalamic content of SS and GHRH was related to age and not to gender as GHRH content increased and then reached a plateau by postnatal day (PND) 30, while SS content increased linearly with age in both male and female rats. The percent of GHRH content released from the cultures in 20 min reached adult levels by PND 10, but the percent release of SS only matured after PND 30. These results suggest that the age-related change in GH secretion reflects a shift in the ratio of release of GHRH and SS (Ge et al., 1989).

2. SOMATOSTATIN GENE

2.1. Somatostatin Gene

Somatostatin (SS) is synthesized as part of a larger precursor molecule. Its biologically active forms, somatostatin-14 (SS-14) and somatostatin-28 (SS-28), arise by post-translational cleavage of prosomatostatin (proSS), which in turn is derived from a larger precursor called preprosomatostatin or preproSS (Goodman et al., 1984). The gene encoding

rat preproSS is 1.2 kilobases in length and is interrupted within the proSS coding sequence by a single intron of 630 bases.

2.2. Somatostatin Gene Expression in Development

Somatostatin mRNA has been detected in the neurons of embryonic rats by northern blot (Almazan et al., 1989), *in situ* hybridization (Burgender, 1994; Baram and Shultz, 1991) and radiodensitometric preproSS cDNA hybridization assay (Zingg et al., 1984). The SS gene has been detected at embryonic day (ED) 14 (Burgunder, 1994; Almazan et al., 1989; Baram and Shultz, 1991) although Zingg et al. (1984) using tissue from younger fetuses and a cDNA hybridization method, detected the genetic transcript by ED7. Adult levels of SS mRNA in the rat brain appear to be reached between postnatal day (PND) 11 and 14 (Burgunder, 1994 and Zingg et al., 1984) although there is a transient increase in SS mRNA in both male and female rats at the ages of 25 and 35 days respectively (Argente et al., 1991). In the adult hypothalamus, SS mRNA is localized in the paraventricular nucleus and lining the third ventricle wall from the optic chiasm to the ventromedial nucleus. The SS gene distribution in the adult neocortex, pyriform cortex, and amygdala and the absence of expression in the cerebellum, corresponds with the location of peptide immunoreactivity (Arentzen et al., 1985). The SS gene is also expressed in astrocytes in the cerebellum that appear by ED20 and fall 7-fold by PND 8. In contrast, SS mRNA levels in cortical astrocytes are detected by ED20 and increase steadily to adult levels (Shinoda et al., 1992).

SS may have a role in development as it is initially expressed at different times in various central nervous system (CNS) areas. Baram and Shultz (1991) were able to detect

SS mRNA in the amygdalo-hippocampal complex, the dorsolateral thalamus and distinct zones of the parietofrontal cortex, by ED16. SS mRNA is expressed along the third ventricle near the hypothalamic periventricular nucleus between ED14 and ED16. Burgunder (1994) has reported that by ED19, SS gene levels were higher in the ventral structures (i.e. pyriform cortex, ventral caudate putamen, amygdala, hypothalamus, zona incerta) compared to dorsal ones (in particular neocortex and dorsal caudate putamen). The onset of SS mRNA expression in different brain areas may be related to the role the SS peptide may play in that system. SS neurons in the cortex are thought to be inhibitory interneurons (Burgender, 1994), and a functioning inhibitory system may be important to the development of certain cortical brain areas. The differences in initial SS mRNA appearance in distinct CNS regions may also be simply a reflection of early or delayed comparative maturation. Burgender et al. (1994) noted that the relative delayed maturation of parts of the hippocampus may account for the delayed maturation of SS mRNA in that area.

2.3. Regulation of Somatostatin Gene Expression

Several neuropeptides and neuromodulators that can modulate SS secretion are also able to regulate gene expression. SS gene expression can be upregulated by potassium-induced depolarization (Tolon et al., 1994), growth hormone (Zeitler et al., 1990), growth hormone-releasing hormone (Zeytin et al., 1988), testosterone (Chowen-Breed et al., 1989; Zeitler et al., 1990; Chowen et al., 1993; Werner et al., 1988), estradiol (Werner et al., 1988), dexamethasone (Nakagawa et al., 1992; Lam et al., 1993) and N-methyl-D-aspartate (NMDA) receptor activation (Rage et al., 1994). SS mRNA levels are likely to be decreased

by gamma-aminobutyric acid (GABA) since bicuculline, a GABA antagonist, augments SS gene expression by suppressing GABA tone (Rage et al., 1994).

Secretion of SS is increased by many agents that stimulate adenylate cyclase activity suggesting that cyclic adenosine 3', 5'- monophosphate (cAMP) is a second messenger for SS release (Tolon et al., 1994). SS gene expression can also be regulated by cAMP. cAMP has been shown to influence SS transcription at a sequence (29 to 60 base pairs in length) upstream from the transcriptional initiation site (Goodman et al., 1990). This sequence, designated the cAMP-responsive element (CRE), is a classical enhancer sequence, stimulating transcription at a distance and orientation independent manner. The SS CRE sequence is also essential for basal (constitutive) and tissue specific expression (Dixon et al., 1990; Montminy et al., 1990; Goodman et al., 1990). Montminy and Bilezikjian (1987) used DNA-affinity chromatography to purify a SS CRE-binding protein (CREB) from PC12 cells and Yamamoto et al. (1988) isolated the protein in brain tissue. CREB is a 43-kDa protein that stimulates transcription of genes by binding to DNA as a dimer. CREB dimerization and binding are enhanced by phosphorylation. Protein kinase-A and protein kinase C can both phosphorylate CREB and therefore may hold a regulatory role in SS transcription (Dixon et al., 1990; Montminy et al., 1990; Goodman et al., 1990).

2.4. Somatostatin Biosynthesis and Postranslational Processing

The direct translational product of the SS gene is preprosomatostatin (preproSS). PreproSS serves as the precursor product for a family of somatostatin peptides. PreproSS is 116 amino acids in length with a molecular weight of 12, 727 (Goodman et al., 1984;

Benoit et al., 1990). The pre-region of preproSS is the hydrophobic amino-terminal that is involved in the transport of the polypeptide through the endoplasmic reticulum (Goodman et al., 1984). The amino-terminal leader sequence also contains the positive sorting signal that organizes the peptide into the secretory pathway responsible for proteolytic maturation (Sevarino et al., 1990). Following removal of the pre-region the 92 amino acid SS precursor, prosomatostatin (proSS, MW 10, 389) is formed (Goodman et al., 1984; Benoit et al., 1990). Somatostatin-14 and somatostatin-28 are the most common final cleavage products of proSS. Somatostatin-14 (SS-14) is located at the carboxyl-terminal of proSS and is cleaved from the precursor at a dibasic site (Arg-Lys) (Goodman et al., 1984). Somatostatin 28 (SS-28) is cleaved at a single basic amino acid (Arg) and contains the amino acid sequence of SS-14 in its carboxyl-terminal end (Benoit et al., 1990). SS-28 is more abundant in the gut and SS-14 is more abundant in the hypothalamus suggesting that different endoproteolytic enzymes are responsible for the selective cleavage of the precursor to release the specific mature peptides (Cohen et al., 1984). The amino-terminal of SS-28, $SS\text{-}28_{[1\text{-}12]}$ is also a peptide product that can be cleaved from proSS but its biological function is not known (Goodman et al., 1984; Benoit et al., 1990). Other peptides of higher molecular weight than SS-14 and SS-28 (25K and 4K) have been located in the hypothalamus (Zingg and Patel, 1979). SS-14, followed by SS-28 are the predominant forms in all neural tissues, (Zingg and Patel, 1979; Patel et al., 1980; Benoit et al., 1984). Although most of the other members of the somatostatin family, including proSS, have been shown to be secreted, only SS-14 and SS-28 have shown demonstrable biological activity so far (Zingg and Patel, 1979; Patel et al., 1980;

Benoit et al., 1984).

3. Somatostatin Peptide Storage

3.1. Ontogeny of Somatostatin Peptide

Immunoreactive SS peptide has been detected in rat fetuses by radioimmunoassay (McGregor et al., 1982; Shiosaka et al., 1982; Khorram and McCann, 1984; Nobou et al., 1985; Gabriel et al., 1989) or immunocytochemistry (Shiosaka et al, 1982) and has advanced the knowledge of the peptide's ontogeny. Somatostatin is present in the rat brain during development and its appearance follows a caudal to rostral sequence (McGregor et al., 1982). Cells containing the SS peptide first appear on embryonic day (ED)14 in the hypothalamus (McGregor et al., 1982) and are located in the primordium of the area surrounding the fasciculus mammilothalamicus (Shiosaka et al., 1982). Khorram and McCann (1984) reported that SS positive neurons are first detected in the hypothalamus at ED16. With the use of radioimmunoassay, they observed a further increase in SS content on postnatal day (PND) 10, with the levels falling significantly on day 15, only to rise again to day 10 levels on day 21. Nobou et al. (1985) measured detectable levels of SS in extracts taken from embryos three days before birth and found that hypothalamic SS content increased steadily with age and reached levels 10 fold higher than embryonic measurements by PND 60. Gabriel et al. (1989) reported that SS content in juvenile rat hypothalami continued to rise steadily between PND 25 and 90. SS levels in the median eminence of male rats reached adult levels by PND 35 compared to female rats where maximal levels were not reached until

90 days of age (Gabriel et al., 1989). Shiosaka et al. (1982) used immunocytochemistry (ICC) to detect SS positive neurons in the periventricular nucleus (PeVN) on ED19. They reported that SS positive cells in the PeVN reached maximal levels by birth and decreased in number as the rats grew. Shiosaka's group observed SS positive fibers in the anterior commissure, suprachiasmatic nucleus and median eminence on ED20 and a fiber network in the ventromedial hypothalamus a day earlier.

The ontogeny of SS distribution in extra hypothalamic sites has also been studied (Nobou et al., 1985; McGregor et al., 1982). SS peptide content in the cortex, brainstem, hippocampus, cerebellum, olfactory bulb and amygdala experiences a rapid post-natal increase. Adult levels are approached and often exceeded between PND 14 and 30 (Nobou et al., 1985; McGregor et al., 1982). SS content in the brainstem and cortex emerge by ED14 whereas the appearance of SS in the hippocampus, cerebellum, olfactory bulb and amygdala does not occur until birth or just before it (Nobou et al., 1985; McGregor et al., 1982).

The ontogeny of SS peptide and SS mRNA are related, with the mRNA generally appearing before or on the same day as the peptide. Both SS mRNA (Burgender, 1994; Almazan et al., 1989; Baron and Shultz, 1991) and peptide immunoreactivity (McGregor et al., 1982) can be detected for the first time in the brains of fetuses by ED14. In certain brain areas like the hypothalamus, amygdala and hippocampus there is a lag between the appearance of SS mRNA and the peptide. In the hypothalamus, SS mRNA can be detected as early as ED14 (Baram and Shultz, 1991), two days before the peptide was detected by Khorram and McCann (1984). The lag between the initial detection of SS mRNA and SS

peptide is even larger in the amygdala and the hippocampus as SS mRNA is expressed by ED16 (Baram and Shultz, 1991) but the peptide is not detectable until after birth (Nobou et al., 1985; McGregor et al., 1982). The time lapse between the detection of the gene and the peptide suggests that, in these areas, transcriptional mechanisms may appear before translational ones.

3.2. Peptide Distribution

3.2.1. Hypothalamus

3.2.1.1. Neurons

The hypothalamus contains approximately 28% of the somatostatin found in the adult rat brain (Brownstein et al., 1975). Several studies confirm that in the rat most of the SS-containing perikarya are found in the PeVN (Kobayashi et al., 1977; Elde et al., 1984; Finley et al., 1981; Bennett-Clarke et al., 1980; Johansson et al., 1984). Elde et al. (1984) found the most prominent collection of SS immunoreactive (SSir) perikarya in the PeVN area dorsal to the caudal portion of the optic chiasm. Kobayashi et al. (1977) reported 25-fold higher SSir in the retrochiasmatic area than the amount in the preoptic area, second only to the density found in the median eminence. Johansson et al. (1984) reported that in the PeVN, up to one hundred SSir cell profiles could be seen on each side of the third ventricle extending from the optic chiasm to the anterior parts of the paraventricular nucleus (PVN). At more rostral levels, the majority of SSir cells were seen adjacent to the dorsal half of the ventricle. At mid-levels SSir was present all along the ventricle whilst caudally the main group once again occupied a more dorsal position. Bennett-Clarke et al. (1980) also

observed that SSir cells located along the third ventricle varied in their antero-posterior distribution. At the level of the suprachiasmatic nucleus, SSir staining divided into dorsal and ventral groups of cells. Lightly stained SS neurons appeared scattered throughout the dorsal portion of the PeVN, whereas neurons in the ventral group exhibited a dark stain and clustered in compact groups near the ventricle ependyma (Bennett-Clarke et al., 1980). At the PVN level, dorsal and ventral divisions end and SSir perikarya line the entire third ventricle wall (Johansson et al., 1981; Bennett-Clarke et al., 1980). Finley et al. (1981) also observed SSir positive cell bodies adjacent to the ventricle at the suprachiasmatic nucleus level, but extending to the beginning of the ventromedial nucleus (VMN). Other hypothalamic sites that contain SSir include the PVN, arcuate nucleus (Arc), VMN, ventral premammillary nucleus and the dorsolateral aspect of the medial forebrain bundle (Johansson et al., 1984; Elde et al., 1984; Kobayashi et al., 1977; Brownstein et al., 1975).

3.2.1.2. Fibers

The most dense aggregation of SSir fibers and terminals in the hypothalamus was in the external zone of the median eminence with some fibers extending into the internal layer (Bennett-Clarke et al., 1980; Finley et al., 1981; Johansson et al., 1984; Elde et al., 1984). The Arc and the VMN contain the next highest concentration of immunoreactive fibers followed by the anterior nucleus, parts of the medial and lateral preoptic nuclei, the ventral premammillary nucleus and the lateral part of the mammillary complex (Finley et al., 1981; Johansson et al., 1984). Johansson et al. (1984) could detect additional SSir fibers in the VMN, supraoptic nucleus and the medial and posterior mammillary nuclei. Unlike

Johansson's group, Finley et al. located fibers in the PVN, dorsomedial nucleus, posterior nucleus, dorsal premammillary nucleus, the organum vasculosum of the lamina terminalis (OVLT) and the subfornical organ. Elde et al. (1984) also detected SSir fibers in the OVLT.

3.2.1.3. Connectivity Studies

Although SS is heterogeneously distributed throughout the hypothalamus, the connection of SS-containing neurons has been examined by tracing studies that follow the retrograde transport of substances injected into the median eminence (ME). Such strategies have revealed that certain subsets of SS-containing neurons project to the median eminence (Kawano and Daikoku, 1988; Ishikawa et al., 1987; Merchenthaler et al., 1989). These studies utilize the double staining method that combines the immunocytochemical staining of SS and the use of the retrograde tracer horse radish peroxidase (Ishikawa et al., 1987), biotinylated wheat germ agglutinin (Kawano and Daikoku, 1988) or lectin wheat germ agglutinin (Merchenthaler et al., 1989). With the use of two separate primary antibodies and different coloured end products, this technique can identify neurons that contain SS and the retrograde tracer transported from the axon terminals in the ME to the cell body in the hypothalamus (Kawano and Daikoku, 1988; Ishikawa et al., 1987; Merchenthaler et al., 1989). Ishikawa et al. (1987) showed that the SS neurons that also project to the median eminence were widely distributed in the PeVN, extending from the OVLT to the ME. The largest number of such cells was identified at the level of the suprachiasmatic nucleus in the anteroposterior axis and several double-labeled cells were observed in the preoptic suprachiasmatic nucleus and the PVN (Ishikawa et al., 1987). Kawano and Daikoku (1988)

detected neurons labeled for biotinylated wheat germ agglutinin and SS in the rostral periventricular region. The cells were distributed rostrocaudally from the preoptic area level to the anterior edge of the median eminence. Kawano and Daikoku reported that although SSir labeled neurons were numerous in the Arc, none of the cells were labeled with biotinylated wheat germ agglutinin. Merchenthaler et al. (1989) demonstrated that at least 70% of SS neurons in the anterior periventricular area and the PVN project to the ME. SSir perikarya located in the VMN and Arc did not contain lectin wheat germ agglutinin and therefore did not project to the ME. SS may function as a local neurotransmitter in these areas (Kawano and Daikoku, 1988). Collectively, these results suggest that neurons in the PeVN and PVN, but not those in the Arc and VMN, innervate the ME and are involved in releasing SS into the portal system to regulate growth hormone secretion from the anterior pituitary (Kawano and Daikoku, 1988; Ishikawa et al., 1987; Merchenthaler et al., 1989).

3.2.2. Extra hypothalamic Sites

In addition to hypothalamic sites, somatostatin is widely distributed in other areas of the CNS. SS containing perikarya and fibres are present in olfactory related areas and the telencephalon. The thalamus, mesencephalon and rhombencephalon contain dense SS fibre networks but few SS neurons (Elde et al., 1984; Johansson et al., 1984; Finley et al., 1981; Bennett-Clarke et al., 1980). Most SS neurons in these extra hypothalamic sites do not project to the hypothalamus and are not involved in the regulation of the growth hormone axis (Johansson et al., 1984; Finley et al., 1981).

4. SOMATOSTATIN PEPTIDE RELEASE

4.1. Physiological Somatostatin Release

Studies concerning the *in vitro* release of basal and stimulated SS, have shown that the secretion of the peptide is dependent on the transient release of intracellular calcium stores and second messenger systems linked to calcium (Iversen, et al., 1978; Maeda and Frohman, 1980; Richardson et al., 1983; Berelowitz et al., 1978; Honnegger et al., 1991; Cugini et al., 1991) Iversen et al. (1978) perfused 200 µm thick hypothalamic slices and observed that basal and 50 mM KCl-induced SS release was dependent on calcium, because when calcium was omitted from the superfusion solution the basal efflux was somewhat reduced and there was no response to the 6 min pulse of high potassium at all. Maeda and Frohman (1980) incubated blocks of hypothalamic tissue with 60 mM KCl or the depolarizing agent veratridine (1×10^{-5} M) after determining basal release for 20 min. SS and thyrotropin-releasing hormone (TRH) release was increased by membrane depolarization, and this was abolished when calcium was removed from the media indicating that the release of SS and TRH by hypothalamic fragments involves a Ca^{++}-dependent membrane depolarization process (Maeda and Frohman, 1980). Richardson et al. (1983) used dispersed cell cultures made from adult rat hypothalami, and observed that membrane depolarization, induced by either KCl 56 mM or ouabain (the Na^+, K^+-ATPase inhibitor) 1×10^{-6} M or greater, markedly stimulated SS release. Incubation at 4° C, or in the presence of EDTA 0.05 M or the calcium channel blocker verapamil at a concentration of 50 µM, abolished these stimulatory effects. Berelowitz et al. (1978) also showed that 56 mM KCl-

induced SS release could be blocked with verapamil or Ca^{++} free media. The rat *in vitro* model used incubated hypothalamic fragments (30 min incubations).

Honegger et al. (1991b) used a statically incubated acute rat hypothalamic explant system to determine whether Ca^{++} acts as a second messenger in the regulation of both SS and GHRH release. The calcium-entry antagonist, verapamil, antagonized K^+-stimulated SS and GHRH release in a dose dependent fashion, with maximal inhibition reached at 1×10^{-9} M. The calcium ionophore, A23187, significantly stimulated SS and GHRH release but this effect was not blocked by verapamil. Thapsigargin, which increases the efflux of Ca^{++} from calciosomes, did not affect either SS or GHRH release at 1×10^{-5} M or 1×10^{-4} M. Honegger et al. concluded that calcium influx was crucial for depolarization -induced SS and GHRH release. Calcium entrance in response to A23187 was independent of verapamil-sensitive calcium channels. The lack of a Thapsigargin treatment effect suggests that increased intracellular Ca^{++} from intracellular stores is not equivalent to an increase in Ca^{++} influx (Honegger et al., 1991b).

Cugini et al. (1991) investigated the role of signal transduction systems in the secretion of SS and GHRH from perfused rat hypothalamic fragments. Responsiveness to depolarization in a concentration-dependent manner was shown by the stimulation of SS and GHRH release after a brief 10 min pulse of high K^+ medium. There was also a marked stimulation of SS and GHRH release by forskolin (an adenylate cyclase activator) and db cAMP (cAMP analog) in a concentration-dependent manner, suggesting that a stimulatory role of the adenylate cyclase-protein kinase-A pathway in both SS and GHRH release.

Cugini's group demonstrated concentration-dependent stimulation of SS and GHRH release in hypothalamic fragments by A23187, suggesting a role for acute changes in cytosolic calcium in the regulation of secretion of these two peptides. The protein kinase-C activator, PMA, did not stimulate basal SS or GHRH release but it did have stimulatory effects on the forskolin-stimulated release of the peptides. This suggests a role for protein kinase-C modulation of stimulated, but not basal, hypothalamic SS and GHRH release in perfused hypothalamic fragments. The additive effect of A23187 on forskolin-induced SS release suggests intracellular interactions of the calcium-calmodulin system with adenylate cyclase/protein kinase A -stimulated release. In contrast, forskolin-stimulated GHRH release was not increased by the addition of A23187. Cugini et al. concluded that these observations implicate a rich and varied role for the signal transduction mechanism in the release of SS and GHRH, and hence in the regulation of GH secretion. They noted that there was a potentially important role of cAMP-dependent pathways, a modulating role for protein kinase-C, and an independent role for cytosolic calcium fluxes on SS and GHRH release.

4.2. Regulation of Somatostatin Release

Although membrane depolarization and intracellular calcium movement are essential in the secretion of SS from neurons in the PeVN, other physiological mechanisms are capable of modulating the release of SS into the hypophyseal portal blood system. Components of the GH system, different catecholamine, acetylcholine, and Corticotropin releasing hormone have all been shown to alter SS secretion (Epelbaum et al., 1986; Berelowitz et al., 1981; Chihara et al., 1979; Aguila et al., 1992; Richardson et al., 1981;

Peterfreund and Vale, 1984; Mitsugi et al., 1990).

The ability of somatostatin to inhibit its own secretion has been demonstrated by various experimental studies. SS analogs can inhibit the release of hypothalamic somatostatin in a dose dependant manner (Richardson and Twente, 1986; Epelbaum et al., 1986). By performing ultrastructural ICC on ultra thin sections of rat hypothalamus, Epelbaum et al. (1986) observed that in the PeVN, perikarya and dendrites of labelled SS neurons showed frequent apposition of their limited membranes. Classical synapses were also observed between SS-containing axonal processes, and labelled perikarya or dendrites. The ability of SS analogs to inhibit SS secretion correlated well with the occurrence of SS-SS synapses in the PeVN, suggesting that SS exerts negative feedback control on its own release through autoreceptors located on the perikarya or dendrites of SS-containing neurons. Beaudet et al. (1995) used *in situ* hybridization to detect intensely labelled SS receptors in the PeVN, distributed beneath the ependymal border of the third ventricle. The correspondence of receptor localization with SS-containing neurons (Beaudet et al., 1995; Johansson et al., 1984) and the presence of high affinity binding sites in the hypothalamus (Srikant and Patel, 1984) support the concept of SS autoregulation.

Results from *in vivo* (Mitsugi et al., 1990) and *in vitro* (Richardson et al., 1988) studies have shown that GHRH may have stimulatory effects on SS release. Mitsugi et al. (1990) showed that the icv administration of GHRH, produced a significant increase in the portal plasma concentration and secretion rate of SS, suggesting that GHRH centrally influences SS secretion into hypophyseal portal blood. Richardson et al. (1988) used

dispersed adult rat hypothalamic cells to show that GHRH caused a dose-dependent (1-100 nM) stimulation of SS in static 1 hr incubations. Attempts to demonstrate a possible anatomical connection between SS and GHRH have been made. Tannenbaum et al. (1990) correlated the autoradiographic distribution of SS receptors with the immunocytochemical distribution of GHRH-containing neurons in the hypothalami of adult rats. By electron microscopy, Horvath et al. (1989) demonstrated synaptic contacts between GHRH-containing nerve terminals and SS-containing dendrites. Taken together these studies suggest that a reciprocal interaction exists between GHRH- and SS-containing neurons (Horvath et al., 1989; Tannenbaum, 1990).

Growth hormone suppresses its own secretion by stimulating SS release (Aguila and McCann, 1993; Sato et al., 1989; Sheppard et al., 1978; Minami et al., 1992). The GH-induced release of SS involves an increase in *c-fos* expressed in SS-containing neurons the PeVN and Arc (Minami et al., 1992) and stimulation of the cholinergic system (Torsello et al., 1988). Somatomedins (peripheral GH effectors) have been shown to mimic the effects of GH on SS (Becker et al., 1995; Berelowitz et al., 1981). Berelowitz et al., demonstrated that both somatomedin-C and GH stimulated SS released from intact medial basal hypothalami tissue blocks, suggesting that there may be a dual mechanism for the negative feedback of GH on SS release; one mediated by GH itself and the other through its peripheral effector, somatomedin-C.

Somatostatin release is stimulated by physiological concentrations of dopamine (Richardson et al., 1983a; Gomez-Pan and Rodriguez-Arnao, 1983; Kitajima et al., 1989;

Chihara et al., 1979) and the dopamine antagonist, metoclopramide has been shown to abolish this (Richardson et al., 1983a). In dispersed hypothalamic cells, ß-adrenergic antagonists have been shown to inhibit ouabain-stimulated SS release (Richardson and Twente, 1990) without affecting basal secretion (Gomez-Pan and Rodriquez-Arnao, 1983; Richardson and Twente, 1990).

Serotonin (5-hydroxytryptamine/5-HT) has not been shown to affect SS release *in vivo* (Chihara et al., 1979), however it has been found to inhibit SS release in two different *in vitro* systems (Peterfreund and Vale, 1984; Richardson et al.,1981). Kiss et al. (1988) using a combination of electron microscopic autoradiography and ICC provided morphological evidence for a direct contact between serotonergic elements and SS neurons of the anterior PeVN, that project to the ME. Synaptic connections between labelled serotonergic nerve endings and SSir elements were observed by Kiss et al. (1988), however Descarries' group have reported that most serotonin contacts and possible actions in the cerebral cortex are via non-synaptic contacts (Seguela, 1989). Although serotonin binding sites to the S1 (5-HT_1) receptor subtype have been detected in the hypothalamus (Laruelle et al., 1988; Biegon et al., 1982), only the S2 (5-HT_2) receptor subtype has been localized on SS immunoreactive neurons in the cortex, (Bowen et al., 1989; Cross et al., 1984) but not in the hypothalamus (Laruelle et al., 1988). Direct correlations between SS immunoreactivity and binding sites to the S2 serotonin receptor ligand [^{3}H]ketanserin, have been studied primarily in postmortem Alzheimer brains where there is a selective loss of SS neurons containing S2 serotonin receptors in the frontal (Cross et al., 1984) and temporal cortices

(Bowen et al., 1989; Cross et al., 1984). Whether serotonin-SS interactions are mediated by S1 receptors in the hypothalamus, S2 receptors that correlate with SS immunoreactivity in cortical areas, or by another hypothalamic serotonin receptor subtype must be clarified by further studies.

Corticotropin releasing hormone, a component of the hypothalamic-pituitary-adrenal axis, has been shown to modulate SS release. The icv administration of 5 μg of corticotropin-releasing hormone (CRH) increased the portal plasma concentration and secretion rate of SS (Mitsugi et al., 1990). Light microscopic studies of vibratome sections, double-labelled for CRH and for SS, suggested the presence of reciprocal synaptic interactions between neurons containing CRHir and those containing SSir in the parvocellular PVN and in the anterior periventricular area (Hisano and Daikoku, 1991).

5. INTERLEUKINS

5.1. Interleukin-1

The CNS and the immune system both have major roles in the maintenance of homeostasis. For many years it was thought that the two acted independently because the brain appeared isolated from the effects of the immune system by the presence of the blood brain barrier and by the absence of a lymphatic drainage system (Fontana et al., 1984; Reichlin, 1993). However, it is now clear that neural and endocrine factors modify immunological function, whilst the chemical messengers that mediate many immune system functions can in turn affect the CNS (Imura et al., 1991; Reichlin, 1993; Ader et al., 1990).

Research into the function of the family of immunological chemical messengers known as cytokines has greatly advanced our understanding of CNS-immune system interactions.

The acute phase response of the immune system is triggered in response to infection, burns, trauma or neoplasia. This response is also triggered by the exposure to an endotoxin or bacterial lipopolysaccharide (LPS). LPS is a complex glycolipid and a component of the outer membrane of most Gram negative bacteria (Elmquist et al., 1993). The acute phase response initially consists of an increase in certain plasma proteins as well as the mobilization and activation of phagocytic mononuclear macrophages (Smith, 1991). Major cytokines, interleukin-1ß (IL-1ß) and tumor necrosis factor (TNF) and interferon 1 (INF 1), which mediate natural immunity (protection against viral or bacterial infections) are produced during the acute phase response and act to co-ordinate central and peripheral host defense mechanisms (Dinarello and Thompson, 1991).

IL-1 is a term that is applied to two cytokine isoforms, IL-1α and IL-1ß, and one antagonist, IL-1ra (IL-1 receptor antagonist) (Dinarello and Thompson, 1991). IL-1α and IL-1ß show less than 30% structural homology to each other but both isoforms produce similar biological effects and share the same receptor (Imura et al., 1991; Smith, 1991). Both IL-1 polypeptides are synthesized as precursors of approximately 33 kDa that are proteolytically cleaved in order to generate the mature 17 kDa proteins (Smith, 1991)

There are two IL-1 receptors, known as IL-1R type I and type II (IL-1RtI and IL-1RtII, respectively). IL-1RtI (80 kDa) is expressed primarily on T cells, but can be detected on endothelial cells, keratinocytes, heptacytes and fibroblasts. IL-1RtII (68 kDa) is highly

expressed in B-cells and to a lesser degree on neutrophils and bone marrow. IL-1ß appears to have a greater affinity for type II receptors whereas IL-1α binds with greater selectivity to the type I IL-1 receptor subtype. (Dinarello and Thompson, 1991; Cunninghan and De Souza, 1993) IL-1ra binds to both receptor subtypes.

5.2. Interleukin-1ß in the Brain

5.2.1. Peripheral LPS or IL-1ß Effects in the Brain

The neuroendocrine and immune system link has been characterized by investigations examining mechanisms whereby peripherally generated cytokines modulate the activity of the central nervous system. For example, the findings that iv injections of IL-1ß increased corticotropin-releasing hormone (CRH) levels in the hypophyseal portal blood, and that subsequent elevations in plasma adrenocorticotropic hormone (ACTH) levels were blocked by the immunoneutralization of CRH (Sapolsky et al., 1987) have suggested that IL-1ß acts within the CNS in order to stimulate the hypothalamic-pituitary-adrenal axis (Rivest et al., 1992). Furthermore, peripheral administration of IL-1ß exerts other central effects such as the induction of slow-wave sleep (Katsuura et al., 1988).

Coceani et al. (1988) demonstrated that iv endotoxin or natural IL-1ß given as a bolus (0.8 mg/kg) or continuous infusion produced fever in conscious cats. Coceani also showed that icv but not iv injection of pyrogenic doses of bacterial endotoxin (LPS) led to the appearance in the cerebrospinal fluid of IL-1ß activity. They concluded that blood-borne IL-1ß was likely to act at a discrete site outside the blood-brain barrier, possibly at the OVLT. Centrally formed IL-1ß may instead initiate general fever-promoting and fever-related events

(Coceani et al., 1988).

Fontanna et al. (1984) showed that an extract prepared from dissociated brain cells of mice injected previously with LPS, was found to contain a factor that increased the proliferation response of thymocytes (thymocyte proliferation was used as an assay for IL-1ß). The concentrated brain extracts from the LPS-treated mice, produced fever in control mice. Taken together, these data suggest that the pyrogenic actions were mediated by peripheral IL-1ß gaining access to the CNS or by IL-1ß produced locally in the brain (Fontanna et al., 1984).

The expression of *c-fos*, an immediate early proto-oncogene, is upregulated during an increase in synaptic activity, and therefore *c-fos* and its protein product, Fos, are used as markers of cellular activity (Rivest et al., 1992; Sagar et al., 1995). Rivest et al. (1992) demonstrated that both iv and icv injections of IL-1ß increased plasma ACTH levels, but only the icv infusion of the cytokine increased *c-fos* expression in the paraventricular nucleus (PVN) and the arcuate nucleus (Arc) of the hypothalamus. The effect of icv, but not iv, IL-1β injections on *c-fos* suggest that IL-1β induced neuronal activation is not initiated by peripherally-derived IL-1β that crosses the BBB, but by IL-1β secreted locally in the brain. Sagar et al. (1995) used immunocytochemistry (ICC) to observe that iv LPS induced Fos expression in oxytocin labelled neurons in the PVN and somatostatin positive neurons in the PeVN. The increase in Fos protein suggests that these hypothalamic neurons are activated by peripherally administered LPS. Other hypothalamic areas that show LPS-induced Fos nuclear immunostaining include; the dorsomedial nucleus, the Arc, the ME and the

circumventricular organs (the OVLT, the subfornical organ and the area postrema). LPS-induced Fos immunoreactivity was attenuated by pretreatment with indomethacin or dexamethasone (Sagar et al., 1995). The effects of central IL-1β and indomethacin on c-fos gene expression and Fos protein content, respectively, suggest that both IL-1β and prostaglandin may be important candidates for the central mediation of peripherally-derived LPS activation of the brain. Elmquist et al. (1993) observed that two hours after intra peritoneal (ip) LPS administration, robust Fos-like immunoreactivity was observed in the several brain nuclei including the hypothalamic paraventricular and supraoptic nuclei, and the nucleus of the solitary tract in the brainstem. The nucleus of the activated brainstem nucleus sends inputs into the hypothalamus (Richardo and Koh, 1978) and may be involved in its activation/regulation by LPS. Together these data suggest that peripheral LPS administered iv (Sagar et al., 1995) or ip (Elmquist et al, 1993) has the ability to activate hypothalamic neurons which may contain SS. The increase in Fos protein expressed in the circumventricular organs (Sagar et al., 1995) and the nucleus of the solitary tract (Elmquist et al, 1993) suggests that these areas may be involved in the peripheral transduction of the respective iv and ip LPS signal into the brain.

5.2.2. Interleukin-1α and β Receptors in the Brain

Peripheral and centrally-derived IL-1ß can function through IL-1 receptors that have been localized in the CNS (Katsuura et al., 1988; Haour et al., 1994; Cunningham et al., 1992). Katsuura et al. (1988) identified a single type of high affinity binding site for IL-1ß in rat hypothalamus (dissociation constant or $K_d = 1.0 \pm 0.2$ nM) and cerebral cortex (K_d =

1.3 ± 0.2 nM), but not in the pituitary. The maximum binding capacity (B_{max}) in the hypothalamus (B_{max} = 75.4 ±10.8 fmol/mg protein) was 4 times greater than in the cerebral cortex (B_{max} = 17.2 ± 1.5 fmol/mg protein). Binding studies also revealed that in the hypothalamus, about 100 times greater concentrations of IL-1α than IL-1ß were needed to displace bound ^{125}I-IL-1ß to the same extent (Katsuura et al., 1988). It is unlikely that such high IL-1α concentrations would occur in the brain, suggesting that, in contrast to peripheral subtypes, IL-1α does not bind to hypothalamic IL-1ß receptors (Katsuura et al., 1988). Cunningham et al. (1992) used *in situ* histochemistry in mouse brains to show the high intensity localization of type I IL-1 receptor mRNA in the dentate gyrus, raphe nucleus and choroid plexus of the lateral, third and fourth ventricles. Cunningham's group did not observe type I IL-1 receptor mRNA in either the PVN or ME suggesting that IL-1ß effects in the hypothalamus are mediated through the type I receptors distributed in the choroid plexus of the third ventricle, or by a currently unidentified receptor subtype. Haour et al. (1994) demonstrated that brain IL-1 receptor density was down-regulated in the dentate gyrus in a dose-dependant fashion, by systemic LPS administration, providing further evidence of receptor-mediated responses to peripheral IL-1ß.

5.2.3. Interleukin-1ß and Prostaglandins Located in the Brain

Several *in vitro* studies illustrated that IL-1ß and prostaglandins were produced in the CNS and may be involved in the central effects of cytokines in the brain. Navarra et al. (1993) showed that prostaglandin E_2 (PGE_2) and LPS stimulate bioactive IL-1ß release from rat hypothalamic explants. IL-1ß can also stimulate the release of PGE_2 from the

hypothalamus (Navarra et al., 1992; Pozzoli et al., 1992; Blatteis et al., 1990). The IL-1ß induction of PGE_2 release suggests that prostaglandins may play a mediatory role in the hypothalamic effects of IL-1ß (Navarra et al., 1992). Breder et al. (1988) have implicated neurons as a possible source of central IL-1ß. An antiserum directed against human IL-1ß was used to stain the human brain immunocytochemically for IL-1ß immunoreactive (IL-1ßir) neuronal elements. The densest accumulations of IL-1ßir fibres were found in the periventricular regions that participated in anterior pituitary control. IL-1ßir fibres were found throughout the PeVN, Arc, PVN, and the ME (Breder et al., 1988).

Astrocytes and microglia are potential non-neuronal sources of centrally-derived IL-1ß (Fontanna and Grob, 1984; Reichlin, 1993). LPS can stimulate astrocytes to secrete IL-1ß (Fontanna and Grob, 1984) and LPS can induce an increase in the expression of IL-1ß mRNA in glial cells (Lieberman et al., 1989). IL-1ß has also been shown to induce PGE_2 release from rat astrocyte cultures (Katsuura et al., 1989). Microglia cells that are embryologically and functionally related to macrophages and astrocytes (Reichlin, 1993), can also secrete IL-1ß when activated (Reichlin, 1993; Giulian et al., 1986; Righi et al., 1989).

5.2.4. Interleukin-1α and β Transport Through the Blood-Brain Barrier

The mechanism whereby peripherally originating immunological events are signaled to the CNS is still an issue of speculation and controversy. Currently there are five theories posited in order to explain the transport of the peripheral-central message through the blood brain barrier. The first theory suggests that chronic exposure to various cytokines disrupts the blood-brain barrier and increases the permeability of endothelial cells (Maruo et al.,

1992; Sharief et al., 1992). This theory suggests, that mediators of the immune system may increase their own entry into the CNS and initiate the central response. A second theory suggests that IL-1α and to a lesser extent IL-1ß are transported through the blood-brain barrier by a saturable system (Banks et al., 1989; Banks et al., 1991). A third theory proposes the leakage of cytokines through areas such as the circumventricular organs, that have no blood-brain barrier (Blatteis, 1990). Blatteis (1990) has shown that the ablation of the vascular plexus of the OVLT in the anteroventral third ventricular wall resulted in the suppression of fever evoked by intraperitoneal injection of LPS whereas central injection of IL-1ß did not affect the pyrogenic sensitivity of the animals. The foot processes of astrocytes are close to the blood-brain barrier, and the transport of the message from the processes to the cell bodies inside the barrier could comprise a fourth possible mechanism for the conveyance of the peripheral-central message; blood borne cytokines may come into contact with foot processes and stimulate the release of prostaglandins from the astrocytes cell bodies (Katsuura et al., 1989) or an immune antigen may be transported by the same retrograde movement, into the CNS (Fontanna and Fierz, 1985). The release of astrocytic prostaglandins or the presentation of the immune antigen inside the CNS may stimulate the production of central cytokines (Katsuura et al., 1989; Fontanna and Fierz, 1985) . A fifth mechanism for the transportation of immunological-CNS messages suggests, that peripheral cytokines, through neuronal signal mediation, may induce central cytokine production. Autonomic preganglionic neurons receive direct projections from brain stem nuclei, hypothalamic nuclei and limbic forebrain structures (Ader et al., 1990). Extensive ascending

and descending connections exist between these areas which have been shown to link some endocrine systems with neuroendocrine and autonomic systems (Ader et al., 1990). The hypothalamus and limbic system structures are regions that are affected by cytokines and may be the central components of a neuronal circuit that responds to peripheral immune signals (Ader et al., 1990).

6. Interleukin-1ß Effects on Somatostatin

Cytokines have been shown to alter hypothalamic function (Mandrup-Poulsen et al., 1995; Imura et al., 1991; Ader et al., 1990) and several studies have demonstrated that cytokines are capable of modulating the growth-hormone axis (Honegger et al., 1991; Kasting and Martin, 1982; Peisen et al., 1995; Scarborough, 1990).

McCaffery et al. (1970) noted that 22 out of 120 children with inflammatory bowel disease were severely growth retarded. Farthing et al. (1981) observed that children with Crohn's disease and retarded growth had a stunted GH secretion response to sleep and insulin-induced hypoglycemia compared to normal children. Farthing's group also noted that in some of his patients the 5 hr mean growth hormone levels were reduced compared with levels previously reported in normal short children, although the pulsatile pattern of GH secretion was preserved. Raised basal GH secretion during starvation and protein-caloric malnutrition in Crohn's-growth retarded children, suggests that the GH malsecretion was unlikely due simply to malnutrition, but that an endocrine dysfunction contributed to the problem (Farthing et al., 1981). Although the suppression of the GH axis may not be the sole

or direct pathology involved in the growth retardation observed in this study, it does indicate that chronic immune activation has an effect on the physiology of the GH axis.

GH may also affect the function of the immune system (Ader at al., 1990; Mandrup-Poulsen et al., 1995) suggesting that the regulation of the GH neuroendocrine and immune systems are interrelated. Mandrup-Poulsen et al. (1995) noted that in GH-deficient children, an increased IL-1ß and IL-2 production was suppressed with GH substitution therapy. Insulin-like growth factor I administration appeared to inhibit mononuclear IL-1ß synthesis (Mandrup-Poulsen et al., 1995). Ader et al. (1990) observed that GH deficiencies have been associated with abnormal cellularity of the bone marrow and thymus, and depressed T-cell function and antibody responses. Ader et al. (1990) also noted that administration of GH to GH-deficient animals afforded some restoration of immune system function.

In an attempt to further examine the immunological-neuroendocrine GH link, several studies in rats have investigated the effect of IL-1ß and endotoxin on GH release. Rettori et al. (1987) observed that following the icv injection of 5 ng of IL-1ß, GH plasma values increased 5 min after the injection and remained elevated for the duration of the 2-hour experiment. The site of action in these experiments was unclear but the hypothalamus was a likely site in view of the low dose of IL-1ß used (Imura et al., 1991). Endotoxin effects on GH secretion in humans are also stimulatory (Ader et al., 1990).

Kasting and Martin (1982) measured plasma GH levels in freely behaving adult rats for 6 hr on three consecutive days. Kasting and Martin observed that the normal pulsatile GH release observed on the first day was abolished on the second day by iv endotoxin

treatment. On the third day, however, GH secretion was greater than on the initial control day. Kasting's group also observed that the suppression of GH secretion by endotoxin was reversed in all animals by anti-somatostatin serum. Kasting and Martin speculated on the rebound in GH secretion and the possible role of SS. They surmised that the endotoxin induced a large increase in SS release in the ME and almost completely suppressed GH release, as passive immunoneutralization of endogenous SS indicated. Therefore the rebound release may be due to either depletion of stores of releasable SS in ME nerve terminals or a refractory response of somatotrophs perhaps due to downregulation of receptors.

Peisen et al. (1995) examined the long-term effects of LPS and IL-1ß on GH secretion in juvenile rats. Using the same time frame, they also looked at the effects of endotoxin on body weight and GH release. The results of the *in vivo* component of their study revealed that iv LPS treatment caused significantly less body weight gain for 2 days. Thereafter, these animals exhibited parallel weight gain but never reached the weights observed in the saline controls. During the first day of weight gain suppression, GH pulsatility was completely abolished and GH baseline levels, mass (total plasma GH secreted/5 hr) and peak frequency were decreased. By the second day of LPS administration, a rebound in GH secretion occurred as GH baseline and GH mass was significantly increased above control levels. By day 3, there was no significant difference between GH levels measured in LPS-treated animals and those of the controls (Peisen et al., 1995).

The greatest LPS-induced changes in GH secretion occur during the first 24 hrs of endotoxin administration whereby GH release is initially suppressed and then rebounds

above normal levels. It is during this time that body weight gain is first decreased, never to catch up to saline controls (Peisen et al., 1995). A dysfunction in somatostatin secretion, as suggested by Kasting and Martin (1982), may cause the observed effects on GH secretion and perhaps even the inhibition of body weight gain observed by Peisen (although a GHRH influence cannot be ruled out). Peisen et al. (1995) and Honegger et al. (1991) have examined the acute effects of IL-1ß on SS secretion in juvenile and adult rats, respectively. Honegger et al. (1991) observed that 20 min incubation with IL-1ß stimulated SS secretion from acute hypothalamic rat explants. The effects of IL-1ß were antagonized by the cylco-oxygenase inhibitor, indomethacin. Peisen's *in vitro* experiments with medial basal hypothalamic explants, demonstrated that SS secretion was increased by 30 min incubations with either LPS or IL-1ß and that the effect was reversed by the IL-1 receptor antagonist. These results agree with the acute suppression of plasma GH in adult (Kasting et al., 1982) and juvenile rats and the reduction of somatic growth in juvenile rats exposed to infectious material (Peisen et al., 1995).

Neither study was able to address the effects of IL-1ß on SS during the longer period when GH appears to return to normal levels, but the deleterious effect on growth is not recovered. Acute explant models are restricted to the study of short time points due to limitations in tissue viability. Scarborough et al. (1989) were able to detect IL-1ß induced SS release from fetal dissociative cultures, during 2-5 day incubation, but no significant SS effects at time points less than 24 hours were found (Scarborough, 1990). This lack of a 24 hour SS response suggests that the study of intermediate time periods (in hours) may not be

achieved using dissociative cultures which may require 24 hours to recover from the mechanical and enzymatic manipulations inherent to the technique.

7. Purpose Of Study

The purpose of this study is to examine SS release following moderate term exposure to IL-1ß (i.e., 2, 12 and 24 hr). A demonstration of the SS response to the cytokine exposure during the interval between short term and longer term experiments, may advance our knowledge concerning the period where the GH axis may be most affected. The mediatory role of prostaglandins in IL-1ß effects on SS secretion will also be investigated.

This study will use hypothalamic slice cultures harvested from neonatal rats (adapted from Gahwiler, 1988) to observe the IL-1ß effect on SS release during the intermediate time points. The advantages of this model are its degree of tissue organization, its accessibility and the ease with which the cultures are maintained for longer periods of time compared to acute explant experiments. The method retains a certain degree of tissue organization and the culturing system is described as organotypic in order to emphasize the maintenance of characteristic properties unique to the tissue of origin (Gahwiler, 1988). Organotypic cultures are explant tissue dissected from the donor brains as opposed to dissociative cultures where the cells are dispersed and allowed to resettle (Gahwiler et al., 1991). The cultures retain many, but not all, of the glial-neuronal and neuronal-neuronal connections that may be necessary for the coordination of a response to a variety of perturbations. However this *in vitro* model is limited by the loss of afferent serotonergic or noradrenergic contacts, and

the absence of extrahypothalamic effectors such as somatomedins and GH that might have *in vivo* influences on somatostatin/IL-1ß interactions.

II METHODS

1. SLICE EXPLANT CULTURES

Neonatal hypothalamic slices were cultured by a technique modified from Gahwiler. (1988). Four day old male rat pups were decapitated without anesthetic in a manner approved by the Animal Care Committee, Memorial University of Newfoundland. In order to dissect out the hypothalamus, two parasagittal cuts through the skull and skin beginning at the foramen magnum, extending rostrally through the lateral part of the skull just above the ears, and converging between the eyes were made. The skull was then peeled back to expose the brain (Gahwiler, 1988). A spatula was used to separate the brain from the olfactory bulbs and cranial nerves. The brain was then removed from the skull and placed on its dorsal surface in a petri dish containing several drops of cold Gey's balanced salt solution (Gibco) supplemented with 5 mg/ml glucose.

With the use of a dissecting microscope, the hypothalamus was isolated from the brain using razor blade knives to make five successive cuts . (All blades were washed in acetone, rinsed in tap water and 95% ethanol and then stored in 70% ethanol just before culturing began). A transverse cut was made approximately 2 mm rostral to the optic chiasm, followed by a second transverse cut through the peduncular mammillary nucleus (Sherwood and Timiras, 1970) in order to dissect away the cerebellum. The block of tissue was placed on its anterior surface and a cut was made approximately 5.2 mm from the ventral surface of the brain, through the thalamic nuclei. The tissue block was again placed on its dorsal surface and two sagittal cuts (lateral to the optic tracts) removed the lateral cortex,

isolating a hypothalamic portion of tissue approximately 4.8 mm long and 5.2 mm deep.

The hypothalamus was partitioned into 400 µm slices using a Sorvall tissue chopper. The tissue was attached to a sterilized plastic disc of Aclar foil (Proplastic) by placing it (on its dorsal surface) into a 20 µl drop of chicken plasma (Cocalico), followed by the addition of 20 µl of thrombin (1000 NIH units/ml: E Merck) which was mixed with the plasma. The plasma/thrombin mixture clots within minutes and serves to stick the tissue to the plastic disc. After coagulation a drop of glucose enriched Gey's solution was put on the hypothalamus in order to keep the tissue moist. The block was then chopped into 400 µm slices.

The chopped hypothalamus was taken off the Aclar foil with a spatula and placed in a petri dish containing supplemented Gey's solution. Under the dissecting microscope, the slices were illuminated by transmitted light and quickly separated and placed in individual drops of Gey's solution. The slices were then stored at 4 °C for at least 1 hour or until all the brains for that culture day had been dissected into slices. This refrigeration period seems to benefit the survival of the cultures, possibly allowing for cut membranes to close and proteolytic enzymes to diffuse away from the tissue (Gahwiler et al., 1991).

Slices were attached to glass coverslips by embedding them in another plasma/thrombin clot. The chicken plasma (20 µl) around the individual slice was coagulated by the addition of 20 µl of supplemented Gey's solutions containing 20 NIH units/ml thrombin. The mixture was then spread around and under the slice and left to coagulate for at least 1 hour. After coagulation the coverslips were placed into plastic tubes

(Nunc) containing serum containing culture medium (SCM) consisting of 50% Basal Medium Eagle's with Earles' Salts (Gibco), 25% Earles' balanced salt solution (Gibco) and 25% heat inactivated horse serum (Gibco), supplemented with 6.5 mg/ml glucose (Gahwiler, 1988). The tubes were tightly capped and then inserted into a roller drum within a 37 °C dry incubator. The tubes were rotated in the drum at a speed of 12 revolutions/hour.

After four days in culture the SCM was decanted and replaced with serum free culture medium (SFM) consisting of DMEM/F-12 (Gibco) supplemented with an insulin, transferrin, selenium mixture (5 µg/ml, 5 µg/ml, and 5 ng/ml respectively); a mixture of 25 U/ml penicillin, 25 µg/ml streptomycin and 50 µg/ml neomycin, 100 mM putrescine, 1 µM triiodothyronine (T_3) (Sigma), 1 µg/ml arachidonic acid and 0.5 mg/ml docosahexanoic acid (Nu Chek Prep) (Wray et al., 1991).

Neonatal brain tissue is very soft and sags when positioned on its dorsal surface. The anatomy is therefore different from what might be observed *in situ* or in fixed tissue. A sterotaxic atlas of a fixed 10 day old rat brain (Sherwood and Timiras, 1970) was used to estimate the relative differences in morphology that would occur in the cultures compared to fixed brain slices. In this system four slices were usually cultured and the preoptic area including the paraventricular nucleus and the anterior portions of the periventricular nucleus were likely to be located in one of these four slices.

2. SOMATOSTATIN RADIOIMMUNOASSAY (RIA)

2.1 Parameters

The somatostatin radioimmunoassay was adapted from the procedure developed by Penman et al. (1979). In this study, the somatostatin assay was downsized from the 600 µl final assay volume used by Penmen et al., to 300 µl in order to compensate for lower amounts of sample collected from the cultures. This assay was conducted in 6 x 38 mm polystyrene tubes, compared to the standard 12 x 75 mm size, and therefore antibody dilutions and tracer amounts had to be titrated in order to find the new optimal amounts for the smaller tubes. A titration 'checkerboard' was performed to examine the effects of various dilutions of the primary antibody, the secondary antibody, and the tracer amount on the standard curve of the RIA. At a constant secondary antibody dilution of 1:16, the tracer concentration was tested at 1500 CPM, 3000 CPM and 6000 CPM with primary antibody dilutions of 1:25 000, 1:30 000 and 1:35 000. The ability of the secondary antibody to bind to different concentrations of primary antibody at a standard tracer dilution of 3000 CPM, was also tested. The standard curves were constructed with the standard concentrations of 2, 8, and 63 pg/tube in order to determine the effect of changes in antibody dilutions on the linear area of the curve. The titrated primary and secondary antibody dilutions were 1:30 000, 1:35 000, and 1:45 000 and 1:16, 1:24, 1:32, respectively.

Serial dilutions of experimental incubation samples were compared to a standard curve constructed from synthetic SS peptide in order to assess the parallism between the SS secreted from the cultures and the commercial cylic peptide. The detection limit, percent

recovery of unlabelled exogenous peptide, average non-specific binding and average maximal binding of the RIA were also calculated (n=8).

Primary antibody specificity was assessed extensively by Penman et al. (1979) who have used the same primary antibody as the one used in this RIA. Penman et al. (1979) determined that the primary antibody showed no cross-reaction with any of the following hypothalamic, pituitary or gastrointestinal peptides and proteins: thyroid stimulating factor, human growth hormone, ß-lipotrophic hormone, γ-lipotrophic hormone, follicle stimulating hormone, ß-melanocyte stimulating hormone, met-enkephalin, oxytocin, thyrotrophin-releasing hormone, porcine insulin, glucagon, secretin, substance P, gastrin-17, cholecystokinin, vasointestinal polypeptide, bombesin, gastric inhibitory polypeptide and motilin. The antibody was therefore considered specific to somatostatin.

2.2 Assay

Different concentrations of IL-1ß were incubated with the hypothalamic cultures for 2 hr, 12 hr and 24 hr in order to examine the effect of incubation time on somatostatin release. The amounts of somatostatin released into the supernatant were measured by a four day sequential RIA adapted from Penman et al. (1979). All standards, antibodies and radioactive peptides were diluted in assay buffer containing 0.05 M sodium phosphate buffer, pH 7.4 and 0.4% human serum albumin (wt/vol). The tubes used in the assay were plastic polystyrene tubes with the dimensions 6 mm x 38 mm (Bellco Glass Inc). Standard curves were constructed using eight serial dilutions of cyclic somatostatin (Bachem California) between 125 - 0.977 pg/tube. The standards were measured in triplicate and each tube

contained 25 µl of standard. Four (4) Non Specific Binding (NSB) tubes initially received 75 µl of releasing medium (RM). 25 µl RM was added to four Reference (REF) or B_{max} tubes that measure the maximal binding of the tracer to the primary antibody. 25 µl samples from release experiments containing an unknown content of somatostatin were measured in duplicates. All tubes, except the four Total Count (TC) tubes (which contained tracer only), received an additional 75 µl of RM. 50 µl of rabbit somatostatin antiserum (R10: kindly donated by Dr. A. Grossman) was added to each tube (except TC and NSB) at an initial dilution of 1:30 000 and incubated for 24 hr at 4 °C. 50 µl of tracer containing 3000 CPM of ^{125}I-^{11}Tyr-somatostatin (Amersham) was added to all tubes and incubated for 24 hr at 4 °C. Separation of bound from free ^{125}I-^{11}Tyr-somatostatin was carried out by double antibody precipitation. Sheep anti-rabbit immunoglobulin (1:16; Harlan Bioproducts for Science, 50 µl) and normal rabbit serum (1:200; DAKO, 50 µl) were incubated with all tubes except TC for 24 hr at 4 °C. The tubes (except TC) were transferred into centrifuge buckets adapted for the smaller tubes and centrifuged at 2000g for 1 hr. The supernatant containing free radioactivity was then aspirated and discarded. The TC tubes were combined with the rest of the tubes and the pellet (bound radioactivity) was counted in a gamma counter. This procedure is a competitive binding assay using ^{125}I-^{11}Tyr-somatostatin. The more labelled SS tracer counted in the pellet, the less SS was present in the RM.

2.3 Statistical Analysis

Due to the variation in somatostatin release among different cultures, data was 'normalized' by expressing the experimental values as a percentage of basal levels measured

from the same slice culture. The 'normalized' data was subjected to the Wilk-Shapiro/Rankit Plot procedure to examine whether the data conformed to a normal distribution. The time course data was not distributed normally, therefore the nonparametric Wilcoxon Paired Signed Rank Test was used to detect significant differences between basal values and experimental group percentages. Data were expressed as mean ± SE and significance was set at $p < 0.05$.

The Kruskal-Wallis one way analysis of variance by ranks was used for multiple comparisons of the three IL-1ß dose effects on slice one, slice two, slice three or slice four, separately. This nonparamentric analysis of variance was also used to examine the differences in the indomethacin-IL-1ß experiments. Significantly different means, detected by a comparison of mean ranks in the analysis of variance procedure, were subjected to the Wilcoxon Signed Rank Test in order to examine pairwise comparisons. Data were expressed as mean ± SE and significance was set at $p < 0.05$.

3. Time Course of IL-1ß Effects on Somatostatin

Nine male neonatal rats (4 days old) were used for each time point. Three rats per litter from three different litters (3 rats x 3 litters = 9 rats) were grouped into a single time point. Four slices from each brain were cultured and used in the somatostatin time course. Hypothalamic slice cultures were washed for 50 min in 750 µl of releasing medium consisting of Earle's balanced salt solution (Gibco) containing (mM): $CaCl_2$ $2H_20$, 1.8; KCl, 5.3; NaCl, 117.0; $MgSO_4$, 0.8; $NaH_2PO_4H_20$, 1.0; D-glucose, 5.6; phenol red, 0.03, which

was further supplemented with 0.1% bovine serum albumin (Sigma), 100 KIU Aprotinin (Bayer), 10 mM HEPES (Fisher Scientific), 0.17 mM ascorbic acid (Sigma) also an extra 5.6 mM D-glucose (Fisher Scientific). The cultures were exposed to 2 hr of releasing medium and the supernatant was collected. Basal levels of SS were assayed. The slices were then incubated with releasing medium in the presence of 1×10^{-9} M, 1×10^{-10} M or 1×10^{-11} M recombinant human interleukin-1 (R&D Systems) 2 hr, 12 hr or 24 hr.

After basal samples were collected, IL-1ß containing medium was added to the cultures. 2 hr before the end of the 12 and 24 hr time points, fresh IL-1ß medium was applied. By restricting the time period of assayed material to 2 hrs, the effects of time on peptide release that are independent of IL-1ß are conserved and equal in all three time points (Fig. 1). IL-1ß concentrations are expressed in molar values (M) or as units of activity per ml (U/ml). The activity of IL-1ß is time-dependent in normal immunological reactions (Vilcek and Lee, 1992), therefore the 2 hr pulse of new IL-1ß at the end of the longer time points maintained IL-1ß activity levels similar to that experienced in the 2 hr time point.

Six male neonatal rats (4 days old) were used as controls for each time point. Three rats were taken from each of two litters. Four slices from each brain were cultured and used as controls slices for the 2hr, 12 hr and 24 hr time points. Control slices were incubated in parallel to slices exposed to IL-1ß. These 'sham' cultures were added to the study to examine if experimental manipulations would affect basal somatostatin release. Determining that the observed effects on SS secretion were due to IL-1ß and not a result of tissue lost during medium changes and other nonspecific manipulations of the cultures, was a crucial control.

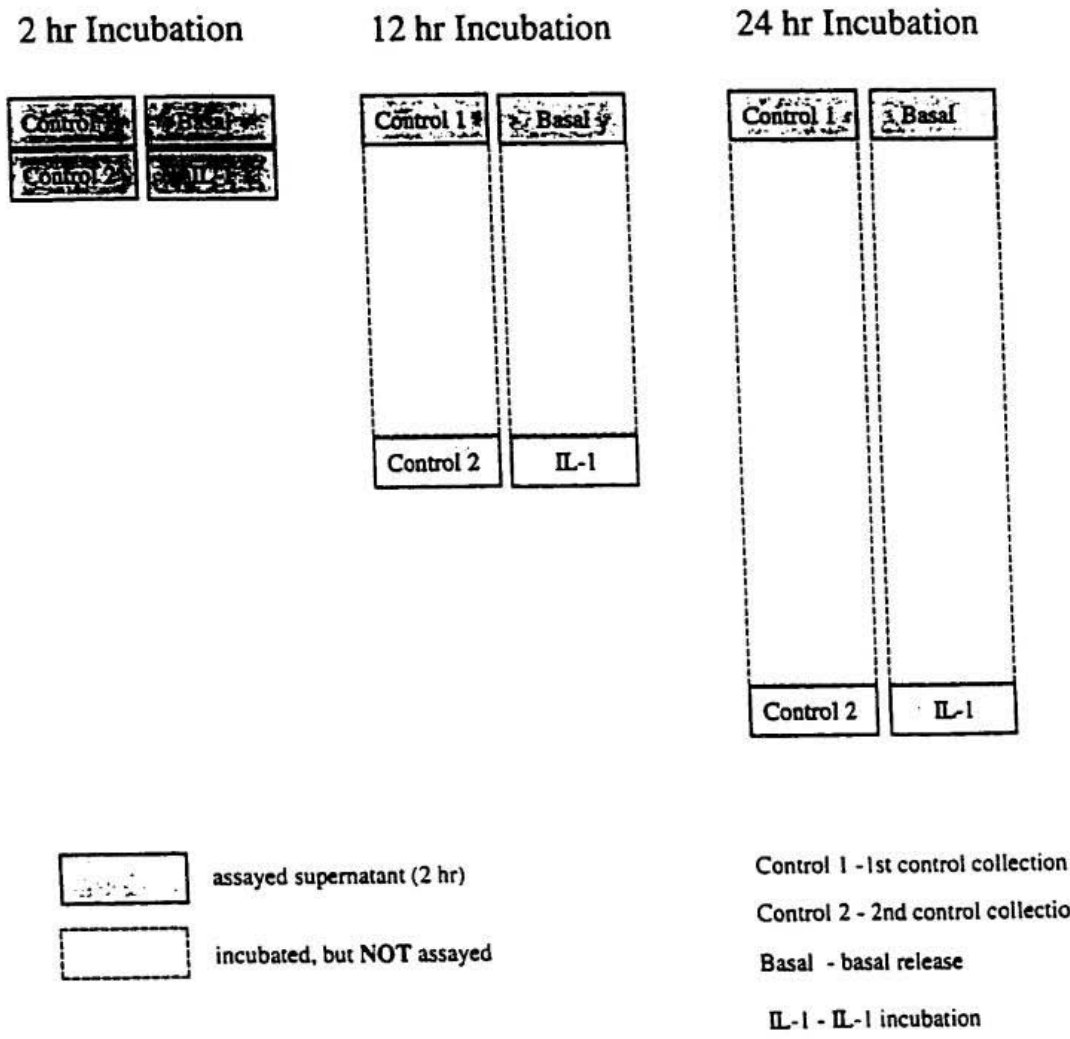


Fig.1 Conceptual diagram of the time course experimental design. The diagram illustrates that control slices were incubated in parallel with slices exposed to IL-1β. The shaded squares represent the medium that was incubated with the cultures and measured by radioimmunoassay for somatostatin. White blocks represent the medium that was incubated with the cultures, discarded and replaced with fresh medium.

4. Indomethacin Antagonism of IL-1ß Effects on Somatostatin

Release experiments designed to examine the effect of the prostaglandin biosynthesis inhibitor, indomethacin (Sigma), on IL-1ß induced somatostatin release were adapted from the time course protocol. Two groups of seven male rats (4 days old) were used to examine the indomethacin/interleukin-1 interaction. Siblings were selected from various litters in groups of threes and fours. In the IL-1ß experiments, cultures were derived from four slices taken from each brain (slice 1, slice 2, slice 3 and slice 4). In the indomethacin experiments, only cultures derived from slice 1 and slice 4 were included because IL-1ß did not significantly alter somatostatin secretion from slice 2 or slice 3. (Further description provided in the Results chapter, section 3 entitled; somatostatin release experiments.) One group of cultures was incubated with releasing medium for 2 hr (basal release), then SFM containing 10 µg/ml indomethacin for 10 hr, followed by another 2 hr incubation in releasing medium containing 10µg/ml of indomethacin (12 hr total incubation). In this group of cultures we examined the effects of indomethacin on basal release. The second group of slices was exposed to a basal incubation followed by an incubation with 1×10^{-9} M IL-1ß plus 10 µg/ml indomethacin, in order to determine whether indomethacin, by blocking prostaglandin synthesis, would affect the ability of IL-1ß to affect SS secretion. The 12 hr time point was the intermediate incubation time and 1×10^{-9} M IL-1ß proved to be the optimal dose.

5. HISTOLOGY

The peroxidase-antiperoxidase (PAP) immunocytochemistry (Sternberger, 1986) was used in this study in order to identify peptide storage in hypothalamic neurons in culture. The cultures were adhered to coverslips by creating a chicken plasma clot over and around the tissue. The plasma clot which is essential for the growth of the cultures created problems with immunocytochemical procedures. Long wash periods were required to remove the fixative and wash away the secondary antibody which was observed in this study to react nonspecifically with the plasma clot. The plasma clot is not as strong as other adhesive matrixes like paraffin which are commonly used in immunocytochemistry. The cultures often washed off the tissue, therefore the compromise wash period of 4 x 10 min was selected. Due to the large loss of culture tissue during the procedure, the distribution of SS was examined in paraffin-embedded neonatal brain slices. This would allow us to observe the localization of SS in the tissue from which the cultures were harvested. The DAKO SS antibody was quantified on adult rat pancreatic tissue therefore adult rat pancreas was used positive controls for the immunocytochemistry.

5.1 Immunocytochemistry on Cultures

Twelve male neonatal rats (four days old) were divided into two groups. One group of six rat brains were cultured and then stained for somatostatin after one day *in vitro*. The other set of six rat brains were cultured and stained after the seventh day *in vitro*. As in the somatostatin time course design, groups of three rats were litter mates and four slices per brain were cultured.

The culture medium of 1 day old and 7 day old cultures were decanted and replaced with Bouin's Fixative (v/v:10% formalin, 5% glacial acetic acid and 75% saturated picric acid) for 24 hr at 4 ° C. The cultures were rinsed twice with 0.01 M sodium phosphate buffer containing 0.9% NaCl, pH 7.4 (PBS) for 10 min, 1 hr and then the cultures were left in PBS overnight at 4 ° C. Following a 4 x 10 minute wash, the slices were incubated with 0.06% hydrogen peroxide in PBS for 15 minutes in order to block endogenous peroxidase. After another wash, the cultures were incubated with PBS (pH 7.4) containing 1% Triton X-100 for 20 min. Following another set of washes, the cultures were transferred from the culture tubes to individual petri dishes and incubated with 10% normal swine serum (DAKO X901) in 0.01 M PBS for 30 min. The primary, secondary and PAP antibodies were all diluted in PBS containing 1% normal swine serum. The blocking serum was removed and somatostatin antisera (DAKO A566, 1:100), normal rabbit serum (DAKO X902 , 1:300) or glial fibrillary acidic protein antibody (DAKO Z334, 1:1000) were incubated with the cultures for 48 hr at 4 ° C. Following a 4 x 10 min wash, the cultures were incubated with the secondary antibody, swine anti-rabbit immunoglobulin (DAKO Z196, 1:800) for 30 min at room temperature, washed and then incubated with rabbit PAP (DAKO Z113, 1:100) for another 30 min at room temperature. The PAP antibody was removed by 4 x 10 min washes with PBS and then 3,3'-diaminobenzidine (DAB: 0.4 mg/ml, Sigma) in 0.05 M tris saline (pH 7.6) was reacted with the peroxidase. The DAB-peroxidase reaction was stopped by washing 4 x 10 min with distilled water, after a specific brown coloured stain was observed in the tissue or until nonspecific staining appeared in the serum control. The cultures were

dehydrated by 5 min incubations in 40%, 70%, 95% and three 100% ethanol successively, followed by a 5 min xylene wash. Cultures were then mounted under their coverslips in Permount.

5.2 Confirmational Immunocytochemistry on Paraffin Embedded Hypothalamic Tissue

To confirm storage of somatostatin in hypothalamic neurons, sections from neonatal rat brains and adult rat pancreas were stained with an SS antiserum (DAKO). Paraffin embedded, microtome cut sections (10 μm) from four three day old male rats and five adult male rats (300 g - 350 g) were stained for SS.

5.2.1 Preparation of Tissue

Four neonatal rats (four days old) were transcardially perfused with ice cold heparanized saline (2.5 Units/ml), followed by ice-cold 10% buffered formalin (100 ml of 40% formaldehyde, 3.5 g NaH_2PO_4, 6.5 g Na_2HPO_4, 900 ml water). The heads were removed and postfixed in the same preservative at 4° C for an additional 24 hours. The olfactory bulbs and the cerebellum were dissected away and the tissue was stored in 10% buffered formalin until embedded. The tissue was embedded in paraffin in an automatic apparatus (Tissue-Tek VIP) and cut into 10 μm sections on a microtome. Every section was kept. The slices were baked in an oven at 60° C for 1 hour, allowed to cool and then stained for SS.

To obtain the pancreas tissue for the ICC positive control, five male adult rats were sacrificed using an overdose of 65 mg/ml sodium pentobarbitol (MTC Pharmaceuticals). The adult rats were perfused with the same protocol as the neonatal rats. The pancreas was

removed and sectioned into smaller pieces. The fixation, embedding and tissue cutting procedures were identical to those used for the neonatal rats except every tenth pancreatic section was kept. Like the neonate tissue, the pancreas sections were also baked in an oven at 60° C for 1 hour, allowed to cool and then stained for SS.

5.2.2 Immunocytochemistry

The PAP method was also used to identify SS-containing cells in paraffin-embedded hypothalamic brain slices. The primary antibody was a commercial antiserum (DAKO A566) raised in rabbit. The staining capacity of A566 was listed as labelling D cells of the endocrine mammalian pancreas and cells of the parvicellular region of the hypothalamus (DAKO specification sheet). The SS antibody was first tested on adult pancreas tissue with a 48 hour incubation period and a dilution of 1:800. Once positive staining was achieved in the pancreas, neonatal rat brain tissue was stained with the same antibody for 72 hours at a 1:400 dilution.

5.2.2.1. Adult Rat Pancreas Tissue

The following protocol localized SS in the pancreas with no staining in the negative reagent control sections. The slides were rehydrated through a series of three xylene washes, followed by serial dilutions of ethanol (100%, 95%, 80%, 70% and 40%). The slides were then incubated for 5 min in distilled water followed by 0.6% hydrogen peroxidase in 0.01 M sodium phosphate buffer, pH 7.4 (PBS) for 15 min in order to block endogenous peroxidase. After another 3 washes in 0.01M PBS, the cultures were exposed to 0.01 M PBS containing 4% triton X-100 (Sigma) for 15 min in order to render the tissue more permeable. The slices

were washed and then incubated in 0.05% protease (Sigma) diluted in 0.01 M PBS, for 15 min. After the wash step the slides were incubated for 30 min with 10% normal swine serum in order to block nonspecific binding sites. Slides were incubated for 48 hours at 4°C with anti-SS (1:800) or normal rabbit serum (1:1200, DAKO) which served as a negative control (All primary antibodies were diluted in PBS containing 0.03% triton X-100). After 3 x 5 min washes in 0.01M PBS the slices were incubated at room temperature for 1 hr with the secondary antibody (swine anti-rabbit, DAKO, 1:500), washed and incubated with rabbit PAP (1:100; DAKO) for 1 hr at room temperature. Following another wash step, the peroxidase was reacted with 3,3'-diaminobenzidine (DAB: 0.4 mg/ml, Sigma) in 0.05 M Tris saline (pH 7.6) with 1% nickel chloride and monitored until there was specific staining observed or before nonspecific staining appeared in the serum control. The addition of the metal chloride to the DAB solution resulted in a blue coloured stain. The DAB reaction was stopped by washing 3 x 5 min with distilled water. The tissue was dehydrated by 2 min incubations in 40%, 70%, 80%, 95%, 100% and 100% ethanol successively, followed by a 2 min xylene wash. Coverslips were added using Permount.

5.2.2.2. Neonatal Rat Brain Tissue

The neonatal tissue protocol was modified from the procedure used to stain for SS in the adult rat brain sections. The neonatal rat SS peptide content is known to be less than adult rat levels (Khorram and McCann, 1984; Nobou et al., 1985; Gabriel et al., 1989) and as a consequence four changes were made in order to improve specific staining. 1) The slides were pretreated (after rehydration) with 0.06% protease in 0.05 M tris saline (pH 7.5)

for 10 min. followed by a 5 min incubation with ice-cold tris saline (pH 7.5) to stop the protease action; protease pretreatment has been shown to increase antibody penetration into paraffin embedded tissue and augment staining better than triton X-100 (Finley et al., 1982). 4% triton X-100 pretreatment was not used on neonatal tissue. 2) The primary antibodies were diluted in 0.01 M PBS containing 0.03% triton X-100 and 1% normal swine serum in order to decrease non specific binding. 3) The dilutions for anti-SS, normal rabbit serum and swine anti-rabbit serum were decreased to 1:400, 1:600 and 1:200, respectively, in order to enhance the exposure of the primary antibody to the antigen and therefore intensify staining. 4) The primary incubation times were increased from 48 hr to 72 hr.

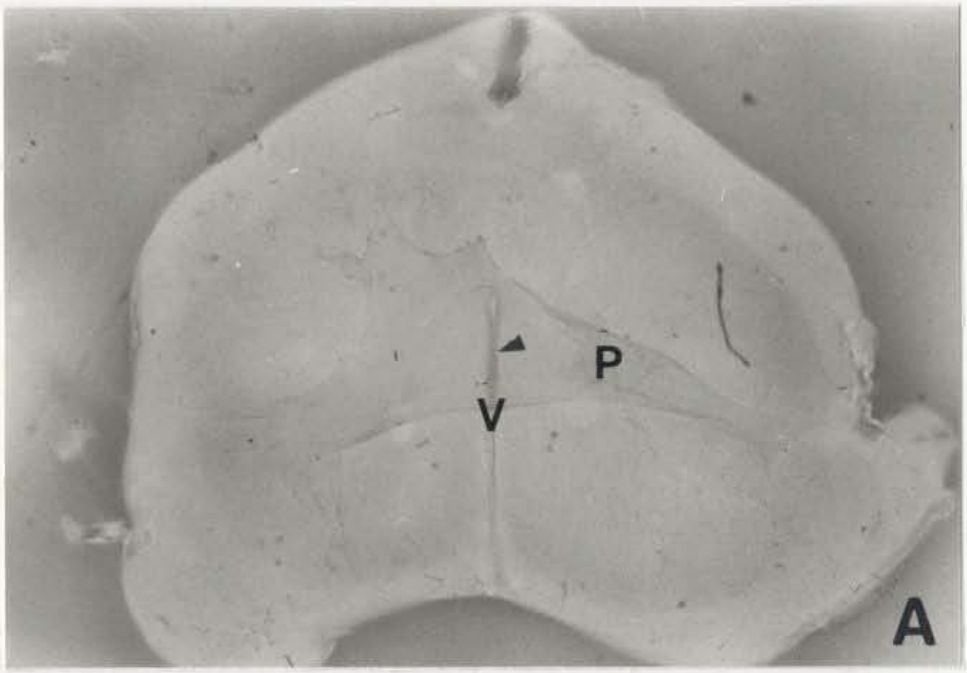
III RESULTS

1. SLICE EXPLANT CULTURES

Immunocytochemical staining of the cultures was attempted in order to examine somatostatin-containing neurons. Astrocytes were also stained for identification and differentiation from neurons. Analysis of staining in the one day old cultures was impaired by the thickness of the tissue and a nonspecific reaction of the secondary antibody with the chicken plasma clot used to embed the cultures. In the older cultures, slices that were used as immunocytochemical controls were often lost in the wash steps, therefore any statements regarding 'staining' in the cultures are estimates rather than direct comparisons. The pictures of the 'stained' cultures were included in the thesis in order to examine the morphological changes in the cultures over time.

At the first day *in vitro* (DIV1), the cultures retained the shape and relative thickness (400 μm) of the original slice. There appeared to be a number of cells stained for somatostatin situated around the third ventricle (Fig. 2A). By DIV7, the cultures had flattened into a thin layer of cells and spread out into the plasma clot, to occupy a larger area. At DIV7, what appeared to be ependymal cells derived from the third ventricle, had aggregated to form a centralized zone of cells (Fig. 2B). On either side of the ependymal cells, a layer of dispersed cells had spread out to occupy the space between the plasma clot and the thicker portions of tissue (Fig. 2B). The DIV7 cultures also contained a migratory zone of cells that appeared to have moved from the initial culture into the plasma clot (Fig. 2B). At a higher magnification (10X) the dispersed cells appeared to be neurons stained for

somatostatin (Fig. 3A) and a different culture stained with the astrocyte marker, glial fibrillary acidic protein, (Fig. 3B) showed that the migratory zone may have contained astrocytes.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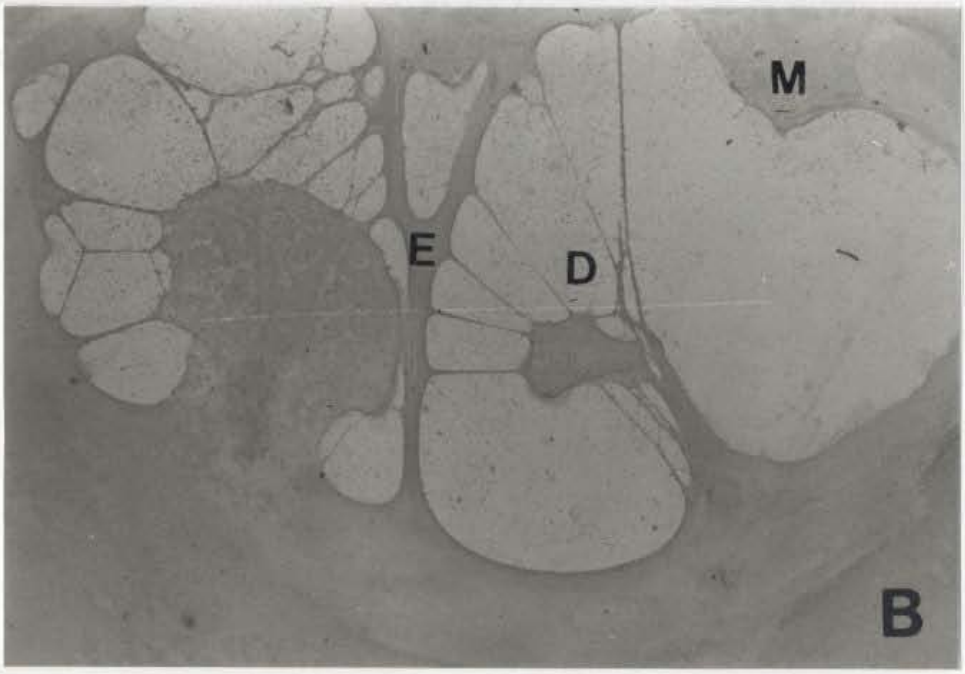
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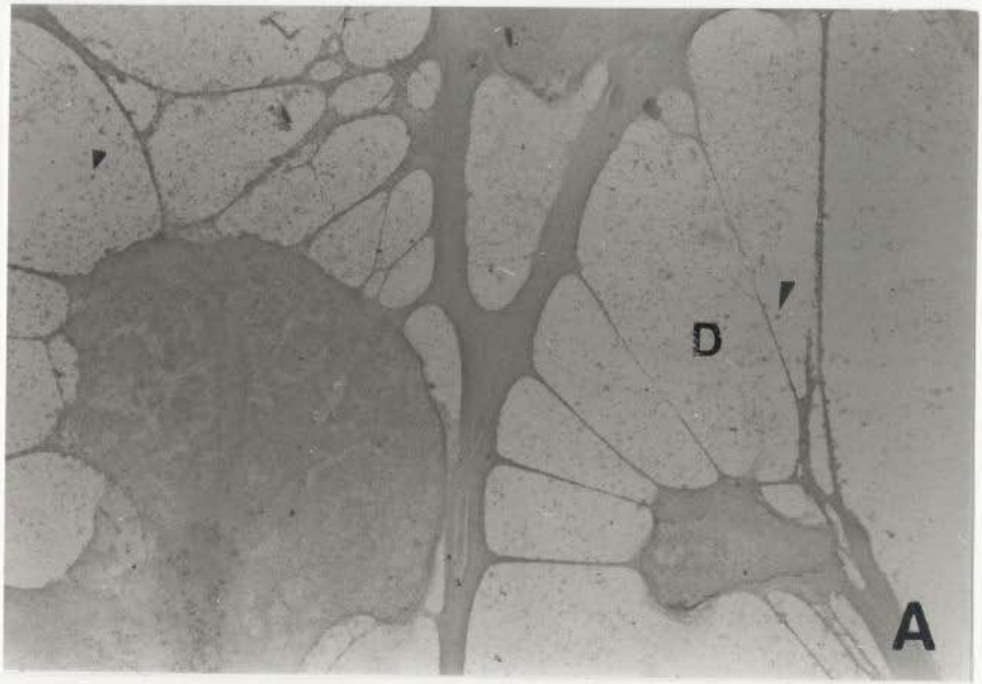
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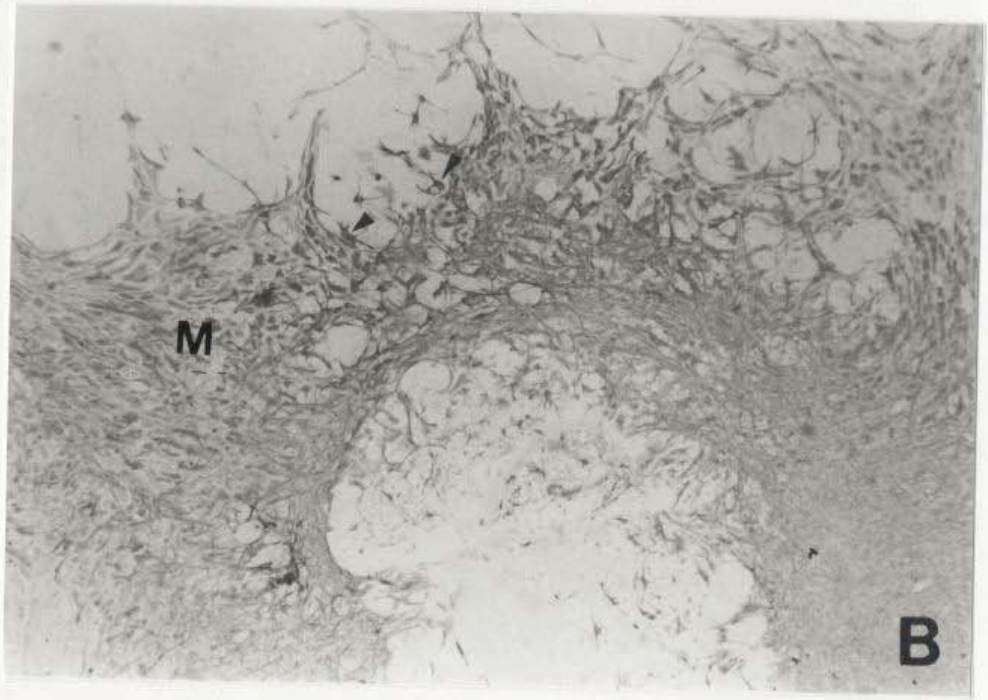
M
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2. SOMATOSTATIN RADIOIMMUNOASSAY

2.1. Dilution Titration

The variation of the primary antibody dilution and the tracer amount yielded an optimal standard curve at an antibody concentration of 1:30, 000 and a tracer amount of 3000 CPM (initial dilutions), when the secondary antibody dilution was held constant at 1:16 (Fig. 4). The most favorable primary-secondary antibody interaction was achieved at concentrations of 1:30 000 and 1:16, respectively (Fig. 5).

2.2. Parallelism

The radioimmunoassay of the serial dilutions of culture-incubated releasing medium, showed relative parallelism to the cyclic peptide standard dilutions used in the standard curve (Fig. 6).

2.3. Sensitivity, Percent Recovery and Binding Capabilities

The detection limit or sensitivity of the radioimmunoassay, defined as the precision of the zero standard by Ekins and Newman (1970), was 2.1 ± 0.2 pg/tube (SEM). The mean recovery of exogenous somatostatin was 91.9 ± 12.1 %. The average amount of nonspecific binding was 5 ± 0.003 % of total counts. The maximal binding of tracer to the primary antibody in the absence of unlabelled peptide was between 15 - 25 % of total counts. The somatostatin antibody is therefore able to functionally measure up to 25% of the iodinated somatostatin that it encounters in the samples and other standard radioimmunoassay tubes.

Dilution Titration of the Primary Antibody and the Tracer

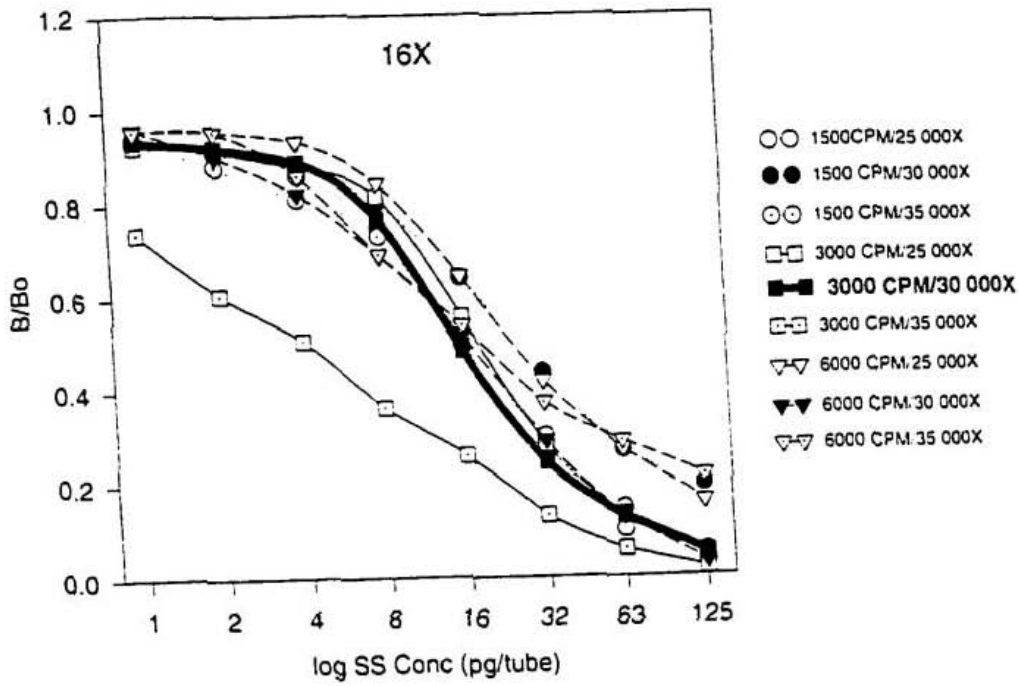


Fig. 4 Dilution titration of the primary antibody and tracer in the somatostatin radioimmunoassay. The Y-axis represents the fraction of bound tracer in each standard tube (B) compared to the maximal tracer binding to the primary antibody (Bo) measured in the Reference (REF) tubes. The X-axis represents the log concentration of each of the 8 standard tubes (serial dilutions from 125 - 0.977 pg/tube). The curves were constructed by holding the dilution of the secondary antibody constant at 16X and titrating the primary antibody dilutions and tracer amounts. The most favorable dilution curve (black squares, thick solid line) occurred at a tracer amount of 3000 CPM and the primary antibody dilution of 30 000X. This curve was the best because it encompassed a large range in the Y-axis and the slope of the curve was steep allowing for greater assay sensitivity.

Dilution Titration of the Primary and the Secondary Antibodies

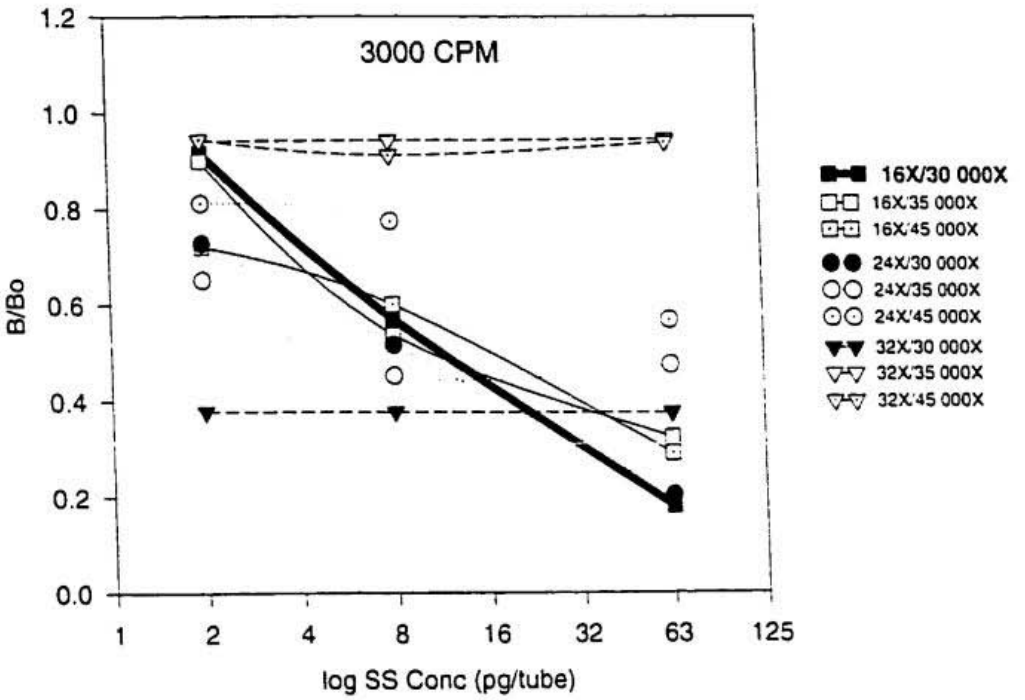


Fig. 5 Dilution titration of the primary and the secondary antibodies in the somatostatin radioimmunoassay. The axis and experimental design are similar to Fig. 6 except that the tracer is held at 3000 CPM and the primary and secondary antibody dilutions are titrated. Only the three standard concentrations (2, 8 and 63 pg/tube) that comprised the slope of the standard dilution curve were used to determine the most favorable curve. The most favorable primary and secondary antibody interaction was achieved at the dilutions of 30 000X and 16X respectively (black squares, thick solid line). This curve displayed the steepest gradient which translated into a sensitive assay with the ability to differentiate between closely set concentrations.

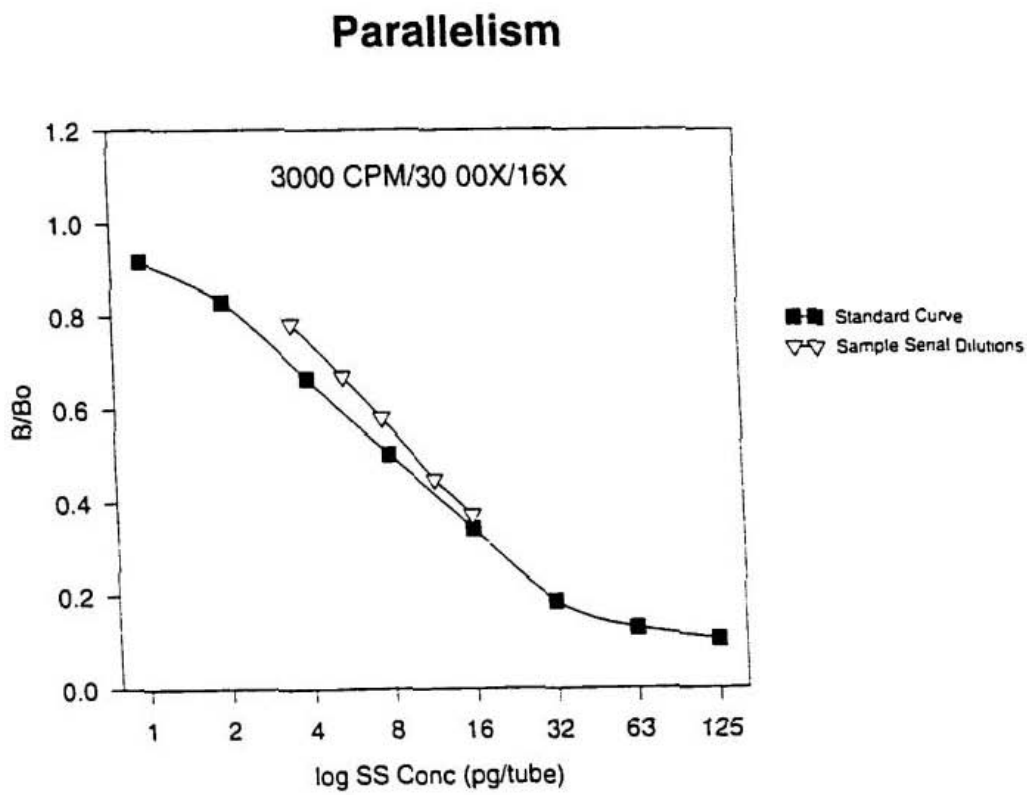


Fig. 6 Comparison of the serial dilution curve of the commercial peptide used in the somatostatin radioimmunoassay to that of samples collected from the cultures. The optimal tracer amount, primary dilution and secondary dilution was 3000 CPM, 30 000X and 16X, respectively. Parallel serial dilution curves existed between the commercial somatostatin peptide (black squares) and the samples containing secreted peptide from the cultures (white triangles). The observed parallelism demonstrated that both peptides were the same isoform and were specific to the primary antibody used in the radioimmunoassay.

3. Somatostatin Release Experiments

Although three different concentrations of IL-1ß were used, there was no dose response observed in this hypothalamic slice culture system. Only the cultures derived from the most rostral slices and incubated with the 1 x 10^{-9} IL-1ß and 1 x 10^{-11} IL-1ß for 12 hrs (135.24 ± 29.66 % and 45.18 ± 11.99 %, respectively) were significantly different ($p < 0.05$) in the Kruskal-Wallis one way analysis of variance followed by the Wilcoxon Signed Rank test pairwise comparison statistical procedure. This difference only occurred when one set of cultures showed a nonsignificant increase above the amounts of basal SS secretions.

3.1. 2 hr Incubations

There was no difference observed in the amount of SS released from the cultures, between two successive basal collections (Fig. 7A). The 2-hr incubation 1 x 10^{-9} M IL-1ß decreased the amount of SS secreted into the medium to 49.18 ± 8.12 % of basal levels (mean ± SE) in the fourth, most caudal slice only (Fig. 7B). None of the slices incubated with 1 x 10^{-10} M IL-1ß exhibited a significant alteration from basal secretion levels (Fig. 7C). When exposed to 1 x 10^{-11} M IL-1ß for 2 hours only the most rostrally-derived cultures exhibited a significant decrease in released SS to 58.67 ± 7.73 % (mean ± SE) of basal concentrations (Fig. 7D).

3.2. 12 hr Incubations

SS levels remained unchanged among the 12 hr controls (Fig. 8A). The SS amounts were significantly decreased to 68.37 ± 10.68 % and 50.10 ± 4.72 % of basal levels (mean ± SE) when cultures were incubated for 12 hr with 1 x 10^{-9} M IL-1ß (Fig. 8B) and 1 x 10^{-10}

M IL-1ß (Fig. 8C), respectively. The 12 hr incubation with 1 x 10^{-11} M IL-1ß induced a significant inhibition in SS secretion to 45.18 ± 11.99 % of basal concentration (mean ± SE) in the first slice only (Fig. 8D).

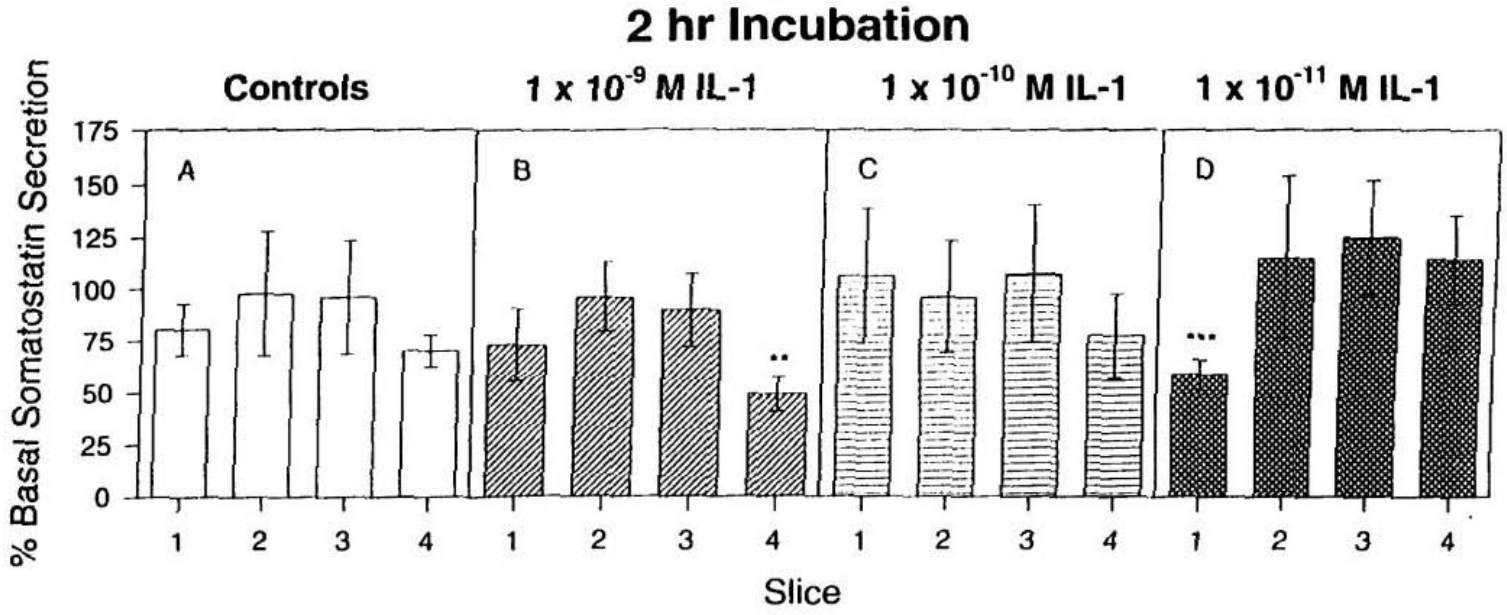


Fig. 7. The effect of 2 hr incubations with three different IL-1ß concentrations on the amount of *somatostatin secreted from* hypothalamic slices cultures. The release *experiments were conducted* after the cultures were grown for 4 days *in vitro*. There were two successive collections taken from the cultures. The first collection served as a measurement of basal somatostatin secretion and the second collection *was either* another basal collection for control purposes or *releasing medium containing* the various concentrations of IL-1ß. The y-axis represents *the experimental* second collection expressed as a percentage of basal levels of somatostatin secreted into the media. The x-axis represents the brain slice number from which the cultures were derived . The slices are *numbered in a rostral - caudal order*. In A) there is no significant difference *between the first* and second control collections (n = 6 rats) suggesting that the experimental procedure itself does not affect somatostatin release. In B) only the most caudal-derived slice cultures (n = 8 rats) exhibited a *significant decrease in* somatostatin released when exposed to *1 x 10⁻⁹ M IL-1ß for 2 hr*. In C) the IL-1ß dose of 1 x 10^{-10} M (n = 9 rats) did not significantly alter *somatostatin* secretion compared to basal levels. In D) the 2 hr incubation with 1 x 10^{-11} M IL-1ß induced a significant inhibition of somatostatin release in the most rostral slice (n = 9). Statistical differences *are shown by asterisks:* *, $p < 0.05$; **, $p < 0.02$; ***, $p < 0.01$ (Wilcoxon *Paired Signed Rank* Test).

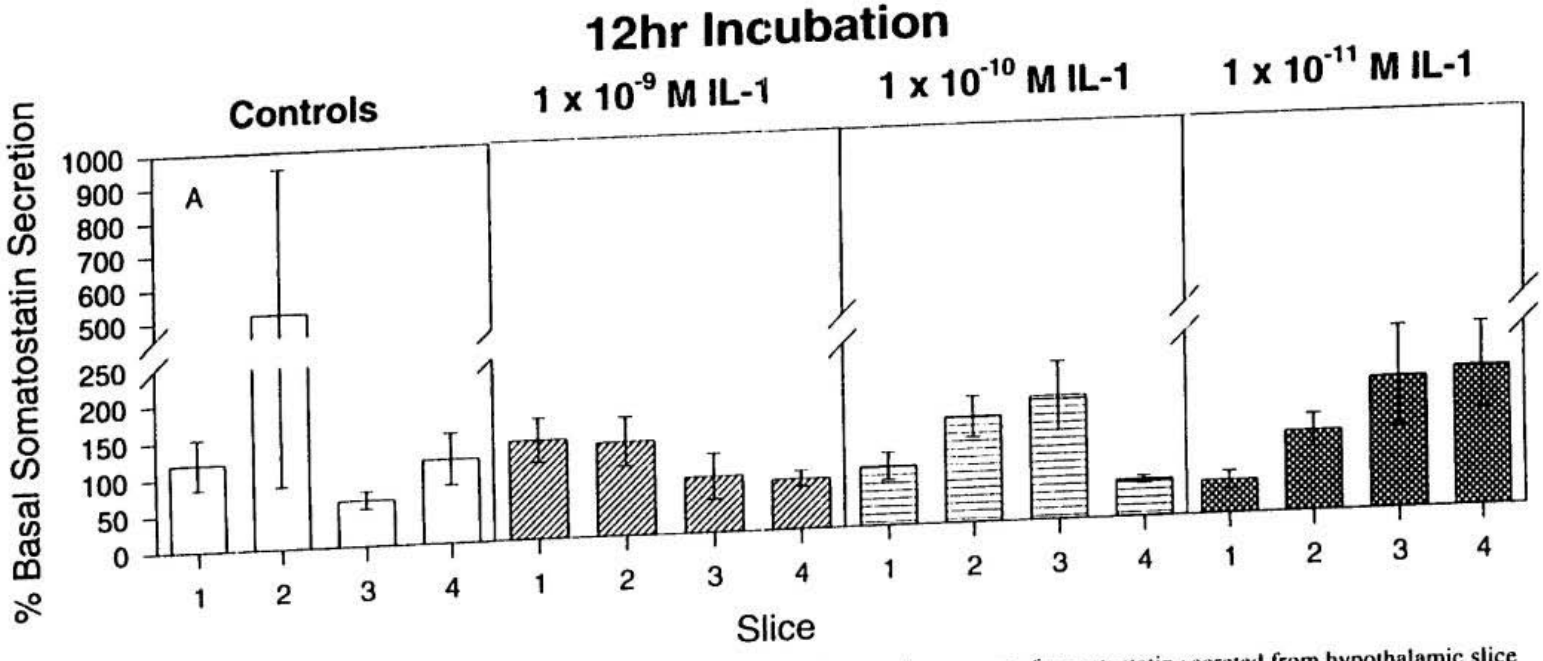


Fig. 8 The effect of 12 hr incubations with three different IL-1ß concentrations on the amount of somatostatin secreted from hypothalamic slice cultures. The release experiments were conducted after the cultures were grown for 4 days *in vitro*. The y-axis represents experimental values expressed as a percentage of basal somatostatin secretion. The x-axis represents the brain slice number (rostral to caudal) from which the cultures were derived. In A) none of the control slice cultures (n = 6 rats) showed any significant difference from the first basal collection although slice 2 exhibited marked variability due to two cultures with very high amounts of SS release. In B) only cultures derived from slice 4 (n = 9 rats) showed a significant decrease in SS secretion when exposed to 1 x 10^{-9} M IL-1ß-for 12 hr. In C) the fourth slice (n = 9 rats) also showed a significant IL-1ß induced inhibition of SS secretion in response to 1 x 10^{-10} M IL-1ß. In D) it was the cultures harvested from slice 1 (n = 9 rats) that showed any significant changes from measured basal SS levels when exposed to 1 x 10^{-11} M IL-1ß. Statistical differences are shown by asterisks: *, $p < 0.05$; **, $p < 0.02$; ***, $p < 0.01$ (Wilcoxon Paired Signed Rank Test).

3.3. 24 hr Incubations

There was no difference in SS release between control cultures incubated with medium alone for 24 hr (Fig. 9A). SS release was not affected by 24 hr incubations with any of the IL-1ß concentrations tested (Fig, 9B, 9C and 9D).

3.4. 12 hr Indomethacin and IL-1ß Incubations

The 12 hr incubation with 10 µg/ml indomethacin affected the basal release of SS from slice 1, the most rostrally-derived slice, as there was a significant decrease to 47.43 ± 15.51 % (mean ± SE) of basal measurements (Fig. 10A) in these culture slices. Fig. 10B demonstrated that 1 x 10^{-9} M IL-1ß combined with 10 µg/ml indomethacin did not significantly affect the amount of SS secreted from slice 1 or slice 4. One way analysis revealed that there was no significant difference in the amount of SS released in response to indomethacin alone and indomethacin plus IL-1ß. When analysis of variance and pairwise comparisons were made to the 12 hr 1 x 10^{-9} M IL-1ß data from the time course, there were no significant differences between IL-1ß alone, indomethacin alone and IL-1ß plus indomethacin incubations with one exception. The exception occurred when there was a trend that the IL-1ß incubation was higher than basal levels in slice 1 (135.24 ± 29.66 % of basal secretions, mean ± SE) and the indomethacin incubation was significantly lower than basal values (47.43 ± 15.51 % of basal secretions , mean ± SE).

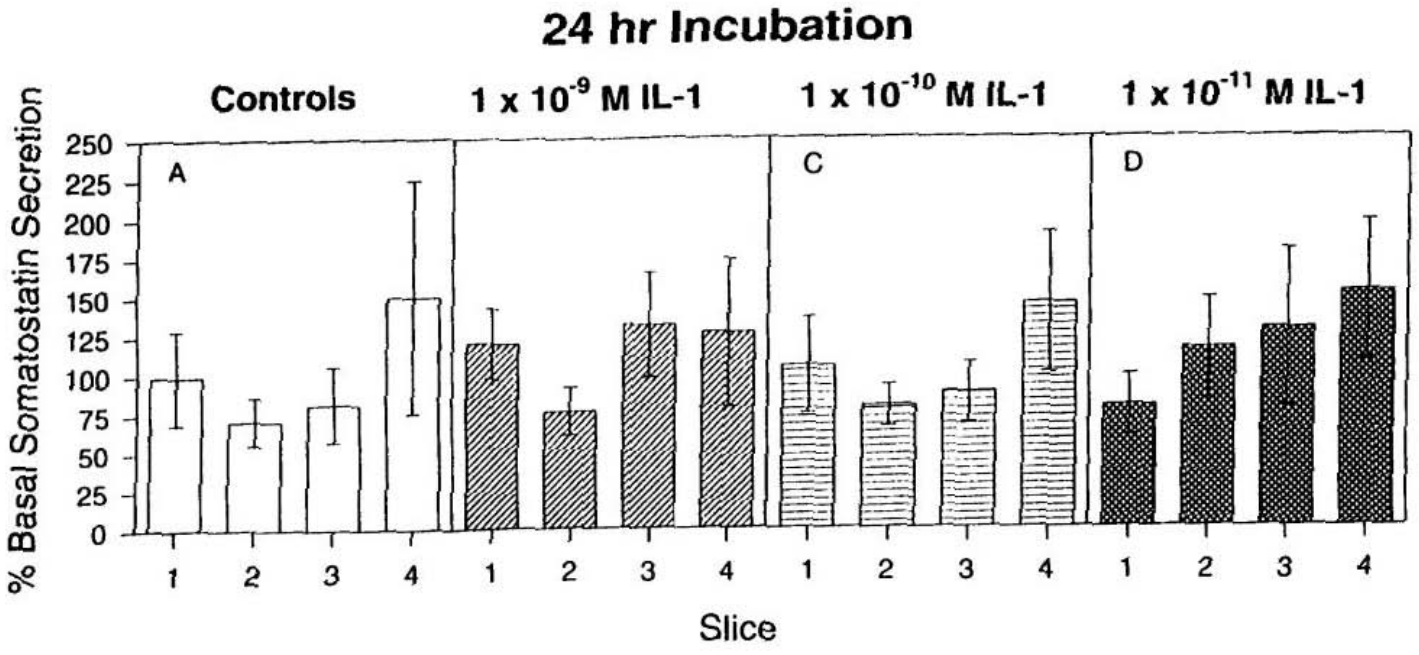


Fig. 9 The effect of 24 hr incubations with three different *IL-1ß concentrations on* the amount of somatostatin secreted from hypothalamic slice *cultures. The release* experiments were conducted after the cultures were grown for 4 days *in vitro*. The y-axis represents experimental values expressed as a percentage of basal somatostatin secretion and the x-axis represents *the rostral-caudal brain slice* number from which the cultures were derived. *In A) exposure of* the cultures (n = 6 rats) to two successive incubation of media alone for 24 hr total did not cause any significant change in the amount of SS release. In B) the 24 hr incubation with 1 x 10^{-9} M IL-1ß did not *significantly affect the* secretion of SS from any of the cultures. In C) the exposure to *1 x 10^{-10} M IL-1ß did not* effect SS secretion. In D) no significant difference *in SS secretion was* measured in response to the 24 hr incubation with 1 x 10^{-11} M IL-1ß either. Slices from 9 rats were used in each group of cultures exposed to IL-1ß. Statistical differences are shown by asterisks: *, $p < 0.05$; **, $p < 0.02$; ***, $p < 0.01$ (Wilcoxon Paired Signed Rank Test).

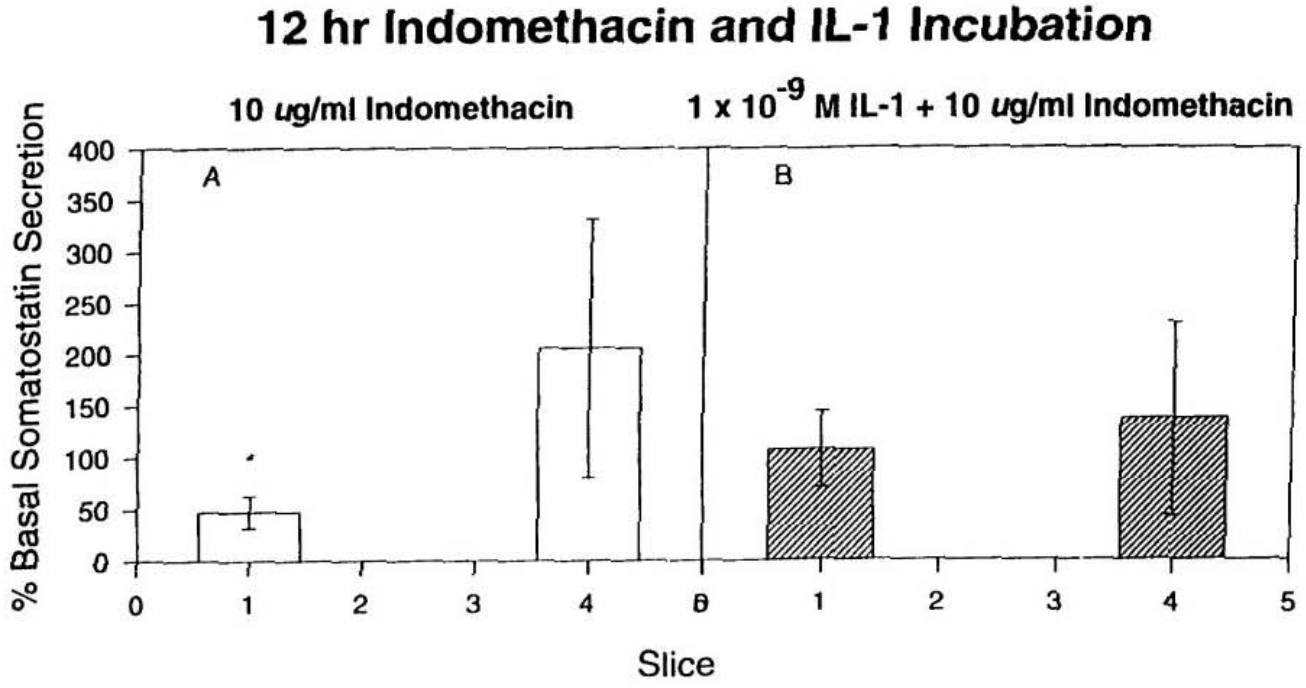


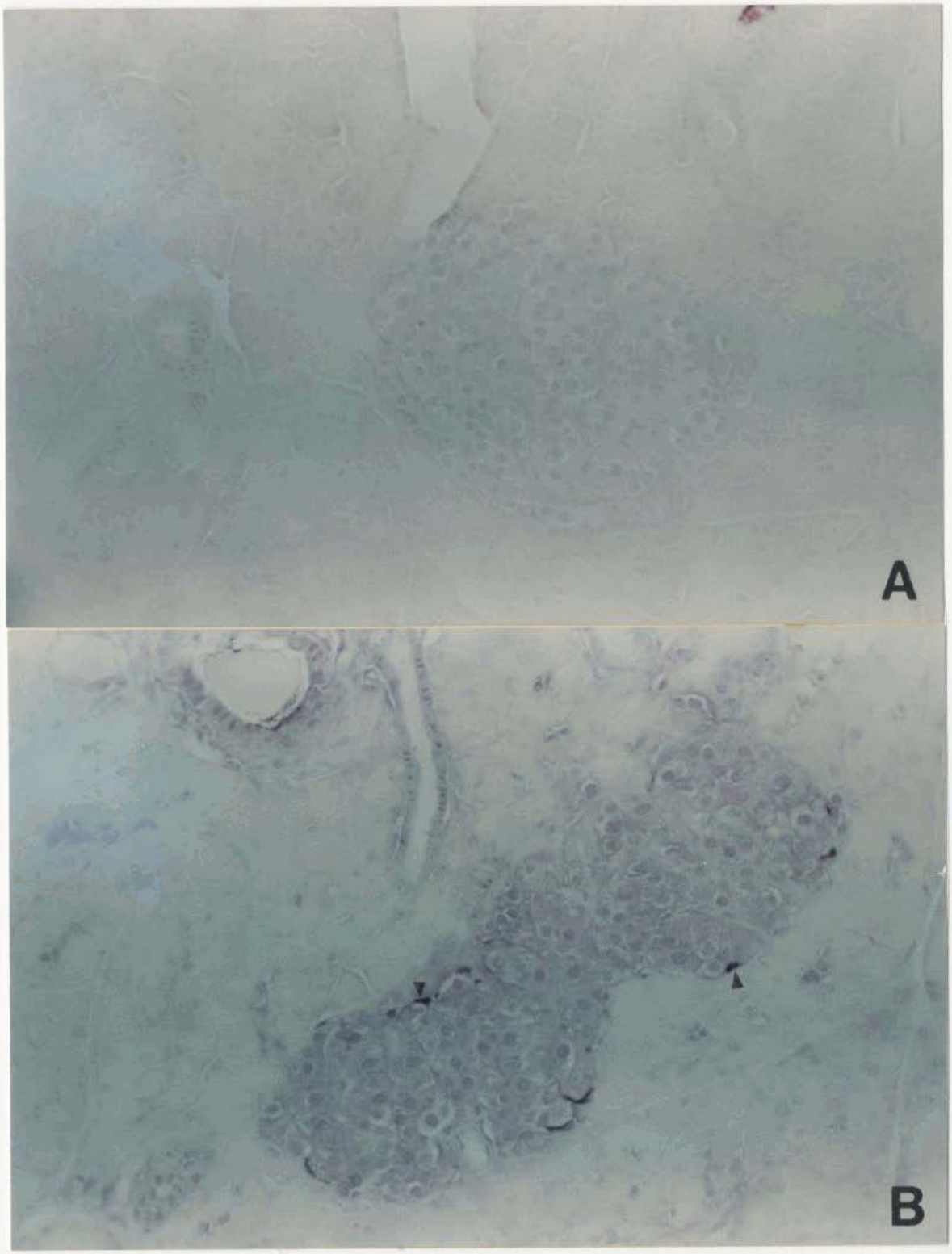
Fig. 10 The effect of 12 hr incubations with indomethacin alone and IL-1β combined with indomethacin, on the amount of somatostatin secreted from hypothalamic slice cultures. The release experiments were conducted after the cultures were grown for 4 days *in vitro*. In A) the 12 hr incubation with the prostaglandin inhibitor, indomethacin (n = 7 rats) was able to inhibit the amount of somatostatin released from the first slice only. In B) indomethacin plus 1 x 10^{-9} M IL-1β did not alter the amounts of somatostatin secreted from cultures derived from either the first of the fourth slices (n = 7 rats). One way analysis of variance showed that the inhibition of somatostatin secretion by indomethacin was statistically indistinguishable from the inhibition of the peptide by indomethacin plus IL-1β. Statistical differences are shown by asterisks: *, $p < 0.05$; **, $p < 0.02$; ***, $p < 0.01$ (Wilcoxon Paired Signed Rank Test).

4. SOMATOSTATIN IMMUNOCYTOCHEMISTRY

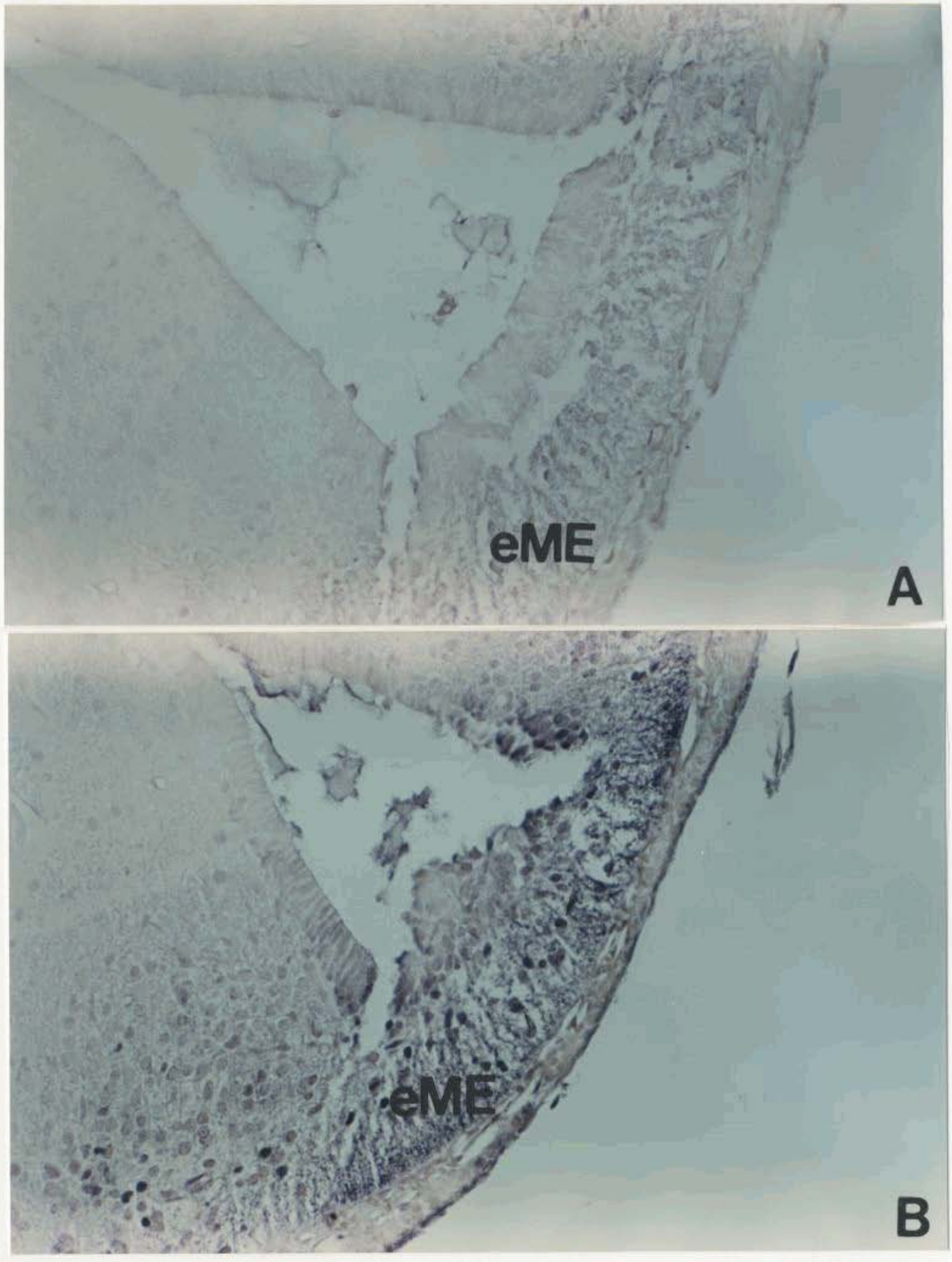
The location of SS peptide storage in the hypothalamus was verified by the immunocytochemical staining of SS-containing neuronal fibres in neonatal tissue. Formalin-fixation and paraffin embedding provided good preservation of the tissue and SS antigen sites in neonatal brains and adult pancreas. A titration of primary antibody concentrations revealed that the 1:800 and 1:400 dilutions produced the best staining in the adult pancrease and neonatal rat brain, repectively. A dark blue stain attributable to the Nickel chloride addition in the DAB reaction, identified SS immunoreactivity. Control slices were incubated with normal rabbit serum in order to determine whether a reaction caused by the nonspecific binding of the secondary antibody to the tissue has occurred. Preincubation of the SS antibody with the SS peptide as a control measure was not attempted due to time and economic restraints. Staining was considered specific to SS-containing cells if no corresponding color appeared in the reagent control. SS immunoreactive staining was achieved in this study, coinciding to results described by Bennett-Clarke et al. (1980), without administering the axonal transport blocker colchicine to any of the animals.

Immunocytochemistry was conducted on pancreas slices first, because the SS antibody (DAKO) was characterized in this tissue by the manufacturer and the pancreas could, thus, serve as a positive control. The slices were incubated with the SS antibody or the serum control for 48 hr at 4 ° C. Dark blue colored SSir cells were distributed around the outer edges of the islets of Langerhans which comprise the endocrine pancreas (Fig. 11B). There was no corresponding staining observed in the negative control (Fig. 11A).

Pancreas tissue was included as the positive control in each run of brain tissue tested. Staining in brain tissue was judged specific only if there was staining in the pancreas positive control and no staining in the normal rabbit serum reagent control.


A
B

To achieve staining in the neonatal brain, the primary antibody concentration was increased from 1:800 to 1:400 and the incubation time was extended to 72 hr at 4 ° C. The protease pretreatment changes detailed in the methods section were enough to produce consistent SS staining of the ME in neonatal rats. In this study staining was limited to the neonatal ME as no SSir was detected in cell bodies (possibly due to the insensitivity of the SS antibody. The varicosities of SS-containing fibres stained intensely in the external zone of the ME (Fig. 12B). No corresponding staining was observed in the negative control (Fig. 12A).


eME
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IV DISCUSSION

1. Slice Explant Cultures

Slice explants of tissue derived from the hypothalami of 3-day-old rats, were grown in culture. The age of the donor rat appears to be important as Gahwiler and Knoppel (1990) have reported that the survival of neurons was usually superior with tissue derived from young postnatal or even fetal animals but the requirement for organotypic tissue organization would favour the use of older animals. Slices taken from 3-day-old rats provided a good compromise for the preparation of hypothalamic cultures as most of the cytoarchitecture is already present, yet the cells are still capable of some differentiation, maturation and migration which may help the neurons survive (Gahwiler, 1988). Peisen et al. (1995) and Honegger et al. (1991) derived their acute slice explant systems from juvenile and adult male rat brain tissues, respectively. Hypothalami were dissected out, halved and incubated in polyethylene vials containing medium, in an atmosphere of 95% O_2. The incubation tubes were kept in a shaking water bath at 37° C. The neurons and other cells in these hypothalamic fragments taken from older animals are probably near the end stages of differentiation and migration (Gahwiler and Knopel, 1990). The fragments would therefore have lost most of the survival ability that was present in the cells derived from the younger tissue used in this study. The dissections inherent to the hypothalamic fragment method used by Peisen and Honegger cause a complete loss of afferent innervation to the hypothalamus. Although factors that may be released by remote afferent fibres are also absent in our slice culture system (due to similar slicing procedures), the neurons are embedded in a

microenvironment that, as far as cell types are concerned, seems closely to resemble the natural one (Gahwiler et al., 1991). In contrast, the hypothalamic fragments are suspended in medium, therefore necrosis would begin at the tissue edges in contact with an "unfamiliar " environment. Coupled with the problem of oxygen penetration to the fragment's inner layers, the brain tissue used in the acute explant methods is progressively dying from the moment of dissection. Peisen et al. and Honegger et al. were therefore unable to examine incubation times longer than a couple of hours (2.5 and 2 hours, respectively). Our slice cultures, in comparison, continued to survive and metabolize the culture medium well beyond seven days. Our hypothalamic culture system is therefore not limited to 2 hour incubation times, and can be used to study the effects of various test substances during a longer time frame.

The dissociated culture system used by Scarborough et al. (1989) could also sustain longer incubation times than acute explant systems. The primary monolayer cultures were prepared by mechanoenzymatic dissociation of fetal day 17 rat diencephalon (Scarborough et al., 1989). Scarborough's method could sustain incubations from 2 to 5 days, but they could observe no effect between 0 and 48 hours. The dissociation involved in this technique would interrupt the synaptic contacts, axonal and dendritic position, and the relative arrangement of the neurons in relation to one another. The fetal age of the donor animals allows for the reestablished of these contacts, possibly over the 48 hours where no effect was observed. The slice culture system used in this study is suitable to examine incubation periods between the 2 and 24 hours that the acute explant and dissociative culture models

cannot observe. A defining feature of slice cultures prepared with the roller-tube technique is that they flatten during the first week *in vitro* to form a thin layer of cells, yet they retain much of the organization one observes in the same tissue *in vivo* at the cellular level. Anatomically, this organization is expressed in the cytoarchitecture of cell populations, the morphology of single cells, the patterns of connections formed and the transmitter phenotypes (Gahwiler et al., 1991). Gahwiler also noted that physiologically, the organotypic organization is manifested in the neuron's repertoire of voltage-dependent ionic conductances, neurotransmitter receptors, and second-messenger systems.

The thinning of the cultures may be the result of several mechanisms including the death of injured cells at the cut surfaces of the slice, spreading due to the mechanical rotation of the tubes in the roller drum and the gradual migration of cells. The cultures are grown in 25% serum-containing medium (SCM) for three days, then switched to serum free medium (SFM). Tominanga et al. (1994) and Wray et al. (1993) used the same technique in order to examine the suprachiasmatic nucleus and observed that culturing in SCM promoted the uniform spreading and thinning of the cultures and improved cell survival. Tominanga et al. (1994) provided a detailed description of glial cells in the migratory zone of the cultures which agreed with the observation made in this study that astrocytes may migrate into the chicken plasma clot and represent the majority of cells in the migratory zone. Clarke and Gillies (1988) and Davidson and Gillies (1993) using dissociative fetal models, showed that cultures grown entirely in SCM exhibit less KCl-induced somatostatin release than cultures grown in SFM. Davidson and Gillies (1993) speculated that this effect could be due to the

influence of factors in the serum exerted directly on the neurons and that it is important to remember that although serum promotes the growth and survival of many cells *in vitro*, it is an undefined and unphysiological cocktail containing many biologically active substances which would not normally come into contact with brain cells. Davidson and Gillies also noted that the suppression of neuronal SS release in SCM could be due to an influence of the glial cells on neuronal responses, as these cells undergo marked proliferation in the presence of serum. As a compromise, this study followed the methods of Bertini et al. (1993) and Wray et al. (1991) and grew the cultures first in SCM and then switched to SFM to remove the unknown factors in the serum in hopes of achieving a more 'physiological' release of peptides.

2. Somatostatin Radioimmunoassay

The primary antibody, secondary antibody and tracer concentrations were similar to the values used by Penman et al. (1979). Although the volumes used in this assay were half that of Penman et al. (1979), that did not appear to affect the standard curve parameters.

The parallelism between serial dilutions of samples from the culture and the standard curve suggested that the peptide released from the cultures was immunologically indistinguishable from the standard somatostatin tetradecapeptide.

The detection limit, percent recovery and nonspecific binding amounts are comparable with the values of somatostatin assays quoted in the literature (Epelbaum et al., 1977; Patel et al., 1980; Arimura et al., 1975; Kronheim et al., 1976; Penman et al., 1979).

The maximal binding of 15-25% was lower then that of Epelbaum et al. (1977), who reported 40-60% maximal binding. The lower maximal binding may be a reflection of the affinity of the specific batch or lot of primary antibody used in the assay. Differences in ionic interaction in the smaller tubes (6 x 38 mm) used in this assay may also have affected maximal binding more than the other assay parameters.

Penman et al. (1979) reported that the primary antibody (R10) did not cross react with any of the substances tested, indicating that it binds specifically to somatostatin. Cross-reaction studies with SS analogs revealed that the SS antibody would still bind to analogs missing key amino acid components in the N- and C-terminals suggesting that the antibody binds to a site near the central part of the molecule (Penman et al., 1979).

3. Somatostatin Release Experiments

Although a dose response trend was evident in slice 4 cultures, no significant dose response relationship was observed in any of the cultures. The concentrations used in this study (1×10^{-11} M, 1×10^{-10} M) are close to physiological IL-1ß concentrations in blood (Mandrup-Poulsen et al., 1995) whereas the 1×10^{-9} M IL-1ß concentration is reached during bacterial meningitis (Mustafa et al, 1989). The slice 1 cultures showed no evidence that increasing doses of IL-1ß could produce a greater inhibition in SS release. Only the lowest IL-1ß concentration caused a significant decrease in SS secreted from slice 1 during either the 2 or 12 hr incubations (Fig. 7D and Fig. 8D). Cultures derived from slices 2 and 3 did not respond to any of the IL-1ß concentrations used in this study. We did observe a trend

toward a dose response relationship in slice 4 cultures, however. During the 2 hr incubation, only the highest dose of IL-1ß produced a significant inhibition of SS secreted from slice 4 (Fig 7A). With the 12 hour incubation, the two highest doses induced a significant decrease in SS released from slice 4, but the lowest concentration did not (Fig. 8).

The variability between the different slices is the most striking aspect of the culture method used in this study. The different slices appear to contain distinct subpopulatons of SS neurons with their own neurochemical sensitivities. Slice 1 neurons are likely to contain the preoptic area whereas slice 4 neurons are most likely derived from the paraventrilcular (PVN) and periventricular (PeVN) nucleic (Sherwood and Timiras, 1970). Honegger et al. (1991) also observed different responses to IL-1ß depending on the anatomical boundaries of their hypothalamic explant fragments. SS secreted by mediobasal hypothalamic (MBH) explants (defined by the authors as hypothalamus fragments rostral to and excluding the paraventricular and periventricular nucleus), was not altered by any of the IL-1ß doses used in that study. In our study, slice 1 cultures derived from similar preoptic area boundaries as the MBH fragments, were inhibited by IL-1ß, but in an unphysiological manner. If Honegger et al. could have extended their 30 min incubation to 2 or 12 hr, they may have observed an unphysiological response to IL-1ß as well. SS neurons are scattered throughout the hypothalamus with neuronal groups aggregating in the preoptic and anterior hypothalamus (Honegger et al., 1991). The cultures derived from the first three slices in this study and the MBH explants used by Honegger et al. (1991) all secreted measurable basal amounts of SS. This suggests that although the subpopulation of SS neurons in the preoptic area and the

scattered SS neurons throughout the hypothalamus are functional, these neurons may not contribute to the hypothalamic SS response to IL-1ß. In contrast, slice 4 cultures and Honegger's hypothalamic fragments that contained the PVN and PeVN, were sensitive to IL-1ß modulation. Merchentaler et al. (1989) and Daikoku (1988) showed that SS neurons from these areas innervate the ME. Their data implies that PVN and PeVN SS neurons are probably involved in releasing SS into the portal system to regulate growth hormone secretion from the anterior pituitary. Collectively, these results indicate that IL-1ß may alter pituitary growth hormone secretion by modulating the amount of SS secreted from neurons distributed in the PVN and PeVN.

Although there was a trend toward a dose response relationship in slice 4 cultures, this effect was not significant. The lack of a significant dose response may be attributed to an inherent variability in the amount of inducible SS secreted from different cultures. SS secreted from slice 4 cultures in response to the 12 hr exposure of 1×10^{-9}M and 1×10^{-10} M IL-1ß fluctuated from 58 to 79% and 45 to 55%, respectively. One source of variability that may alter SS secretion is the number of astrocytes present in each culture. Tominga et al. (1994) reported that the approximate number of astrocytes in their organotypic slice cultures varied between one and three thousand cells. (Tominga et al. used the same technique as this study to examine the changes in vasopressin mRNA in the suprachiasmatic nucleus.) The differences in inducible SS released from each culture may therefore be due to the diversity in astrocyte content. Pares-Herbute et al. (1988) observed that the presence of astrocytes altered the amounts of SS secreted from dissociative fetal rat cortical cultures. They

compared the basal and forskolin-induced release of SS from untreated cultures to those treated with cytosine arabinoside (Ara C), a mitotic inhibitor of non-neuronal cells. Ara C decreased the presence of astrocytes (GFAP positive cells) from 30% of total cellular content to 6%. The untreated group of cultures secreted less basal SS than the Ara C-treated group, despite having similar SS cellular content amounts. The authors observed that in the neuron-rich, Ara C-treated preparation, forskolin was not able to stimulate SS release whereas forskolin did induce a dose dependent increase in SS secreted from untreated cultures. Pares-Herbute and colleagues restored forskolin responsiveness to Ara C treated cultures by incubating them with media obtained from separate glial rich cultures. Although the authors did not expand on a possible negative relationship between glial cells and basal SS neuronal function, it still is plausible. Davidson and Gillies (1993) provided indirect evidence that astrocytes may suppress SS release from neurons. Davidson and Gillies, also using a dissociative fetal cortex model, showed that cultures grown in medium containing serum exhibited less KCl-induced somatostatin release than cultures grown in medium without serum. They observed that astrocytes undergo marked proliferation in the presence of serum. The authors noted that the suppression of neuronal SS release in serum containing medium could be due to an influence of the glial cells on neuronal responses. Together these results suggest that astrocytic substances may interfere or enhance SS neuronal function depending on the general stasis of the cell, or the specific second messenger system envolved in the neuronal activation.

3.1. 2 hour and 12 hour Incubations

None of the controls at 2, 12 or 24 hr showed a significant difference between the first and second collection of basal SS secretion, suggesting that the experimental manipulation of the cultures did not effect SS release

3.1.1. 1 x 10^{-9} M IL-1ß

The highest dose of human recombinant IL-1ß (1 x 10^{-9} M) caused a significant decrease in the release of SS from slice explant cultures during 2 hr and 12 hr incubations. Significant inhibition was only observed in the fourth slice in both the 2 hr and 12 hr incubations. Slice 4 was likely to contain the PVN and the rostral periventricular area (Sherwood and Timiras, 1970). These areas have been shown to innervate the ME (Merchenthaler et al., 1989; Kawano and Daikoku, 1988) and may release SS into the portal blood system to regulate GH secretion. This IL-1ß induced inhibition was in contrast to the acute stimulation of SS release reported by Honegger et al. (1991) and Peisen et al. (1995) and the chronic release augmentation observed by Scarborough et al. (1989) and Scarborough, 1990. The difference between the results found in this study and others will be discussed in the 12 hr indomethacin section. Peisen et al. (1995) and Scarborough et al. (1989) also used 1 x 10^{-9} M IL-1ß while Honegger used 100 U/ml as the highest dose. 1 x 10^{-9} M IL-1ß is in the concentration range observed by Mustafa et al. (1989) who measured an average of 0.9 ±1.2 x 10^{-9} M IL-1ß in the cerebral spinal fluid of 106 infants and children with bacterial meningitis.

The rebound in GH secretion shown by Kasting and Martin (1980) after repetitive iv

LPS treatment over a period of three days, is consistent with the IL-1ß induced inhibition in SS secretion observed in this study. Kasting and Martin reported that the increase in GH secretion was a consequence of more frequent secretory surges and shortened trough periods. Trough periods in GH secretion are a function of SS inhibition (Tannenbaum et al., 1991) and the decrease in SS release in response to IL-1ß observed in this study may mediate the shortened GH trough periods observed by Kasting and Martin (1980).

3.1.2. 1 x 10^{-10} M IL-1ß

The response to 1 x 10^{-10} M IL-1ß was more variable than the 1 x 10^{-9} M reaction. The 2-hr incubation with the intermediate dose of cytokine did not affect SS release, and the 12-hr incubation attenuated of release in the fourth slice. The 1 x 10^{-10} M dose of IL-1ß corresponds with levels of the cytokine reached during infection. Dinarello and Thompson (1991) reported that circulating IL-1ß levels, during infections in humans, rarely exceeded 5 x 10^{-10} M and during experimental endotoxemia, IL-1ß levels reach a maximal concentration of 1.5 - 2.0 x 10^{-10} M. The 2 hr, 1 x 10^{-10} M IL-1ß incubation did not produce a significant effect on the cultures in this study. It is possible that any effect of IL-1ß may not have been large enough to overcome the variability in SS release from the cultures or the measurement variability of the radioimmunoassay. The 2-hr incubation with IL-1ß may also release cytokines and other substances that may antagonize the effects of IL-1ß (Scarborough, 1990) and may mask its influence on SS release. The 12 hr incubation of IL-1ß at 1 x 10^{-10} M, attenuation of SS release is in contrast to the expected facilitation of SS secretion reported by Honneger et al. (1991), Peisen et al. (1995) and Scarborough et al.

(1989). The possible mechanism of action by which the 12 hr exposure to the cytokine inhibits SS release will be discussed in section 3.3 entitled: 12 hr indomethacin and IL-1ß incubation.

3.1.3. 1 x 10^{-11} M IL-1ß

The 2 hr and 12 hr incubation with 1 x 10^{-11} M IL-1ß caused an inhibition in the release of SS from the first slice only. These cultures are likely to contain the medial and periventricular preoptic nuclei (Sherwood and Timiras, 1970). The preoptic area has been shown to contain SSir neurons (Finley et al., 1981; Bennett-Clarke et al., 1980) and endotoxin-inducible immunoreactive Fos proteins (Elmquist et al., 1993; Sagar et al., 1995). Ishikawa et al. (1987) have shown that SS neurons in the preoptic area also innervate the ME, although to a lesser extent than the PeVN. Mandrup-Poulsen et al. (1995) have noted that cytokines usually exert their physiological actions at lower molecular concentrations than most hormones (1 x 10^{-12} M - 1 x 10^{-15} M).

Bernardini et al. (1989) observed a similar paradoxical effect when they exposed rat hypothalamic explants to the arachidonic acid metabolite, LTB_4. LTB_4 stimulated CRH release from the explants, but only at the lowest concentration used in the study. Bernardini and colleagues proposed that the CRH neurons were hypersensitive to the lowest LTB_4 concentration because the dose was well below the affinity concentration range of its receptor. They suggested that the hyperresponsiveness was due to denervation of the isolated PVN. The cultures used in this study also arise from denervated tissue. SS neurons in slice 1 cultures may also be hypersensitive to the 1 x 10^{-11} M IL-1ß concentration. Katsuura et al.

(1988) have identified a 1 x 10^{-9} M dissociation constant for rat hypothalamic IL-1ß receptors.

Bernardini et al. also speculated that the stimulation of CRH at such low LTB_4 concentrations may be related to a synergistic effect with other endogenous (tissue) arachidonic acid metabolites. Activation by IL-1ß involves a complex signal cascade system with many synergistic and antagonistic components (Smith et al., 1991). The IL-1ß signal cascade may involve other cytokines (Smith et al., 1991) or arachidonic acid metabolites (Navarra et al., 1990) that contribute to the transduction of the cytokine signal in vivo, but may be absent in vitro. The absence of an intermediary signal component would explain the significant yet unphysiological response of the slice 1 cultures to IL-1ß. These results suggest that preoptic area-derived SS neurons may be responsive to IL-1ß but via a different mechanism than the ME-innervating SS neurons in slice 4.

3.2. 24 hr Incubations

IL-1ß did not affect SS release at any of the concentrations used when incubated for 24 hr. Peisen et al. (1995) speculated that the catchup growth observed after 48 hr may have been caused by a development of tolerance to LPS, and therefore the lack of a 24-hr effect may be caused by a tolerance to IL-1ß. Fresh medium was applied 2 hours before the end of the 24 hour period to conserve the effect of time on peptide release in all three time points. The SS neurons may have become desensitized by the length of the first IL-1ß incubation and may have been insensitive to the subsequent two hour IL-1ß pulse at the end. A second possibility is that IL-1ß or SS may have become unstable during the 24 hour incubation.

Proteolytic degradation of IL-1 ß by enzymes in the media may have resulted in the observed lack of effect during the 24 hour time point. However, the SS peptide was more stable during this time point as no significant difference between the 24 hour control incubations was apparent (Fig. 9A). This suggests that most of the IL-1ß actions on SS are initiated and completed during time periods less than 24 hours. It is also possible that the effects of IL-1ß during periods longer than 24 hr, may be mediated by growth hormone-releasing hormone and not SS. Scarborough et al. (1989) observed a dose-dependent increase in SS mRNA and SS peptide release only after 24 hr and speculated that long term (> 24 hr) IL-1ß exposure was neurotrophic. Taken together with the results of this study, it is possible that the site of acute IL-1ß effects is on SS secretion from granular stores, and the chronic effects of IL-1ß (according to Scarborough) are localized on SS biosynthesis. This leads to the speculation that at 24 hr, IL-1ß is predominantly acting on SS mRNA and peptide stores, and not at the level of releasable SS, hence the lack of effect observed between basal and IL-1ß incubated SS secretion from the cultures.

3.3. 12 hr Indomethacin and IL-1ß Incubations

Indomethacin is a potent inhibitor of the cyclooxygenase enzyme that synthesizes arachidonic acid metabolites such as the prostaglandins and the thromboxanes. Due to this ability, indomethacin has been frequently used in the study of prostaglandin function in the brain. Indomethacin administration has been shown to attenuate many actions of IL-1ß on the central nervous system (Sagar et al., 1995; Navarra et al., 1990). Prostaglandins have consequently been considered an important mediator of IL-1ß function in the brain. Navarra

et al. (1992) have shown that IL-1ß specifically stimulated prostaglandin E_2 (PGE_2) release from hypothalamic explants. Since prostaglandin binding sites are predominantly distributed in the hypothalamus (Dray et al., 1989), prostaglandins probably hold an intermediary role in the brain area. The slice cultures were therefore exposed to indomethacin to examine the role of prostaglandins in the IL-1ß/SS interaction. Since cultures derived from slices 2 and 3 did not respond to IL-1ß, they were not included in the indomethacin experiments. 10 µg/ml indomethacin was incubated with slice 1 and slice 4 cultures for 12 hr to examine its effect on basal SS release. The divergent response patterns between the two cultures continued as basal SS secretion was decreased in slice 1 cultures but not in their slice 4 counterparts (Fig. 10A). The significant inhibition of SS release by indomethacin suggests that prostaglandins (PGs) may be necessary to maintain basal SS release from SS neurons in the preoptic area. Navarra et al. (1992) have observed that the calcium ionophore, A 23187 and 28 mM KCl stimulated the release of PGE_2 from hypothalamic explants. The authors speculated that the calcium-mediated and potassium-stimulated release of PGs, implies that prostaglandins may play a role in neuromodulation. If PGs are involved in the basal secretion of SS, then its removal by indomethacin may have interfered with spontaneous SS release from slice 1 cultures. The 12 hr exposure to indomethacin did not alter the amount of basal SS released from slice 4 cultures. This suggests that basal SS release from SS neurons in these slices are less dependent on PG synthesis than slice 1 neurons. Basal SS release from slice 4 cultures could also be independent of PG synthesis entirely, under the conditions encountered in this study.

The coincubation of indomethacin (10 µg/ml) with IL-1ß (1×10^{-9} M) did not significantly alter SS release compared with basal levels in slice 1 cultures. Although this IL-1ß dose did not affect SS release by itself, it did appear to negate the effects of indomethacin on basal SS release. This suggests that the 1×10^{-9} M IL-1ß concentration may initiate other mechanisms besides PGs that also sustain basal SS in this subpopulation of neurons. What these other mechanisms could be and the specific role of the IL-1ß/SS interaction in the preoptic area, must be defined by further research.

The concurrent incubation with indomethacin reversed the 1×10^{-9} M IL-1ß suppression of SS release in slice 4 cultures. Prostaglandins may therefore be an important component of the inhibitory IL-1ß cascade. However, Honegger et al. (1991) reported that IL-1ß stimulated SS secretion through a dose dependent mechanism, during 30 min incubations. Honegger et al. (using the same 10 µg /ml concentration as this study) showed that indomethacin abolished the SS stimulation in response to IL-1ß. Taken together these two studies show that indomethacin, presumably through the inhibition of prostaglandin synthesis, can paradoxically both promote and decrease the secretion of SS. It is possible that the IL-1ß-induced stimulation of SS secretion may in fact be involved in the eventual inhibition of SS release. We propose that the SS released during the 12 hr (and 2 hr) exposure to IL-1ß is sufficient to activate autoreceptors on the cell surface. The activation of these autoreceptors would inhibit subsequent SS secretion. Therefore SS may be involved in a negative feedback loop to control its own secretion at the hypothalamic level (Richardson and Twente, 1986). We speculate that the effect of decreased SS on the GH axis

is a secondary consequence of this central feedback loop.

IL-1ß 's liberation of prostaglandins (PGs) from astrocyte stores would be the initiating step in this central feedback loop. Hartung et al. (1989) observed that recombinant IL-1ß evoked the production of the eicosanoids, thromboxane B2 and PGE from rat astrocyte cultures. Hartung proved that the stimulatory effect was specific since it could be eliminated by the heat-inactivation of the molecule or by its neutralization with an anti-IL-1 antibody. PG release from the astrocytes in culture was evident by 1 hr. This suggests that PGS would be present to initiate a SS signal cascade within the 2 hr and 12 hr period proposed by our study.

The next step in the cascade, following astrocyte PG release, would be the involvement of the eicosanoid in the SS release stimulatory cascade. Honegger et al. (1991) demonstrated that IL-1ß stimulated SS release from hypothalamic explant fragments. Honegger et al. observed that the specific cyclo-oxygenase inhibitor naproxen, at 0.1 µg/ml attenuated the IL-1ß-induced stimulation of SS. The coincubation with either a 1 µg/ml dose of naproxen or 10 µg/ml indomehtacin completely abolished the IL-1ß stimulation of SS. Honegger et al. also investigated the effects of other arachidonic acid metabolites on SS secretion. Arachidonic acid can also be metabolized by the enzyme lipo-oxygenase to form the leukotrienes. Honegger showed that neither the 1 nor 10 µg/ml doses of the lipo-oxygenase inhibitor, BWA4C significantly altered IL-1ß stimulated SS release, suggesting that lipo-oxygenase products are not involved in the SS response to IL-1ß. Honegger et al. concluded that IL-1ß effects on the SS secretion may be mediated via cyclo-oxygenase

stimulation of PGE synthesis. PGs may also stimulate SS release indirectly. IL-1ß has been shown to alter the secretion of CRH though a mechanism dependent on eicosanoid synthesis (Navarra et al., 1990). Bernardini et al. (1989) showed that $PGF_{2\alpha}$ and the thromboxane A_2 receptor agonist U-49, 619, stimulated the release of CRH from explanted rat hypothalami in culture. These studies suggest that various cyclo-oxygenase metabolites can stimulate CRH. Since CRH is a known regulator of SS release (Peterfreund and Vale, 1984) the peptide may posses an intermediary role in the IL-1ß/SS system. Collectively these data suggest that PGs may act directly or indirectly on SS neurons to increase the secretion of SS.

The release of SS into the extracellular space may interact with SS autoreceptors resulting in the observed decrease in SS. Richardson and Twente (1986) examined the pattern of SS release from hypothalamic cell suspensions over a 3 hr time period. SS release did not increase in a linear pattern, whereas another peptide hormone, thyroid releasing hormone did. A nonlinear secretion pattern suggests that a specific negative feedback system is regulating SS release. Richardson and Twente also examined the effect cell density on SS release. An increase in hypothalamic cell density decreased the quantity of SS released per unit number of cells whereas a decrease in cell density increased SS secretion. Exogenous SS added to the cell suspension dose-dependently inhibited both basal SS secretion and SS release stimulated by the Na^+/K^+ ATPase inhibitor, ouabain. Epelbaum et al. (1986) also demonstrated that SS can inhibit its own release in during their 1.5 hr perfusion of hypothalamic explants. Epelbaum et al. showed that the SS analog D-Trp8 SS, caused a dose-dependent inhibition of 25 mM K^+-induced SS release from anterior periventricular

tissue but not from the ME. This supports our observation that anatomically distinct SS neuronal subpopulations have different neurochemical sensitivities. The SS self inhibition observed by these authors occurs within a similar time frame as this study suggesting a common mechanism of action. Collectively these and our data suggest that SS, progressively released over 2 or more hours, could diminish its own release. The demonstration of high affinity SS binding sites in the hypothalamus (Srikant and Patel, 1984) and the distribution of SS receptors near SS neurons in the PeVN (Beaudet et al., 1995) suggests that SS exerts negative feedback control on its own release through autoreceptors. The presence of SS-SS synapses in the hypothalamus (Epelbaum et al., 1986) further supports the notion of SS autoregulation. We speculate that this SS induced SS suppression could be one of the key mechanism responsible for the IL-1ß-induced suppression of SS released observed in this study. The observed effect would provide the means for the cytokine to stimulate directly and indirectly inhibit SS secretion form the slice 4 cultures.

IL-1ß did not alter the secretion of SS from slice 4 cultures during the 24 hr incubation. The lack of a 24 hr effect could be due to desensitization of the SS autoreceptors. In a review paper, Epelbaum et al. (1989) reported that the depletion of endogenous SS with cysteamine, increased the affinity and number of SS binding sites in cortical synaptosomes. The authors commented that the down regulation of SS receptors in response to increased SS released has never been reported, but the possibility of SS receptor desensitization is still a distinct possibility. Peisen et al. (1995) also speculated that a tolerance to LPS may be responsible for the attenuated rate of growth observed in their rats.

Desensitization may also have occurred during any receptor-mediated step of the IL-1ß signal transduction to SS neurons.

The key mechanism in our theory of IL-1ß-induced SS inhibition, is the activation of SS autoreceptors. Conclusive support of our theory would have been provided by the addition of a SS receptor antagonist to the experiments. If the coadministration of a SS receptor antagonist blocked the IL-1ß effect, then SS autoreceptors could be considered as one of its mechanisms of action. Richardson and Twente (1986) demonstrated the specificity of their SS self inhibition with a SS analog. They showed that Ala^9-SS (a SS analog with reduced bioreactivity) did not affect SS secretion, even at 1 nM concentrations. The use of this partial SS receptor agonist or any other full SS receptor antagonist was not conducted in this study, however, due to time and fiscal restraints.

4. Somatostatin Immunocytochemistry

The release of somatostatin from granules in SS neurons of hypothalamic slice cultures was verified by the immunocytochemical localization of SS peptide storage in the tissue from which the cultures were harvested (four day old rats). The staining observed in the neonatal hypothalami processed in this study verified the work of Shiosaka et al. (1982) who conducted a detailed immunocytochemical study on fetal, neonatal and adult rats. Unfortunately the immunocytochemistry conducted on the slice cultures themselves did not produce consistent results to compare to the paraffin embedded tissue.

The differences in primary antibody incubation time and pretreatment protocols used

in this study reflect the dissimilarity in the amount of SS available to bind the antibody. The SS antibody (DAKO) was developed to detect SS in the pancreas and produced specific staining in both the protocols used. The SSir detected in the D cells of the pancreas corresponded to staining observed by Ensinck et al. (1978) and Nilaver and Kozlowski (1989) and was observed whether the primary antibody was incubated for 48 hr or 72 hr and was independent of the type of pretreatment used. Sternberger (1986) reported that the necessary incubation time of the primary antibody depends on the antigen availability. Increasing the incubation time to 72 hr for the neonate brains allowed for better penetration and accessibility of the antibody to the antigen in the tissue. The variety of incubation periods required for the brain compared to the pancreas may be a function of the amount of SS antigen present in the different tissues or the success of the pretreatment procedures to penetrate the different organ tissues.

The storage of imunoreactive SS in neurons in neonatal rats' brains is less than in adult rat brains (Khorram and McCann, 1984; McGregor et al., 1982). Although SS staining in other neonatal brain areas was achieved by Shiosaka et al. (1982), in this study consistent staining was limited to the neonatal ME. SS distribution in cell bodies was not observed. It is possible that the primary antibody and the protocol used on the neonate brains were only able to detect the highest amount of SSir, which happened to be the ME (Elde et al., 1984; Johansson et al., 1981; Finley et al., 1981; Bennett-Clarke et al., 1980). The increase in primary antibody titre and incubation time and the protease pretreatment may have further adjusted the availability of the antibody to the SS antigen (Sternberger, 1980) so that it could

detect the lower amounts of SS antigen present in the neonatal tissue.

The pretreatment of both the neonatal brain and the adult pancreas slices with protease may have helped increase the antibody permeability as Finley and Petrusz (1982) have demonstrated that proteolytic treatment improved SS staining in paraffin-embedded rat brains. Finley and Petrusz speculated that the reason for improvement of staining after proteolytic digestion of the tissue, was that the protease must somehow unmask antigenic sites within the tissue by dissolving some fixation -induced cross-linkages formed between proteins and allow the antibody to penetrate the tissue much more effectively than would otherwise occur.

5. Conclusion

In conclusion, 2 hr incubations and 12 hr incubation with IL-1ß at concentrations consistent with those achieved physiologically, caused a significant inhibition of SS release from neonatal slice explant cultures specifically derived from known hypothalamic sources of somatostatinergic ME innervation. All three IL-1ß doses inhibited SS secretion during the 12 hr incubations whereas the 2 hr IL-1ß effect was only achieved at the 1×10^{-9} M and 1×10^{-11} M concentrations. The storage of SS was verified by immunocytochemistry in formalin-fixed, paraffin-embedded neonatal hypothalami. The inability of the indomethacin plus IL-1ß incubation to inhibit SS release suggests that chronic IL-1ß exposure may modulate SS secretion through a complex signal cascade system that involves prostaglandin release and or synthesis. The IL-1ß induced attenuation of SS secretion corresponded to the

rebound in GH secretion observed after an initial decrease in release in response to long term exposure to endotoxin. The deleterious effect of this cytokine-neuroendocrine interaction on somatostatin and the growth hormone axis is an area of interesting speculation and must be elucidated by further research.

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