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Kyrkouli, Stavroula E., Ph.D.

City University of New York, 1989

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GALANIN AND FEEDING BEHAVIOR: RELATION TO NOREPINEPHRINE AND NEUROPEPTIDE Y

by

STAVROULA E. KYRKOULI

A dissertation submitted to the Graduate Faculty in Psychology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

1989

This manuscript has been read and accepted for the Graduate Faculty in Psychology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

June 6, 1989
Date

Richard J. Bodnar
Chair of Examining Commitee

June 6, 1989
Date

Herbert D. Saltzstein
Executive officer

Sarah Leibowitz

Thomas Frumkes

Nori Geary

James Gibbs

Supervisory Committee

The City University of New York

Abstract

GALANIN AND FEEDING BEHAVIOR:

RELATION TO NOREPINEPHRINE AND NEUROPEPTIDE Y

by

Stavroula E. Kyrkouli

Adviser: Professor Richard Bodnar

The neuropeptide, galanin (GAL), is widely distributed in the gut and brain. It is most concentrated in the hypothalamus and, in particular, the paraventricular nucleus (PVN) where receptor sites for ^{125}I-GAL may also be found. This peptide has recently been found to elicit feeding in satiated rats after medial hypothalamic administration. Moreover, it is found to coexist with norepinephrine (NE) in the PVN, a site and neurotransmitter believed to be important in the control of natural feeding behavior.

Based on these findings, this dissertation investigates the possibility that GAL influences food intake through its actions in the PVN and, in particular, via its direct interaction with the noradrenergic system localized in this nucleus. The results strongly suggest that GAL induces feeding, in part, through the release of endogenous NE in the PVN. This is in contrast to the action of another hypothalamic peptide, neuropeptide Y (NPY). While this

peptide is also a strong stimulant of food intake and coexists with NE in the PVN, the results suggest that it acts independently of, or possibly even antagonistically with, this catecholamine.

These results were obtained through three experiments, that involved brain cannula-mapping procedures, pharmacological tools, and the microdialysis technique in combination with high performance liquid chromatography (HPLC) with electrochemical detection (ED).

1) The brain cannula-mapping experiment, including tests with GAL in 14 areas of the brain, demonstrated that GAL's action in the brain is anatomically specific. Its site of action, like that of NE, is found to be the PVN and, in particular, the parvicellular division of this nucleus. In the PVN, GAL's effect is shown to be behaviorally specific, involving a selective increase in food intake with no change in other food related- behaviors.

2) The pharmacological experiment involved PVN administration of the α-receptor blockers, phentolamine (general α), rauwolscine (specific α_2) and prazosin (specific α_1), in combination with GAL as well as NE and NPY. This experiment demonstrated that GAL-induced feeding, similar to NE-stimulated feeding, depends specifically upon intact α_2-receptor sites. Further, experimentation with the catecholamine-synthesis inhibitors, α-methyl-p-tyrosine and Fla-63, suggested that GAL's action also depends upon the

release of endogenous NE. This is in contrast to NPY, which is still effective in eliciting feeding in the presence of α_2-receptor antagonists, and whose action is actually potentiated by catecholamine-synthesis inhibitors.

3) The microdialysis experiment examined the impact of PVN injection of GAL on extracellular levels of NE, collected via a microdialysis probe in the PVN and analyzed by HPLC. This experiment demonstrated that PVN administration of GAL enhances endogenous levels of NE, suggesting that GAL potentiates the release of this catecholamine. Unlike GAL, however, NPY failed to stimulate NE levels in the PVN but in fact tended to decrease extracellular NE, suggesting a possible inhibitory influence on this amine.

These findings with GAL and NPY suggest the existence of two types of peptide-amine interactions in the PVN. These are: a cooperative interaction, whereby GAL stimulates the release of NE and through this amine regulates feeding; and an antagonistic interaction, whereby NPY and NE may actually suppress each other's action. In light of other findings, it is proposed that GAL has a specific physiological function in modulating natural feeding behavior, particularly at the onset of the animal's active (dark) cycle. At this time, GAL is believed to play a role specifically in the potentiation of carbohydrate ingestion, acting through the enhanced release of NE in the PVN.

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General Introduction

Multiple classical neurotransmitters and neuropeptides are now implicated in the control of feeding behavior. Research over the last ten years has focused on the hypothalamus, and in particular the paraventricular nucleus (PVN), as a critical area in the mediation of these effects (Leibowitz, 1988). The purpose of the present series of experiments is to investigate the function of the neuropeptide, galanin (GAL), in the control of feeding behavior.

Galanin is the first member to be discovered in a new peptide family (Tatemoto, Rokaeus, Jornvall, McDonald & Mutt, 1983). This peptide has recently been found to elicit eating after injection into the PVN (Kyrkouli, Stanley & Leibowitz, 1986), and it is also known to coexist with the catecholamine (CA), norepinephrine (NE), in the hypothalamus (Skofitsch & Jacobowitz, 1985; Levin, Sawchenko, Howe, Bloom & Polack, 1987). The question is, where and through what mechanism does GAL act in the brain to modulate feeding behavior. Is its primary site of action in fact within the PVN, where NE through α_2-noradrenergic receptors is most effective in stimulating feeding (Leibowitz, 1978; Goldman, Marino & Leibowitz, 1985)? Further, does GAL interact directly with PVN NE in this process, enhancing its release and then activating the local α2-receptor sites? Finally,

can the action of GAL be distinguished from that of another peptide, neuropeptide Y (NPY), which is also known to stimulate feeding (Clark, Kalra, Crowley & Kalra, 1984; Stanley & Leibowitz, 1984), as well as coexist with NE in the PVN (Holets, Hokfelt, Rokaeus, Terenius & Goldstein, 1988; Levin et al., 1987)?

Three experiments have been conducted to address these questions.

Experiment 1, a brain cannula-mapping study, investigated GAL's site(s) of action in the brain and also examined whether its effect on feeding is behaviorally specific.

Experiment 2, a pharmacological study with receptor antagonists and NE synthesis inhibitors, examined whether the feeding effect of GAL is dependent upon the integrity of α-adrenergic receptors and endogenous stores of NE. The results of these tests with GAL were compared with the effects of these agents on the feeding response stimulated by either NE or NPY.

Experiment 3, a biochemical study using the microdialysis technique, examined the impact of PVN injection of GAL on local NE levels in freely-moving rats. These results were compared with the effects of NPY on this amine.

The following background section reviews the role of the hypothalamus, and in particular of the PVN, in the

control of feeding behavior and then describe evidence implicating GAL, NE, as well as NPY, in the control of eating.

A) Hypothalamus and Feeding Behavior

Ingestive behavior is believed to be controlled by both peripheral and brain factors that interact in the initiation, as well as termination, of a meal. Peripheral organs found to be important in food intake regulation include the liver, small intestine, stomach, pancreas, and adrenal glands (Smith & Gibbs, 1979). Signals from the periphery reach the brain via two routes: neural and hormonal. The main mediator of neural messages is the vagus, which receives information from visceral organs, penetrates the brain at the level of the medulla, and travels through the medulla to terminate in the nucleus of the solitary tract (Powley & Berthoud, 1986). Hormonal mediators include the pancreatic hormones, insulin and glucagon; several brain-gut hormones, e.g. cholecystokinin and bombesin; and the steroid hormones, e.g. corticosterone and estrogen (Gibbs & Smith, 1984). In addition to being active in the periphery, each of these hormones is also believed to have direct impact in systems in the brain (Leibowitz, 1988).

Within the brain, the region most extensively studied in relation to feeding is the hypothalamus. This structure,

which is particularly rich in classical neurotransmitters and peptides, has been demonstrated to have an essential function in the control of food intake and appetite for nutrients. The interest in the hypothalamus originated from observations that destruction of the lateral hypothalamus (LH) results in aphagia and weight loss (Anand & Brobeck, 1951), while electrical stimulation of the same area induces feeding in non-deprived rats (Anand & Dua, 1955). Complementary effects have been observed in the ventromedial hypothalamus (VMH), where electrolytic lesions cause hyperphagia and obesity (Hetherington & Ranson, 1942), and electrical stimulation inhibits feeding in food-deprived animals (Anand and Dua, 1955). These early findings gave rise to what became known as the "dual center" theory. This theory postulated that feeding behavior is controlled by two centers in the brain: the VMH "satiety center" and the LH "feeding center" (Stellar, 1954).

Advances in techniques and procedures, however, have shifted attention away from the "hypothalamocentric" view of feeding behavior and have moved it towards analyses of multiple sites, not only within the hypothalamus but also in extrahypothalamic and peripheral systems which are implicated in the control of feeding behavior. Current views (Sclafani & Kirchgessner, 1986; Grill, 1986) recognize the inadequacy of the "dual center" theory and emphasize the essential role of the medial and lateral hypothalamus, in

relation to forebrain, hindbrain, as well as peripheral endocrine and autonomic systems.

Our knowledge of the role of the hypothalamus in the control of feeding has been greatly advanced by the development of new neuroanatomical and neurochemical techniques. The use of these techniques, in combination with more classical experimental procedures, has allowed investigators to postulate that this structure plays an integrative role in relation to ingestive behavior (Leibowitz, 1988). Such information was obtained, among others, from: 1) anatomical studies, describing neural connections between the hypothalamus and neuroendocrine/autonomic systems (Swanson & Sawchenko, 1980); 2) microinjection studies, showing the effects of classical neurotransmitters, peptides or drugs in specific hypothalamic as well as extrahypothalamic sites (Leibowitz & Stanley, 1986); 3) detailed behavioral studies, examining the effects of various neurochemical substances on temporal patterns of eating behavior and on ingestion of the specific macronutrients, carbohydrate, protein and fat (Shor-Posner et al., 1985); 4) endocrine studies, showing close interaction between circulating hormones and brain neurochemical systems (Leibowitz, 1988); 5) biochemical studies, measuring the activity of neurotransmitters and receptors in distinct brain sites as a result of physiological manipulations, e.g. food deprivation and

refeeding (Jhanwar-Uniyal & Leibowitz, 1986); and 6) recent microdialysis studies which, in combination with an HPLC with ED, measure levels of neurotransmitters and their metabolites in discrete brain areas of freely behaving animals (Stanley et al., 1989).

These and related studies have revealed that the hypothalamus plays an integral role in receiving and integrating multiple inputs (metabolic, hormonal, neurogenic, thermal and cortical factors) reflecting the nutritional status of the organism, and then in sending output signals for behavioral and physiological adjustments that accomodate the energy and nutrient needs of specific tissues (Leibowitz, 1988).

B) Hypothalamic Paraventricular Nucleus and Feeding Behavior

Within the hypothalamus, the PVN is distinguished as a critical site involved in the control of food intake (Leibowitz, 1978). In addition, this nucleus, through its neuroendocrine and autonomic connections, is also found to be involved in numerous other functions, including control of hormone secretion (Antoni et al., 1983; Leibowitz et al., 1988; Merchenthaler, Vigh, Petrusz & Schally, 1983; Mezey & Kiss, 1985; Lechan & Jackson, 1982), body fluid homeostasis (Zimmerman, 1983), analgesia (Kordower & Bodnar, 1984), gastrointestinal functions (Shiraishi, 1987), cardiovascular

functions (Ciriello & Claresu, 1980), and pineal melatonin synthesis (Klein et al., 1983). The neurocircuitry of the PVN has been extensively studied. These anatomical findings, suggesting a key function for the PVN in energy homeostasis, will be reviewed in the following sections.

1) Neurocircuitry of the PVN

Nucleus. The PVN is a densely-packed, wing-shaped nucleus, that lies on either side of the third ventricle in the rostral area of the hypothalamus and contains about 10,000 neurons (Swanson & Sawchenko, 1983). The evidence obtained from retrograde tracer studies, combined with immunohistochemical results, has led to the concept of PVN cellular compartmentalization, a notion that is important for understanding the function of this nucleus in relation to its afferent and efferent projections. This nucleus is composed of distinct magnocellular (oxytocinergic and vasopressinergic) and parvicellular divisions (Swanson & Kuypers, 1980). In particular, the magnocellular division of the PVN has been subdivided into four parts: anterior, medial, posterior medial (oxytocinergic) and posterior lateral (vasopressinergic). The parvicellular division is formed of six parts: periventricular, anterior, medial dorsal, medial ventral, lateral and dorsal.

Afferents. The PVN receives afferent projections from a

variety of cell groups in the brainstem, hypothalamus and telencephalon (Cunningham & Sawchenko, 1988; Luiten, ter Horst & Steffens, 1987; Swanson & Sawchenko, 1983). Projections from each of these levels reach or influence cells in both divisions of the PVN, and each projection ends in a distinctive way with respect to the cytoarchitectonic subdivisions of this nucleus.

Projections to the PVN from the lower brainstem include the following: 1) the NTS in the ventromedial medulla, which projects to the parvicellular part of the PVN via a pathway that is primarily noradrenergic (A2 cell group) and transmits visceral afferent information from the vagus and glossopharyngeal nerves; 2) the parabrachial nucleus, which receives massive afferents from the NTS, projects to the medial parvicellular division of the PVN, and, like the NTS noradrenergic projection, relays visceral sensory information to the forebrain; 3) non-noradrenergic cells in the NTS, which project to the ventrolateral medulla (A1 cell group) and then to the parvicellular division of the PVN, as well as the magnocellular division through which visceral afferent stimuli influence vasopressin secretion; 4) the pontine locus coeruleus (A6 cell group), which receives a substantial projection from the A1 region and projects to the most medial parts of the parvicellular division of the PVN, a pathway believed to mediate in part the feeding response induced by PVN administration of NE; and 5) the

dorsal and median nuclei of the raphé, which send input to the PVN via a pathway that is primarily serotonergic and may provide the neural substrates for the feeding suppressive effect observed after injection of serotonin into the PVN.

Additionally, the PVN receives inputs from the other hypothalamic nuclei. It has been proposed that these projections provide a substrate for local integration of hypothalamic neuroendocrine and autonomic responses (Swanson & Sawchenko, 1983). Most of these short afferents terminate preferentially in regions of the nucleus that project primarily to the median eminence and, to a lesser extent, autonomic centers in the brainstem and spinal cord. Specifically, projections from the preoptic area, suprachiasmatic nucleus, ventromedial, anterior and lateral hypothalamus terminate in the parvicellular division of the nucleus, while projections from the dorsomedial and medial preoptic nuclei end in the magnocellular division. The arcuate and supraoptic nuclei, in contrast, project to both divisions.

Limbic structures involved in the regulation of feeding (Wyrwicka, 1988) also project to the PVN. These include parts of the septal region (primarily the lateral nucleus) and also the amygdaloid complex (primarily the medial nucleus), which sends pathways to the PVN via the bed nucleus of the stria terminalis (Swanson & Sawchenko, 1983) and/or the ventral premammillary nucleus (Luiten, ter Horst

& Steffens, 1987).

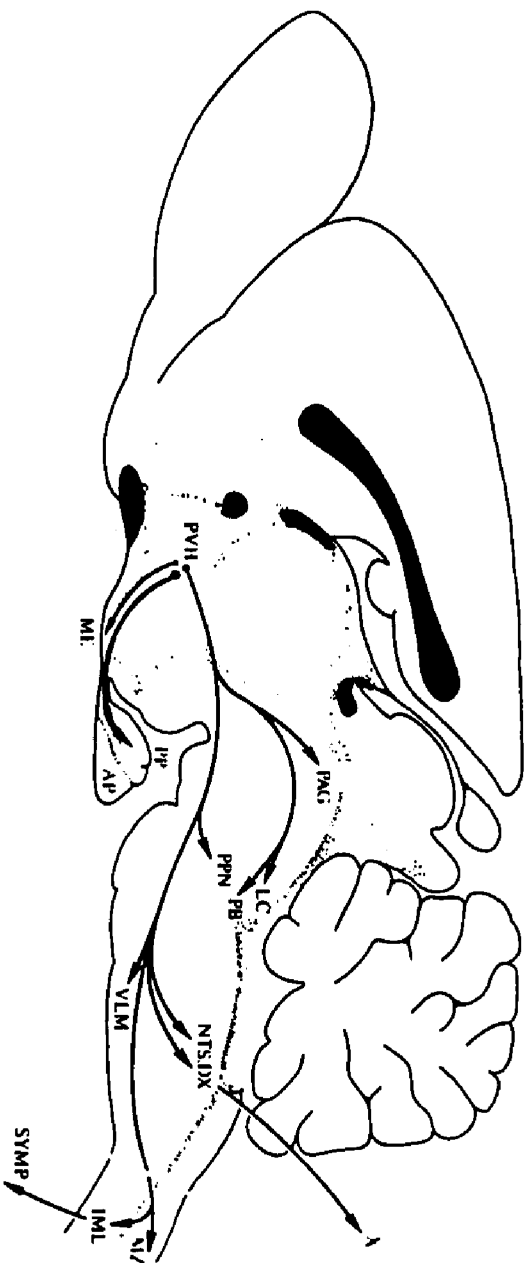
Efferents. There is strong evidence to suggest that anatomically distinct cell groups in the PVN project to the posterior pituitary, median eminence, and autonomic centers in the brainstem and spinal cord (Swanson & Kuypers, 1980; Swanson & Sawchenko, 1983; Kiss, 1988; Luiten, ter Horst & Steffens, 1987). These outputs, outlined in Fig. 1, may be summarized as follows.

First, cells from the magnocellular division project to the posterior pituitary. From here, they control the release of the peptide hormones, oxytocin and vasopressin, into the general circulation. There is evidence to suggest that both of these hormones may have a role in modulating food intake (Leibowitz, 1988; Verbalis, McCann, McHale & Stricker, 1986).

Second, cells from the medial-dorsal parvicellular division of the PVN project to the external zone of the median eminence. By secreting releasing factors into the hypophyseal portal system, these cells influence the release of hormones from the anterior pituitary. Corticotropin releasing factor, for example, is known to be concentrated in PVN neurons and to activate the secretion of adrenocorticotropic hormone (ACTH) from the pituitary. This hormone then activates the adrenal glands, which release corticosteroids (e.g. corticosterone) (Lechan, Nestler, Jacobson & Reichlin, 1980; Merchenthaler, Hynes, Vigh,

Figure 1. A summary of the major neuroendocrine and descending projections of the PVN in the rat; reproduced by permission from Swanson, Sawchenko & Lind (1986). Abbreviations: AP, anterior pituitary; DX, dorsal motor nucleus of the vagus nerve; IML, intermediolateral column; LC, locus coeruleus; ME, neurohemal zone of the median eminence; MZ, marginal zone; NTS, nucleus of the solitary tract; PAG, periaqueductal gray; PB, parabrachial nucleus; PP, posterior pituitary; PPN, pedunculopontine nucleus; SYMP, sympathetic nervous system; X, vagus nerve.

Figure 1



Schally & Petrusz, 1984), and, in turn, influence the release of hormones (insulin, glucagon) from the pancreas. Through these steroids and peptide hormones, the PVN is believed to participate in metabolic homeostasis, in addition to controlling feeding behavior (Leibowitz, 1988).

Third, cells from the medial-ventral parvicellular division of the PVN project predominantly to cell groups in brainstem and spinal cord. As indicated in Fig. 1, these fibers preferentially innervate regions associated with the autonomic nervous system, including the Edinger-Westphal nucleus, parabrachial nucleus, nucleus of the solitary tract, and dorsal motor nucleus of the vagus nerve, as well as the periaqueductal gray, locus coeruleus, and sensory trigeminal nuclei. These cells contribute about 30% of all axons descending from the hypothalamus (Schwanzel-Fukuda, Morell & Pfaff, 1984). Through these descending projections, the PVN may influence ingestive behavior (Crawley & Kiss, 1985; Weiss & Leibowitz, 1985; Sawchenko, Gold & Leibowitz, 1981), as well as gastrointestinal functions (Rogers & Hermann, 1985; Shiraishi, 1987), cardiovascular functions (Ciriello & Claresu, 1980), analgesia (Kordower & Bodnar, 1984), and melatonin synthesis in the pineal gland (Klein et al., 1983).

The PVN is unique within the hypothalamus, in that it contains three functionally distinct neural systems, namely, the magnocellular neurosecretory cells, the parvicellular

neurosecretory cells, and the "autonomic" cells. Through these efferent projections, the PVN is now proposed to have a pivotal role in integrating a variety of autonomic, neuroendocrine and behavioral functions (Swanson & Sawchenko, 1980; Kiss, 1988; Leibowitz, 1988).

While a good deal is now known about the parvicellular and magnocellular cells of the PVN, there is considerably less information about other cell types in the PVN, which send efferent projections to hypothalamic nuclei, namely, the arcuate nucleus, lateral, dorsomedial and ventromedial hypothalamus (Swanson & Sawchenko, 1983). In addition, interneurons are also present in the nucleus (Van den Pol, 1982), the function of which is yet to be determined.

2) Neurochemistry of the PVN

As demonstrated by immunohistochemical studies, this nucleus contains an exceptional variety of neuroactive substances and their receptor sites. Among others, the PVN is known to contain particularly high concentrations of GAL (Skofitsch & Jacobowitz, 1985; Ch'ng et al., 1985), NE (Swanson & Sawchenko, 1983; Cunningham & Sawchenko, 1988) and NPY (Allen et al., 1983; Hokfelt et al., 1983). Neuronal fibers containing these substances originate from the hindbrain, midbrain as well as hypothalamic areas, and they project to different regions of the PVN. The following

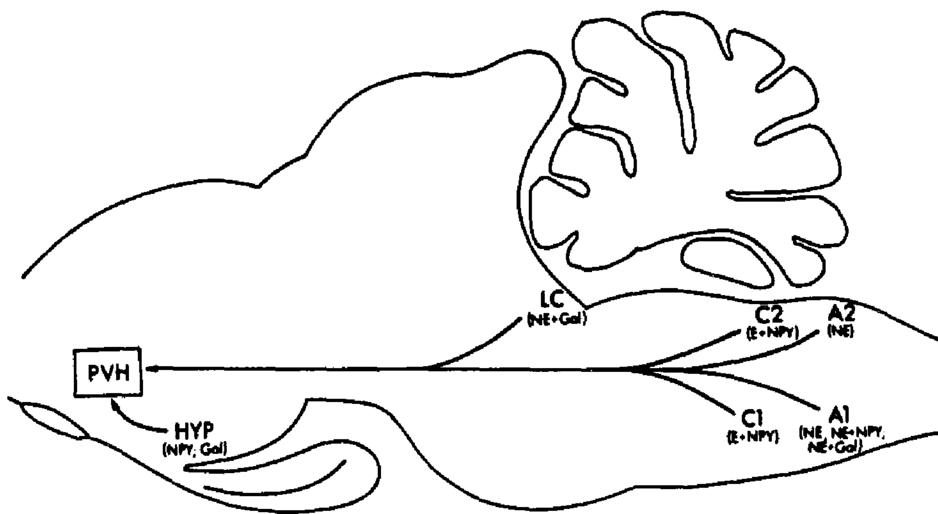
section focuses on the PVN innervation by fibers immunoreactive to these substances.

Galanin. This 29-amino acid peptide (Tatemoto, Rokaeus, Jornvall, McDonald & Mutt, 1983) is widely distributed in the periphery and the brain (for review see Rokaeus, 1987). Within the brain, highest concentrations of GAL have been found in the hypothalamus and, in particular, the PVN, supraoptic nucleus and median eminence. In addition, particularly large amounts of GAL have been demonstrated in the arcuate nucleus, locus coeruleus, caudal spinal trigeminal nucleus, and nucleus of the solitary tract (Melander, Hokfelt & Rokaeus, 1986; Ch'ng et al., 1985; Skofitsch & Jacobowitz, 1985). Likewise, receptors for radiolabeled GAL are heterogeneously distributed throughout the brain. Moderate and dense binding have been observed, among other areas, in the anterior and paraventricular hypothalamus, amygdala, septum, nucleus accumbens, and ventral tegmental area (Skofitsch, Sills and Jacobowitz, 1986; Melander, Fuxe, Harfstrand, Eneroth & Hokfelt, 1987).

The galaninergic input to the PVN (Fig. 2) is predominantly confined to its parvicellular division (Levin et al., 1987), while the magnocellular portion of this nucleus is sparsely innervated by GAL fibers. Within the parvicellular PVN, these fibers innervate two functionally distinct areas: 1) the anterior and periventricular parts, which are known to project to the median eminence and

Figure 2. Schematic drawing of a saggital section through the rat brain, showing the organization of, and coexisting peptides contained within, ascending catecholaminergic inputs to the PVN (PVH); reproduced by permission from Levin, Sawchenko, Howe, Bloom & Polack (1987). Ascending epinephrine-containing pathways (E) arise from the C1 and C2 cell groups, each of which also expresses NPY immunoreactivity. Noradrenergic (NE) inputs arise from the A1, A2, and A6 cell groups in the pons and medulla. Subpopulations of neurons in the A1 region express NE alone, NE plus NPY, or NE plus GAL. In addition, both GAL and NPY immunoreactivity are contained within substantial populations of non-noradrenergic, non-adrenergic cells in the hypothalamus (HYP) that project to the PVN.

Figure 2



contain several of the releasing- or release-inhibiting hormones of the hypothalamus; and 2) the dorsal parvicellular part and the ventral aspect of the medial parvicellular part, which project to autonomic cell groups of the medulla and spinal cord. Both sets of fibers originate from the A1 (medulla) and A6 (locus coeruleus) noradrenergic cell groups, where GAL coexists with NE (Levin et al., 1987; Holets et al., 1988).

In addition to these long ascending GAL-containing fibers, there are several hypothalamic nuclei which are found to send galaninergic inputs to the PVN (Levin et al., 1987). The most prominent of these projections originates in the dorsomedial hypothalamus, although a considerable number are also found in the lateral hypothalamus, arcuate nucleus and medial preoptic area.

Norepinephrine. As indicated above and illustrated in Fig. 2, the noradrenergic innervation to the PVN originates from three cell groups (Cunningham & Sawchenko, 1988; Swanson & Sawchenko, 1983): 1) the A1 cell group of the ventrolateral medulla; 2) the A2 cell group of the dorsomedial medulla; and 3) the A6 (locus coeruleus) cell group of the dorsolateral pontine tegmentum. The A1, A2 and A6 cell groups each project to cytoarchitectonically and biochemically distinct parts of the PVN as follows: 1) projections from the A1 region are found primarily in those parts of the magnocellular division that contain

vasopressinergic neurons; 2) projections from the A2 region are distributed primarily throughout the parvicellular division, particularly the medial dorsal part that contains CRF-immunoreactive neurons. A less-dense A2 projection to the magnocellular division of the PVN can also be found; and 3) fibers originating from the locus coeruleus (A6) are distributed almost exclusively to the parvicellular region of the PVN, with the most prominent input localized to the periventricular zone.

Neuropeptide Y. The brain concentration of NPY, a 36-amino acid member of the pancreatic polypeptide family (Tatemoto, Carlquist & Mutt, 1982), is higher than the concentration of any other peptide (Allen et al., 1983). The largest amounts of NPY have been found in the hypothalamic PVN and arcuate nucleus. Additionally, particularly large amounts of this peptide have been demonstrated in the suprachiasmatic nucleus, median eminence, dorsomedial nucleus and paraventricular thalamic nucleus (for review, see Gray & Morley, 1986). Receptor binding sites for NPY have been localized in several hypothalamic nuclei, including the PVN, as well as in the cortex, hippocampus, amygdala, and septum (Martel, St-Pierre & Quirion, 1986).

This peptide's innervation to the PVN (see Fig. 2) comes from the following sources (Sawchenko et al., 1985; Harfstrand et al., 1986; Levin et al., 1987; Bai et al., 1985): 1) the noradrenergic A1 (medulla) and A6 (locus

coeruleus) cell groups, where NPY coexists with NE and perhaps also with GAL; 2) the C1, C2 and C3 adrenergic cell groups of the medulla, where NPY coexists with epinephrine; and 3) the hypothalamic arcuate nucleus, a projection devoid of NE. The NPY-containing fibers from the lower brainstem project most densely to the anterior and medial regions of the parvicellular division of the PVN, areas known to send efferents to the neurohemal zone of the median eminence. Somewhat less dense inputs are seen in the: 1) dorsal and lateral parts of the parvicellular division, the principal known outputs of which are to autonomic centers in brainstem and spinal cord; 2) the periventricular part, which also appears to project primarily to the hypophysiotropic zone of the median eminence; and 3) the magnocellular division, which contains predominantly oxytocinergic neurons.

3) Neurochemistry of feeding with focus on the PVN.

A variety of substances are known to modulate feeding behavior when administered into the PVN. Some of these neurochemicals are found to stimulate food intake, while others have an inhibitory effect on this behavior. Eating-stimulatory substances which are active in the PVN include: NE (Leibowitz, 1978), γ-aminobutyric acid (GABA) (Grandison & Guidotti, 1977), several opioid peptides (β-endorphin, morphine, D-Ala-Metenkephalinamide) (for review see Levine,

Morley, Gosnell, Billington & Bartness, 1985), NPY (Clark et al., 1984) and GAL (Kyrkouli et al., 1986). Eating-inhibitory substances active in this nucleus include: serotonin (5-HT) (for review see Leibowitz, Weiss & Shor-Posner, 1988), neurotensin (Stanley, Hoebel & Leibowitz, 1983), calcitonin (DeBeaurepaire & Freed, 1983), and corticotropin-releasing factor (CRF) (Krahn, Gosnell, Levine & Morley, 1984). For the monoamines, NE and 5-HT, the PVN is believed to be a primary site of action. For the peptides, in contrast, (with GAL yet to be tested) the PVN appears to be only one of several hypothalamic or even extrahypothalamic sites that can respond to their local administration.

This dissertation is focused on GAL's effects on feeding and this peptide's possible relationship with two other feeding stimulatory substances, namely, NE and NPY. The following sections will provide background information on the physiological effects of these substances, particularly those related to the feeding process.

a) Galanin and feeding

Galanin influences a variety of physiological and endocrine systems. It alters pancreatic (for review, see Ahren, Rorsman & Berggren, 1988) and anterior pituitary hormone levels (Ottlecz, Samson & McCann, 1986; Melander, Fuxe, Harfstrand, Eneroth & Hokfelt, 1987) and causes

changes in gut activity (Ekblad, Hakanson, Sundler & Wahlestedt, 1985). There is little information to date on GAL's behavioral effects. Recently, it has been implicated in analgesia (Post, Alari & Hokfelt, 1988; Cridland & Henry, 1988) and memory (Sundstrom, Archer, Melander & Hokfelt, 1988; Chan-Palay, 1988). This peptide's coexistence with other peptides (vasopressin, substance P, cholecystokinin) (Rokaeus, Young & Mezey, 1988; Ju, Melander, Ceccateli, Hokfelt & Frey, 1987) and classical neurotransmitters (norepinephrine, serotonin, acetylcholine) (Levin et al., 1987; Melander, Staines & Rokaeus, 1986) suggests that it may interact with a variety of neurochemical systems.

Recently, GAL has been implicated in the control of feeding. Specifically, it was discovered that administration of GAL into the PVN of satiated rats, at doses ranging between 30 and 300 pmol, produces a dose-dependent stimulation of feeding behavior (Kyrkouli et al., 1986). A temporal analysis of this feeding response indicated that the average latency to eat is approximately 6 min, with many rats starting to eat within 2-3 min. The animals eat without interruption for approximately 30 min and consume an average of 3-4 g of food. Injections of GAL into sites proximal to the PVN (nucleus reuniens and perifornical hypothalamus), or into the periphery, fail to produce this response, giving some indication that GAL is acting within the brain and possibly in a localized region.

More recently, a study from our laboratory, in rats maintained on macronutrient diets (Tempel, Leibowitz & Leibowitz, 1988), revealed that GAL has a specific pattern of effect on diet selection that varies across the circadian cycle. Galanin significantly potentiates carbohydrate intake, while producing no change in protein consumption. This effect occurs predominantly at the onset of the rats' active (dark) cycle and is absent at the end of the dark period. In addition to potentiating carbohydrate intake, GAL also stimulates the consumption of fat. This increase in fat intake is particularly strong at the end of the nocturnal cycle, when GAL is ineffective in stimulating carbohydrate consumption.

b) Norepinephrine and feeding

In 1960, Grossman discovered that NE injected into the hypothalamus elicits a strong feeding response in satiated rats (Grossman, 1960). This effect of NE is now well established in the rat and is known to occur in a variety of other species as well (Leibowitz, 1980).

Site of action and neural substrates. Extensive cannula-mapping studies have demonstrated that the feeding stimulatory effect of NE is localized to the PVN (Leibowitz, 1978). Hypothalamic but not extrahypothalamic injections of this amine, at low doses (25 pmol), induce food intake, and the amount eaten increases, while the latency to eat

decreases, as the injection site approximates the PVN. Moreover, electrolytic lesions of this nucleus strongly attenuate feeding elicited by ventricular injection of NE (Leibowitz, Hammer & Chang, 1983).

The neural projections mediating this response are believed to originate from noradrenergic neurons in the dorsal pons (locus coeruleus and subcoeruleus, A6 cell group), and also possibly from medullary noradrenergic (or adrenergic) neurons that course through the dorsal pontine region (Leibowitz & Brown, 1980). The output of this system has been shown to return to the hindbrain via a dorsal periventricular route and then continue towards the dorsal medulla and the peripheral autonomic nervous system (Sawchenko, Gold & Leibowitz, 1981; Weiss & Leibowitz, 1985). The NE-induced feeding response is found to be dependent upon an intact vagus nerve, in particular its coeliac branch (Sawchenko et al., 1981).

α2-noradrenergic receptors. Pharmacological studies have revealed that the feeding stimulatory effect of NE is mediated through α_2-noradrenergic receptors located specifically within the PVN. Feeding is elicited by PVN or peripheral injections of α_2-noradrenergic agonists, but not by β-adrenergic, dopaminergic, serotonergic, or cholinergic agonists. Conversely, food intake stimulated by PVN injections of exogenous NE, by drug-induced release of endogenous NE, or by selective α_2-noradrenergic agonists, is

attenuated by α_2-noradrenergic antagonists but not by antagonists of other types of receptors (Goldman et al., 1985; Leibowitz, 1980). Biochemical studies have shown the PVN to contain particularly high levels of α_2-adrenergic binding sites (Leibowitz, Jhanwar-Uniyal, Dvorkin & Makman, 1982).

Behavioral analysis. Behavioral analysis of the feeding response induced by PVN injection of NE revealed that this effect is dose-dependent, the effective doses being between 25 and 500 pmol. The average latency to eat is 2-3 min, with feeding occurring within less than 30 sec in some animals. The subjects eat continuously for about 15-20 min and consume an average of 3-4 g of food (Leibowitz, 1975; 1980). Meal pattern analysis reveals that NE increases the size of the meal consumed, without affecting the number of meals after injection (Shor-Posner, Grinker, Marinescu & Leibowitz, 1985).

Macronutrient intake. Studies using a macronutrient self-selection feeding paradigm have revealed that NE specifically enhances ingestion of carbohydrate, while causing only a small increase in fat ingestion and no change or a decrease in protein intake (Leibowitz, Brown, Tretter & Kirchgessner, 1985). This effect is most pronounced at the start of the dark cycle and is also observed after PVN administration of the α_2-agonist clonidine (Yee, MacLow, Chan & Leibowitz, 1987). The opposite effect, namely, a

deficit in carbohydrate ingestion, occurs in response to local infusion of agents which block NE synthesis (Yee et al., 1987) or destroy noradrenergic neurons (Shor-Posner, Azar, Jhanwar-Uniyal, Filart & Leibowitz, 1986).

Interactions with corticosterone. The action of PVN NE in stimulating carbohydrate ingestion is closely associated with the activity of the adrenal hormone, corticosterone. Specifically, hypophysectomy and adrenalectomy abolish the feeding response induced by PVN α_2-noradrenergic stimulation, while thyroidectomy and gonadectomy have no effect (Bhakthavatsalam & Leibowitz, 1986; Leibowitz, Roland, Hor & Squilari, 1984; Roland, Bhakthavatsalam & Leibowitz, 1986). This response is restored by subcutaneous injections or implants of corticosterone. Furthermore, NE infusion directly into the PVN, in contrast to other hypothalamic nuclei, dose-dependently increases blood levels of corticosterone (Leibowitz, Sladek, Spencer & Tempel, 1988), while infusion of 6-hydroxydopamine (6-OHDA) into the PVN, which sharply reduces its noradrenergic innervation, inhibits adrenocorticotropin responses to neural stimulation (Feldman, Conforti & Melamed, 1986). A circadian rise in corticosterone at the onset of the dark cycle (Krieger & Hauser, 1978) coincides with a peak in endogenous NE in the PVN (Stanley, Schwartz, Hernandez, Hoebel & Leibowitz, 1987) and a surge in the rats natural preference for carbohydrate.

Possible relation between the actions of GAL and NE in

the PVN. While not yet directly tested, the possibility that GAL stimulates food intake by interacting with the PVN noradrenergic feeding-control system receives indirect support from the following evidence: 1) GAL coexists with NE in the PVN, as well as in brainstem noradrenergic neurons which innervate this nucleus (Levin et al., 1987; Holets et al., 1988) and which appear to mediate NE's feeding stimulatory effect (Leibowitz & Brown, 1980); 2) GAL induces a strong feeding response when injected into the PVN, a primary site of action for NE (Leibowitz, 1978); 3) GAL enhances the ingestion of carbohydrate at the start of the dark period (Tempel et al., 1988), precisely when NE is also most effective in inducing consumption of this macronutrient (Tempel, Tomkow & Leibowitz, 1988).

c) Neuropeptide Y and feeding

Neuropeptide Y has been implicated in various functions. It has been shown to influence circadian rhythms (Albers & Ferris, 1984), to modulate brain control of cardiovascular parameters (Fuxe et al., 1986; Fuxe et al., 1983), and to alter the release of pituitary and adrenal hormones (Leibowitz et al., 1988; Wahlestedt et al., 1987; Harfstrand et al., 1986). Similar to GAL, NPY has been found to coexist with several classical transmitters and peptides in neurons of the periphery, as well as the brain. Specifically, it has been colocalized with NE and

epinephrine (Sawchenko et al., 1985; Levin et al., 1987), as well as with 5-HT (Guy, Bosler, Dusticier, Pelletier & Calas, 1987; Blessing, Howe, Joh, Oliver & Willoughby, 1986), GABA (Hendry et al., 1984) and enkephalin (Pruss et al., 1986).

Evidence strongly suggests that central NPY plays a role in the regulation of food intake. Clark et al. (1984) first demonstrated that NPY injection into the third ventricle elicits a feeding response in satiated rats. While this effect was also observed after lateral ventricular administration of this peptide (Levine & Morley, 1984), other studies showed that NPY injection directly into the PVN evokes a larger feeding response than ventricular injection, with lower doses needed to produce this effect (Stanley & Leibowitz, 1984). These findings suggested a hypothalamic site of action for NPY, a proposal supported by a subsequent brain-cannula mapping study (Stanley, Chin & Leibowitz, 1985). This report demonstrated that several hypothalamic areas, the PVN in addition to the LH and VMH, are sensitive to the feeding stimulatory effect of NPY, in contrast to extrahypothalamic sites which exhibited no response.

Behavioral analysis of the NPY-induced feeding response has revealed that this effect is dose-dependent; a small dose of 24 pmol produces a significant increase, and a 235 pmol dose causes a peak effect. The average latency to eat

is approximately 10 min. In the lower end of the dose range, the feeding consists of a single meal (approximately 4 g), completed within 1 hour. Higher doses produce greater intake initially (15 g in 1 hour) and then a continued eating response which over the next 4 hours causes the rats to ingest the same amount of food they would normally eat over a 24-hr period (Stanley & Leibowitz, 1985). Furthermore, chronic PVN injection of NPY, over a 10-day period, causes rats to double their daily food consumption and gain weight at a rate of more than 10 g per day (Stanley, Kyrkouli, Lampert & Leibowitz, 1986).

As with GAL, there is indirect evidence to suggest that the feeding response induced by NPY may be related to that elicited by NE. Neuropeptide Y and NE coexist in medullary neurons (A1 cell group) that innervate the parvicellular part of the PVN (Everitt et al., 1984; Sawchenko et al., 1985; Holets et al., 1988) and that have a role in mediating NE's feeding stimulatory effect (Leibowitz & Brown, 1980). Moreover, PVN injection of NPY, like NE, is particularly active at the start of the dark cycle, when it elicits specifically the ingestion of carbohydrate (Stanley, Daniel, Chin & Leibowitz, 1985; Tempel et al., 1988). The NPY-induced feeding response, like that of NE, depends on circulating levels of corticosterone (Stanley, Lanthier, Chin & Leibowitz, 1986; Kalra, Dube & Kalra, 1988). Furthermore, NPY and NE in the PVN are both effective in

stimulating the release of this hormone (Leibowitz et al., 1988; Wahlestedt et al., 1987).

While these findings suggest that NPY and NE act though common neural substrates to stimulate feeding, other evidence indicates that their actions may be dissociable. Specifically, it was shown that the NPY-induced feeding response remains intact after administration of phentolamine, a general α-receptor blocker, which antagonizes the feeding response elicited by NE (Stanley & Leibowitz, 1985). Additionally, it was demonstrated that destruction of the A1 and A2 noradrenergic projections to the hypothalamus, using the CA neurotoxin 6-OHDA, fails to affect the NPY innervation to this structure (Gustafson & Moore, 1987), suggesting that hypothalamic NPY innervation arises predominantly from intrinsic NPY-producing neurons, rather than from medullary neurons where NE and NPY coexist. Thus, it is evident that NPY may not act simply through the release of endogenous NE to elicit feeding. Although some mode of interaction with this monoamine may occur, the nature of this interaction, the conditions under which it occurs, and its possible physiological significance, remain to be characterized.

C) Rationale

The present series of three experiments has two

specific objectives: First, to investigate the phenonemon of feeding elicited by central injection of GAL, and second, to relate GAL's effects on feeding to those elicited by the coexisting neurotransmitters, NE and NPY.

Experiment 1: Galanin's sites of action in the brain. Through tests with direct injection of GAL into the PVN, this peptide, like NE, has recently been found to potentiate feeding behavior. With this discovery, and the knowledge that NE acts primarily through the PVN as opposed to other hypothalamic areas, we asked whether GAL is similar to NE in having a focused site of action within the PVN, or whether it acts upon multiple sites within the hypothalamus or even outside this structure. This question provides the basis for the first experiment. This experiment investigated GAL's site(s) of action in the brain, to determine whether its effects are anatomically specific. It also examined the behavioral specificity of GAL's effect, at the brain site of greatest sensitivity.

To test for anatomical specificity, GAL was microinjected into several hypothalamic and extrahypothalamic sites, and its effects on food intake were measured. Brain areas tested included: 1) sites that have traditionally been implicated in the control of feeding (e.g. VMH, perifornical/lateral hypothalamus, amygdala); 2) sites that surround the PVN (e.g. nucleus reuniens, anterior hypothalamus) and thus may help to define the precise area

of sensitivity; 3) sites (e.g. nucleus accumbens, dorsomedial nucleus, ventral tegmental area) that exhibit high levels of GAL immunoreactivity and/or dense radioligand binding; and 4) sites within the PVN, namely, the parvicellular and magnocellular division, that have distinct cytoarchitecture and neuroanatomical connections. In addition to covering a broad range of brain areas in the rostro-caudal as well as medial-lateral dimension, this experiment tested two doses of GAL in each site, in order to reveal sufficient information to determine relative sensitivity of the different areas.

To test for behavioral specificity, this part of the experiment examined whether GAL's stimulatory effect on food intake occurs without simultaneous effects on other behaviors besides eating. For these tests, GAL was injected into the PVN, and the animals were observed for a variety of behaviors, namely, eating, drinking, grooming, rearing, resting, sleeping and low or high levels of activity.

Experiment 2: Pharmacological analysis of GAL-induced stimulation of feeding: comparisons with NE and NPY. This experiment focused on defining the nature of the interaction between GAL and NE. Since these two neurochemical substances are known to coexist within the PVN, and NE in this nucleus is known to be a potent stimulant of feeding, the possibility exists that GAL may act, in part, through the release of endogenous NE.

In this experiment, pharmacological tools were used to examine whether GAL's effect on feeding, similar to that of NE, is dependent upon intact α_2-receptors, and also whether GAL acts through the release of endogenous NE. Three receptor blockers and two synthesis inhibitors were tested. The receptor antagonists used were: phentolamine (PHT), a general α-blocker; rauwolscine (RAU), a specific blocker of the α_2-type receptors, and prazosin (PRA), a specific blocker of the α_1-type receptors. The CA-synthesis inhibitors used were: α-methyl-p-tyrosine (α-MpT), a tyrosine hydroxylase inhibitor which blocks the synthesis of dopamine, and Fla-63, a dopamine-β-hydroxylase inhibitor which more selectively blocks the synthesis of NE and epinephrine. The doses used were chosen based on pilot experimentation.

To compare with GAL, another peptide, NPY, was similarly tested in this experiment. Evidence indicates that NPY, similar to GAL, coexists with NE in the PVN, where it is also found to potentiate feeding. While there are few pharmacological and biochemical studies of GAL's actions on endogenous noradrenergic systems in the periphery or brain, extensive work with NPY in the periphery indicates that this peptide generally acts independently of α_2-noradrenergic receptors and endogenous NE stores, and actually produces an inhibition of NE release from presynaptic terminals (Hakanson, Wahlesdedt, Ekblad, Edvinsson & 1986). The

question is, does NPY in the hypothalamus have a similar mode of interaction with this CA in its stimulatory effect on feeding, and can this interaction, as revealed by the results with the pharmacological agents, be distinguished from that detected for GAL?

Experiment 3: Impact of GAL and NPY on PVN NE levels. Using an HPLC with ED, this experiment directly measured the impact of PVN administration of GAL on extracellular levels of NE in the PVN. For comparison, similar tests with NPY were also conducted. For these tests, we employed the microdialysis technique (Hernandez, Stanley & Hoebel, 1986), which allows brain neurotransmitters and their metabolites to be collected and measured in freely moving animals. The microdialysis probes used here have a 6000 molecular weight cut off, thus excluding the measurement of peptides which are composed of larger molecules.

In summary, these experiments investigate the functional interactions between three anatomically and physiologically related neurochemical substances, the peptides, GAL and NPY, and the amine, NE, in the PVN. These neurochemical mechanisms are believed to provide excellent model systems for studying, in localized brain areas, the precise nature of the peptide-amine interactions and their potential physiological function in the control of ingestive behavior and related endocrine and metabolic processes.

General Methods

Subjects. Subjects were adult, male, Sprague-Dawley albino rats (obtained from Charles River Breeding Labs). The male rat is considered the best animal model for the study of ingestive behavior since its natural patterns of food ingestion and its neural substrates of feeding have been extensively studied and characterized. Moreover, its pharmacological responses are found to be very similar to those observed in humans.

The subjects used in these experiments weighed between 350 and 400 g at the time of surgery. They were housed individually in standard wire-mesh stainless steel cages, measuring 43.2 x 22.9 x 19.1 cm, located in a temperature-controlled room (approximately 22°C). The animals were kept on a 12/12 hr light-dark cycle, with lights onset at 7.00 a.m.

Diets. Animals were maintained and tested either on Purina rat chow pellets (experiment 2, α-adrenergic receptor antagonists) or on a sweetened diet consisting of 46% Purina rat chow, 37% sucrose and 17% Carnation evaporated milk (in all other experiments). Food and water was available ad libitum.

While in the first part of the pharmacological experiment the animals were tested on pellets, the other experiments used the sweetened diet which has been shown to

have particular advantages for the study of ingestive behavior. Specifically, in addition to being nutrionally complete and ensuring proper growth and body weight gain, it is particularly palatable to the rats and easy to eat. Thus, this diet permits robust feeding responses and, consequently, minimizes the possibility of false negative results. Also, because of its texture, it minimizes spillage.

Surgery. Subjects were stereotaxically implanted with unilateral cannulae under Metophane anesthesia. Tail pinching was used by the experimenter to ensure that the animals were completely anesthetized before the operation started. The guide cannulae were constructed from 26-gauge, stainless steel tubing, cut to a length of 18.0 mm. They were targeted 1.0 mm dorsal to each brain region tested, to minimize damage to that structure. The cannula was fixed in place on top of the skull, using acrylic cement and six stainless steel machine screws that penetrated the bone. Cannula patency was maintained by a 33-gauge, stainless steel obturator ground flush with the guide cannula, which was kept in place at all times except during testing.

The animals were given ten days of post-operative recovery prior to testing, during which time they were acclimated to the testing conditions through frequent handling and mock-injections. The mock injections were similar in every respect with the drug-injections, except

that no drug was infused into the animal's brain. The advantage of this method was that it acclimated the animals to the testing conditions without the risk of tissue damage which may result from repeated infusion of drugs.

Drug Administration. Drug tests were conducted every 2-3 days, an interval found to permit full recovery from drug effects and yield the most stable test scores. The drugs were administered directly into the brain area, through a 33-gauge, 19-mm long injector which extended 1.0 mm beyond the guide cannula. Drugs were injected in solution using a 1-μl Hamilton syringe, in a relatively small volume of 0.3 μl to minimize tissue damage. They were infused over a period of 10 sec, while the injection volume was monitored by the movement of a small bubble through a polyethylene tubing which connected the injector to the syringe. Drugs were dissolved in sterile physiological (0.9%) saline (experiment 2, α-adrenergic receptor antagonists) or Ringer's solution (all other experiments). While saline has traditionally been used as a drug vehicle, Ringer's solution is more appropriate for intracerebrally injected drugs, since its composition (189 mM NaCl, 3.9 mM KCl, and 3.37 mM $CaCl_2$) is similar to that of the cerebrospinal fluid (CSF).

Tests were conducted during the light phase of the day/night cycle starting at approximately 1:00-2:00 pm. At this time, there is essentially no spontaneous feeding, thus, the conditions are ideal for the study of

neurochemical substances that stimulate feeding. Each rat had repeated tests, generally 3-7 tests/condition, and these test scores were averaged to reflect the rat's eating response. One hour prior to testing, the animals were given fresh food to ensure maximal satiety.

The following drugs were used: 1) The peptides, galanin (Peninsula) and neuropeptide Y (Peninsula); 2) The monoamine, l-norepinephrine-d-bitartarate (Sigma); 3) The relatively selective α-receptor blockers, phentolamine HCL (Geigy), rauwolscine HCL (Roth) and prazosin HCL (Pfizer); and 4) The CA-synthesis inhibitors, α-MpT methyl ester (Sigma) and Fla-63 (Astra).

Histology. When the behavioral testing ended, the animals were sacrificed with an overdose of Nembutal (100 mg/kg) and perfused intracardially with isotonic saline followed by 10% buffered formalin. Brains were removed and coronal sections of 100 μm were cut and stained with cresyl violet. The histological material was examined microscopically, to determine the locus of the cannula tip as well as the extent of damage created by implanting the cannula into the brain. From projections of the stained sections, the injection site (identified as a dark mark on the tissue approximately 1 mm ventral to the cannula tip) was drawn relative to nearby brain areas. The atlas of Konig and Klippel (1974) was used as a guide.

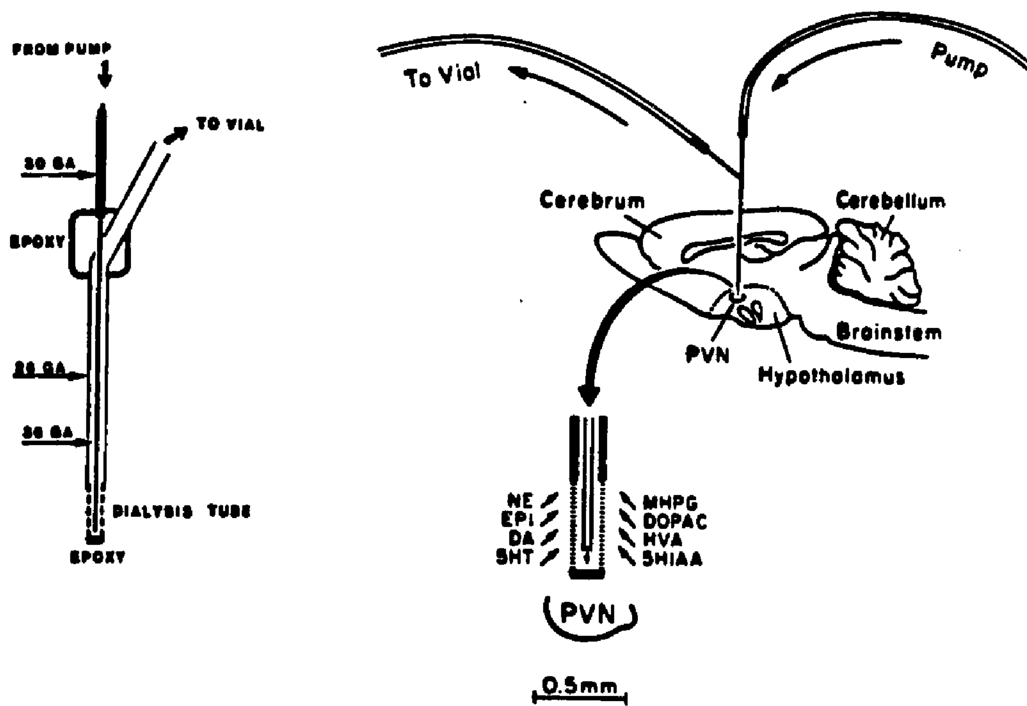
Microdialysis technique. The microdialysis probe,

illustrated in Fig. 3, is conceptually similar to a push-pull cannula, except that a hollow 200-μm diameter, semi-permeable membrane is glued to the tip of the probe that is inserted into the brain. This membrane prevents the artificial CSF from contacting the brain tissue, while allowing neurochemicals to pass through. As described by Hernandez and his colleagues (1986), the efficiency of absorption (ratio between concentration of neurochemicals outside the dialysis probe to the concentration in the dialysate) ranges between 6 and 9%, depending upon the specific neurochemical analyzed. This dialysis probe has several significant advantages over the loop-type dialysis probe developed by Ungerstedt (1982). The most important advantage is its small size, which allows it to be used in small brain areas, e.g. the PVN, without substantial tissue damage normally obtained with the loop-type probe.

The probe was constructed as described by Hernandez et al. (1986). It consisted of a 25-mm long, 26-gauge stainless steel tubing, with a 35 degree bend about 10 mm from the top. A small hole was made at the outside of the bend. Cellulose dialysis tubing, with an O.D. of 200 μm (Spectrum Co.), was glued to the inside of the outer tube so that 2.0 mm of the cellulose protrudes; the tip was then occluded with epoxy cement. The inner tubing was a 25-mm long stainless steel tubing, with an O.D. of 125 μm. This tubing was inserted into the hole at the bend on the outer tubing,

Figure 3. In the left panel is a diagram of a concentric microdialysis probe. Fluid flows down through a 36-gauge tube (reinforced with a 30-gauge tube) and up the 26-gauge tube. With the fluid come the neurochemicals that diffuse in through the dialysis tube, which is 0.2 mm wide and 2.0 mm long (dotted lines). The right panel illustrates the microdialysis probe implanted into the brain, in direct proportion to the PVN where neurotransmitter release is being measured.

Figure 3



until it almost touched the epoxy at the occluded end on the dialysis tube. For protection, a piece of 30-gauge stainless steel tubing was soldered onto the inner tubing where it projected out of the bend. The hole was sealed and tubes fixed together with epoxy putty.

Perfusion technique. Prior to use, Ringer's solution was filtered through a 0.22 μm filter and degassed under a vacuum for 30 min. A pump-driven, gas-tight 2.5 ml syringe perfused Ringer's solution through the probe via polyethylene tubing (PE-20) at a flow rate of 2 μl/min. The dialysate flowed into the probe and then out through PE-10 tubing, to a 400-μl microcentrifuge vial clipped to the PE-20 tubing. The probe, which was inserted directly into the guide cannula, was connected via a counterbalanced swivel arm at the top of the experimental chamber to the syringe pump. This allowed the subjects unrestricted movement throughout the entire experiment. After passing through the probe, the dialysate containing the neurochemicals to be analyzed, passed through a short piece of PE-10 polyethylene tubing to the collection vial, which was attached by a clip to the top of the animal's headpiece. These vials, 250 μl microcentrifuge tubes, could be removed and replaced with minimal effect on the subjects' ongoing behaviors. Consecutive 20-min (40 μl) samples were collected. Once removed, the vials were kept on ice (usually less than 10 min.) until the dialysate sample was injected into the HPLC

(discussed below). To inject, the contents were drawn into a 100-μl Hamilton syringe and then injected directly into the injection port of the HPLC.

HPLC technique. The HPLC used consisted of a BAS 400 with dual LC-4B amperometric detectors and dual glassy carbon electrodes, a temperature controller (30 C), and a phase 11, ODS, 3 μm, 3.2 mm bore column. The mobile phase contained 116.8 mM NaOH, 144.7 mM monochloroacetic acid, 100 μm EDTA, 1.38 mM 1-octaneosulfonic acid and 2% v/v acetonitrile at pH 3.1 and was pumped through the system at a rate of 1.0 ml/min. For detection of the neurochemicals, the electrode potential was set at 710 mV, and output current at 1 nA was set to cause a full scale pen deflection on the dual pen chart recorder.

Standards containing 50 pg/20 μl of NE were injected at the beginning of each test session. Norepinephrine eluted with a retention time of 1.8 min. Its minimum detectable amount, with a signal to noise ratio of 4:1, was approximately 1 pg (6 fmoles). The main criterion used for concluding that the unknown peak was NE, was its coelution with the standard NE. An additional criterion was obtained from a split sample analysis. At the end of several experimental sessions, a sample was collected and split in half. One half was analyzed as it was, and the other half was analyzed after adding a small amount of NE from the standards (7.5 pg/3 μl). Then the peaks from the two

samples, having been identified as NE individually, were directly compared to each other to ensure that they coeluted.

Statistics. All experiments were designed for within-group comparisons. Data were analyzed either with one-way ANOVA or with a repeated measures ANOVA, followed by Duncan's multiple range test for comparisons across the different groups. For comparisons between two treatments of the same group, a students' t-test for dependent means was used.

Experiment 1. Galanin's site(s) of action in the brain

Research over the past decade with central microinjections of putative neurotransmitters has revealed dramatic effects of various peptides on eating behavior. While in most cases these peptides are found to have an inhibitory effect on food intake, an excitatory response has been reported for two peptide families, namely, the opioid peptides (for review, see Levine et al., 1985) and pancreatic polypeptides (Clark et al., 1984; Stanley & Leibowitz, 1984). Recently, the neuropeptide GAL has also been found to stimulate food intake (Kyrkouli et al., 1986).

Galanin is widely distributed in the gut and brain. It has its highest concentrations, however, in the hypothalamus and, in particular, the PVN (Skofitsch & Jacobowitz, 1985; Ch'ng et al., 1985) where GAL receptor binding sites have also been found (Melander et al., 1987). Injections of GAL directly into the PVN elicit a strong feeding response in a dose-dependent manner (Kyrkouli et al., 1986). In a preliminary mapping study, GAL was found to produce no effect after injection into the periphery or into a site 1 mm dorsal to the PVN (nucleus reuniens) or lateral to this nucleus (perifornical hypothalamus). This suggested that GAL acts within the brain and perhaps in a specific area to stimulate food intake.

The galaninergic innervation to the PVN has been shown

to originate from brainstem catecholaminergic neurons (A1 and A6 cell groups), where this peptide coexists with NE (Levin et al., 1987; Holets et al., 1988). This CA has been found to potentiate feeding by acting primarily within the PVN, as opposed to other hypothalamic or extra-hypothalamic areas (Leibowitz, 1978). In light of the discovery that GAL, like NE, stimulates food intake after direct administration into the PVN, the present experiment was designed to investigate whether GAL is similar to NE in having a focused site of action within this nucleus, or whether it acts upon multiple sites within the hypothalamus or even outside this structure. In addition to determining whether GAL's effects on feeding are anatomically specific, this experiment also investigates whether these effects are behaviorally specific at the brain site of greatest sensitivity.

Methods

Subjects and Surgery. Ninety eight male, Sprague-Dawley rats were used in this experiment. Rats were implanted with unilateral cannulae aimed at one of 14 brain areas tested. Table 1 lists these sites and their respective coordinates. There were between 3 and 16 animals with cannulae aimed at each injection site.

Procedures. One hour prior to testing, subjects were given fresh food to ensure maximal satiety. Subsequently,

Table 1

Stereotaxic coordinates for each injection site

N	Site	A/P	L	V
Hypothalamic:				
7	Anterior hypoth. (AH)	A 7.5	0.7	8.2
16	Paraventricular nu. (PVN)	A 6.8	0.4	7.4
4	Periventricular area (PVS)	A 6.8	0.4	7.9
4	Perifornical hypoth. (PFH)	A 6.8	1.3	7.4
8	Dorsomedial nucleus (DMN)	P_β 2.6	0.5	8.3
4	Ventromedial nucleus (VMH)	P_β 2.6	0.5	9.0
6	Posterior hypoth.(PH)	A 4.6	0.4	7.6
Extrahypothalamic:				
3	Nucleus accumbens (ACB)	A 9.6	1.3	7.1
8	Diagonal band (DB)	A 8.5	0.8	7.9
9	Nucleus reuniens (NR)	A 6.8	0.4	5.9
3	Amygdala (AMYG)	A 5.8	3.5	8.2
4	Ventral tegment. area (VTA)	A 3.2	0.8	8.0
15	Third ventricle (V III)	A 7.1	0.0	7.8
4	Fourth ventricle (V IV)	P 3.1	0.0	6.7

The incisor bar was 2.5 mm below the interaural line. Abbreviations: A/P, anterior or posterior to interaural line; P_β, posterior to bregma; L, lateral to midsagital sinus; V, ventral to skull surface.

they were injected with GAL (0.1 or 0.3 nmol/0.3 μl vehicle) or its vehicle (Ringer's solution), and food intake was measured 1 hr postinjection. The tests were conducted at approximately 2:00 to 3:00 p.m. during the day cycle. The drug doses and vehicle were administered in a counterbalanced order (ABBA).

Behavioral Testing. During the one hour test period following injection of GAL (0.1 nmol) or vehicle, the rats with cannnulae aimed at the PVN, at 2-min intervals, were observed for a variety of behaviors. These included: 1) eating; 2) drinking; 3) grooming; 4) sleeping; 5) resting (lying immobile with eyes open); 6) low levels of activity (upper body movements); or 7) high levels of activity (locomotion). Two observers, with similar training in scoring these behaviors, were used for this behavioral analysis.

Statistics. The drug data were analyzed by repeated measures ANOVA, with comparisons to the control group made by Duncan's multiple range test. The behavioral observations were analyzed by Student's t-test for dependent means. P was less than 0.05 or 0.01.

Results

The results in Figs. 4, 5 and 6 demonstrate that the PVN is the most responsive hypothalamic site to GAL

Figure 4. Food intake (mean ± S.E.M.) by satiated rats 1 hour after injection of GAL (0.1 or 0.3 nmol) into one of several hypothalamic sites (For abbreviations, see Table 1). *$p<0.05$, **$p<0.01$ relative to vehicle score of 0.2-0.7 g, by Duncan's Multiple Range Test.

Figure 5. Food intake (mean ± S.E.M.) by satiated rats 1 hour following injection of GAL (0.1 or 0.3 nmol) into one of several extra-hypothalamic sites (For abbreviations, see Table 1). *$p<0.05$, **$p<0.01$ relative to vehicle by Duncan's Multiple Range Test.

Figure 6. Schematic representation of central injection sites for GAL and its stimulatory effect on feeding behavior in satiated rats. Each site is represented by a circle with a number indicating the magnitude of the average feeding response (to the nearest gram) after an injection with GAL. Saggital sections and abbreviations are derived from the Pellegrino, Pellegrino & Cushman (1979) atlas of the rat brain.

EFFECTS OF GALANIN
IN DIFFERENT HYPOTHALAMIC SITES

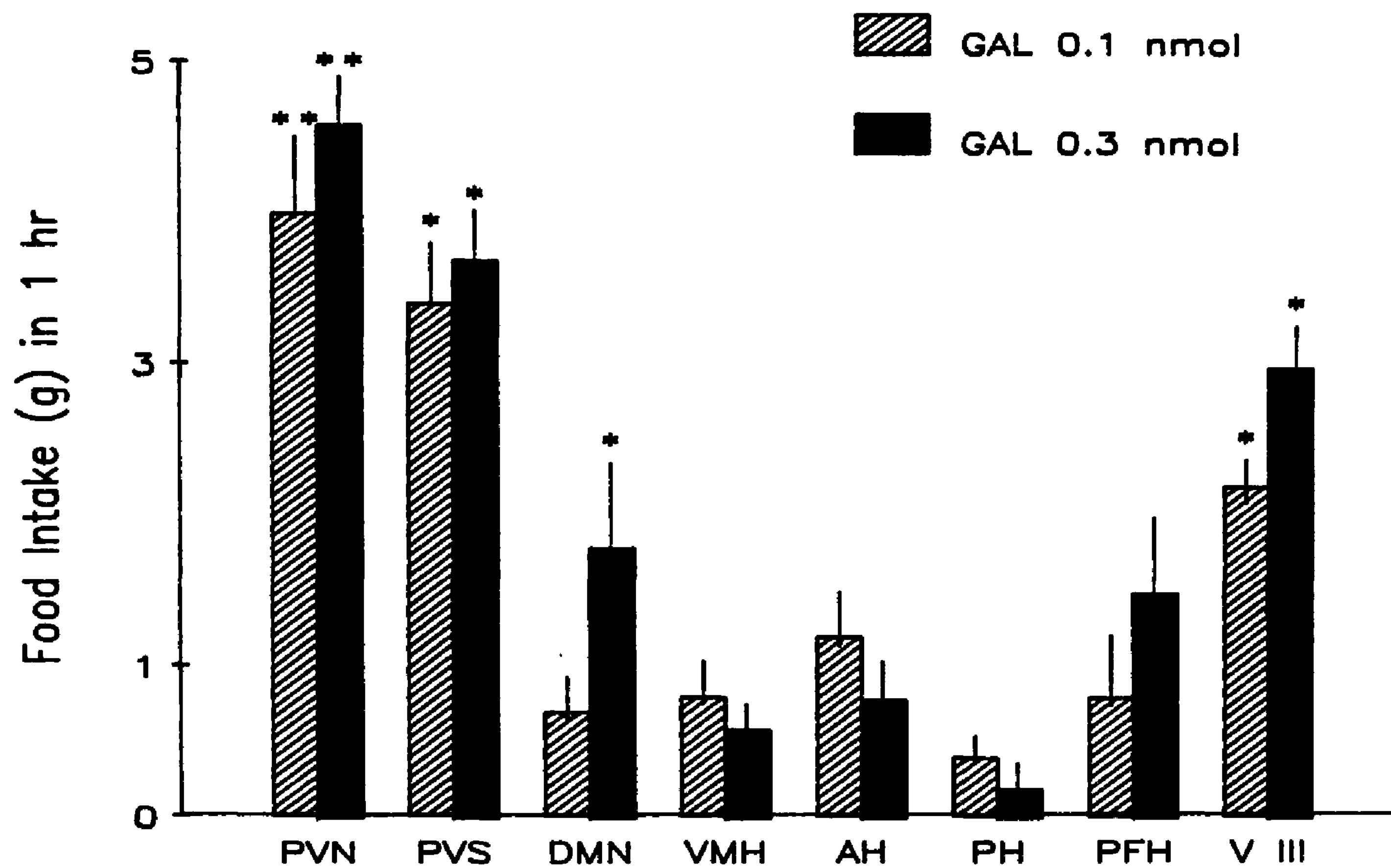


Figure 4

EFFECTS OF GALANIN
IN EXTRA-HYPOTHALAMIC SITES

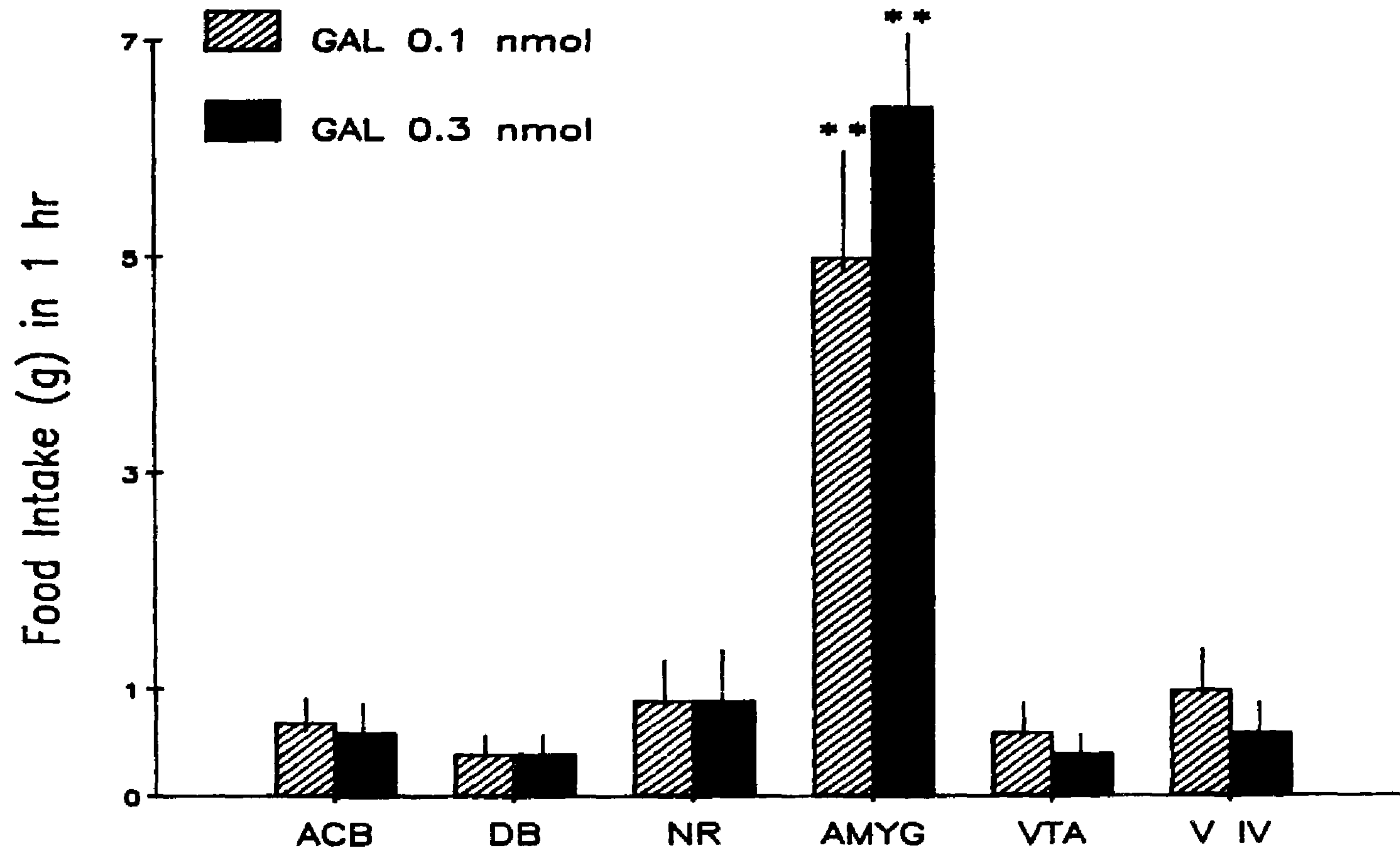
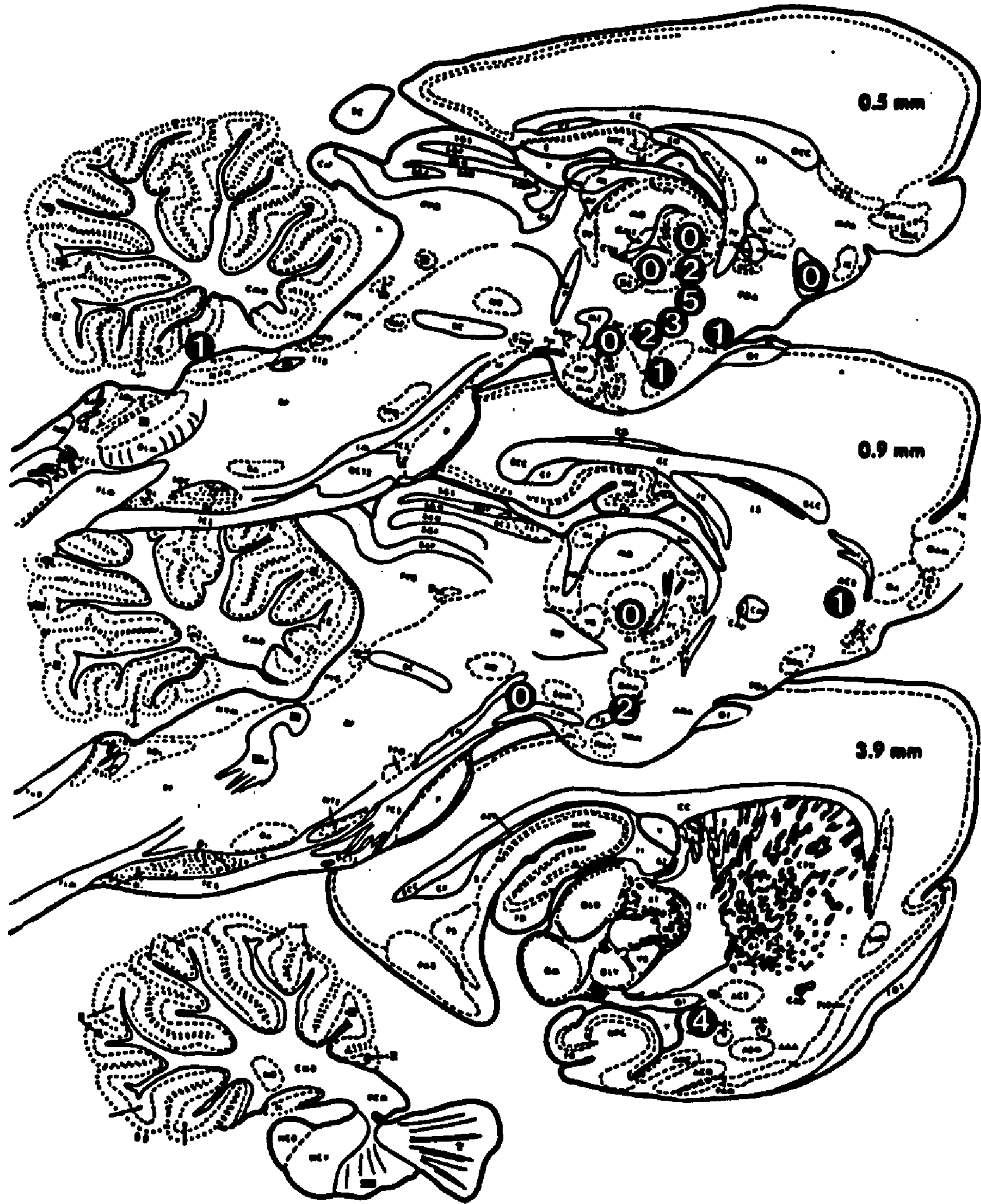


Figure 5

Figure 6



administration. Consistent with our previous findings (Kyrkouli et al., 1986), GAL injected into this nucleus reliably stimulated eating [$F(2,45)= 36.3$, $p<0.001$]. The average feeding responses were 4.0 g (0.1 nmol) and 4.6 g (0.3 nmol), and the scores of the individual rats (n=16) ranged from 2.9 to 7.9 g, relative to vehicle baseline scores of 0.2 to 0.7 g. These baseline scores were comparable in magnitude in all brain areas tested. All animals in this group had an injection site located within the borders of the PVN according to the Konig and Klippel atlas (1974). This area extended between A 5910 and A 5150 μ in the rostral-caudal dimension and L 0.2 to L 0.6 μ in the medial-lateral dimension. The PVS injection site, which is located within approximately 0.2 mm lateral to the V III both within the PVN and up to 1.2 mm ventral to the ventral border of this nucleus, was found to be similarly responsive to local administration of GAL [$F(2,9)= 4.9$, $p<0.05$]. An average feeding score of 3.7 g was obtained with 0.3 nmol of GAL.

In contrast to the PVN and PVS, other medial hypothalamic nuclei, namely, the DMN, AH, VMH and PH, were generally unresponsive to either dose of GAL (Figs 4 and 6). While the VMH, AH and PH yielded no eating in response to local GAL injection, the DMN, located approximately 0.3 mm posterior to the caudal border of the PVN, produced a small response of 1.8 g ($p < 0.05$) at the highest GAL dose of 0.3

nmol. The animals included in this group had cannulae terminating between A 4890 and A 4110 μ. In the lateral hypothalamus, specifically in the area of the fornix (PFH), GAL was similarly without effect, producing a non-significant response of 1.5 g. This site lies approximately 1.0 mm lateral to the lateral border of the PVN.

With one exception, the extrahypothalamic sites tested, including the ACB, DB, VTA, and NR, were each found to be unresponsive to GAL administration (Figs 5 and 6). The cannulae of the animals which were included in each of these groups terminated as follows: ACB, between A 9410 and A 8380 μ; DB, between A 8620 and A 7470 μ; VTA between A 2180 and A 1760 μ; and, NR between A 5910 and A 5150 μ, between 0.3 and 1.5 mm dorsal to the dorsal border of the PVN.

As figures 5 and 6 demonstrate, the only extrahypothalamic site that yielded a significant effect was the AMYG, where GAL produced strong feeding responses of 5.0 and 6.4 g at the two doses tested [$F(2,6)= 17.1$, $p<0.01$]. Histological analysis revealed that two of the three cannulae terminated in the medial amygdaloid nucleus (Fig. 7), which yielded maximum scores of 4.8 and 6.5 g. A third was located in the basal nucleus, pars lateralis, yielding a slightly stronger response of 8.4 g.

In light of the responsiveness of the PVN and PVS, it is not surprising that the V III also yielded a strong effect of 3.0 g (Fig. 4). This injection site was located

Figure 7. Photomicrograph of a frontal section of the rat brain showing the injection site for an animal with a cannula aimed at the medial amygdaloid nucleus.

Figure 7

in the ventricle approximately 0.3 mm anterior to the rostral border of the PVN. This response to V III injection contrasts to the lack of effect of GAL in the V IV (Figs. 5 and 6), which would be expected to spread caudally and minimally into the rostral hypothalamus.

These results clearly distinguished the PVN and PVS as being most sensitive to GAL's stimulatory effect on food intake. In light of these findings, a more detailed histological analysis was conducted on the injection sites in these two nuclei, in relation to the individual rats' responses to GAL administration. As described above, animals with cannulae in these two medial sites had mean scores of 4.6 g (PVN) and 3.7 g (PVS) after GAL (0.3 nmol) administration. In Fig. 8, brain photomicrographs (frontal sections) are presented showing the injection sites for 8 of the rats with cannulae terminating in or around the PVN. Figure 9 contains a diagrammatic representation of these injection sites and their respective behavioral sensitivity to GAL.

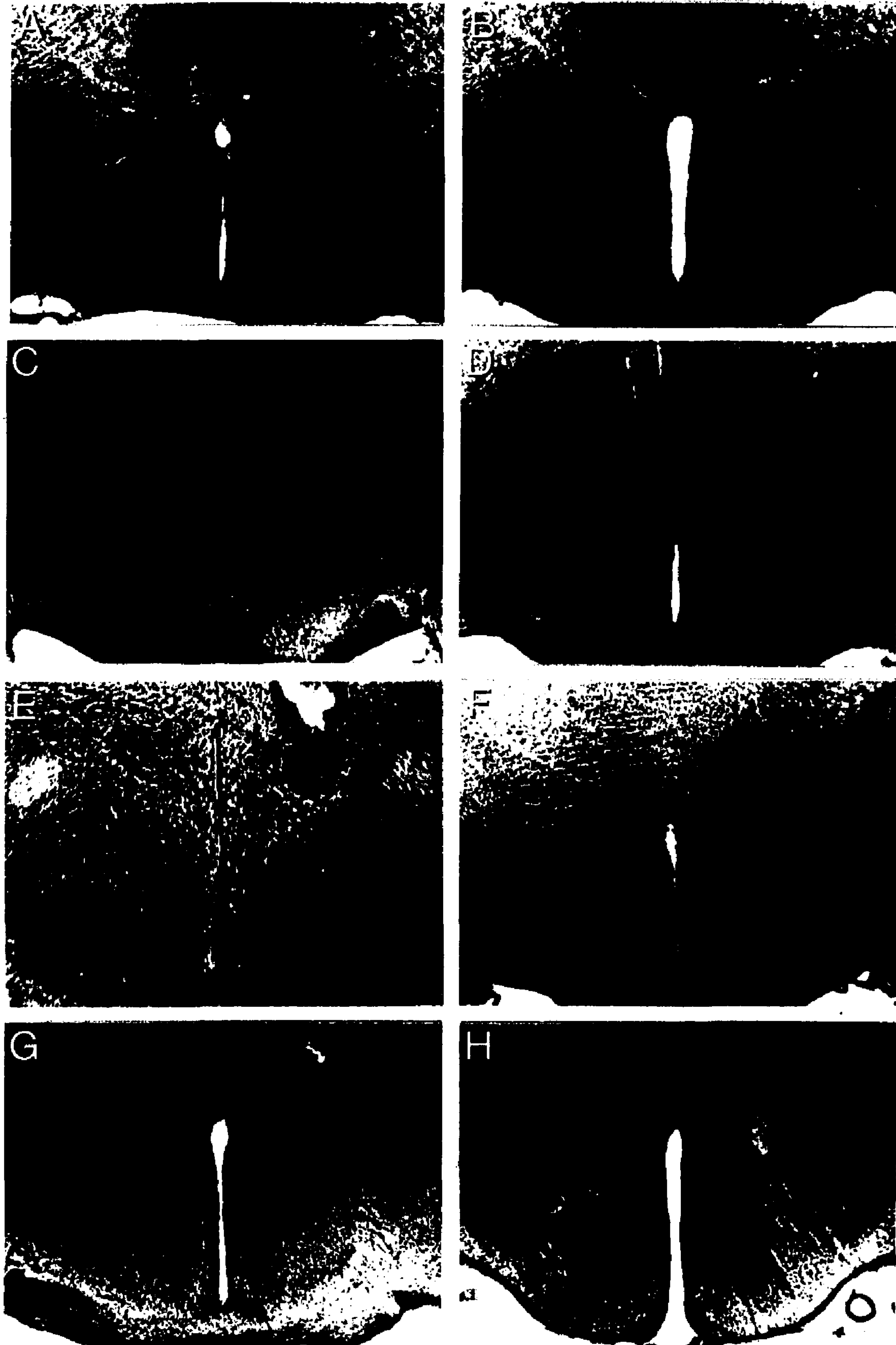
As illustrated in these figures, food intake was greatest (5.6±0.6 g) when the GAL injection site was in the parvicellular part of the medial PVN (A 5660 μ, n=6), 0.2 to 0.3 mm lateral to the V III (Fig. 8, D). This response decreased, however, as the injection site moved away from this area in every direction, namely, anterior (Fig. 8, A and B), posterior (H), lateral (G), dorsal (C) and ventral

Figure 8. Photomicrographs of frontal sections of the rat brain, showing representative injection sites (indicated by arrows) for animals with cannulas aimed at hypothalamic sites at the level of or near the PVN. The sites and the respective feeding responses after injection of GAL (0.3 nmol) are: A, just anterior to the rostral border of the PVN (A 6360μ), 0.5 g; B, at the rostral border of the PVN (A 6060μ), 3.1 g; C, just dorsal to the PVN (A 5660μ), 0.6 g; D, within the parvicellular portion of the PVN (A 5660 μ), 5.9 g; E, within the magnocellular portion of the PVN (A 5660), 1.9 g; F, ventral to the PVN (A 5660 μ), 2.9 g; G, dorsolateral to the PVN (A 5340), 0.5 g; and H, in the DMN (4110 μ), 0.5 g.

Figure 9. Frontal diagrams of the rat brain based on the atlas of Konig & Klippel (1974), illustrating average food intake (in grams) after injection of GAL (0.3 nmol) into several hypothalamic sites in and around the borders of the PVN.

Figure 10. Percentage of time (mean ± S.E.M.) exhibiting eating, drinking, grooming, rearing, sleeping, resting, and low or high levels of activity by rats given PVN injections of GAL (0.1 nmol) or vehicle. * $p<0.05$, ** $p<0.01$ relative to vehicle by t-test for dependent means.

Figure 8



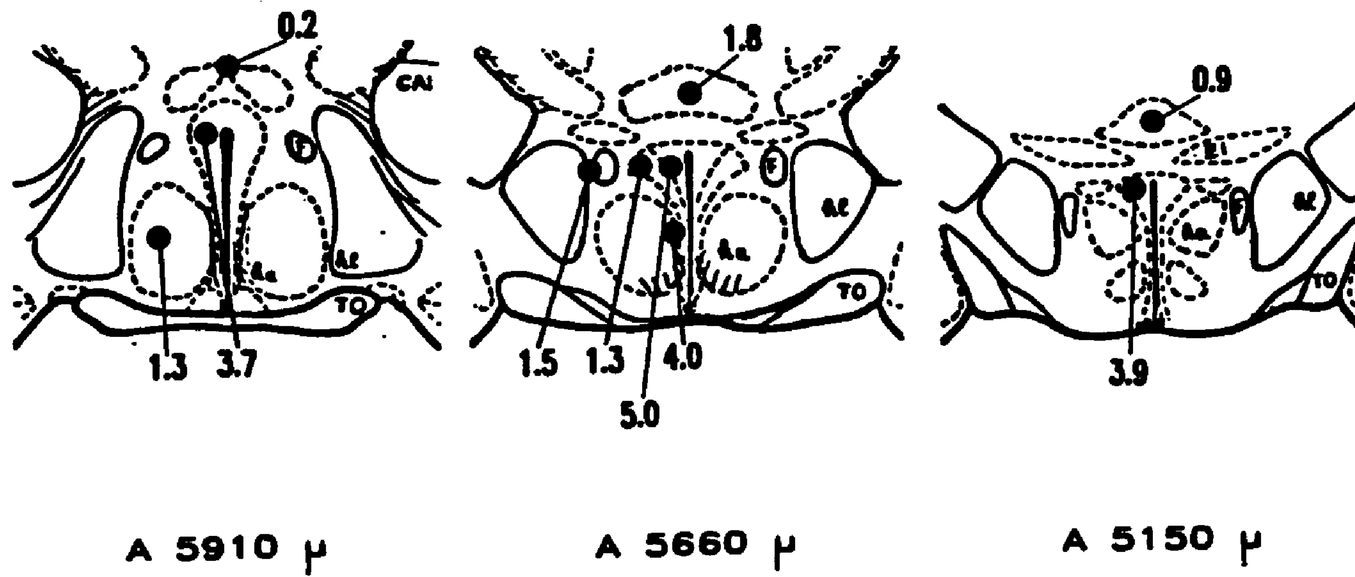


Figure 9

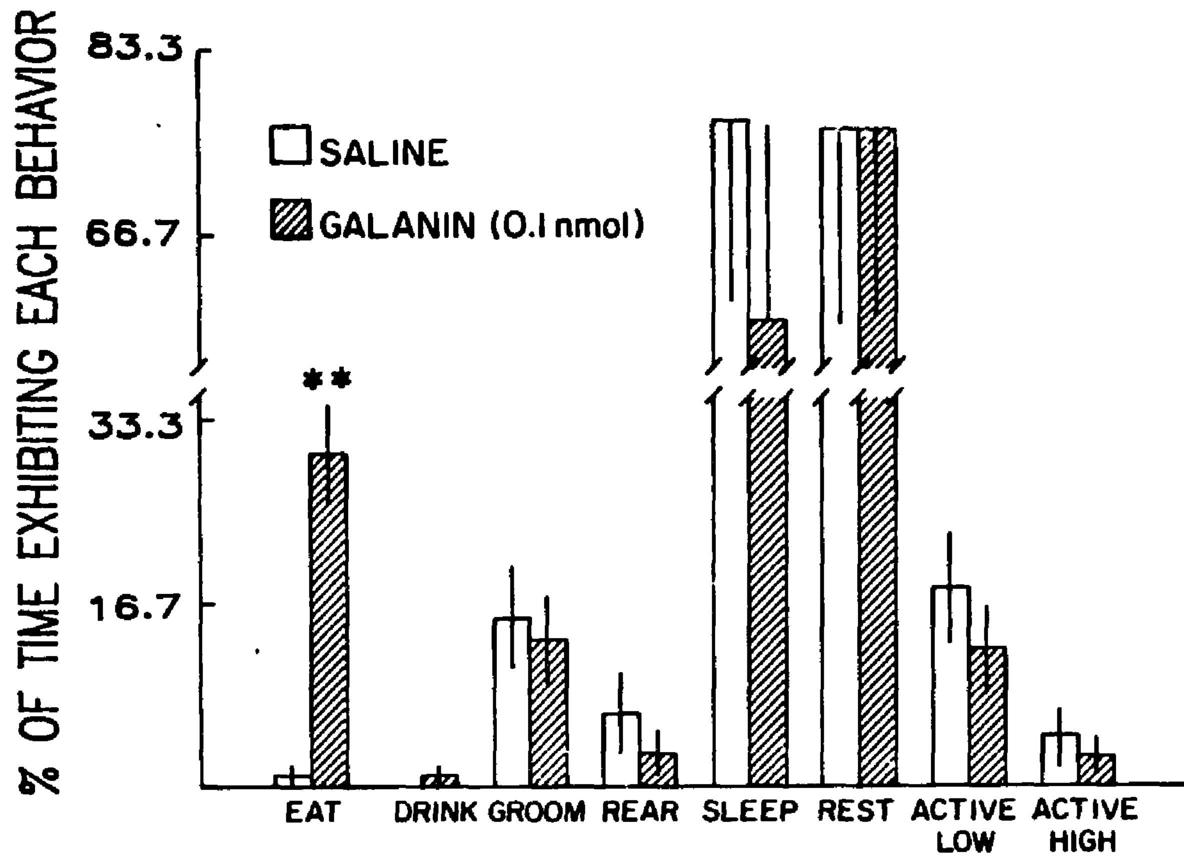


Figure 10

(F). It appeared smaller (4.0 g) as the injection site moved up to 1.0 mm ventrally, into the area of the PVS. It was drastically reduced, to 1.3 g, with GAL administration into the magnocellular (lateral) PVN (n=4) located, 0.5 to 0.6 mm lateral to the V III and approximately 0.3 mm lateral to the parvicellular portion of this nucleus (E). The anterior (n=3) and posterior PVN (n=3) produced a similar, fairly strong response of 3.7 g (A 5910 μ) and 3.9 g (A 5150 μ), respectively. Feeding was also considerably attenuated or lost when the injection site moved into the nucleus reuniens (n=4), which lies approximately 0.5 mm dorsal to the PVN. Precisely at the level of the PVN, where the strongest eating response was observed (5.6 g), the nucleus reuniens injection site yielded a small, non-significant response of 1.8 g (n=4). There was no response whatsoever as this dorsal cannula moved approximately 0.4 mm either anteriorly (n=2) or posteriorly (n=3).

Figure 10 illustrates the percentage of time that the animals spent engaging in various behaviors following an injection with GAL (0.1 nmol) or its vehicle into the PVN. As shown in this figure, the eating response elicited by GAL was the only response reliably affected by this peptide. Detailed analysis of this eating response showed its average latency to be approximately 6 min, ranging from 1 to 15 min. Galanin specifically increased the percentage of time spent eating, from less than 1 min after vehicle as compared to

almost 9 min after GAL ($t=7.8$, $p<0.01$). This response appeared to occur independently of changes in any other behaviors, namely, drinking, grooming, rearing, sleeping, resting, low and high levels of activity.

Discussion

These results demonstrate that the food intake induced by central injection of GAL is anatomically localized and behaviorally specific. Within the hypothalamus, only the PVN and the closely situated PVS yielded a significant eating response to GAL. This response was not detected in nearby medial or lateral hypothalamic sites, nor was it seen in extrahypothalamic areas, with the exception of the AMYG. With injection into the PVN, feeding behavior was observed without apparent changes in other food-associated behaviors.

Anatomical specificity. Detailed histological analysis revealed that the most sensitive tissue to GAL's stimulatory effect on feeding lies within the borders of the PVN, as opposed to along its lateral, ventral or caudal edges. Specifically, it distinguished the parvicellular portion of this nucleus as the area which yields the strongest feeding response of 5.6 g. More ventral and lateral sites produced smaller and more variable responses. A smaller response of 4.0 g was observed from the closely situated PVS. A considerably weaker effect of 1.8 g was observed in the DMN

localized 0.7 mm posterior to the middle part of the PVN. The reduced sensitivity of the PVS and the DMN may reflect either a reduced number of GAL receptor sites situated at these sites or the spread of GAL from the injection site into the PVN where GAL sensitivity is greatest. While a definitive choice between these two alternatives may not be made with certainty at this point, the latter possibility appears likely since GAL could have easily diffused, perhaps via the V III, from either the PVS or DMN into the PVN itself. The insensitivity of the VMH, however, which lies approximately 0.3 mm ventral to the ventral border of the DMN and 0.7 mm caudal to the middle part of the PVN, clearly marks the limit of this diffusion. In the medial-lateral axis, the spread of GAL appears to be even more limited or non-existent. This is indicated by the finding that the magnocellular portion of the PVN, localized approximately 0.2-0.3 mm lateral to the parvicellular part, yields only a small response of 1.3 g. Similarly, a response of 1.5 g was seen in the PFH, around 1.0 mm lateral to the middle part of the PVN.

Anatomical studies (Levin et al., 1987; Melander et al., 1986; Skofitsch & Jacobowitz, 1985) have demonstrated an intense innervation of the PVN and PVS by GAL-immunoreactive fibers, which may provide the physiological substrate for feeding induced by this peptide. A major portion of the galaninergic fibers to the PVN originate in

the pontine locus coeruleus (A6) and medullary (A1) noradrenergic cell groups (Levin et al., 1987; Holets et al., 1988). These cells innervate the parvicellular division of the PVN, and in particular its medial part, a region associated with autonomic-related outputs of the nucleus (Swanson & Sawchenko, 1983). These GAL-containing fibers, in contrast, do not project to the magnocellular division of the PVN. These findings are consistent with the behavioral results of the present study, showing that the parvicellular division of the PVN is uniquely sensitive to GAL's stimulatory effect on feeding, in contrast to the magnocellular PVN where responsiveness to GAL is considerably reduced. Consistent with this evidence is the finding of receptor binding studies, showing that galaninergic receptors also exist within the PVN (Melander et al., 1987). However, since other hypothalamic nuclei, including the DMN and VMN, also receive dense GAL innervation and contain GAL receptors, the unique responsiveness of the PVN suggests that the cells within this structure that are acted upon by GAL, are specifically involved in controlling the feeding response.

The efferent projections of this neurocircuit have not yet been revealed. It is interesting, however, that a dense population of GAL-containing cells in the adjoining magnocellular division of the PVN descends towards the midbrain periventricular grey (Gray & Magnuson, 1987), along

a course similar to that identified for the efferent projection of the noradrenergic feeding stimulatory system (Weiss & Leibowitz, 1985).

That neurons within the PVN provide the neural substrates mediating GAL's stimulatory effect on feeding is also supported by a recent electrolytic lesion study in our laboratory (Kyrkouli et al., 1989). Results from this investigation indicate that the destruction of this nucleus, and in particular its parvicellular division, attenuates or abolishes the eating response produced by V III GAL administration. Likewise, electrolytic lesions of the PVN have been shown to disturb the animals' response to NE (Leibowitz, et al., 1983).

Most intriguing was the finding of this study that GAL injected into the AMYG resulted in a strong feeding response. This unexpected finding deserves some discussion in light of evidence implicating this structure in the control of food intake (Wyrwicka, 1988). Anatomical studies have reported particularly high concentrations of GAL-immunoreactive neurons in the AMYG (Rokaeus et al., 1984; Melander, Hokfelt & Rokaeus, 1986), as well as ^{125}I-GAL binding sites (Skofitsch, Sills & Jacobowitz, 1986), which may provide the neural substrates for the effects observed in this study. Moreover, a GAL pathway, originating from the central amygdaloid nucleus and terminating in the midbrain periaquaductal gray, has also been revealed (Gray &

Magnuson, 1987).

This relatively localized effect observed with GAL injection stands in contrast to the more anatomically diffuse response detected with NPY (Stanley et al., 1985) and the opioid peptides (Levine et al., 1985; Stanley, Lanthier & Leibowitz, 1989). Specifically, NPY appears to be generally effective in multiple hypothalamic areas, including the PVN, VMH and LH, although it is generally ineffective in extrahypothalamic sites. Similarly, with opiate agonists, brain-cannula mapping studies have revealed strong feeding responses with injection into several hypothalamic, as well as extrahypothalamic, sites. While these results clearly distinguish GAL, as a peptide with a relatively localized site of action, the basis for this distinction remains to be determined.

Behavioral specificity. Behavioral analysis of PVN injections of GAL revealed that this peptide selectively enhanced feeding without affecting any other behaviors. Following the meal induced by GAL administration, the animals engaged in a sequence of behaviors, including, drinking, grooming, rearing, low levels of activity, resting and sleeping, which have been characterized as the normal postprandial satiety sequence (Antin, Gibbs, Holt, Young & Smith, 1975). These findings strongly suggest that GAL in the PVN elicits feeding in a primary and specific manner.

The failure of GAL to elicit drinking behavior is in

contrast to the effects of NE (Leibowitz, 1975a, b) as well as NPY (Stanley et al., 1985), both of which have previously been shown to induce a small but significant increase in this behavior. While this difference between these neurotransmitters remains to be explained, the available evidence allows one to differentiate the drinking response after NPY and NE from their stimulatory action on feeding. Specifically, the drinking response induced by NE is thought to be mediated by both α- and β-adrenergic receptors, while the eating response involves only α-receptors. Additionally, the latency to drink is shorter (< 1 min) than the latency to eat (1-3 min) (Leibowitz, 1975). Similarly, NPY's action on drinking may be anatomically distinguished from its action on feeding, since this peptide failed to elicit food intake after administration into the medial preoptic area as well as lateral hypothalamus, both of which were sensitive to its feeding stimulatory effects.

Possible relation between GAL and NE. Evidence suggests that to stimulate feeding, GAL may interact with the PVN noradrenergic system. Specifically, GAL has been shown to coexist with NE in the PVN as well as in brainstem noradrenergic neurons innervating this nucleus (Levin et al., 1987), which is particularly rich in GAL immunoreactivity as well as receptor binding sites for this peptide (Melander et al., 1987). In addition to being GAL's primary site of action, the PVN has also been found in

extensive brain-cannula mapping studies to be exceptionally responsive to NE's stimulatory effect on feeding as well (Leibowitz, 1978; Mattheus, Booth & Stolerman, 1978). In these studies, NE, similar to GAL, was found to be most effective in the parvicellular division of the PVN (Leibowitz, 1978), with the amount eaten increasing and the latency to eat decreasing with greater proximity of the injection site to the middle portion of the PVN. These findings, together with the mapping results of this report, support the possibility that GAL interacts with NE in the PVN to elicit feeding.

Experiment 2. Pharmacological analysis of GAL-induced stimulation of feeding behavior: comparisons with NE and NPY

Evidence suggests that GAL may stimulate feeding by interacting with NE in the PVN. To directly test this hypothesis, the present study examined whether the feeding response induced by GAL in the PVN depends upon intact α_2-receptors, similar to that of NE (Goldman et al., 1985; Schlemmer, Elder, Casper & Davis, 1981; Yee et al., 1987). Further, to examine whether GAL's effect depends upon the release of endogenous NE, this experiment also studied the impact of CA-synthesis inhibitors on GAL-induced feeding.

Similar to GAL, the peptide NPY is found to coexist with NE in the PVN (Everitt et al., 1984; Sawchenko et al., 1985; Holets et al., 1988), as well as induce a strong feeding response in satiated rats (Stanley & Leibowitz, 1984). To understand the relation of this peptide to NE, as well as to compare its action with that of GAL, this experiment also examined the impact of α-receptor antagonists and CA-synthesis inhibitors on the feeding response induced by PVN NPY injection.

Methods

Subjects and Surgery. Adult male Sprague-Dawley rats were implanted with unilateral cannulae aimed at the PVN.

With the incisor bar at -2.5, the stereotaxic coordinates were: 6.8 mm anterior to the interaural line, 0.4 mm lateral to the midsaggital sinus, and 7.4 mm dorsal to skull surface. Subjects were maintained and tested on pellets (α-adrenergic receptor antagonists) or sweetened mash diet (CA-synthesis inhibitors).

Drugs. The drugs used in these experiments were: 1. The agonists GAL (0.3 nmol), NE (6.3 nmol), and NPY (78 pmol); 2. The α-noradrenergic antagonists PHT (30, 60, and 120 nmol), RAU (2.6, 26.0, and 260 pmol), and PRA (0.5, 5.0, and 50 nmol); 3. The tyrosine hydroxylase inhibitor α-MpT (0.5, 5.0, 25, and 50 nmol) and the dopamine-β-hydroxylase inhibitor Fla-63 (0.05, 0.5, and 50 nmol). Most drugs were dissolved in bacteriostatic saline, water, or Ringer's solution. Rauwolscine was dissolved in 0.1% sodium bisulfate and Fla-63 in 10% tartaric acid solution. Galanin, NE, NPY, and Fla-63 were injected in a volume of 0.3 μl, while the other drugs in 0.5 μl. To enhance solubility, some of the drugs were gently heated and/or sonicated.

Procedures. One hour prior to testing, animals were given freshly prepared food to minimize possible spontaneous eating during the testing period. Subsequently, they were injected with one of the antagonists, and food intake was measured 30 min later to determine whether these agents had any effects of their own on feeding behavior. Galanin, NE, NPY or their respective vehicles were injected 30 min

following administration of the receptor blockers or 3 hrs after the CA-synthesis inhibitors. Food intake was then measured 1 hr later. With one exception, each animal was tested with the different agonists but only one of the antagonists, at all doses indicated. The exception was PRA, which, to maximize the reliability of the results, was administered to the same animals that received tests with RAU.

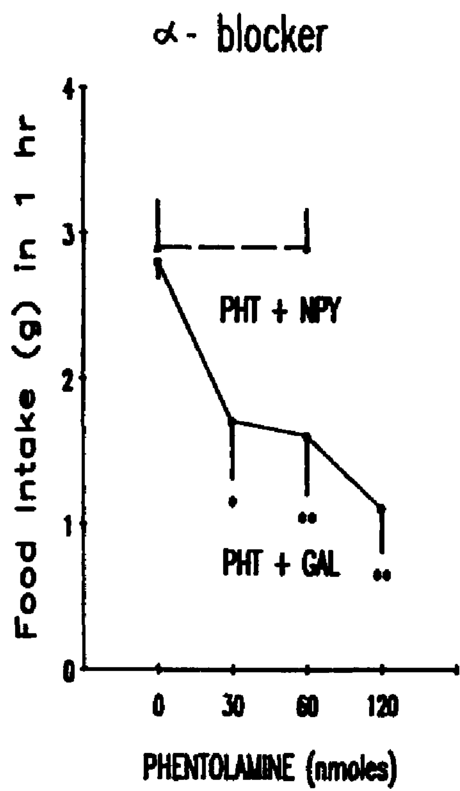
Statistics. The data were analyzed by one way ANOVA, with comparisons to the control group made by Duncan's multiple range test. P value was less than 0.05.

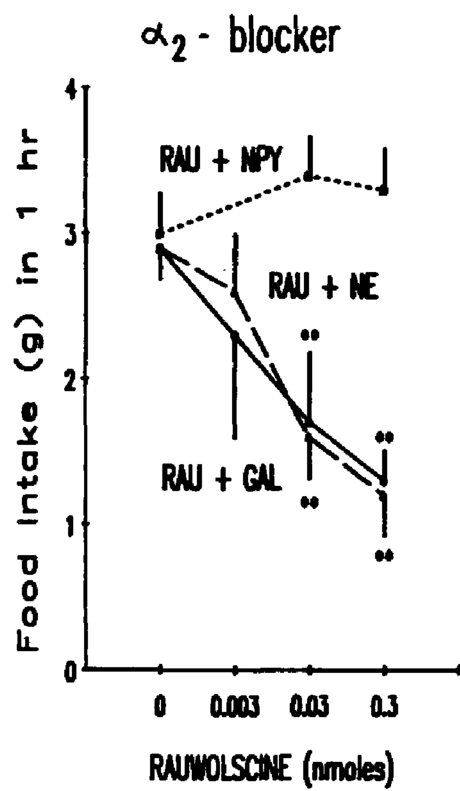
Results

α-adrenergic receptor antagonists. This experiment examined the impact of α-noradrenergic receptor blockers on the feeding response induced by GAL. Figure 11 shows the effects of PVN injections of these antagonists, and of their respective vehicles, on feeding induced by GAL (0.3 nmol), NE (6.3 nmol) or NPY (78 pmol). Tests with injection of the vehicles alone invariably yielded low baseline feeding scores ranging from 0.3 to 0.8 g.

As shown in the left panel of Fig. 11, the general α-noradrenergic antagonist PHT, at 30, 60, and 120 nmol, dose-dependently suppressed GAL-induced feeding (2.8 g), producing a maximal blockade of nearly 90% [$F(3,36)=5.41$,

Figure 11. Impact of PVN injections of the general α-blocker PHT (left panel), the α2-blocker RAU (middle panel) or the α1-blocker PRA (right panel) on the feeding response induced by local administration of GAL (0.3 nmol), NE (6.3 nmol) or NPY (78 pmol) in satiated rats. * $p<0.05$, ** $p<0.01$ by Duncan's New Multiple Range Test.





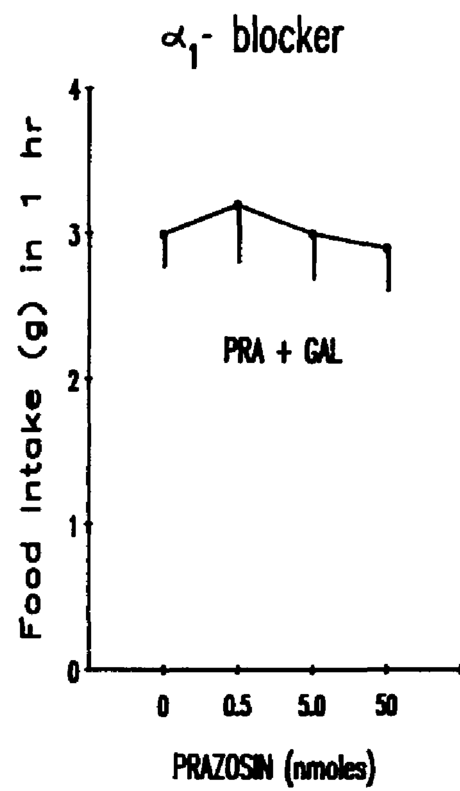


Figure 11

$p<0.01$]. This blocking effect was pharmacologically specific, since it failed to be detected with PHT in combination with NPY. At a relatively high dose (60 nmol) that produced a reliable ($p<0.01$), 75% suppression with GAL, PHT failed to have any effect on NPY-induced food intake.

The specific α_2-receptor antagonist RAU (middle panel of Fig. 11), at doses of 0.03 and 0.3 nmol, also reliably suppressed GAL-stimulated feeding (2.9 g). This blockade occurred in a dose dependent manner, with RAU at the highest dose producing an 80% reduction in the rsponse [GAL $F(3, 54)= 11.26$, $p<0.001$]. At the same doses, RAU similarly reduced the feeding response elicited by NE (3.0 g), causing a maximal suppession of approximately 85% [$F(3,63)= 13.88$, $p<0.001$]. In contrast, RAU did not alter NPY's stimulatory effect on food intake (3.0 g).

The α_1-antagonist PRA, at a wide range of doses tested (0.5, 5.0, and 50 nmol), was clearly ineffective in reducing the feeding response produced by GAL (Fig. 11, right panel). In a previous study (Goldman, Marino & Leibowitz, 1985), this antagonist at similar doses was also found to have no impact on NE-induced feeding.

In an attempt to relate the effects of these three agonists in the PVN, we compared the magnitude of their feeding responses induced in individual animals. These comparisons revealed a positive correlation ($r=+.410$, $p<0.03$) between the effectiveness of GAL and NE in the PVN.

In contrast, there was no significant correlation between NE and NPY or between NPY and GAL.

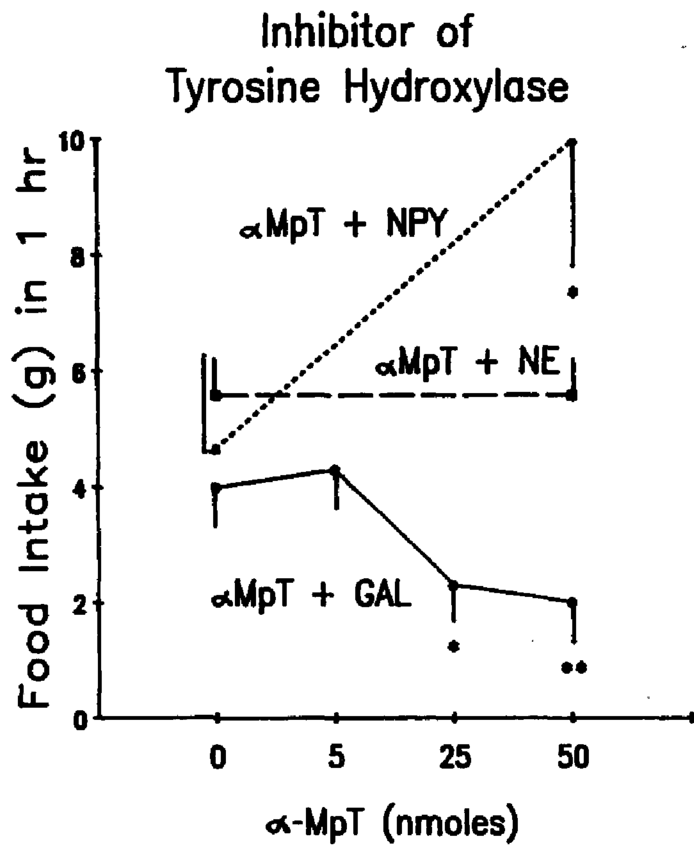
The different vehicles for the antagonists, as well as the antagonists at the doses used, had little effect of their own on the rat's baseline feeding behavior. In each case, the antagonist + saline treatment produced a feeding score that was comparable in magnitude (0.4 to 0.8 g) with the feeding response observed after injection of saline alone.

Observations of the subjects' behavior during the tests failed to reveal gross behavioral changes following the injections of these receptor antagonists or their vehicles. The animals engaged in a sequence of behaviors (upper body movements and locomotion, grooming, rearing, resting and sleeping), comparable to that observed after saline administration.

Catecholamine-synthesis inhibitors. This experiment studied the impact of CA-synthesis inhibitors on the feeding responses observed after PVN administration of GAL. For control purposes, the effects of these agents on the feeding responses induced by NE and NPY were also examined. Figure 12 shows the effects of the centrally administered tyrosine hydroxylase inhibitor, α-MpT, and of the dopamine-β-hydroxylase inhibitor, Fla-63, on the feeding effects of GAL (0.3 nmol), NE (6.3 nmol) and NPY (78 pmol). α-Methyl-p-

Figure 12. Impact of PVN injections of the CA-synthesis inhibitors, α-MpT (left panel) and Fla-63 (right panel), on the feeding response induced by local administration of GAL (0.3 nmol), NE (6.3 nmol) or NPY (78 pmol) in satiated rats. * $p<0.05$, ** $p<0.01$ by Duncan's New Multiple Range Test.

Figure 13. A summary of the effects of the α-receptor blockers PHT (α), RAU (α2) and PRAZ (α1), and the CA-synthesis inhibitors α-MPT and Fla-63 on the feeding responses induced by NE, GAL or NPY. ↓, suppression; ↑, increase; 0, no change; -, not tested.



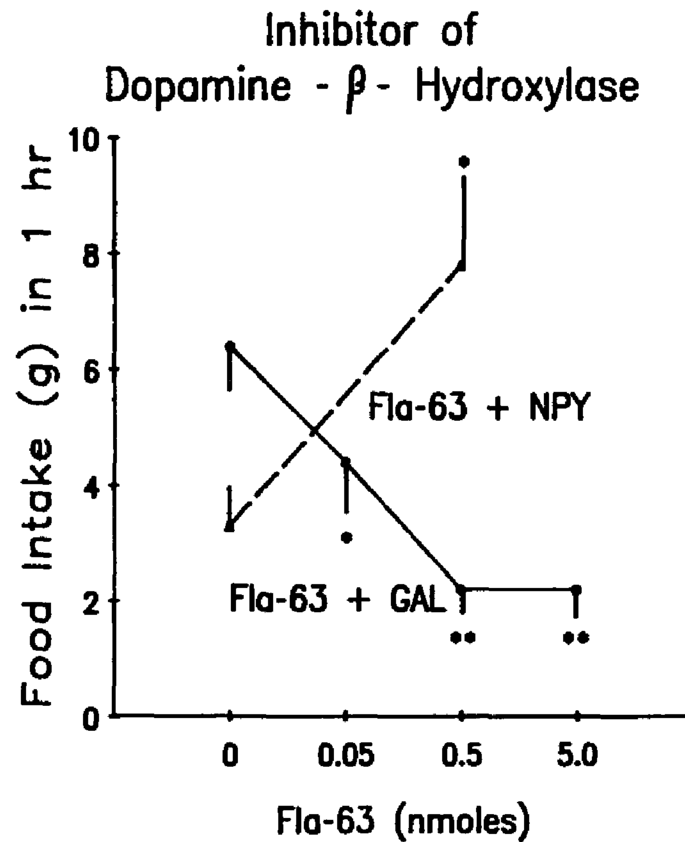


Figure 12

	NE	GAL	NPY
Receptor Antagonists			
PHT (a)	↓	↓	O
RAU (a2)	↓	↓	O
PRAZ (a1)	O	O	—
CA Synthesis Inhib.			
a—MPT	O	↓	↑
Fla—63	O	↓	↑

Figure 13

Tyrosine dose-dependently reduced the GAL-induced feeding response of approximately 4.0 g [$F(3,52)= 4.4$, $p<0.01$] (Fig. 12, left panel). At the highest dose of 50 nmol, a maximum suppression of approximately 70% was seen. This is in contrast to the effect of α-MpT on the action of NE or NPY. At the maximum dose of 50 nmol, α-MpT failed to reduce the feeding response (5.6 g) produced by exogenously administered NE, and it actually significantly enhanced, by over 100%, the eating response (4.7 g) elicited by NPY in the PVN ($t=2.5$, $p<0.05$; Fig. 12, left panel).

The dopamine-β-hydroxylase inhibitor, Fla-63, also reliably suppressed GAL-induced food intake [$F(3,44)= 13.89$, $p<0.001$] (Fig. 12, right panel). Central administration of Fla-63, at doses of 0.05, 0.5, and 5.0 nmol, dose-dependently reduced the rats' food intake by up to 85%. However, consistent with α-MpT's impact on NPY, Fla-63 (0.5 nmol) caused a significant enhancement of the feeding response elicited by NPY, increasing it from 3.3 up to 7.9 g ($t=4.7$, $p<0.05$).

Similar to the receptor blockers, these pharmacological agents failed to cause any gross alterations in the subjects' typical behavioral repertoire. When tested alone, neither the synthesis inhibitors, nor their vehicles, had any effect of their own on the rats' feeding behavior, as previously reported (Goldman et al., 1985).

Discussion

These results, summarized in Fig. 13, demonstrate that the functional integrity of PVN α_2-receptors and the availability of endogenous NE in the PVN are critical to the feeding stimulatory action of locally-injected GAL. This is in direct contrast to the results obtained with NPY, another feeding-stimulatory peptide which acts normally in the presence of an a_2-receptor antagonist and is actually facilitated in its actions by blockade of endogenous NE synthesis. These findings reveal different forms of peptide-amine interactions in the PVN.

α_2-Noradrenergic System. Several pharmacological studies have suggested that the feeding stimulatory effects of NE are mediated through α_2-receptors located in the PVN. Peripheral or central administration of the α_2-receptor agonist clonidine has been shown to elicit a strong feeding response (Lichtenstein, Marinescu & Leibowitz, 1984; Schlemmer et al., 1981; Yee et al., 1987). Moreover, the feeding induced by peripheral injections of clonidine is suppressed by systemic administration of the α_2-antagonist yohimbine, but not by the α_1-antagonist PRA (Schlemmer et al., 1981). More recently, Goldman et al., (1985), using central drug injections, demonstrated that the feeding elicited by PVN injection of NE or clonidine is blocked by local injections of the α_2-antagonists, yohimbine and RAU.

This NE-induced feeding response is also attenuated by the general α-blockers PHT and phenoxybenzamine, but is not affected by the specific α_1-blockers, PRA and corynanthine.

Although early evidence suggested that clonidine's physiological effects are mediated through presynaptic α_2-receptors (Langer, 1981), subsequent studies argue for the involvement of postsynaptic α_2-receptors in clonidine's actions, as well as in numerous physiological phenomena. In particular, the PVN α_2-receptors mediating eating behavior appear to be postsynaptic, since their responsiveness to noradrenergic stimulation is unaffected or even enhanced by drug manipulations or lesions that destroy presynaptic NE terminals or block NE synthesis (Goldman et al., 1985; Leibowitz & Brown, 1980). Furthermore, PVN 6-OHDA lesions, which destroy noradrenergic terminals, result in long-term attenuation of carbohydrate intake, a macronutrient specifically enhanced following NE or clonidine administration (Shor-Posner et al., 1986). Destruction of the PVN (Leibowitz et al., 1981) or of its efferents to the hindbrain (Weiss & Leibowitz, 1985), similarly diminishes the feeding response produced by central injection of NE.

GAL-Induced Feeding. An interaction between GAL and NE was first suggested by the finding that they are colocalized in the PVN (Skofitsch & Jacobowitz, 1985). Subsequently, behavioral studies have revealed several similarities in

their actions. Specifically, both substances stimulate feeding when injected into the PVN (Kyrkouli et al., 1986; Leibowitz, 1978), and the responses elicited by these neurochemicals in individual animals are found to be positively correlated in magnitude (Tempel et al., 1988).

Against this background, it is now demonstrated that GAL elicits a feeding response which can be attenuated specifically by α_2-receptor antagonists but not by α_1-antagonists. Furthermore, the feeding stimulatory effect of GAL is blocked by the CA-synthesis inhibitors, α-MpT, a dopamine-synthesis inhibitor, and Fla-63, a more selective NE and epinephrine synthesis inhibitor. This is similar to the effects produced by drugs which are known to enhance synaptic availability of endogenous NE but contrasts with their inability to antagonize the direct postsynaptic action of exogenous NE. Together, these findings suggest that GAL may produce its effect on eating behavior, in part, through the activation of endogenous NE.

This proposal is supported by the findings of the previous experiment, distinguishing the PVN, and in particular its parvicellular region, as the site of action for GAL. This experiment revealed that GAL, similar to NE, produces its strongest response after injection directly into the PVN and is relatively or totally ineffective in eliciting feeding in other hypothalamic or extrahypothalamic sites. It is also supported by findings demonstrating a

presynaptic excitatory interaction between GAL and NE in peripheral sympathetic nerves (Rokaeus, 1987), as well as in the hypothalamus (Melander et al., 1987).

NPY-Induced Feeding. Neuropeptide Y, similar to GAL and NE, also stimulates feeding when injected into the PVN. The question is whether this peptide, like GAL, acts in close association with NE. This study suggests that it does not and that NPY and NE stimulate feeding by acting through different neural substrates. Specifically, it was found that, unlike GAL, NPY-elicited feeding is not dependent upon PVN α_2-receptors nor apparently on endogenous NE stores in the PVN. Consistent with this evidence are anatomical studies dissociating NPY and NE containing projections in the PVN. While the PVN is very dense with NPY, which to some extent coexists with NE (Chronwall et al., 1985; Everitt et al., 1984), approximately 50% of its NPY-containing fibers originate from non-noradrenergic, non-adrenergic neurons of the arcuate nucleus (Chronwall et al., 1985). In light of this evidence, it will be important to distinguish which subgroup of NPY-containing neurons is involved in the feeding response.

Evidence further dissociating NPY from NE comes from cannula-mapping studies. These experiments have demonstrated that NE's stimulatory action on feeding is localized in the PVN area (Leibowitz, 1978). This is in contrast to NPY's

feeding response, which can be found in several hypothalamic cell groups but generally not extrahypothalamic areas (Stanley et al., 1985). Other pharmacological studies, consistent with these results, have reported that the eating response produced by PVN NPY administration is unaffected by local injection of general α-noradrenergic or specific α_2-noradrenergic receptor antagonists (Kalra et al., 1988; Levine & Morley, 1984; Stanley & Leibowitz, 1985). It may, in fact, be potentiated by peripheral administration of an α_2-receptor antagonist (Kalra et al., 1988). Additionally, experiments in other systems indicate that α_2-noradrenergic blockers, which attenuate the effects of NE in the hindbrain or periphery, fail to alter NPY's physiological actions (Fuxe et al., 1986; Lundberg & Hokfelt, 1986; Pernow, 1988; Stjarne & Lundberg, 1986). These findings suggest that NPY acts through its own receptors, independently of postsynaptic α_2-receptors.

In addition to their autonomous action on the postsynaptic membrane, evidence obtained in other systems once again indicates that NPY can act independently of presynaptic NE stores (Fuxe et al., 1986; Hokfelt et al., 1986; Lundberg & Hokfelt, 1986; Pernow, 1988; Stjarne & Lundberg, 1986). In a study of feeding behavior, brainstem knife cuts which strongly reduced hypothalamic NE and EPI levels, actually increased the feeding stimulatory effect of NPY (Sahu, Kalra, Crowley & Kalra, 1988). These findings are

in agreement with the results obtained in this study, showing that pharmacological agents which block the synthesis of NE or EPI in the PVN fail to attenuate the feeding response produced by NPY. In fact, as with the knife-cut study (Sahu et al., 1988), this response is significantly enhanced in the absence of NE, possibly arguing for an antagonistic interaction between NPY and NE. This interaction may occur either presynaptically, where NE inhibits the release of NPY, or postynaptically, where NE interferes with the receptor action of NPY.

Additionally, the antagonistic interaction between NPY and NE suggested here is supported by several in vitro experiments with this peptide. Specifically, NPY has been found to exert an inhibitory influence on NE release from the sympathetic, as well as central, nervous system (Serfoso, Bartfai & Vizi, 1986; Yokoo, Schlessinger & Goldstein, 1987). Furthermore, this peptide is effective in potentiating the inhibitory action of α_2-receptor agonists on NE release, an effect which can be reversed by α_2-receptor blockade (Martire, Fuxe, Pistritto, Preziosi & Agnati, 1986). This inhibitory action of NPY on NE release is believed to be mediated through presynaptic α_2-autoreceptors. Additional support comes from in vivo experiments with NPY. Neuropeptide Y decreases NE turnover in the PVN and, in particular, its parvicellular portion (Harfstrand et al., 1986), while injection of this peptide

into the ventricle decreases CA utilization (Vallejo, Carter, Biswas & Lightman, 1987).

In summary, these experiments provide direct indication that GAL in the PVN elicits feeding by acting, at least in part, through the activation of endogenous NE. This is in contrast to NPY, which appears to act independently of NE. The precise subcellular mechanisms involved are not known at this point. Further experimentation is needed to reveal the biochemical processes which mediate the GAL- and NPY-induced feeding responses.

Experiment 3. Impact of GAL and NPY on PVN NE levels

Pharmacological experiments have revealed that the feeding response induced by injection of GAL into the PVN is dependent upon intact α_2-noradrenergic receptors, similar to NE-stimulated feeding. Futhermore, these studies have demonstrated that GAL's stimulatory effect on feeding is abolished after pharmacological manipulations known to deplete endogenous NE stores. These findings suggested that GAL stimulates food intake through the release of NE in the PVN. To directly address this possibility, the present experiment, using the microdialysis technique in combination with an HPLC with ED, measured local extracellular NE levels after GAL administration into the PVN of freely-behaving rats. For comparison, the effects of NPY on NE levels were also measured.

The dialysis technique provides the possibility to monitor endogenous neurotransmitters in the extracellular fluid of freely-behaving animals. While a variety of techniques, e.g. push-pull cannula or cup, voltammetry, have been used to measure in vivo release of brain neurochemicals, the dialysis technique has several advantages over these other procedures (L'Heureux, Dennis, Curet & Scatton, 1986). For example, compared to the push-pull cannula, the dialysis probe is smaller and, therefore, more suitable for the study of small brain areas, e.g. PVN.

Also, this probe in contrast to the push-pull cannula, avoids mechanical damage of the tissue since the fluid flow is contained within the dialysis tube. Additionally, the dialysis probe has a higher recovery range for monoamines and metabolites. This technique may also be preferred to _in vivo_ voltammetry, which is powerful in monitoring extracellular levels of neurotransmitter metabolites but is limited in the determination of the neurotransmitter themselves. The dialysis probes employed in the present experiment (Hernandez et al., 1986) had a further advantage; in contrast to other types of cannulas which require permanent implantation, the dialysis probe could be removed and, therefore, minimize problems resulting from gliosis (Hamberger et al., 1982).

Methods

Subjects and Surgery. Six male Sprague-Dawley rats weighing 360-385 g were implanted with 10 mm long, 21-gauge guide cannulas aimed 2.5 mm dorsal to the PVN. With the incisor bar 3.0 mm above the interaural line, the coordinates used were: 6.0 mm anterior to the interaural line, 0.4 mm lateral to midsaggital sinus, and 4.0 mm ventral to the skull surface. Animals were housed individually in microdialysis chambers and had food and water available at libitum.

Procedures. Microdialysis probes were inserted into the guide cannulas 24 hrs prior to testing, to allow for adaptation in the microenvironment surrounding the tip of the probe (Benveniste, Drejer, Schousboe & Diemer, 1987). Tests were started at approximately 1:00 pm. Each subject had between one and three tests. During a one-hr interval prior to drug administration, three consecutive 20-min samples were collected which were used to establish baseline measurements. Subsequently, the microdialysis probe was removed, and GAL (0.3 nmol), NPY (78 pmol), or Ringer's solution was injected directly into the PVN, in a volume of 0.3 μl. The probe was then put back to place, and after a 10-min latency to allow the brain extracts to reach the microcentrifuge vial, 20-min samples were collected over the next 2 hrs. Food intake was measured 1 hr postinjection.

Statistics. Data were analyzed by repeated measures ANOVA, with comparisons to the control group made by Duncan's multiple range test ($p<0.05$ or $p<0.01$).

Results

This experiment examined the impact of GAL on PVN extracellular NE levels of freely moving rats, as measured by the HPLC microdialysis technique. The results demonstrate that this peptide, in contrast to NPY, is effective in potentiating levels of NE in this nucleus.

Norepinephrine baseline values, during the hour prior to GAL or aCSF injection, were 3.0 ± 0.8 and 1.9 ± 0.5 pg, respectively. These values represent the average scores of the three consecutive 20-min samples collected before the injection. Throughout this 1-hr baseline period, NE values were relatively stable, ranging between 1.2 and 4.5 pg. As can be seen in Fig. 14, injection of aCSF caused the levels of NE to increase from 1.9 to 4.5 pg. This increase was brief, occurring only within the first 20-min interval. After this time, extracellular NE returned to baseline levels and remained relatively stable, at approximately 1.0 pg, throughout the remainder of the 2-hr measurement period. The initial transient rise in NE appears to be due to the disruption of the internal milieu by the removal and subsequent reinsertion of the probe. This is indicated by pilot tests conducted with animals which received no injection but simply had their probes removed and which demonstrated that this process alone is sufficient to cause the change in NE.

Figure 14 reveals a rise in NE levels after an injection of GAL into the PVN. Statistical analysis via a two-way ANOVA revealed a significant difference between treatments [$F(1,16)= 6.74$, $p<0.02$], with NE levels increasing from 3.0 to 6.3 pg immediately following an injection of GAL ($p<0.01$), as well as across the different time-points ($p<0.01$). There was also an interaction between

Figure 14. Levels of extracellular NE (in picograms) as a function of time (minutes) following an injection of GAL (0.3 nmol) or vehicle into the PVN of satiated, freely-moving rats. * $p<0.05$, ** $p<0.01$ as compared with baseline levels by Duncan's Multiple Range Test.

Figure 15. Levels of extracellular NE (expressed as percentage of baselines) as a function of time (minutes) following an injection with GAL, NPY or vehicle into the PVN. * $p<0.05$, ** $p<0.01$ as compared with baseline levels by Duncan's Multiple Range Test.

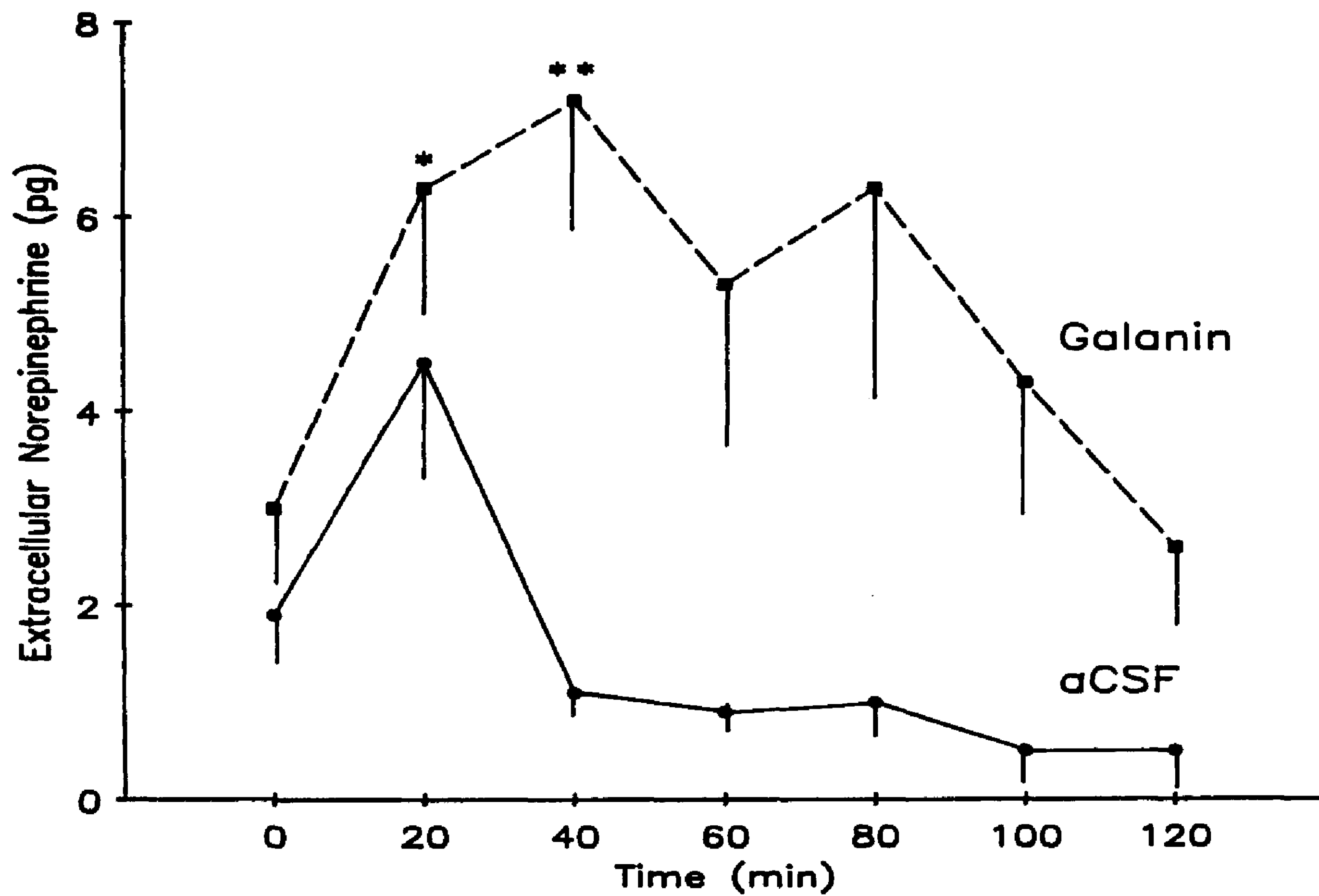


Figure 14

Microdialysis Study of PVN Norepinephrine After Galanin And Neuropeptide Y Injection

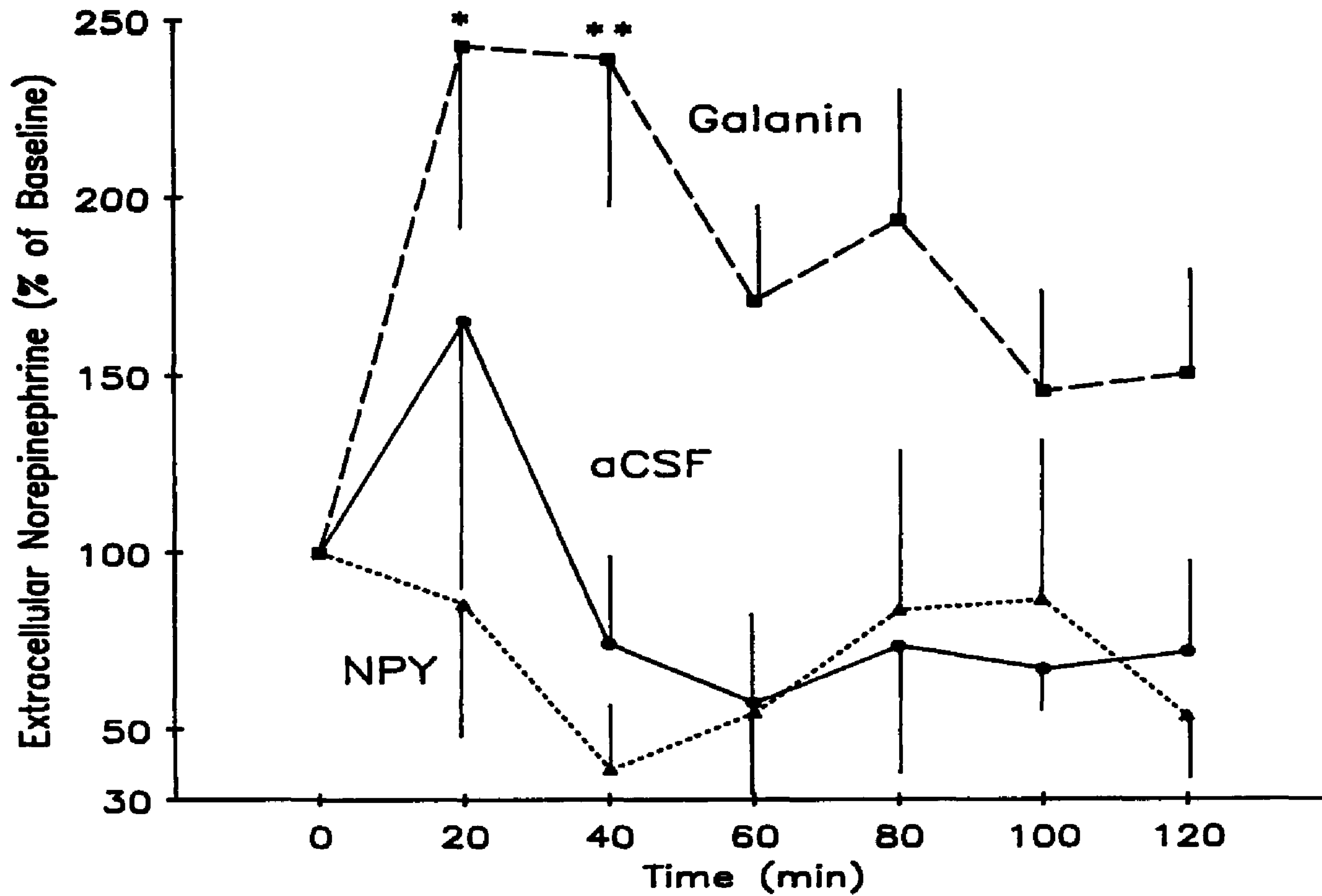


Figure 15

drug and time ($p<0.03$). Norepinephrine remained at high levels for 80 min postinjection. After this time, NE levels started declining and they reached baseline levels at the end of the second hour. This increase in NE was observed in all animals tested.

In contrast to GAL, PVN injection of NPY (78 pmol) failed to increase, and actually tended to reduce, extracellular NE levels in the PVN. This effect is depicted in Fig. 15, where data were plotted as percent change for comparison purposes. As this figure demonstrates, NE levels decreased to approximately 50% of its baseline levels (from 2.6 to 2.0 pg) 40 min after PVN injection of NPY. This suppression did not reach statistical significance, although it was observed in 80% of the animals tested. After this point, NE returned to baseline levels and remained relatively stable during the remainder of the 2-hr measurement period.

An example of the histological results is depicted in Fig. 16, which shows the degree of tissue damage generally produced by passage of the dialysis probe through the PVN. All animals included in the group had the probe passing through the PVN or immediately adjacent to its borders (A 6260 to A 5150 μ, in the rostral-caudal dimension; L 0.1 to 0.6 μ, in the medial-lateral dimension). In most cases, the tip of the probe extended beyond the ventral borders of this nucleus (approximately 0.1 to 0.5 mm).

Figure 16. Representative photomicrograph of a frontal section of the rat brain showing the tip of the dialysis probe passing through the periventricular division of the parvicellular portion of the PVN. The probes of the other animals in the group also passed through the PVN at the same medial level or further lateral.

Figure 16

Food intake measurements 1 hr following an injection with GAL or NPY revealed that these peptides significantly enhanced feeding behavior, as previously demonstrated (Kyrkouli et al., 1986; Stanley et al., 1984). An injection of aCSF, in contrast, failed to produce a response (0.3 ± 0.1 g). Injections of GAL and NPY resulted in feeding responses of approximately 3.0 and 4.0 g, respectively ($t=4.7$, $p<0.01$ for GAL; $t=4.4$, $p<0.02$ for NPY). Correlations between the feeding responses and the levels of extracellular NE after GAL injection yielded a positive correlation ($r=0.60$). This did not quite reach statistical significance due to the small number of animals in the group ($n=6$).

Discussion

This preliminary study examined the impact of GAL and NPY, two peptides which similarly stimulate feeding, on endogenous NE levels in the PVN. The results demonstrate that these neurochemicals differentially affect extracellular NE, as measured via the microdialysis/HPLC technique. Whereas GAL reliably enhances levels of this amine, NPY fails to alter it. In the following sections, these findings will be discussed in relation to published literature on GAL and NPY.

GAL-NE Interactions. The pharmacological experiment

demonstrated that GAL-elicited feeding is dependent upon intact α_2-adrenergic receptors, as well as presynaptic stores of NE. This suggests that GAL acts to stimulate feeding through the release of endogenous NE in the PVN. Consistent with this hypothesis, this study reveals a significant increase in extracellular NE, as a result of local injections with GAL. Other biochemical studies have also reported a stimulatory effect of GAL on noradrenergic function. This has been demonstrated in peripheral sympathetic nerves (Ohhashi & Jacobowitz, 1985), as well as in the median eminence (Melander et al., 1987).

An important question at this point is whether the increase in NE observed in the PVN reflects a direct effect of GAL on this amine, or whether this phenomenon is secondary to the feeding response that simultaneously occurs following administration of this peptide. Some preliminary evidence argues that GAL's effect on NE is primary. This is suggested by the results obtained in one additional subject whose food was removed just before the testing session. In this rat, GAL in the abscence of feeding caused a dramatic increase in PVN NE levels, from a baseline of 1.0 pg to 9.1 pg, 40 min after an injection. Further evidence supporting a direct effect of GAL on NE levels can be found in the results obtained with NPY. This peptide, failed to increase NE release. Thus, it appears that the presence of a feeding response is, by itself, not sufficient for potentiating PVN

NE.

If we accept that GAL has a primary effect on NE release, the next issue to be addressed is the intracellular mechanism for this release. From evidence about coexistence relationships in other systems (Changeux, 1986; Hokfelt et al., 1986), one may speculate on several possibilities. Specifically: 1) GAL may affect the release of NE directly by modulating autoreceptors that control its release; 2) GAL may affect the release of NE indirectly through an interneuron, which in turn modulates presynaptic autoreceptors; 3) GAL may enhance NE's postsynaptic effects, by inhibiting either its re-uptake or the action of its degradative enzyme; and 4) GAL may facilitate the action of NE by acting on the postsynaptic membrane via receptor-receptor interaction. These forms of interaction have been described for other peptides, e.g. NPY and acetylcholine (Lundberg & Hokfelt, 1986), NPY and NE (Lundberg & Hokfelt, 1986), CCK and DA (Agnati et al., 1986), and calcitonin-gene-related-peptide and substance P (Hokfelt et al., 1986).

NPY-NE Interactions. The results of the present study are consistent with the findings of the previous experiment, which have demonstrated that NPY acts independently of α_2-receptors and presynaptic stores of NE. They are also consistent with biochemical studies which suggest that NPY interacts with NE in some antagonistic manner. Specifically,

in the periphery as well as the brain, NPY fails to enhance the turnover of NE and may, in fact, depress its release (Fuxe et al., 1986; Pernow, 1988; Stjarne & Lundberg, 1986; Westfall et al., 1988). At low doses, NPY injection into the ventricles reduces NE utilization in the hypothalamus and specifically in the PVN (Harfstrand et al., 1986; Vallejo et al., 1987).

The antagonistic interaction between NPY and NE may take place at the postsynaptic membrane and may be expressed by a receptor-receptor interaction. This kind of interaction between receptors has been demonstrated in the lower brainstem, where NPY stimulation modulates the α_2-receptor binding sites and a reciprocal interaction occurs between α_2-adrenergic stimulation and the NPY receptor sites (Fuxe et al., 1986; Agnati et al., 1986). Alternatively, this interaction may take place presynaptically. That is, once released, NPY and NE may exert feedback inhibition on the liberation of their coexisting partner, by acting on presynaptic receptors. In this manner, for example, NPY in the vas deferens is thought to inhibit neurally-evoked contractions by blocking the release of NE and ATP (Lundberg & Hokfelt, 1986). Such mode of action is consistent with the findings of the present study.

In summary, the results of this experiment demonstrate that central administration of GAL enhances extracellular NE levels in the PVN of satiated freely-moving rats. This is in

contrast to NPY, another feeding stimulatory agent that also coexists with NE, but fails to stimulate PVN levels of this amine.

General Discussion

The results of these experiments support a role for central GAL in the control of eating behavior. They demonstrate that GAL stimulates feeding, in part, through its interaction with endogenous NE. Its site of action, similar to NE's, appears to be the PVN and, in particular, its parvicellular region. Galanin's action in the PVN is dependent upon intact α_2-noradrenergic receptors and on presynaptic stores of endogenous NE. Furthermore, PVN injection of GAL increases local extracellular levels of NE, supporting the hypothesis that GAL acts via the release of endogenous NE.

In the absence of specific antagonists for GAL, it is difficult to establish the specific role of this peptide under physiological conditions. However, through direct comparisons with NE's effects, the following hypothesis regarding its physiological function may be formulated.

A) Natural feeding patterns

To understand how GAL may be involved in the control of feeding, it is important to discuss first the different components of natural feeding. Studies of undisturbed, freely-feeding rats maintained on pure macronutrient diets have revealed that patterns of nutrient intake differ

dramatically at different points of the daily cycle (Shor-Posner, Ian, Guerra & Leibowitz, 1987; Tempel et al., 1989). In particular, rats eat their first meal of the night within approximately 10 min after lights go out. In over 80% of the animals, this meal is rich in carbohydrate; it lasts approximately 12 min and is then followed within an hour by the second meal, which is rich in protein. This early pattern changes towards the end of the nocturnal cycle, when preference for carbohydrate is low and protein or fat intake predominate.

B) Role of GAL in relation to NE: When are these neurotransmitters active?

It is proposed that GAL in the PVN, derived from projections ascending from the locus coeruleus (A6) and medullary (A1) noradrenergic neurons, is physiologically active at the start of the animal's active (dark) cycle. At this time, NE is believed to be active in the control of carbohydrate intake (Leibowitz, 1988), and endogenous GAL may be similarly active through the release of NE. It has been demonstrated that PVN injections of NE or clonidine in the rat are most effective in eliciting feeding, and in particular carbohydrate intake, at the onset of the active period (Leibowitz & Shor-Posner, 1986). Moreover, recent evidence indicates that GAL may similarly increase

consumption of carbohydrate and that this effect occurs only at dark onset (Tempel et al., 1988). A neurochemical link between GAL and NE is further supported by the finding of a strong positive correlation between the feeding responses elicited by NE and its coexisting peptide. As indicated above, analyses of natural feeding patterns show carbohydrate to be the macronutrient of choice during the first meal of the active cycle. The present pharmacological and neurochemical results lead us to propose that GAL is physiologically active, along with NE, in controlling the intake of this macronutrient at this time of the circadian cycle.

While little is known about GAL in the brain, evidence in the periphery has linked this peptide to the secretion of insulin from the pancreas (Ahren et al., 1988). With the possibility that central GAL may also have a glucoregulatory function, GAL through PVN $\alpha2$-noradrenergic mechanisms may in fact initiate feeding via NE's relationship with glucose. Circulating glucose has been implicated in the onset of a meal (Luis-Silvestre & Le Magnen, 1980; Camfield & Smith, 1986), and the impact of glucose on NE and its receptors (Leibowitz, 1988), may be linked to the feeding response induced by GAL. Further tests with measurements of blood glucose after GAL injections and also with measurements of PVN GAL after manipulations which lead to a decline in circulating glucose, should help to reveal this

relationship.

It should be noted, however, that besides eliciting the ingestion of carbohydrate, GAL also stimulates fat intake, while producing no change in protein intake. While GAL may exhibit this effect on fat consumption throughout the circadian cycle, it is clearly strongest at the end of the nocturnal period, when GAL is ineffective in stimulating carbohydrate intake. This GAL-induced increase in fat consumption is not well understood at the present time. One hypothesis is that it reflects a smaller and more variable response detected with NE (Leibowitz et al., 1985). Alternatively, it may be linked to GAL's actions on non-noradrenergic systems in the hypothalamus, which are most active at the end of the dark period when fat is the macronutrient of choice (see below).

C) Comparisons with NPY.

The results obtained in the present experiments indicate that NPY in the PVN, which similarly stimulates feeding and coexists with NE, acts quite differently from GAL in terms of its interaction with NE. Rather than operating through endogenous NE, NPY appears to produce its behavioral effects independently of NE and its α_2-receptors and may in fact exert an antagonistic effect on this noradrenergic system.

Rather than acting through the release of NE, NPY may act in a cooperative manner with NE, perhaps strengthening its actions at time of physiological need. In other physiological systems, similar effects of NPY and adrenergic agonists have been observed with ventricular injections and measurements of LH secretion (Allen, Kalra, Crawley & Kalra, 1985); PVN injections and measurements of gastric acid secretion (Humphreys, Davison & Veale, 1988; Shiraishi, 1987); PVN or supraoptic nucleus injections and measurements of corticosterone and vasopressin release (Leibowitz et al., 1988). With regard to feeding, centrally injected NPY acts similarly to the adrenergic agonists in stimulating food intake and also in potentiating carbohydrate ingestion. The possibility of a cooperative interaction between NPY and NE is suggested by the finding that PVN NPY, like NE, is most effective in enhancing carbohydrate ingestion at the beginning of dark (Tempel et al., 1988), a time when the density of α_2-noradrenergic receptors is highest (Jhanwar-Uniyal et al., 1986); in contrast, it is weakest later in the nocturnal period, when there is a decline both, in α_2-receptor density and in preference for carbohydrate. It is also consistent with evidence that PVN NPY, similar to NE, stimulates the release of corticosterone (Leibowitz, 1988; Wahlestedt et al., 1987), which is known to peak at the onset of the dark cycle (Krieger & Hauser, 1978). Evidence suggests that this rise in corticosterone levels is the

product of NPY's interaction with PVN NE (Harfstrand et al., 1986). Further, NPY-induced feeding is dependent upon high levels of circulating corticosterone (Kalra et al., 1988; Stanley at al., 1986).

In addition to this cooperative relationship between NPY and NE, it appears that these two neurochemicals may also interact antagonistically, inhibiting each other's release. This hypothesis is supported by our results with the microdialysis technique (Experiment 3) and is also consistent with other published findings (Fuxe et al., 1986; Pernow, 1988). While the physiological significance of this interaction remains to be determined, there is some suggestion (Leibowitz, 1988; Stjarne & Lundberg, 1986) that, subsequent to the initial cooperation, this peptide and amine act antagonistically, first to terminate the carbohydrate meal and then to switch the animal's preference to the ingestion of other macronutrients, such as protein. A switch from carbohydrate to protein intake has been found to occur in freely feeding animals examined under physiological conditions (Shor-Posner et al., 1987).

D) GAL and NPY in relation to other systems.

1) Interactions with other monoamines. In addition to their interaction with NE, GAL as well as NPY may interact with other PVN feeding neurochemical systems. One possible

candidate is 5-HT, a monoamine known to suppress food intake after administration into the medial hypothalamus (Leibowitz, Weiss & Shor-Posner, 1988). Injections of 5-HT, or drugs that enhance its synaptic availability, into the PVN inhibit specifically the ingestion of carbohydrate, while leaving unchanged or enhancing intake of protein or fat (Shor-Posner, Grinker, Marinescu, Brown & Leibowitz, 1986; Weiss, Papadakos, Knudson & Leibowitz, 1986). The opposite effect, namely, an increase of carbohydrate intake, is observed after manipulations that block the function of 5-HT. These and related findings have led to the proposal that the medial hypothalamic 5-HT antagonizes the PVN α_2-noradrenergic system to control food intake, in particular, carbohydrate intake or the carbohydrate-protein ratio (Leibowitz et al., 1988).

The finding that PVN GAL, as well as NPY, each stimulates the consumption of carbohydrate, a macronutrient specifically suppressed by 5-HT administration into this nucleus, raises the possibility of an antagonistic interaction between these peptides and the PVN serotonergic system. This possibility is supported by anatomical studies, revealing that GAL and NPY each coexists with 5-HT in dorsal raphé neurons innervating the parvicellular division of the PVN (Melander et al., 1986; Blessing et al., 1986; Sawchenko et al., 1983) and also that 5-HT innervates NPY neurons in the hypothalamus (Guy et al., 1988). This hypothesis is also

supported by biochemical studies demonstrating a suppressive effect of these peptides on brain 5-HT metabolism (Vallejo et al., 1987; Fuxe et al., 1988; Sundstrom & Melander, 1988). Additionally, direct evidence for a NPY-5-HT interaction in relation to feeding is provided by a pharmacological study showing that the feeding response induced by PVN administration of NPY is abolished by injection of a serotonergic agonist (Bendotti, Garattini, & Samanin, 1987). Thus, GAL and NPY may each stimulate carbohydrate intake, in part, by inhibiting the activation of the serotonergic system in the PVN, which in turn may potentiate noradrenergic function.

Galanin may also interact with the dopaminergic system. In addition to potentiating carbohydrate consumption at the beginning of the dark cycle, GAL has also been found to stimulate fat intake, with strongest action at the end of the dark cycle. This finding is consistent with anatomical studies demonstrating that this peptide coexists with DA in the hypothalamus and inhibits its release (Nordstrom, Melander, Hokfelt, Bartfai & Goldstein, 1987). Studies with amphetamine, which is believed to act in part through the release of hypothalamic DA (Leibowitz, 1980), have shown this drug to inhibit ingestion of fat as well as protein (Kanarek, Ho & Meade, 1981; Leibowitz, Shor-Posner, McLow & Grinker, 1986). Fat and protein are the naturally preferred macronutrients at the end of the nocturnal cycle, when GAL

is most potent at stimulating fat intake. Therefore, the possibility exists that GAL exerts its effects on fat consumption, in part, by inhibiting the hypothalamic dopaminergic system.

2) Interactions with neuroendocrine systems. Galanin's effects on fat intake may, alternatively, be linked to its influence on the anterior pituitary. This peptide has been found to stimulate the release of growth hormone (GH) in rats (Ottlecz et al., 1986; Murakami et al., 1987; Melander et al., 1987) and humans (Bauer et al., 1986; Davis, Burrin & Bloom, 1987), apparently via GRF secretion (Murakami et al., 1987). Hypothalamic administration of GRF has been shown to stimulate eating (Vaccarino, Bloom, Rivier, Vale & Koob, 1985), and food deprivation induces the release of GH (Driver & Forbes, 1981), while potentiating the ingestion of fat and to a lesser extent carbohydrate (Shor-Posner, Azar, Insigna & Leibowitz, 1985).

3) Interactions with other peptides. Evidence indicates that the feeding-inhibitory peptides cholecystokinin (CCK) and neurotensin (NT) interact with the PVN noradrenergic system to modulate food intake (Myers, 1985; Morley et al., 1985). The effect, in particular, of peripheral CCK is thought to reach the brain by ascending vagal fibers (Smith, Jerome & Cushin, 1981) and then to activate the PVN, via a

relay in the NTS (Crawley & Knas, 1984). The question is whether GAL, by acting via endogenous NE, is itself modulated by these peptides.

Studies have suggested that central and/or peripheral CCK and NT may exert their inhibitory effect on feeding by altering NE's effect in the PVN. This is indicated by findings demonstrating that the stimulation of feeding produced by PVN injection of NE is strongly inhibited by peripheral or PVN administration of either CCK or NT but is not affected by injection of these peptides into other hypothalamic sites (Myers & McCaleb, 1981; Levine, Kneip, Grace & Morley, 1983). Further, infusion of CCK or NT into the PVN affects the endogenous stores of NE in this nucleus (Myers, 1985). It is noteworthy that the direction of this effect (stimulation or inhibition of release) depends on the physiological state of the organism. Specifically, the release of NE is suppressed in food deprived animals but is increased in satiated animals. These findings suggest that PVN terminals which release NE are affected by activation of CCK receptors. Whether or not GAL itself is affected by this interaction, remains to be known. The possibility cannot be excluded, however, that CCK and/or NT influence the release of NE by acting directly on GAL which, in turn, modulates the release of this monoamine.

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