

**HYPOTHALAMIC ACTIONS OF GROWTH HORMONE
SECRETAGOGUES**

MR EKKASIT KUMARNSIT

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DECLARATION

The studies outlined in this thesis were undertaken in the Department of Biomedical Sciences, University of Edinburgh, under the supervision of Professor Gareth Leng. This dissertation has not been submitted for any other qualification at any other university. It is the result of my own work except where specifically stated.

Ekkasit Kumarnsit

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CHAPTER 1
GENERAL INTRODUCTION

1.1 Growth hormone secretion

1.1.1 Introduction

Growth hormone (GH) is expressed specifically in the somatotroph cells of the anterior pituitary gland, and GH secretion by somatotrophs is thought to be central in regulating post-natal growth. In particular, GH increases bone mineral density, especially in longitudinal bones (Longobardi et al., 1999), but GH also influences many bodily processes that influence body composition in humans, such as lipid metabolism (Oscarsson et al., 1989). GH secretion declines progressively during adulthood, and this decline is especially marked in elderly subjects. In severe GH deficiency, an individual's muscle mass, muscle strength and bone mass are decreased, and the relative proportion of total and visceral fat is increased (Johannsson et al., 2000). In elderly subject with hypothalamic-pituitary disease and GH deficiency, total fat mass was significantly higher than in control subjects (Li Voon Chong et al., 2000). Thus, GH promotes skeletal growth and promotes muscle growth rather than fat deposition.

In GH-deficient adults, GH has been applied for therapeutic purposes in order to improve their quality of life. It was reported that recombinant human GH (rhGH) treatment significantly increased lean body mass and decreased fat mass in adults with GH deficiency (Salomon et al., 1989). Many of the actions of GH are mediated through insulin-like growth factor-I (IGF-I), which is increased following GH replacement (Gillberg et al., 2001). In normal adults, plasma IGF-I concentrations, like those of GH, significantly decline with age in both sexes (Yamamoto et al., 1991). Replacement of low-dose GH produces improvements in body composition and in quality of life that can be detected as early as 1 month after the start of treatment, and the beneficial effects continue at 3 months in patients with GH deficiency (Ahmad et al., 2001). Recently, long-term effect of GH replacement was found to prevent the age-related decline in insulin sensitivity in GH-deficient adults (Svensson et al., 2002). Sensitivity to insulin declines with increasing age especially in GH-deficient adults. Additionally, GH treatment in GH-deficient children also results in normal bone mineral density (Fors et al., 2001)

Short stature is commonly caused by impairments of either GH secretion or receptor function. Short stature in children with Prader-Willi syndrome, which may be explained, at least in part, by a decreased GH secretory capacity, can be effectively treated with exogenous GH for one year (Hauffa, 1997). GH treatment to correct physical abnormality has to be continued indefinitely. Linear growth in GH-deficient children is the single most useful parameter available for evaluating the efficacy of GH replacement therapy (Shalet et al., 1998). In children with idiopathic short stature, the causes for growth failure are not definitely established. Plasma GH binding protein activity in children with idiopathic short stature has been reported to be significantly lower than in a control group (Davila et al., 1996). GH binding protein is a soluble form of GH receptor that can bind GH, and the decreased GH binding protein activity in children with idiopathic short stature suggests that they may present a degree of GH insensitivity due to a defect at the GH-receptor level. In adults with GH deficiency, continuation of GH replacement showed improvement in increasing serum insulin-like growth factor-I and lean body mass, whereas discontinuation of the treatment increased total body fat (Norrelund et al., 2000).

GH secretion from the pituitary depends on many factors, including stress (Noel et al., 1972), acute exercise (Bigbee et al., 2000) and stage of menstrual cycle (Faria et al., 1992). In somatotrophs of the anterior pituitary, electron microscopic immunocytochemical studies have demonstrated that GH is stored in large, dense-cored secretory granules (Hirano and Shiino, 1989). In cultured rat somatotrophs perfused with GHRH, GH release and 45 Ca^{2+} efflux appear to be tightly coupled, with the calcium response perhaps slightly preceding GH release (Ohlsson and Lindstrom, 1990). This suggests that GH is released by exocytosis in a calcium-dependent manner like other peptide hormones.

The systemic actions of GH may be mediated by GH itself or by GH-induced release of other peripheral factors, including in particular insulin-like growth factor-I (IGF-I), which is expressed and produced primarily in liver (Butler et al., 2002). In mice lacking a functional GH receptor, IGF-I levels could be as low as 0.2 % of normal levels (Lupu et al., 2001). Under normal conditions, IGF-I is bound in the circulation to specific binding proteins 1-6 (Bach and Rechler, 1996). Most of circulating IGF-I is bound with IGF-binding protein-3 as a complex

whereas the remaining IGF-I circulates either free or bound in a complex with one of five other IGF-binding proteins (Jones and Clemmons, 1995). In children with GH deficiency, serum-concentrations of free IGF-I are significantly lower than those in age- and sex-matched normal children (Wacharasindhu et al., 2000). Conversely, administration of GH significantly increases free IGF-I concentration (Bereket et al., 1997). These data suggest that serum concentrations of total IGF-I are GH-dependent and can be used as a screening tool for GH-deficient status.

Apart from physical improvement, GH therapy with recombinant human GH has been applied to improve mental performance. Low doses of recombinant human GH significantly improve psychological profiles as rated by the Hamilton Depression Scale (HDS) in adult-onset patients with GH deficiency (Takahashi and Chihara, 1999). A significant correlation between increase in IGF-I concentrations and decrease in HDS scores in recombinant human GH-treated patients confirmed the improvement of psychological profiles along with physical changes (Giusti et al., 1998). However, an effect of GH treatment on psychological profiles has not been consistently observed. Standardised psychometric tests have been used to measure cognition and sense of well-being before and after GH therapy. However, following 18 months of GH replacement therapy, there were no significant alterations in cognitive function or quality of life assessed by standardised tests in adult men with acquired GH deficiency (Baum et al., 1998). Another study performed in adults with documented GH deficiency also found no better psychological response, whereas the improvements in increasing IGF-I concentrations, body composition and self-perceived well-being were significant (Carroll et al., 1997).

Thus, GH deficiency, in ageing or in inherited disorders, produces profound physical impairments and possibly also mental impairments. Accordingly, therapeutic strategies to provide remedies for GH deficiency have considerable potential benefits for the quality of life in an ageing population.

1.1.2 GH secretion in dwarfism

Dwarfism can arise by defects in GH production and release, or by defects in downstream signalling. In dwarf (*dw*^{-/-}) chickens, the overall GH mean, amplitude, and baseline concentrations are significantly higher than those of control normal-sized (*Dw*^{+/-}) chickens, and no differences in peak length or peak frequency between genotypes are observed (Buyse et al., 1994). By contrast, in the dwarf mouse strain (*lit/lit*), anterior pituitaries do not release GH or accumulate cyclic adenosine 3' monophosphate (cyclic AMP) in response to human or rat GHRH, whereas dibutyryl cyclic AMP, as well as the adenylate cyclase stimulators forskolin and cholera toxin, markedly stimulate GH release (Jansson et al., 1986). GH-deficient dwarf *lit/lit* mouse is a mutant having a point mutation in the N-terminal ligand-binding domain of the GHRH receptor (Lin et al., 1993). This suggests that the basis of the GH deficiency in the little mouse may be a defect in an early stage of GHRH-stimulated GH release, related either to receptor binding or to the function of the hormone-receptor complex. In Lewis-derived dwarf rats, the percentage of pituitary cells that release GH is substantially lower than in normal rats (Kineman et al., 1989). In addition, the expression of GHRH-R mRNA in pituitary extracts of GH-deficient rats, detected by using RNase protection assay, is markedly reduced in extracts from age-matched male and female dwarf rats with GH-deficiency (Carmignac et al., 1996).

Thus, GH secretion in dwarfs is different from in normals due to a variety of defects in the transduction pathway including defects at the receptor level, cyclic AMP production, and expression of GHRH-R mRNA, as well as a reduction in the total number of GH-secreting cells in the pituitary gland.

1.1.3 Pulsatile pattern of GH release

In all species studied to date, GH is released episodically. This pulsatile pattern of secretion is particularly marked in male rats. (Tannenbaum and Martin, 1976) collected blood samples every 15 min from male rats and demonstrated pulsatile GH secretion at approximately 3-h intervals with peak values higher than 200 ng/ml and basal values lower than 1 ng/ml. This

study also revealed a very constant rhythmic pattern of secretion with a regular time interval between pulses that was unchanged across all phases of a 12-h light dark cycle.

The effects of sex steroid hormones on GH release suggest sexual dimorphism (Gevers et al., 1998). In male rats, plasma concentrations of GH are markedly periodic, with high peak but low basal levels, whereas more continuous secretion and substantially higher basal concentrations are evident in female rats (Eden, 1979). This sex-related difference is believed to be an important contributor to the higher growth rate in males of most mammalian species. In female rats, the interval between GH peaks is shorter than in males and less regularity is observed, especially in old female rats that show very irregular intervals between peaks. (Eden, 1979).

1.1.4 Physiological significance of pulsatile secretion of GH

The pulsatile pattern of GH secretion was hypothesised to be important in regulating GH-mediated processes. Pulsatile treatment with exogenous GH (one pulse per 3 h) resulted in 3- to 5-fold increase in the levels of IGF-I expression in skeletal muscle and rib growth plates of male rats, while, by contrast, continuous infusion with GH was much less effective (Isgaard et al., 1988). Pulsatile intravenous infusion of GH-releasing hormone (GHRH) performed to establish a male type of GH secretory pattern for normal female rats also accelerated growth and increased pituitary GH content, whereas continuous infusion of this GHRH fragment at the same daily dose was ineffective (Clark and Robinson, 1985). Pulsatile GH secretion is believed to be governed mainly by two hypothalamic peptides: a stimulatory factor, GHRH, and an inhibitory factor, somatostatin. These peptide factors reach the anterior pituitary gland via the hypophysial portal circulation after release from nerve endings in the median eminence. Measurement of immunoreactive GHRH and somatostatin concentrations in portal blood found episodic secretion of immunoreactive GHRH, with maximal concentrations during GH secretory episodes, and secretion of immunoreactive GHRH was accompanied by a moderate reduction in portal plasma concentrations of immunoreactive somatostatin (Plotsky and Vale, 1985).

1.1.5 Hypothalamic control of GH secretion

GHRH is synthesised by a sub-population of neurones in the arcuate nucleus (Jacobowitz et al., 1983), and somatostatin by a sub-population of neurones in the periventricular nucleus (Brazeau et al., 1973). These peptides are transported along axons to nerve endings in the median eminence, and released from nerve terminals into the hypothalamo-hypophysial portal blood circulation to influence the anterior pituitary gland.

Okada and co-workers collected serial blood samples from unanaesthetised female rats during electrical stimulation of the periventricular nucleus (Okada et al., 1991). Plasma concentrations were reduced significantly during stimulation, while a large increase in GH secretion followed immediately after the end of the stimulation. (Koibuchi et al., 1986) showed that electrical stimulation of the basolateral amygdala resulted in a significant increase in plasma GH concentration, and this increase was markedly augmented after lesion of the periventricular nucleus. This suggests that the basolateral amygdala provides an excitatory drive to GH secretion, and indicates that the activity of periventricular neurones is inhibitory to this release. Dickson and co-workers (Dickson et al., 1994) showed that electrical stimulation of the periventricular nucleus resulted in inhibition of electrical activity of arcuate neurones that were identified antidromically as projecting to the median eminence, and which were therefore presumed to be neurosecretory neurones. This inhibition was followed with a rebound hyperactivation after the end of stimulation.

The electrical stimulation of the periventricular nucleus could result in the release of somatostatin and thereafter directly modulate GHRH neurones via somatostatin receptors, which have been localised in the arcuate nucleus (Tannenbaum et al., 1998b). Somatostatin released from the periventricular nucleus is believed to mediate its effect on GH secretion via GHRH neurones as well as by actions on somatotrophs. This idea was supported by a study that demonstrated the existence of both GHRH-expressing neurones and somatostatin binding sites in the arcuate nucleus (Bertherat et al., 1992).

Five somatostatin receptor (SSTR) subtypes, SSTR1-5, have been cloned and characterised (Reisine and Bell, 1995). All five subtypes are functionally coupled to inhibition of adenylyl cyclase via pertussis toxin sensitive GTP-binding protein (Patel et al., 1995). SSTR2 comprises SSTR2A and 2B, and can also stimulate tyrosine phosphatase and phospholipase C via SSTR2A (Delesque et al., 1995). The biological roles of individual subtypes are still largely unknown. The distribution of SSTR1 and SSTR2 mRNA-expressing cells in the arcuate nucleus suggests the involvement of these subtypes in regulation of GHRH secretion (Beaudet et al., 1995). Both subtypes are co-localised in GHRH mRNA-containing cells (Tannenbaum et al., 1998b). Given the lack of specific receptor antagonists, antisense oligodeoxynucleotides were used to investigate the specific subtypes that are involved in regulating GHRH secretion. Only the SSTR1 antisense oligodeoxynucleotides diminished the amplitude of ultradian GH pulses without modifying their frequency, whereas SSTR2 antisense oligodeoxynucleotides did not modify the GH secretory pattern (Lanneau et al., 2000). This study supports a preferential role of the SSTR1 receptor subtype in the regulation of GH secretion by somatostatin at the hypothalamus. However, SSTR1 and SSTR2 receptor subtypes may be also involved in other physiological function since they were also expressed in jejunum, stomach, cerebrum, and kidney (Yamada et al., 1992).

In vivo, continuous infusion of GHRH increases nocturnal GH secretion, while the episodic pattern of GH secretion is maintained (Sassolas et al., 1986). This study also found that other parameters, including total sleep time, sleep latency, and duration and timing of the different sleep stages, were not affected by GHRH infusions. On the other hand, pulsatile secretion of GH in rats was abolished by the infusion of a monoclonal antibody specific for rat GHRH (Wehrenberg et al., 1982). The mechanism of action of GHRH was later investigated using MZ-4-71, an antagonistic analogue of human GHRH (hGHRH), to block secretion of GH and IGF-I in rats (Kovacs et al., 1996). Additionally, a competitive antagonist to the GHRH receptor (GHRH-Ant) was demonstrated to suppress pulsatile GH secretion in healthy young men (Jaffe et al., 1993). This confirms the involvement of GHRH in the generation of GH pulses in human. Possible neural pathways that connect the arcuate nucleus and the periventricular nucleus are shown in **fig. 1.1** (Tannenbaum, 1991).

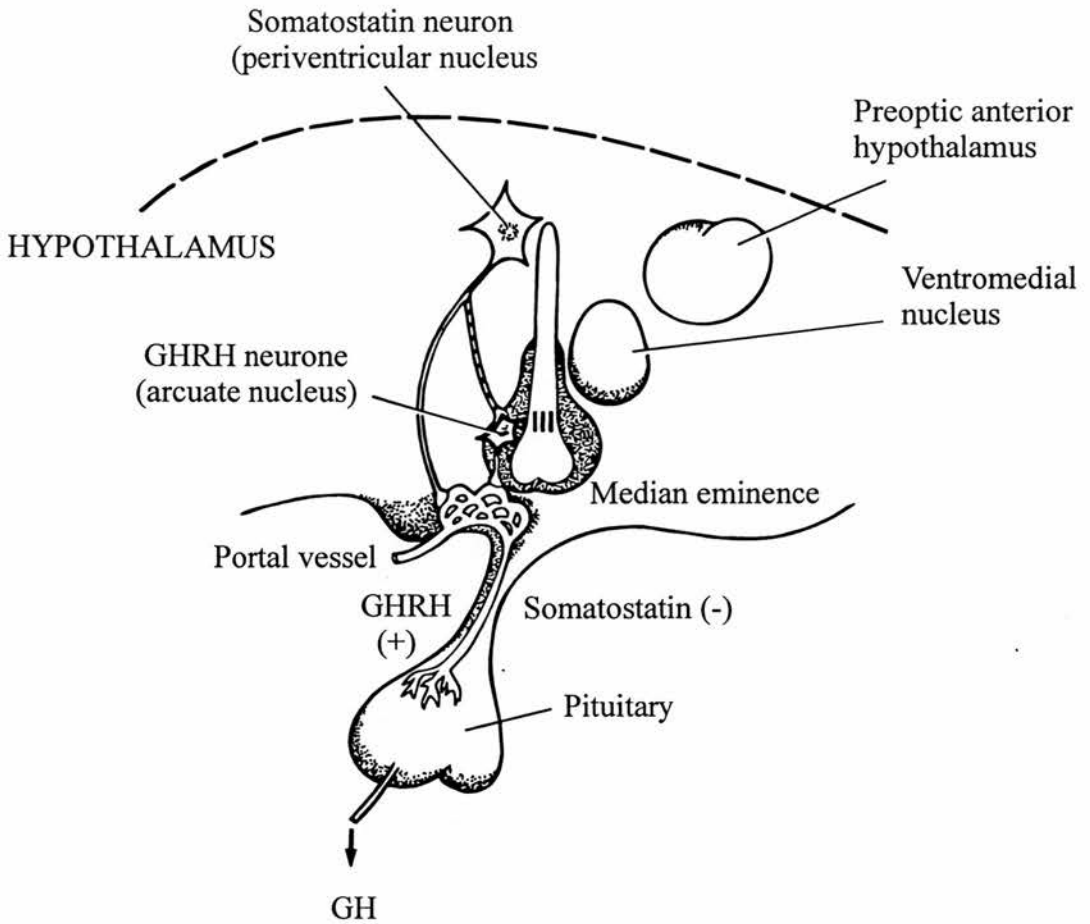


Fig.1.1 . Loci and connections of hypothalamic peptidergic neurones of the GH neuroendocrine axis.:Acta Paediatr Scand [Suppl] 372: 5-16, 1991.

Somatostatin also plays a crucial role in governing the periodicity of GH secretion. To understand the mechanism, pulsatile secretory patterns were established using administration of various involved peptides especially GHRH and somatostatin, in different ways. Application of 10-min GHRH pulses every 3-h cycles to dispersed anterior pituitaries (10^7 cells) generated consistent peaks of GH release, between 200 and 300 ng/min, and these peaks were not altered by continuous co-application of somatostatin (Weiss et al., 1987). However, the same study found that somatostatin withdrawal during GHRH administration elicited markedly higher GH peaks and more total GH release. In another *in vitro* study, the burst of GH release after cessation of perfusion of somatostatin plus GHRH was blocked by the administration of somatostatin during the burst, while the magnitude of the burst was proportional to the concentration of GHRH preceding the withdrawal of somatostatin (Kraicer et al., 1988). Thus the timing and fluctuating pattern of somatostatin release might be key factors that generate periodicity of GH secretion. Such results led to the hypothesis that the magnitude of the GH pulses is set by the amount of GHRH impinging on the somatotrophs, while somatostatin regulates the timing of the episodic bursts of GH secretion.

Somatostatin withdrawal was thus recognised as a potential generator of pulsatile GH release. However, somatostatin withdrawal alone exhibited only a small GH rise (maximum GH 2.9 ± 1.2 $\mu\text{g/l}$) in normal young men (Ho et al., 1993), indicating that somatostatin withdrawal by itself is an ineffective promoter of GH release. Periodic quiescence of somatostatin neurones must be associated with a concomitant GHRH pulse in order to result in a robust GH pulse.

1.2 GH secretagogues

1.2.1 History and development of GH secretagogues

Classically, our understanding of GH release has focussed mainly on the roles of GHRH and somatostatin. However, other neurosecretory pathways may also play an important role in regulating GH release.

In 1976, Cyril Y. Bowers of Tulane University (New Orleans, La., USA) found that some opioid peptide derivatives had weak GH releasing activity (Bowers et al., 1980). Following this observation, many peptide derivatives were synthesised in an attempt to establish more potent GH-releasing peptides. However, the opiate effect on GH release, though obvious, is not highly specific as it also releases prolactin (Shaar et al., 1977). In 1981, peptide analogues of the opiate met-enkephalin were identified that lacked opiate activity, but caused release of GH (Bowers et al., 1981). Thereafter, met-enkephalin was used as a model for newly synthetic compounds.

The effect of met-enkephalin analogues on GH release was also observed in normal men when the analogue DAMME [D-Ala²,MePhe⁴-Met-enkephalin-(o)-o¹, FK 33-824] was shown to enhance GHRH-induced GH release (Delitala et al., 1989). This suggested that the effect of this analogue may be mediated via a different pathway from GHRH.

Conformational energy calculations, computer modelling, structural modification, and biological studies were carried out for new compound synthesis. The met-enkephalin analogue I, Tyr-D-Ala-Gly-Phe-Cys(Et), with an isosteric isomer of methionine in met-enkephalin, was demonstrated to increase serum concentrations of prolactin and GH in male rats, and this effect was reversed by simultaneous treatment with the opiate antagonist naloxone (Nedvidkova et al., 1988). This analogue was more potent than natural met-enkephalin in releasing GH. The inhibition by naloxone suggested the involvement of opiate pathways in the brain rather than an action on the hypophysial-pituitary axis.

Progressively more active analogues were designed. Among a series of GH-releasing peptides (GHRPs), pentapeptide, the His-DTrp-Ala-Trp-DPhe-NH₂ (I) and the hexapeptide, His-DTrp-Ala-Trp-DPhe-Lys-NH₂ (II) exhibit *in vitro* activity at 1-10 ng/ml, and these peptides were also active in releasing GH *in vivo* at low microgram dosages (Momany et al., 1984). These GHRPs lacked any effect on TSH, LH, FSH, and prolactin release.

Due to structural modifications, these enkephalin-derived GH-releasing peptides might have different binding sites from those of opiates. In binding assays, a series of pentapeptides and

hexapeptides GHRPs were found to have low potencies at *mu* and *delta* opioid binding sites receptors in forebrain of rats (Codd et al., 1988). Thus, the changes in structure of GHRPs enhanced GH-releasing activity while decreasing their potency at opioid binding sites.

As new compounds were synthesised that were more potent and more specific in releasing GH from the pituitary gland, these came to be called “GH secretagogues” (GHSs). The chronology and structure of various peptidyl and non-peptidyl GH secretagogues is shown in the **fig. 1.2** (Camanni et al., 1998).

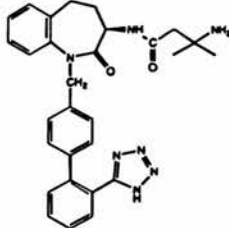
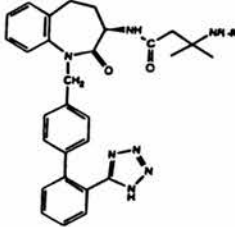
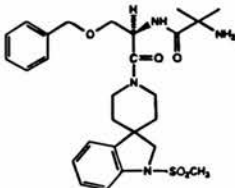
1977	Tyr-D-Trp-Gly-Phe-Met-NH ₂	<u>(D-Trp³)MetEKNH₂</u>
1981	Tyr-D-Trp-Ala-Trp-D-Phe-NH ₂	
1984	His-D-Trp-Ala-Trp-D-Phe-NH ₂	
	His-D-Trp-Ala-Trp-D-Phe-Lys-NH ₂	<u>GHRP-6</u>
1991	Ala-His-DβNal-Ala-Trp-D-Phe-Lys-NH ₂	<u>GHRP-1</u>
1992	His-D2MeTrp-Ala-Trp-D-Phe-Lys-NH ₂	<u>Hexarelin</u>
		<u>L-692,429</u>
1993	D-Ala-DβNal-Ala-Trp-D-Phe-Lys-NH ₂	<u>GHRP-2</u>
1994		
		<u>L-692,585</u>
	R = -CH ₂ CHOH-CH ₃	
1995	INIP-DβNal-D-Trp-D-Phe-Lys-NH ₂	<u>G 7509</u>
	INIP-DβNal-DβNal-Phe-Lys-NH ₂	<u>G 7039</u>
		<u>MK-0677</u>
	GAB-D-2MeTrp-DβNal-Phe-Lys-NH ₂	<u>EP 50885</u>
	GAB-D-2MeTrp-D-2MeTrp-2MeTrp-Lys-NH ₂	<u>EP 51216</u>
1996	IMPR-D-Phe-Ala-Trp-D-Phe(CH ₂ NH)-Lys-ol	<u>NNC26-0194</u>
	Aib-D-2MeTrp-D-2MeTrp-NH ₂	<u>EP 51389</u>

Fig. 1.2. Chronology and structure of various peptidyl and non-peptidyl GH secretagogues.: *Frontiers in Neuroendocrinology* 19,47-72 (1998)

1.2.2 GHRP-6

The synthetic hexapeptide, His-DTrp-Ala-Trp-Dphe-Lys-NH₂ (GHRP-6) was first identified in 1984 (Momany et al., 1984). Its GH releasing activity was very potent *in vitro* and *in vivo* in many species including rat. (Bowers et al., 1984). Intravenous injection or intranasal administration of GHRP-6 caused a marked increase in plasma GH concentrations, and enhanced IGF-I production in healthy male subjects (Hayashi et al., 1991). It was also found to be orally active in normal men at doses of 100 and 300 mg/kg (Bowers et al., 1992).

Membrane fragments from both the hypothalamus and anterior pituitary tissues were found to contain binding sites to GHRP-6 and related peptides, and the bindings of these peptides were significantly correlated to their potencies in releasing GH (Codd et al., 1989).

1.2.3 Pituitary mechanism of GHRP-6

Initially, it was unclear whether GHRP-6 and related peptides stimulated GH release via the same mechanism to that of endogenous GHRH. It was first thought that GHRP-6 acted on the GHRH pathway since it did not elicit GH release in GH-deficient mice, which are presumed to have either a deficit in GHRH or a GHRH receptor malfunction (Bowers et al., 1992). In addition, hexarelin was unable to increase plasma GH concentration in adult men affected with GHRH receptor deficiency (Maheshwari et al., 1999). These *in vivo* blunted GH responses to GHRP indicated the involvement of hypothalamic GHRH for the GHRP effect. Alternatively, the blunted response to GHRP could reflect low or absent pituitary GH stores brought about by chronic GHRH deficiency, rather than a requirement for acute GHRH involvement. However in normal men, synergistic GH release was observed following the administration of maximal doses of GHRP plus GHRH (Bowers et al., 1990). The synergistic action of GHRP and GHRH suggest that they act, at least in part, independently. It proved difficult however to study independent effects of GHRP or related peptides separately from the effects of GHRH and other endogenous factors *in vivo*. A model of acute GHRH deprivation was needed in order to examine the requirement of GHRH for GHRP action *in vivo*. However observations of GH

release at the level of single pituitary cells provided more direct evidence of the cellular effects of GHRP.

1.2.4 GHS and GHRH act on different pathways

Although GHRH and GHS both release GH, their actions appeared to be rather unrelated. GHRP-6 and the later synthesised KP 101, induced GH release when applied to ovine pituitary cells, and the induction was not affected by a GHRH antagonist ([Ac-Tyr1, D-Arg2]-GHRH 1-29) which significantly reduced the effect of GHRH on GH release (Wu et al., 1994). The GH secretory response of pituitary cells desensitises rapidly during continued exposure to either GHRP or GHRH. Dispersed rat pituitary cells that were desensitised to either GHRP-6 or GHRH still showed GH release when challenged with the alternate peptide, showing that there was no cross-desensitisation between them (Blake and Smith, 1991). These findings indicated that GHRPs act via different receptors from GHRH. In support of this, GHRH was demonstrated to have additive stimulatory effects with hexarelin in normal rat somatotrophs, but did not affect hexarelin-stimulated GH release in a GH-secreting rat cell line (GH₁) that lacks GHRH receptors (Giustina et al., 1997). This finding from a GH-secreting rat cell line might not represent the mechanism in normal pituitary cells, but it suggests that non-GHRH-mediated pathways for GHRP action exist. In 12 cell cultures derived from human somatotroph adenomas, GH secretion could be stimulated with GHRP-6 in all 12 cultures, whereas only 6 cultures responded to GHRH (Renner et al., 1994). These observations confirm the existence of a population of GHRP-6-stimulated adenoma cells, which is not dependent on GHRH pathway in releasing GH.

Thus, the *in vivo* synergistic action of GHRP-6 and GHRH suggest that the endogenous GHRH pathway is not the only pathway that stimulates GH release. The idea of another pathway for stimulation of GH release was supported when GHRH antagonists failed to block the induction of GH release by either GHRP-6 or a newly synthesised secretagogue KP-101.

In a rat model in which GHRH function was impaired by treatment with GHRH antiserum, the GH secretagogue hexarelin still stimulated GH mRNA expression in the anterior pituitary (Torsello et al., 1997). From the binding assay, a significant correlation between the binding affinity of secretagogues (MK-0677, L-692,429 and the peptides GHRP-6 and hexarelin) and GH-secretory activity was confirmed; the binding was not displaced by GHRH or by somatostatin, and it showed the properties of a new G-protein-coupled receptor (Pong et al., 1996). It was speculated that GH secretagogues mimic an unidentified natural hormone that regulates GH secretion in concert with GHRH and somatostatin.

The mechanism by which GHRPs elicit GH secretion has been investigated at the level of intracellular signalling pathways in pituitary cells. GHRP-1, a second generation GHRP, (Ala-His-D-beta Nal-Ala-Trp-D-Phe-Lys-NH₂), induced increases in intracellular [Ca²⁺] and GH release in rat pituitary cell cultures like GHRH, but unlike GHRH, GHRP-1 increases GH via a [Ca²⁺]-dependent and cAMP-independent mechanism (Akman et al., 1993). cAMP is one of the second messengers believed to mediate part of the effect of GHRH on GH release since cAMP concentration in rat primary pituitary cell culture was increased by about 3-fold. However, GHRP-6 had no effect on the basal cAMP level (Cheng et al., 1989). No effect of GHRP-6 or other secretagogues on cAMP was observed except GHRP-2 which increased cAMP concentrations; GHRP-2 had an additive effect with GHRH on both cAMP concentrations and GH release, whereas its additive effect with GHRP-6 was only on GH release (Wu et al., 1996). The additive effects of GHRP-2, GHRH and GHRP-6 suggest that these three peptides act on different receptors.

The intracellular mechanism of protein kinase C (PKC) pathway of GHRP can be studied by measuring intracellular free Ca²⁺ *in vitro*. When external Ca²⁺ was removed or when voltage-gated membrane Ca²⁺ channels were blocked, GHRP-6 still caused a transient elevation of intracellular Ca²⁺ in single rat somatotrophs (Herrington and Hille, 1994), suggesting that GHRP stimulates the release of Ca²⁺ from intracellular stores. Additionally, simultaneous measurement of intracellular Ca²⁺ and membrane current revealed that the transient release by GHRP-6 activates a voltage-independent Ca²⁺-activated K⁺ conductance (Herrington and Hille, 1994). This rapid transient phase was followed by a persistent phase. It thus seems likely that

GHRP-6 depolarises somatotrophs, inducing voltage-gated Ca^{2+} entry, while also acting via intracellular second messenger pathways to release Ca^{2+} from intracellular stores, which leads to increasing K^+ conductance and finally transient hyperpolarisation of somatotrophs.

Studies in human somatotrophs have also been performed using cells from human acromegalic tumours. In tumours in which GH secretion was increased by GHRH and GHRP-2 (a second generation GHRP) the cAMP antagonist (Rp-cAMP) blocked the GH response to GHRH but not to GHRP-2, whereas PKC inhibitors attenuated the effect of GHRP-2 on GH secretion but did not affect the GH response to GHRH (Chen et al., 1998). These findings suggest that GHRH increases GH secretion from human GH tumours via the cAMP pathway whereas GHRP-2 increases GH secretion mainly via the PKC pathway.

Based on these findings, GHRPs might act on distinct receptor(s) different from that of GHRH to depolarise pituitary cell membranes that lead to opening of Ca^{2+} channels and to GH release. In addition, the PKC pathway is activated, and Ca^{2+} from intracellular stores is released from intracellular stores, which may also contribute to GH secretion; however intracellular Ca^{2+} has many potential actions, including regulation of gene expression.

1.2.5 Effects of GHRP on GH synthesis

Thus GHRP stimulates release of GH; does GHRP also induce GH synthesis? The expression of GH mRNA, but not prolactin mRNA in monolayer cultures of rat pituitary is increased by exposure to GHRH (Gick et al., 1984). However, GH mRNA expression was also affected by other sex steroids when bovine GH content in primary cultures of normal and transformed tumour cultures of rat pituitary cells was decreased by triiodothyronine (T3) and dexamethasone which did not alter GH secretion (Silverman et al., 1988). On the other hand, estradiol had no effect on bovine GH mRNA expression, but increased bovine GH secretion (Silverman et al., 1988).

Since both GH mRNA expression and GH release are increased by GHRH, it seems plausible that GH release is a consequence of GH synthesis following the induction of GH expression. GHRP was therefore investigated whether it plays a role in the control of GH biosynthesis as well as release. *In vivo*, models of reduced GH mRNA expression were needed to investigate the effects of GHRP. In adult male rats after 5-day pre-treatment with surgical ablation of the mediobasal hypothalamus, hexarelin did not enhance GH secretion, and on day 7 GHRH was significantly more effective than hexarelin; on day 30, by which time the mediobasal hypothalamic function was supposed to recover, hexarelin and GHRH evoked similar rises of plasma GH (Torsello et al., 1996). These results suggest that hexarelin acts, at least in part, on the hypothalamus through the release of an unknown hypothalamic factor that elicits GH release, acting synergistically with GHRH.

In infant rats immunised with GHRH antibodies, hexarelin administration for 3-10 days significantly enhanced the GH response, though mean plasma GH concentrations remained lower than in control group, and treatment with hexarelin for 3 and 5, but not 10 days, restored GH mRNA levels to control values (Torsello et al., 1997). Similar effects were observed in GHRH-antibody passively immunised pups treated with GHRP-6 (Locatelli et al., 1994). Antibodies against GHRH were used to block GHRH function, while leaving GHRH receptors intact. In GH deficiency animal model, impairment could be either at level of GHRH synthesis or GHRH receptor malfunction, but by using GHRH antibodies, changes in plasma GH concentrations should reflect the abilities of hexarelin or GHRP-6 in inducing GH mRNA expression, and thereafter GH release.

These findings suggest that the effects of GHRP on GH release and GH mRNA expression may be unrelated. Moreover, it is likely that GHRP can stimulate GH mRNA expression independently of endogenous GHRH. However, the failure of hexarelin to enhance GH release in adult rats with surgical ablation of mediobasal hypothalamus emphasises the concept that *in vivo*, GHRP acts mostly on the hypothalamus.

1.2.6 Actions of GHRP in the central nervous system

Studies of the effects of GHRP-6 and related peptides have focused on the hypothalamus and the pituitary gland. The hypothalamus was hypothesised to be a central brain region where GHRPs could influence the release of GHRH and somatostatin. Central effects of GHRPs in sheep are inferred by the demonstration of an increase in jugular GH concentrations and a stimulation of GHRH release into hypophysial portal blood following intravenous injection of hexarelin (Guillaume et al., 1994). Measurements of GHRH in the hypophysial portal blood of conscious sheep during infusion of GHRP-6 showed a 50 % increase in GHRH pulse frequency, though the mean plasma GHRH concentrations were not significantly increased. Such findings indicate that GHRP-induced GH stimulation in the sheep involves activation of GHRH neurones in addition to its direct effect on pituitary cells.

Systemic (or central) administration of GHRP-6 resulted in induction of expression of Fos (the protein product of the *c-fos* gene) throughout the ventromedial and ventrolateral regions of the rat arcuate nucleus (Dickson et al., 1993). The induction of Fos expression was highly specific to the arcuate nucleus; no Fos expression was observed in other hypothalamic regions, though since these earliest reports subsequent studies have shown that some secretagogues (though not GHRP-6) induce Fos expression in the supraoptic nucleus, and GHRP-6 and other secretagogues induce some Fos expression in the nucleus tractus solitarii in the brainstem (Bailey et al., 1999b).

The expression of Fos is not secondary to the stimulation of GH secretion, since similar patterns of Fos expression were observed in GH-deficient “little” (*lit/lit*) mice in response to GHRP-6 (Sirinathsinghji et al., 1995), and systemic (or central) injection of GHRH did not result in Fos expression, nor did systemic (or central) injection of IGF-1 (Dickson et al., 1995a). Either intravenous (i.v.) or intracerebroventricular (i.c.v.) injections of GHRP-6 resulted in an increased density of Fos-immunoreactive neurones in the arcuate nucleus, and these effects were similar to those induced by the non-peptidyl secretagogues (L-692,585 and L-692,429), whereas no increase in Fos expression was observed in rats given a systemic injection of a high dose of GHRH (Dickson et al., 1995b). The failure of systemic injection of

GHRH to induce Fos expression in the arcuate nucleus implies that GHRP-6-induced Fos in this region is not mediated by increased plasma concentrations of GH or GHRH.

More recently, the induction of Fos expression in the arcuate nucleus by systemic injection of GHRP-6 was reported to be potentiated in fasted rats and this induction was not altered by co-administration of leptin (Luckman et al., 1999). This study also found that leptin by itself induced Fos immunoreactivity in the arcuate nucleus, though not necessarily in the same neurones as are activated by secretagogues. The detection of electrical responses recorded at the cell bodies of arcuate neurones of rats injected intravenously with GHRP-6 confirms that the arcuate nucleus is a central site of action of GHRP-6 (Dickson et al., 1995b).

The detection of *c-fos* gene expression has been widely used to determine specific central brain regions that respond to compound administration. However, it should not be considered an indicator of a direct site of action, nor should it be considered an infallible marker of activation. Other neurones in the brain that do not express Fos may also be activated. Moreover, neurones expressing Fos might be activated indirectly as a result of depression of activity in inhibitory afferent inputs. Characterising the electrophysiological responses of neuroendocrine arcuate neurones provides more direct evidence for central actions of GHRP-6 or other secretagogues.

1.2.7 Actions of GH secretagogues on secretory neurones in the hypothalamus

Intravenous injection of GHRP-6, resulted in induction of Fos-like immunoreactivity specifically within the arcuate nucleus of rats, as well as stimulating the electrical activity of a population of arcuate neurones of rats that were antidromically identified as projecting to the median eminence (Bailey et al., 1998). Most of the arcuate neurones that expressed Fos in response to GHRP-6 were also labelled with fluorescence retrograde tracer Fluorogold given systemically to label neurones that project outside the blood brain barrier (Dickson et al., 1996). The neuronal activation to the systemic administration of the secretagogue shows that the action is relatively selective, and occurs mainly in putative neurosecretory neurones.

In later experiments it was established that the activity of MK-0677-responsive arcuate neurones was inhibited by systemic somatostatin, whereas the activity of MK-0677-non-responsive and non-neuroendocrine arcuate neurones was not affected (Bailey et al., 1998). These findings indicated that secretagogues act specifically on arcuate neurones that regulate GH secretion from the pituitary gland.

Though the patterns of activation of non-peptidyl secretagogues seen in Fos expression (Dickson et al., 1995b) and electrical activity (Bailey et al., 1998) were similar to that of GHRP-6, the exact mechanism was unclear. L-692,429 is one of a novel non-peptidyl class of GH secretagogues. It was demonstrated to stimulate GH release from rat primary pituitary cells, and this effect was inhibited by GHRP-6 antagonist but not by antagonists to GHRH or opiates (Cheng et al., 1993). Similarly, L-692,400, an antagonist of L-692,429 and GHRP-6, antagonised stimulation of GH release caused by GHRP-6 but did not antagonise GHRH (Smith et al., 1993). Thus GHRP-6 and L-692,429 apparently act at the same receptors. Additionally, L-692,429 was found to increase cytosolic free Ca^{2+} concentration of rat somatotrophs determined by fluorescence ratio imaging (Smith et al., 1993). Such data indicated that the non-peptide secretagogues are mimetics of GHRP-6 based signal transduction.

1.3 Growth hormone secretagogue receptor

1.3.1 Characterisation of the GHS receptor

In 1989, a significant correlation between binding activity of SK&F110679 to membrane fragments of both hypothalamus and anterior pituitary, and the release of GH was obtained (Codd et al., 1989). Since then, a series of secretagogues (GHSs) and their mimetics have been designed to have specific effects on GH release. Consistently, the spiroindoline MK-0677, one of ideal small molecule of GHS, was incorporated with ^{35}S to produce a radioligand with a high specific activity for binding assays (Pong et al., 1996). The binding of this ligand to anterior pituitary membranes is Mg^{2+} -dependent, is inhibited by GTP- γ -S, and is competitively inhibited by benzolactam L-692,429, and by the peptides GHRP-6 and hexarelin, but not by

GHRH and somatostatin (Pong et al., 1996). High-affinity binding was non-competitively inhibited by GTP- γ -S suggesting that the receptor was G-protein coupled. Additionally, the inhibition of [35 S]MK-0677 binding by GHS but not GHRH or somatostatin implied the discovery of a new receptor involved in the control of GH release.

In an attempt to isolate the MK-0677 receptor, digitonin was used to solubilise the receptor from porcine anterior pituitary membranes. The receptor was recovered as a receptor-[35 S]MK-0677-G protein complex that appeared in the digitonin micelle, but the receptor was not successfully isolated (Pomes et al., 1996). In addition, treatment with GTP- γ -S to the solubilised receptor in the digitonin micelle resulted in dissociation of MK-0677 from the receptor, whereas treatment with ATP- γ -S did not (Pomes et al., 1996). This provided evidence that the MK-0677 receptor was coupled to a G protein in the soluble complex.

1.3.2 Cloning of the GHS-R

Identification of binding sites of radiolabelled peptide and nonpeptide GHSs in pituitary and hypothalamic membranes led to attempts to identify, clone and sequence of the GHS receptor (GHS-R). *Xenopus* oocytes injected with Size-fractionated polyadenylated [poly(A) $^{+}$] RNA isolated from swine pituitary, exhibited oscillatory increases in calcium-activated chloride (Cl $^{-}$) currents induced by MK-0677 or GHRP-6 (Howard et al., 1996), indicating expression of the GHS-R. The activation of Cl $^{-}$ currents is presumably based on observations that ligands for the receptor activated IP $_3$ -induced release of intracellular Ca $^{2+}$. However, positive responses from injected oocytes of this study were relatively low. Thus, the use of rat pituitary polyadenylated RNA failed to give a clear response.

Howard and colleagues co-injected synthetic complementary RNA (cRNA) that encodes the bioluminescent-sensitive protein aequorin and cRNA for the G protein α subunit G α 11 to improve the sensitivity of the assay (Howard et al., 1996). With co-injection of G α 11 cRNA and aequorin cRNA, human pituitary, rat pituitary and rat hypothalamus poly(A) $^{+}$ RNA also gave positive responses to MK-0677, whereas poly(A) $^{+}$ RNA from control tissues did not

(Howard et al., 1996). This indicated that a GHS-R, probably coupled to $G_{\alpha 11}$, is present in human pituitary gland. Using this assay, a swine pituitary cDNA library was screened for an MK-0677-inducible signal. Stepwise fractionation of a single positive resulted in the isolation of a single cDNA clone that conferred both MK-0677-evoked aequorin luminescence response and an inward chloride current in *Xenopus* oocytes (Howard et al., 1996).

The nucleotide sequence of the identified clone revealed a full-length swine MK-0677 receptor cDNA-1a, that predicted a protein of 366 amino acids with seven transmembrane (7-TM)-spanning domains, three intra- and extracellular loops, and a G protein receptor triplet signature sequence (Howard et al., 1996). The primary sequence of GHS-R showed that the receptor was distinct from those of other G-protein-coupled receptors. Genomic analysis by Southern blotting was consistent with a single highly conserved gene in human, chimpanzee, bovine, rat, and mouse (McKee et al., 1997). The GHS-R gene has been localised to human chromosome 3Q26.2 and mouse chromosome 3a3 (McKee et al., 1997).

Additionally, cDNA clones that encode a shorter form of the MK-0677 receptor were obtained (McKee et al., 1997). This cDNA is called receptor 1b cDNA that encodes a polypeptide of 289 amino acids that lacks transmembrane domain 6 and 7 of the 1a receptor. Unlike the GHS-R 1a cDNA, when the 1b cDNA is expressed in cells, the resulting protein fails to bind [35 S]MK-0677 and can not be activated by GHS-R ligands (Howard et al., 1996). The functional role of this truncated receptor remains to be defined.

1.3.3 Tissue distribution of the GHS-R

Initial *in situ* hybridisation studies using a cDNA probe selective for GHS-R 1a to sections of Rhesus monkey and rat brain indicated that the receptor is expressed in the arcuate nucleus (Howard et al., 1996). Localisation of the receptor in this area is consistent with the activation of Fos protein expression and electrical responses of neuroendocrine arcuate neurones by GHRP-6 and non-peptidyl GHSs (Dickson et al., 1995b). However, the precise localisations of GHS-R mRNA in rat brain were also performed. The mapping study of GHS-R identified its

expression in pituitary, hypothalamus, hippocampus, substantia nigra, ventral tegmental area, and dorsal and median raphe nuclei (Guan et al., 1997). The expression of the receptor outside the hypothalamic area suggests that it might also be involved in mood, cognition, memory or learning processes. The exact function of expression of the GHS-R gene in areas of the brain other than those involved in GH release is still unclear. Apart from the pituitary gland, the receptor was also found to be expressed in the pancreas, but the expression signal was weak (Guan et al., 1997).

Comparative distribution of mRNA encoding the GHS-R in rat and *Microcebus murinus* (Primate, lemurian) showed significant species differences in the periventricular nucleus, the ventromedial nucleus, the lateral hypothalamic area, and the pituitary gland (Mitchell et al., 2001), but found consistent expression of GHS-R mRNA in the arcuate and ventromedial hypothalamic nucleus. These results may indicate the involvement of GHS-R in the control of GH secretion, feeding behaviour and seasonal rhythmicity among murine species and primates. Especially in the arcuate nucleus, GHS-R is expressed in NPY neurones (Willesen et al., 1999), and *c-fos* mRNA is induced in NPY neurones following GHS administration (Dickson and Luckman, 1997).

In dwarf rats (*dw/dw*) with chronic GH deficiency, GHS-R is up-regulated in the arcuate nucleus, ventromedial hypothalamic nucleus, and in CA1/CA3 regions of hippocampus (Bennett et al., 1997). Administration of recombinant bovine GH continually for 6 days by osmotic minipump completely reversed these changes in the arcuate nucleus and ventromedial hypothalamus, with GHS-R expression falling in both areas to levels significantly below those in normal rats (Bennett et al., 1997). This study also measured the significantly increased GHRH concentrations in dwarf rats, which were normalised by GH administration.

GHS-R mRNA of both 1a and 1b were also detected in human pituitary somatotropinomas removed from patients with acromegaly, and these cultured tumours exhibited strong responses to GHRP-2 in terms of both phosphatidylinositol hydrolysis and GH secretion (Adams et al., 1998). Similarly, GHS-R mRNA was also found in normal human pituitary cultures of foetus

at 18 and 13 weeks of age, and also it has been shown to be functionally responsive to GHS *in vitro* (Shimon et al., 1998).

Ong and colleagues developed a photoreactive derivative of hexarelin by inserting a tyrosine and a Bpa residue (*p*-benzoyl-L-phenylalanine) for iodination and photolabeling respectively (Ong et al., 1998). With this technique, they specifically labelled a protein with an apparent molecular mass of 57000 in human, bovine and porcine anterior pituitary membranes (Ong et al., 1998). This is an identification of a pituitary GHS-R subtype, which is apparently distinct from the recently cloned GHS-R.

1.3.4 GHS-R is expressed in GHRH neurones

In situ hybridisation studies using a cDNA probe selective for GHS-R demonstrated that the receptor is expressed in the arcuate nucleus (Howard et al., 1996). The localisation of expression in this area of the hypothalamus is consistent with electrophysiology experiments and Fos protein immunoreactivity. It was hypothesised that GHS-R-expressing neurones were GHRH-containing arcuate neurones which are neurosecretory. The study using dual chromogenic and autoradiographic *in situ* hybridisation showed extensive overlap with GHRH-expressing neurones in both the arcuate and ventromedial hypothalamic nucleus. In RNase protection assays, localisations of GHS-R were observed mainly in hypothalamus, hippocampus and pituitary (Guan et al., 1997). These data showed expression of GHS-R specifically in many central and peripheral tissues that are generally considered as being involved in GH release, but also in some regions, including ventromedial hypothalamic neurones, that seem unlikely to be specifically involved in GH release.

1.3.5 Most GHS-R-containing neurones are NPY neurones

In the arcuate nucleus, 20-25 % of GHRH mRNA-expressing neurones contained GHS-R mRNA, whereas most of the arcuate GHS-R-mRNA-containing cells did not contain detectable GHRH mRNA (Willesen et al., 1999). This study found that GHS-R mRNA was expressed in

94 % of neurones expressing NPY, 8 % of those expressing proopiomelanocortin (POMC) and 30 % of those expressing somatostatin mRNA. It suggests that while part of secretagogue's effect mediates a direct stimulation of GHRH release, other secretagogue effects are to be expected. The population of *c-fos* mRNA-expressing neurones was also studied using *in situ* hybridisation. In GHRP-6-injected rats, neurochemically identifiable cells expressing *c-fos* mRNA also express NPY (neuropeptide Y) mRNA (51 +/- 4%), GHRH mRNA (23 +/- 1%), tyrosine hydroxylase mRNA (11 +/- 3%), POMC mRNA (11 +/- 2%), or somatostatin mRNA (4 +/- 1%) (Dickson and Luckman, 1997).

NPY is abundant in the hypothalamus, but is mainly synthesised in a sub-population of neurones in the arcuate nucleus (Morris, 1989). The NPY pathway that projects from the arcuate nucleus to the paraventricular nucleus (PVN) is stimulated under conditions of negative energy balance (Beck et al., 1990b). Intracerebroventricular injection of NPY stimulate feeding and increased immunostaining of Fos in several hypothalamic sites, including the parvocellular and magnocellular paraventricular nucleus, and these effects on both feeding and Fos induction were altered by the NPY Y1 receptor antagonist, 1229U91 (Yokosuka et al., 1999). These data suggest the involvement of NPY and the paraventricular nucleus in feeding regulation. The NPY pathways from the arcuate nucleus to the paraventricular nucleus might be involved since injection of secretagogues to the arcuate nucleus exhibited significant orexigenic effects in rats (Torsello et al., 2000). However, only a small proportion of arcuate neurones that project to paraventricular nucleus are activated by injection of GHRP-6 (Honda et al., 1999). Thus the orexigenic effect of this secretagogue may involve other unidentified neural pathways. These effects are unrelated to actions on GH release. The i.c.v. injection of GHRP-6 significantly stimulated eating behaviour in a dose-related pattern, but plasma GH concentrations in response to i.c.v. GHRP-6 fluctuated and are not dose-related in sated, adult rats (Locke et al., 1995).

1.4 Ghrelin

Of the many GHSs, GHRP-6 is the most widely used compound, with potent GH releasing activity both *in vitro* or when injected intravenously into rats (Bowers et al., 1984). In 1993, a group from Merck and Co. reported the first non-peptide GHS, L-692429, a mimic of the GHRP-6 (Smith et al., 1993). Thereafter, an even more potent non-peptide GHS, MK-0677, was synthesised using GHRP-6 as a template (Patchett et al., 1995).

The possibility that GHSs act via an unknown hypothalamic factor (U factor) is still open. It was rather unusual that GHSs had been developed, while neither GHS-R nor an endogenous ligand for this receptor was identified. In 1996, the GHS-R was cloned, and it does not show sequence homology with other G-protein-coupled receptors known so far (Howard et al., 1996). This identification of GHS-R emphasised the existence of a second route of endogenous factor or hormone that regulates GH release. After the identification, a search for its endogenous ligand was actively performed, with the use of the 'orphan receptor strategy'.

1.4.1 Purification and identification of ghrelin

Cells expressing an orphan G-protein-coupled receptor have been used to identify unknown endogenous ligand (for review see Civelli, 1998). The discovery of the GHS-R was a key factor of searching for its novel ligand. Especially, its binding with synthetic GHSs provided a convenient positive control for the assay used to identify the endogenous ligand. Concentrations of intracellular calcium were monitored in order to detect GHS-R stimulation. Because of the presence of GHS-R in the pituitary and the hypothalamus, a novel ligand was thought to be produced in the brain. However, tissue extract from rat brain failed to induce significant changes in intracellular calcium in CHO cell line expressing rat GHS-R (CHO-GHSR62), whereas the stomach extract did (Kojima et al., 1999). The active peptide was further purified from the stomach extract by gel filtration, two ion exchange columns and reverse-phase high-performance liquid chromatography (RP-HPLC). These processes revealed potent intracellular calcium-increasing activity in the fractions of relative molecular mass of

approximately 3,000 (Kojima et al., 1999). Active fractions were further purified by HPLC, followed by RP-HPLC to give pure ghrelin, from *ghre*, a word root in the Proto-Indo-European language for grow.

Kojima and colleagues continued to identify ghrelin. Purified rat ghrelin is a 28-amino acid peptide, and was determined by a protein sequencing to be GSXFLSPEHQKAQQRKESKKPPAKLQPR in which the third residue X was not identified (Kojima et al., 1999). An expressed sequence tag clone revealed that the third residue is a Serine, which was later confirmed in cDNA clones encoding the peptide precursor isolated from a rat stomach cDNA library. However, the synthetic 28-residue peptide (with X = Ser) with the sequence deduced from cDNA analysis did not increase intracellular calcium. Moreover, RP-HPLC showed that purified natural ghrelin had a longer retention time than the synthetic peptide, which means the natural ghrelin has greater molecular weight. Mass spectrometric analysis confirmed that the molecular weight of purified ghrelin (3315) was 126 Da greater than the synthetic peptide (3189). This indicates that Ser 3 in natural ghrelin must be modified by a hydrophobic moiety. Choices of modifications of ghrelin with substances such as a metal, a carbohydrate or lipid, were discussed, and found not convincing. With considerations of molecular weight and elution time from RP-HPLC, the modification was thought to be a hydrophobic compound. Addition of an acyl acid, *n*-octanoic acid (molecular mass 144) was the most probable modification. Then, the hydroxyl group of Ser3 of the synthetic peptide was esterified by *n*-octanoic acid, the resulting modified peptide was of the same molecular weight as purified ghrelin. Moreover, the acyl modification increased the hydrophobicity of the peptide. In the same study, the peptide was then modified at a Ser3 with *n*-octanoic acid, and characterised. The synthetic *n*-octanoylated peptide needed the same retention time as the purified ghrelin to elute on RP-HPLC. Mass spectrometric fragmentation patterns of the synthetic modified peptide and the purified ghrelin were the same. Moreover, these two peptides had the same effect on the GHS-R-expressing cells. These results confirmed the success of the ghrelin synthesis.

Studies using radioimmunoassay combined with RP-HPLC revealed two forms of immunoreactive-ghrelin in rat stomach (Hosoda et al., 2000a). The first form was recognised

by antisera established against the octanoyl-modified portion, whereas the second form was recognised by antisera against the C-terminus portion of ghrelin. The first form of ghrelin was functionally active in increasing intracellular calcium concentration in CHO-GHSR62 cells. The second form eluted from RP-HPLC at the same retention time as that of synthetic non-modified ghrelin. These results indicate that non-modified ghrelin also exists in rat stomach tissue.

In addition, another ligand, des-Gln¹⁴-ghrelin, was also purified and characterised as the second endogenous ligand for GHS-R from rat stomach (Hosoda et al., 2000b). This form is identical to ghrelin except for a missing glutamine at the residue 14. Des-Gln¹⁴-ghrelin also contains *n*-octanoylated serine like ghrelin. It was demonstrated to be produced through an alternative splicing of rat ghrelin gene (Hosoda et al., 2000b). When injected into rats, it stimulated GH release, but the amount of des-Gln¹⁴-ghrelin in rat stomach is relatively low compared with that of ghrelin and des-acyl ghrelin. Thus, *n*-octanoylated full-length ghrelin and des-acyl ghrelin are the two major forms of this hormone in rat stomach.

1.4.2 Physiological functions of Ghrelin

In an assay for GH-releasing activity using primary cultured pituitary cells, ghrelin specifically induced GH release in a clear dose-dependent manner (Kojima et al., 1999). The induction of GH release by ghrelin was specific. No other pituitary hormone was affected *in vitro*. *In vivo*, ghrelin when injected intravenously in anaesthetised rats, increased GH to maximum levels at 5-10 min following the injection (Kojima et al., 1999). Similarly, a single i.c.v. administration of ghrelin to rats increased the plasma GH concentration in a dose-dependent manner, peaking 15-20 min after administration and returning to basal level after 60 min (Date et al., 2000b). However, the GH secretory pattern of adult male rats as shown by episodic bursts was unaltered following central ghrelin (1 µg/rat) administration (Kamegai et al., 2000). No significant difference from the saline control group was observed in plasma leuteinising hormone (LH), thyroid stimulating hormone (TSH) or prolactin following a single i.p. injection of ghrelin at the dose of 30 nM (Wren et al., 2000). However, ghrelin (2 nM), when injected

intracerebroventricularly, increased not only GH secretion, but also increased ACTH secretion and suppressed TSH secretion, and did not alter plasma LH or prolactin (Wren et al., 2000).

1.4.3 The effects of ghrelin on food intake

Intracerebroventricular injection of ghrelin caused a dose-dependent increase in food intake over 4 h, and ghrelin-induced food intake was significantly inhibited by a NPY antagonist, BIBO3304 (Lawrence et al., 2002). Significant stimulation of food intake by ghrelin has been observed at the intraperitoneal dose as low as 1 nM, the dose which yielded plasma ghrelin concentrations not significantly greater than those measured after a 24-h fast (Wren et al., 2001). Food intake induced by i.c.v. injection of ghrelin was more effective than GHRP-6, and was suppressed by an antagonist for GHS-R, [D-Lys-3]-GHRP-6 (Nakazato et al., 2001). Both starvation-induced feeding and food intake in free-feeding rats were suppressed by anti-ghrelin immunoglobulin G (Nakazato et al., 2001). These findings indicate that ghrelin is a powerful, endogenous orexigenic peptide.

A single i.c.v. injection of ghrelin increased in the number of Fos-positive neurones in the hypothalamic arcuate nucleus, the paraventricular nucleus, the dorsomedial hypothalamus, the lateral hypothalamus, and the nucleus of the tractus solitarius and the area postrema of the brainstem (Lawrence et al., 2002). The patterns of Fos induction by ghrelin were similar to those induced by GHRP-6. GHRP-6 induced the same pattern of Fos immunoreactivity in rats with free access to food and in rats that had food withheld (Lawrence et al., 2002). Thus, the activation of these brain regions by GHRP-6 was not secondary to food intake. Ghrelin might also activate these brain regions independently of food intake, according to its identical sites of receptor binding and hypothalamic action to those of GHRP-6. Intranuclear injection of ghrelin was used to compare the orexigenic response between hypothalamic nuclei. After injection of 30 nM ghrelin into the arcuate nucleus of male rats, food intake was significantly greater than in rats given ghrelin microinjections into other nuclei (Wren et al., 2001).

Both repeated i.p. and i.c.v. administration for 7 days increased accumulative food intake with no detectable attenuation of ghrelin-induced feeding on repeated administration, and increased in body weight gain (Wren et al., 2001). Chronic central administration of rat ghrelin (1 μ g/rat every 12 h for 72 h) resulted in a significant increase food intake, but did not affect plasma insulin, glucose, leptin, or GH concentrations (Kamegai et al., 2001b). This chronic central administration of ghrelin also increased both NPY mRNA and agouti-related protein (AGRP) mRNA levels in the arcuate nucleus (Kamegai et al., 2001b). These results indicated that the primary hypothalamic targets of ghrelin are NPY/AGRP-containing neurones. They are also consistent with the finding of co-localisation of GHS-R and NPY mRNA in the arcuate of the rat (Willesen et al., 1999). The involvement of NPY pathway was strongly confirmed when increased food intake by ghrelin was reversed in a dose-dependent manner by co-injection of J-115814, a NPY Y1 receptor antagonist (Shintani et al., 2001). These results support the finding that acute administration of GHRP-6 induced *c-fos* mRNA in the arcuate neurones that also contain GHRH mRNA and NPY mRNA (Dickson and Luckman, 1997).

1.4.4 Tissue distribution of ghrelin

High concentration of ghrelin in rat stomach was a key factor that led to the success of ghrelin purification and identification (Kojima et al., 1999). Since then, *in situ* hybridisation or immunohistochemical analyses have investigated the localisation of ghrelin. Ghrelin-immunoreactive cells were shown to be located in the hypothalamic arcuate nucleus as well as in the stomach (Kamegai et al., 2000). However, it is not definite whether the detection of ghrelin in the hypothalamus reflects local production of ghrelin or ghrelin peptide derived from stomach.

In situ hybridisation also showed the distribution in the rat hypothalamus (Kojima et al., 1999). Northern blot analysis of human tissues revealed that ghrelin mRNA expression was found most abundant in the stomach, and followed by the duodenum, jejunum and lung (Ariyasu et al., 2001). This was correlated to the detection of ghrelin-immunoreactive cells which were abundant from the neck to the base of the rat oxyntic gland, as well as small numbers of ghrelin

cells present in upper small intestine and very small numbers present in the lower small intestine and large intestine (Date et al., 2000a). In rat oxyntic gland, ghrelin was localised on round, compact, electron-dense granules of ghrelin-producing cells which were approximately 30 nm in diameter, smaller than the granules of D cells and enterochromaffin-like cells (Date et al., 2000a). Ghrelin mRNA and peptide were also detected in rat and human pituitary (Kamegai et al., 2001a). No significant amount of ghrelin mRNA was detected in the esophagus, ileum, ileocecum, cecum, colon, rectum, liver, brain, heart, skeletal muscle, thymus, spleen, kidney, placenta, or leukocyte (Ariyasu et al., 2001).

1.4.5 Regulation of ghrelin biosynthesis

In totally gastrectomised patients, plasma ghrelin-like immunoreactivity levels were reduced to 35 % of those in control subjects (Ariyasu et al., 2001). Plasma ghrelin-like immunoreactivity levels were increased by 31 % after 12-h fasting and reduced by 22 % immediately after feeding in human (Ariyasu et al., 2001). It can be concluded that the stomach is a major source of circulating ghrelin and that plasma ghrelin-like immunoreactivity levels reflect feeding states in human.

Both ghrelin mRNA and peptide were not detectable in stomach extracts of rat foetus until 1 day of age, then increased progressively as a function of age through the second and third weeks of life (Lee et al., 2002). The increase in levels of ghrelin expression postnatally might be due to a developmental role of ghrelin to increase food intake and growth after birth. Ghrelin mRNA expression in the stomach is up-regulated upon fasting and after insulin or leptin administration (Toshinai et al., 2001). The concentration of ghrelin peptide in the stomach tissue decreased after fasting and increased after refeeding (Toshinai et al., 2001).

Levels of many neuroendocrine peptides change with age or are influenced by puberty. However, levels of expression of ghrelin mRNA in stomach of male and female rats were similar (Gualillo et al., 2001). In addition, gastric ghrelin mRNA content seems to be relatively stable especially after 40 days of life both in male and female rats (Gualillo et al., 2001). The

independence of sex steroid influence on ghrelin expression was confirmed by unchanged levels of the expression in gonadectomised rats following gonadectomy (Gualillo et al., 2001).

In rat pituitary, ghrelin mRNA was up-regulated following infusion of human GHRH (Kamegai et al., 2001a). The expression of ghrelin in the pituitary might be part of regulation of GH response, whereas the expression in the stomach should be regulation of energy balance.

Since ghrelin and GHSs have been found to have selective site of action in the arcuate nucleus for regulation of GH secretion and energy balance, the objective of this study was to investigate the mechanism of GHS in the arcuate nucleus. To understand how GH secretion is associated with feeding control, interaction between GHS and NPY in the arcuate nucleus was needed to be examined for more clarification. In addition, the ventromedial hypothalamic nucleus has been found to be most sensitive to change of energy balance. Thus, study of effects of these compounds also in this area could be able to explain mechanism of GHS clearer.

CHAPTER 2
IN VITRO ACTION OF GH SECRETAGOGUES ON
NEURONES IN THE ARCUATE NUCLEUS AND THE
VENTROMEDIAL HYPOTHALAMIC NUCLEUS

2.1 Introduction

Growth hormone-releasing peptide-6 (GHRP-6) is one of the earliest compounds that was recognised as a selective and potent growth hormone secretagogue, and its effects are the most well-documented among synthetic secretagogues. Its effects on GH release was confirmed not to be via the same pathway as the endogenous GHRH when a combination of GHRP-6 and GHRH synergistically induced greater release of growth hormone than either treatment alone (Bowers et al., 1984).

Direct evidence that the arcuate nucleus is a site of action of GH secretagogues came first from electrophysiological studies of arcuate neurones. Intravenous injection of GHRP-6 elicited electrical activation of arcuate neurones that were antidromically identified as projecting to the median eminence (Dickson et al., 1995b). Evidence that these effects were specific to the arcuate nucleus came from studies of Fos expression, which showed that, in the hypothalamus, the only region in which GHRP-6 evoked significant Fos expression was the arcuate nucleus. Within the arcuate nucleus, it emerged from subsequent studies that GHRP-6 (and other synthetic secretagogues) induced the same localised pattern of Fos expression whether given systemically or by i.c.v. injection, indicating that the localised nature of Fos expression does not reflect limited penetration of secretagogues into the brain. By contrast with GHRP-6, neither GHRH, nor IGF-1, nor GH itself induced Fos expression in the arcuate nucleus, indicating that the response to GHRP-6 was not secondary to stimulation of GH release.

Identification by double immunohistochemistry (Dickson et al., 1996) or double *in-situ* hybridisation (Dickson and Luckman, 1997) established that, within the arcuate nucleus, GHRP-6 activated a high proportion of neurones that expressed GHRH, but activated a very low proportion of neurones that expressed TH-containing, β -endorphin, somatostatin or POMC. However most of the cells activated by GHRP-6 in the arcuate nucleus did not express detectable GHRH mRNA, but do apparently express NPY. This conclusion was supported by recent studies showing co-localisation of growth hormone secretagogue receptors in NPY neurones in the arcuate nucleus (Willeesen et al., 1999).

The functional identity of the neurones activated by GHRP-6 in the arcuate nucleus though has remained unclear. Certainly as expected, GHRH neurones are activated. The Fos expression that is induced by GHRP-6 is attenuated by a central action of somatostatin, as is secretagogue (MK-0677)-induced excitation of arcuate neurones (Bailey et al., 1998), again indicating a functional association of secretagogue effects with regulation of growth hormone secretion. Somatostatin may therefore be a functional antagonist of GHRP-6 or other secretagogues that act centrally. However not all secretagogue-responsive neurones are inhibited by somatostatin, so is there another functionally independent population in the arcuate nucleus that is activated by secretagogues, and if so, what might its functional role be?

Whether the activation of NPY-expressing neurones in the arcuate nucleus is functionally associated with the effects of secretagogues on GH secretion or is part of an independent physiological response is unclear. NPY is expressed in many different cell populations, so the presence of NPY in activated cells is not a definitive indication of functional identity. An important population of NPY cells in the arcuate nucleus is believed to have a major role in the regulation of food intake, and there have been reports that a sub-population of neurones in the arcuate nucleus which contain NPY are glucose sensitive (Muroya et al., 1999). The role of NPY has been extensively studied both in the control of normal food intake and also in studies of eating disorders; for example, the concentration of NPY in the arcuate nucleus of the hyperphagic obese Zucker rats is much greater than in the lean normophagic rats (Beck et al., 1990a). The effects of NPY on food intake are believed to be exerted principally by a direct projection from NPY cells in the arcuate nucleus to the paraventricular nucleus. However NPY expression may not be confined to this cell population, even within the arcuate nucleus.

Electrophysiological studies, and studies involving co-localisation of Fos expression with fluorogold administered systemically showed that the majority of cells in the arcuate that express Fos in response to secretagogues appear to be neurosecretory neurones, in that they project outside the blood-brain barrier (Dickson et al., 1996). However, electrophysiological studies *in vivo* indicate that arcuate neurones that project to the paraventricular nucleus are not neurosecretory. They do not also project to the median eminence (Honda et al., 1999), and

studies of Fos expression in arcuate neurones identified by retrograde tracing as projecting to the paraventricular nucleus indicate that very few of these neurones are activated by GHRP-6.

Thus it appears that GHRP-6 activates a population of NPY containing cells in the arcuate nucleus, but these appear to be neurosecretory cells rather than the cells that project to the paraventricular nucleus and regulate food intake. In addition, the paraventricular nucleus is probably not the only site of action of NPY in mediating its effects on feeding. In a behavioural study, microinjection of a unique carboxy terminal fragment of NPY into the ventromedial hypothalamus caused significant reduction of eating response induced by NPY (Myers et al., 1995).

Given the specific localisation of GHRP-induced Fos expression to the arcuate nucleus, it was surprising therefore that, when the GHS receptor was cloned and its distribution in the brain mapped, receptor expression was not confined to the arcuate nucleus, but was also prominent in the ventromedial nucleus of the hypothalamus (Bennett et al., 1997). Additionally, extracellular recordings showed the increase in firing rate of ventromedial hypothalamic neurones following bath application of GHRP-6 to hypothalamic brain slices (Hewson et al., 1999). This study used very high concentrations of secretagogue, leaving open the question of whether the arcuate and ventromedial hypothalamus differ in their sensitivity to secretagogues.

GHRP-6 was selected for this *in vitro* study. Due to its potency and selective action in the hypothalamus, it is the most predominant GHS that has been previously used to study mechanism of GH secretion. In this study, L-144,446, the newly synthesised secretagogue was also used for the *in vitro* study in comparison to GHRP-6.

2.2 Aims

As both the arcuate and the ventromedial hypothalamic nucleus mediate effects of GH secretagogues and NPY *in vivo*, this study aimed to investigate the effects of the secretagogue L-144,446, the newly synthesised non-peptidyl compound and GHRP-6 on both brain regions

at the single cell level by using hypothalamic brain slices for extracellular recording. The aim was also to examine whether neurones in these regions respond to somatostatin, NPY and changes in glucose concentrations.

2.3 Methods and materials

2.3.1 Preparation of tissue slices for electrophysiological recording

Male Sprague Dawley rats (200-350 g) were housed and maintained in a controlled environment with 12:12 h dark:light cycle. Water and food pellets were supplied *ad libitum*. All animal experiments in this chapter and the rest of this study were undertaken in full accordance with Home Office requirements for licensing of animal experiments. Rats were decapitated and their brains were quickly removed from skulls. Subsequent dissection was performed at a temperature of 4°C. Rat brains, on ice, were coronally cut by a razor blade through the areas of the optic chiasm and the midbrain to obtain brain chunks containing the whole range of the hypothalamus. These brain chunks were then trimmed with a scalpel blade to remove extra-hypothalamic areas, and the areas that contained the arcuate nucleus and ventromedial hypothalamus were retained. A piece of trimmed rat brain was fixed onto the cold plastic stage with the median eminence side facing the vibratome blade. The stage containing the brain chunk was then fixed into vibratome chamber and submerged under chilled artificial cerebrospinal fluid (aCSF, see **solution preparation**) which was oxygenated with a mixture of 95% O₂ and 5% CO₂. Using a vibratome (Campden Instruments Ltd, UK), the hypothalamic brain chunk was coronally cut into three slices with a thickness of 400 µm. Each slice was finally divided in half symmetrically through the third ventricle. To mark the location of the recording area, two marks on the wall of the third ventricle of each slice were made by using a scalpel blade as shown in **fig. 2.1**. Then slices were maintained in oxygenated aCSF at room temperature for the extracellular recording. The slice preparation starting from decapitating the brain until cutting with Vibratome was completed in no longer than 5 min.

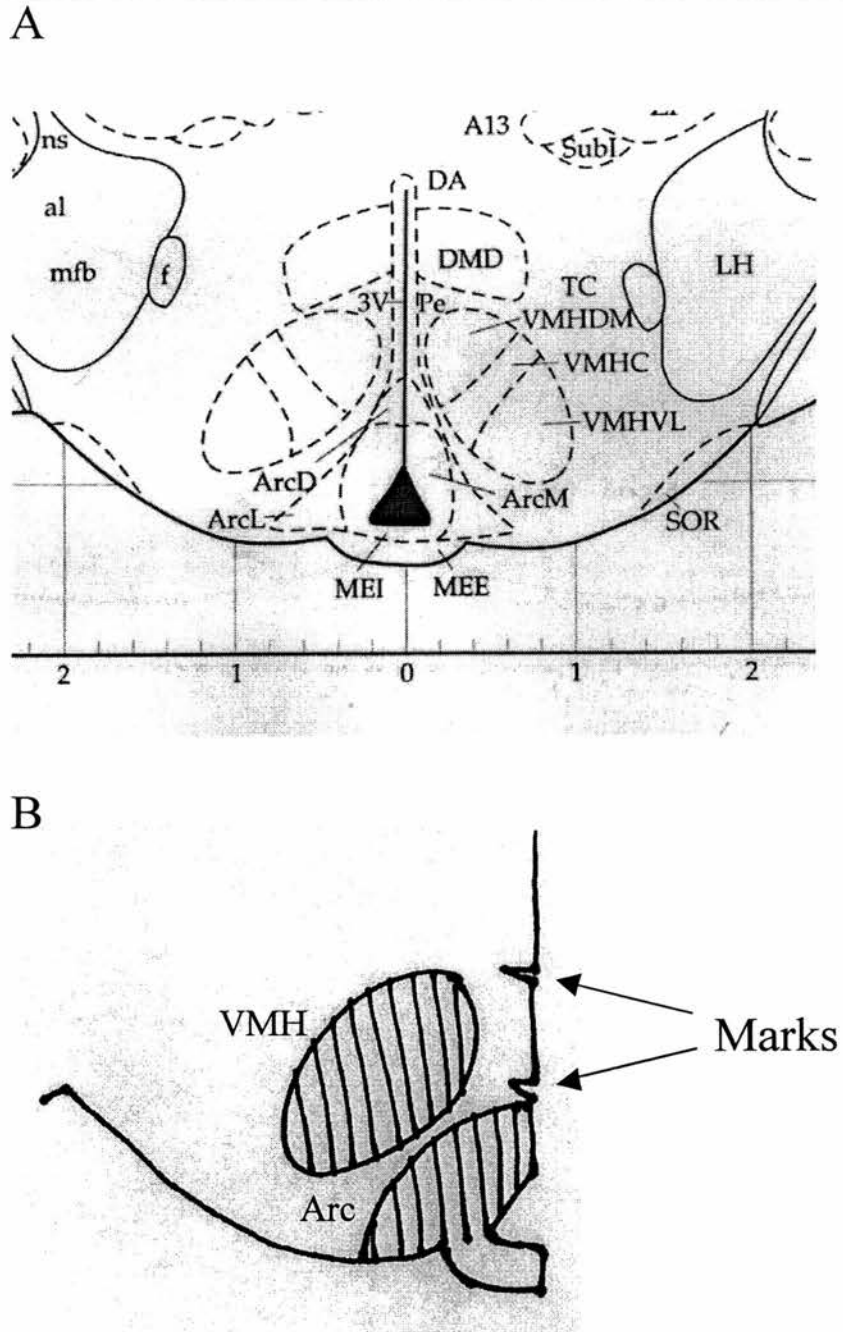


Fig. 2.1. (A) Rat brain atlas (Bregma -2.56 mm, Paxinos and Watson, 1996) used for confirmation of the hypothalamic brain areas for extracellular recording. (B) Schematic diagram showing the regions of the arcuate nucleus and the ventromedial hypothalamic nucleus. The hypothalamic brain slice was divided into two halves bilaterally. Two marks (smallcuts made with a scalpel blade) were made on the wall of the third ventricle, to divide it into one-third parts. These marks were used to guide the placement of microelectrode for recording as indicated in (B).

A coronal section containing the arcuate nucleus and ventromedial hypothalamic nucleus was then placed in an interface recording chamber. It was continuously perfused with oxygenated aCSF fluid at 1.5 ml/min using a peristaltic pump (Gilson, France). The temperature and humidity of the recording chamber were maintained by adjusting an automatic temperature controller (Warner Instrument Corp, USA) to 33°C and supplying with a continuous stream of a mixture of 95% O₂ and 5% CO₂. Each slice was incubated for at least 1 h in the gas-liquid interface chamber before starting recording.

2.3.2 Recording extracellular activity

Microelectrodes were made from Borosilicate glass capillaries (Harvard Apparatus Ltd, UK). Flaming-Brown micropipette puller (Sutter Instrument Corp, USA) was used to pull glass capillaries into microelectrodes with the desired resistance (10-20 MΩ). Microelectrodes were filled with aCSF and then fixed to the amplifier headstage. They were placed visually under the light microscope within either the arcuate nucleus or ventromedial hypothalamic nucleus to enable extracellular single-unit recordings to be made. Tonic firing neurons in both areas were randomly explored. Single-unit activity was amplified, through an Axoclamp 2B amplifier (Axon Instruments, USA). It was filtered and monitored with an oscilloscope and audio amplifier.

Arcuate and ventromedial hypothalamic neurones were identified with guidance from a previous study of their characterised electrophysiological properties (Dickson et al., 1995b; Roesch and Felix, 1984). The spontaneous discharge rate of active neurones was displayed and converted online using a 1401 (analog-to-digital) interface (CED, Cambridge, UK). A height window discriminator (Gould Electronics Ltd, UK) was used to discriminate proper action potential from background level. Signal was then transferred to a PC running Spike 2 software (CED, Cambridge, UK) for analysis and interpretation.

2.3.3 Chemicals

Data collection was commenced after recording a stable period of neuronal baseline activity for at least 15 min. Brain slices were tested with various types of drug which included growth hormone secretagogue (GHS) 144,446 (Merck, UK), growth hormone releasing peptide-6 (GHRP-6, Merck, UK), somatostatin (Sigma, USA), neuropeptide Y (Tocris Cookson Ltd, UK), or NPY Y1 antagonist (L-753044-001J003: Merck, UK). All drugs and antagonist were dissolved in ddH₂O, divided into aliquot parts and frozen until required. Aliquots were diluted to their final concentrations in the appropriate volume of oxygenated aCSF and applied to the slice by switching the perfusion inlet tube to the desired solution by means of three way taps.

In experiments using various concentrations of glucose, the osmolarity of the medium was kept constant by compensatory changes in NaCl (Yang et al., 1999). As well as in the experiment with Ca⁺⁺ removal, the osmolarity was also maintained by compensation with MgSO₄ (Kelso and Boulant, 1982). Each brain slice was used for only one recording in order to avoid prolonged sustaining effect of GHS.

2.3.4 Solution preparation

2.3.4.1 Artificial cerebrospinal fluid (aCSF)

Concentrations and amounts of chemicals

Final concentration (mM)		amount per 1000 ml (g)
125	Sodium chloride	7.305
5	Potassium chloride	0.373
1.25	Potassium dihydrogen orthophosphate	0.170
2.5	Calcium chloride	0.367
1.25	Magnesium sulphate	0.308
26	Sodium bicarbonate	2.16
10	Glucose	1.8

Low calcium aCSF

Final concentration (mM)		amount per 1000 (g)
125	Sodium chloride	7.305
5	Potassium chloride	0.373
1.25	Potassium dihydrogen orthophosphate	0.170
0.25	Calcium chloride	0.0734
1.1	Magnesium chloride	0.102
3.63	Magnesium sulphate	0.893
26	Sodium bicarbonate	2.16
10	Glucose	1.8

2.3.4.2 aCSF with various concentrations of glucose

Chemicals	Final conc. (mM)/amount of chemicals (g) for 1000 ml aCSF		
	20 mM glucose	5 mM glucose	0 mM glucose
Sodium chloride	120/7.61	127.5/7.45	130/7.60
Potassium chloride	5/0.373	5/0.373	5/0.373
Potassium dihydrogen orthophosphate	1.25/0.170	1.25/0.170	1.25/0.170
Calcium chloride	2.5 /0.367	2.5 /0.367	2.5 /0.367
Magnesium sulphate	1.25/0.308	1.25/0.308	1.25/0.308
Sodium bicarbonate	26/2.16	26/2.16	26/2.16
Glucose	20/3.64	5/0.91	0/0

Results

2.3.5 Electrophysiological studies of arcuate neurones

2.3.5.1 Basal activity and activity patterns of arcuate neurones

Stable extracellular recordings were made from 107 spontaneously active neurones in the ventromedial region of the arcuate nucleus of the trimmed rat hypothalamic slice preparation. Each neurone was recorded from a different slice, and was tested with application of secretagogue alone or in combination with other drugs.

All recorded neurones discharged with no conspicuous pattern – in particular no neurones fired phasically, in the manner of vasopressin neurones of the supraoptic (Armstrong and Sladek, 1982) and paraventricular nuclei (Saphier, 1991), and no neurones fired very regularly, in the manner of many neurones in the suprachiasmatic nucleus (Glotzbach et al., 1987). The mean variation coefficient was $112 \pm 4.8 \%$, which confirms their highly irregular firing pattern. The mean spontaneous firing rate of these arcuate neurones was 1.13 ± 0.08 spikes/s (mean \pm SEM) ranging from 0.22 to 4.51 spikes/s. The discharge patterns of these neurones were also studied by analysing the distributions of interspike intervals. The interspike interval distribution of a neurone is influenced by intrinsic neuronal properties, such as the presence of a post-spike after hyperpolarisation or an after-depolarisation, and hence these can be useful in classifying neurones into different functional types. For the neurones analysed, most of them (92 %) had a predominant (modal) interspike interval. The average modal interspike interval in the control period was 0.43 ± 0.04 s, and 16 neurones (15 %) showed a modal interspike interval of less than 100 ms. Of the remainder, 91 neurones (85 %) showed a more dispersed interspike interval histogram with a modal interspike interval greater than 100 ms. Firing patterns with bi-modal interspike interval distributions were also seen in 8 neurones (8 %). The mean firing rates and interspike intervals of neurones that responded and did not respond to secretagogues will be shown in parts of the results of the effects of secretagogues on arcuate neurones.

2.3.5.2 Response of arcuate neurones following administration of L-144,446

Responses to L-144,446 were examined in recordings from 38 arcuate neurones. The response of each neurone to L-144,446 was determined by comparing its mean firing rates in a control period (measured over 10 min immediately before drug administration), and after exposure to L-144,446 (measured from 5 to 15 min following the treatment). This period was selected as including the maximum differences from the control period shown in the average response of all cells before classification. The minute-by-minute firing rates in these two periods were compared statistically using Student's t-test. Two initial arcuate neurones that were stimulated by two different concentrations of L-144,446 are shown in **fig. 2.1.1**. **Figure 2.1.1A** shows a neurone stimulated by L-144,446 (10^{-7} M) for 5 min that increased its firing rate from 1.11 spikes/s to 5.91 spikes/s, more than a 5-fold increase (**fig. 2.1.1A**). **Figure 2.1.1B** shows a different neurone stimulated by a lower concentration of L-144,446 (10^{-8} M) for only 2 min, showing an increase in firing rate from 1.99 spikes/s to 4.15 spikes/s, approximately a 2-fold increase (**fig. 2.1.1B**). After such pilot experiments, the concentration of 10^{-8} M for 2 min was selected to be the condition of the rest of the study of L-144,446. This dose was selected as the lowest dose that would consistently produce clear effects on arcuate neurones.

The effects of L-144,446 were examined on 38 arcuate neurones. To classify neurones as excited, inhibited, or non-responsive, the data for each cell were first normalised by subtracting the mean firing rate in the control value from each minute-by-minute value of firing rate. Mean change in firing rate of each neurone tested were plotted in order of magnitude (**fig. 2.1.2 A**). The population was divided into three groups. Four of the tested neurones (11 %) were significantly inhibited by L-144,446 ($P < 0.02$). The average firing rate of this group decreased by 0.52 spikes/s (24 %; from 2.16 ± 1.09 to 1.64 ± 0.85 spikes/s). Fourteen neurones (37 %) did not show any significant change following administration of L-144,446 ($P = 0.02$). Twenty neurones (53 %) showed a significant (95 %) increase in firing rate after L-144,446 administration ($P < 0.008$). The average firing rate of this group increased from 0.97 ± 0.17 to 1.80 ± 0.33 spikes/s.

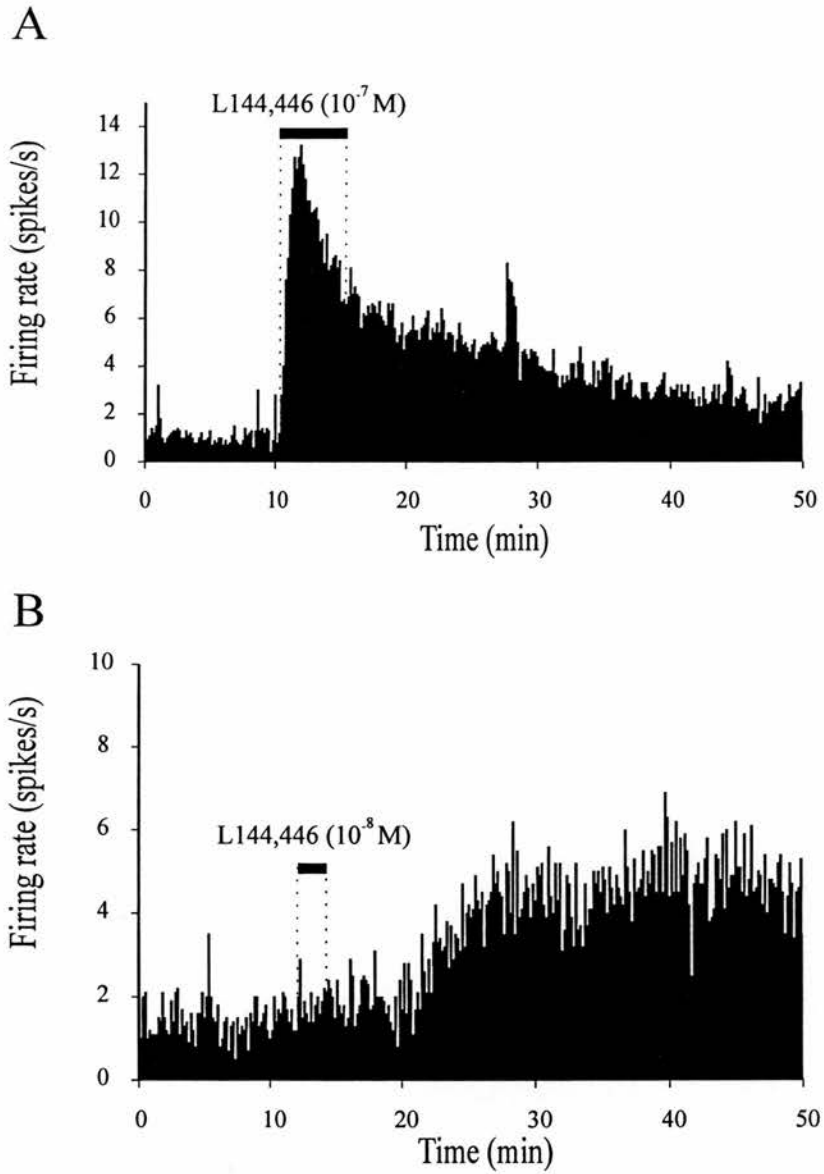


Fig. 2.1.1. Extracellular recordings of the firing rate of arcuate neurones following perfusion of the hypothalamic slices with L-144,446. (A) A neurone was stimulated with 10^{-7} M L-144,446 for 5 min. (B) Another arcuate neurone was stimulated with 10^{-8} M L-144,446 for 2 min. The firing rate was divided into 10-s intervals and is expressed as spikes/s. In each case, the firing rate during the 10-min period taken 5 to 15 min following secretagogue administration (averaged for 60×10 -s intervals) was significantly different from the firing rate of control period taken 10 min before administration (averaged for 60×10 -s intervals; $P < 0.003$, t-test).

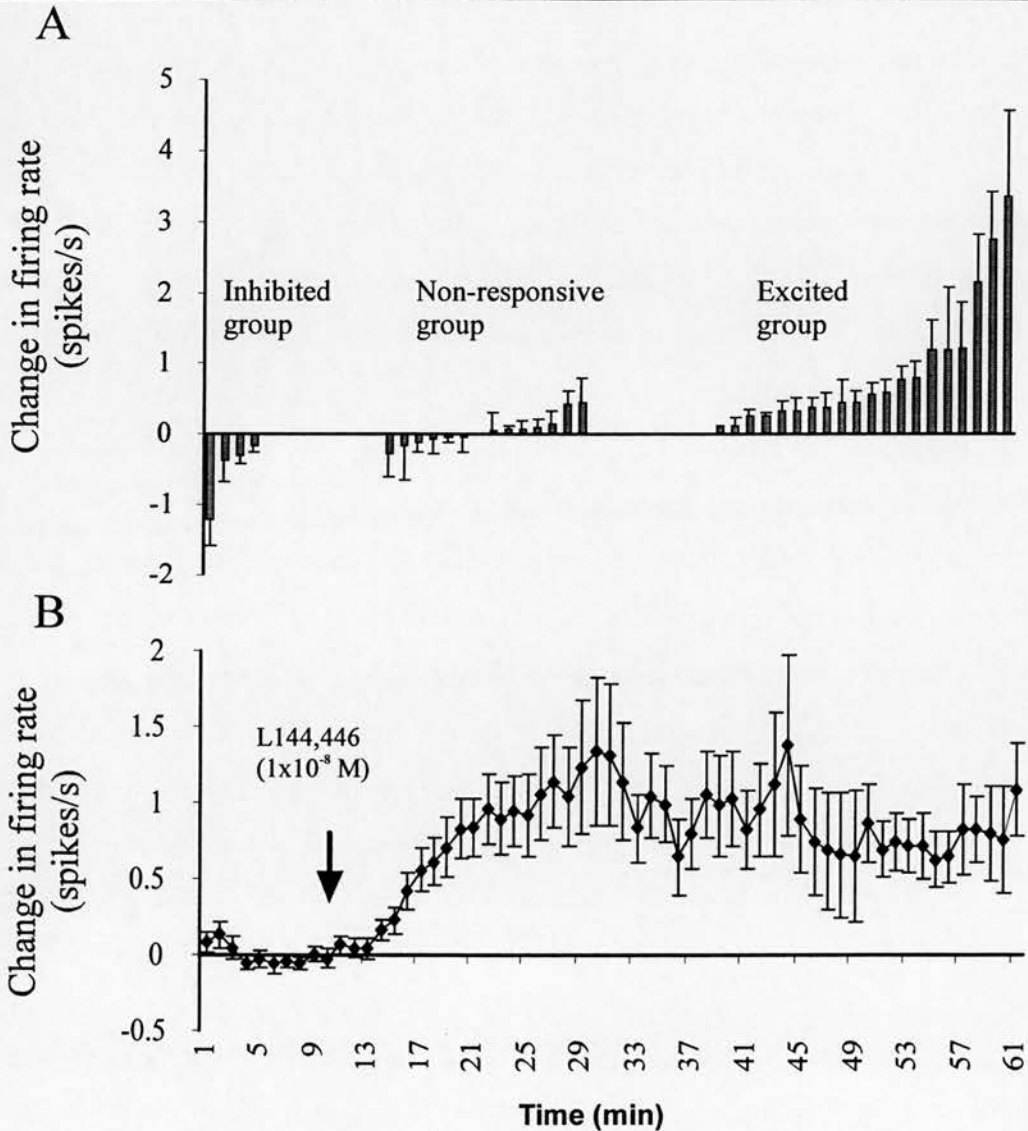


Fig. 2.1.2. (A) Ranking of changes in firing rate of 39 arcuate neurones following L144,446 treatment. Mean firing rate of each individual neurone during the 10-min period after the secretagogue application (10^{-8} M, 2 min) was normalised by subtracting the mean firing rate of the control period (10-min period before the application). Neurones were divided into 3 groups according to their responses. Changes in firing rate after L144,446 treatment were statistically significant in the inhibited group (t-test, $p < 0.02$) and excited group ($p < 0.0075$) but not in the non-responsive group. Each bar represents the change in firing rate (\pm SEM) of one individual neurone following secretagogue administration. **(B) Mean response of the excited group following L-144,446 treatment.** Normalised mean firing rates of the excited neurones were averaged minute-by-minute ($n = 5-18$ per each point).

The basal firing patterns of these three classified groups of the arcuate neurones were then compared. The modal interspike interval of the discharge was taken as the peak in interspike interval histogram of the 10-min period of recording before administration of L-144,446. The modal interspike interval of neurones from these 3 groups was then plotted against mean interspike interval (**fig. 2.1.3**). The excited neurones had mean firing rates which varied between 0.23 and 2.55 spikes/s with a mean \pm SEM of 0.97 ± 0.17 spikes/s. Seventeen of a total 20 excited neurones had uni-modal interspike interval with an average mode of 0.36 ± 0.13 s, whereas the remaining 3 of excited neurones had bi-modal interspike interval distributions. Non-responsive neurones had a mean firing rate of 1.03 ± 0.23 spikes/s and an average modal interspike interval (averaged from 13 neurones with uni-modal interspike interval of a total 14) of 0.18 ± 0.03 s. Four of the inhibited neurones were found to have wide range of mean firing rate, from 0.34 to 4.51 spikes/s with a mean firing rate of 2.07 ± 1.14 spikes/s and an average modal interspike interval of 0.88 ± 0.52 s. It was noticed that all 9 neurones with modal interspike intervals higher than 0.35 s were responsive to L-144,446; 2 were inhibited and 7 were excited. However, the majority of excited and non-responsive neurones had a modal interspike interval of less than 0.5 s and firing rate of lower than 2 s. These two groups of neurones are not simply distinguishable by this analysis.

Further analysis revealed that the inhibited and excited groups had non-significant higher regularity in firing patterns than the non-responsive group (t-test, $p = 0.05$). The variation coefficients of the inhibited and excited groups were 130 ± 24 % and 134 ± 9 % respectively whereas the variation coefficient of the non-responsive group was 158 ± 18 %. The shorter average modal interspike interval in the non-responsive cells may reflect an observation made previously from *in vivo* recordings. Dickson and co-workers (Dickson et al., 1995b) reported that some arcuate neurones *in vivo* fire in clusters of action potentials; these cells display an average modal interspike interval of < 20 ms and very few of these cells are excited by i.v. administration of GHRP-6.

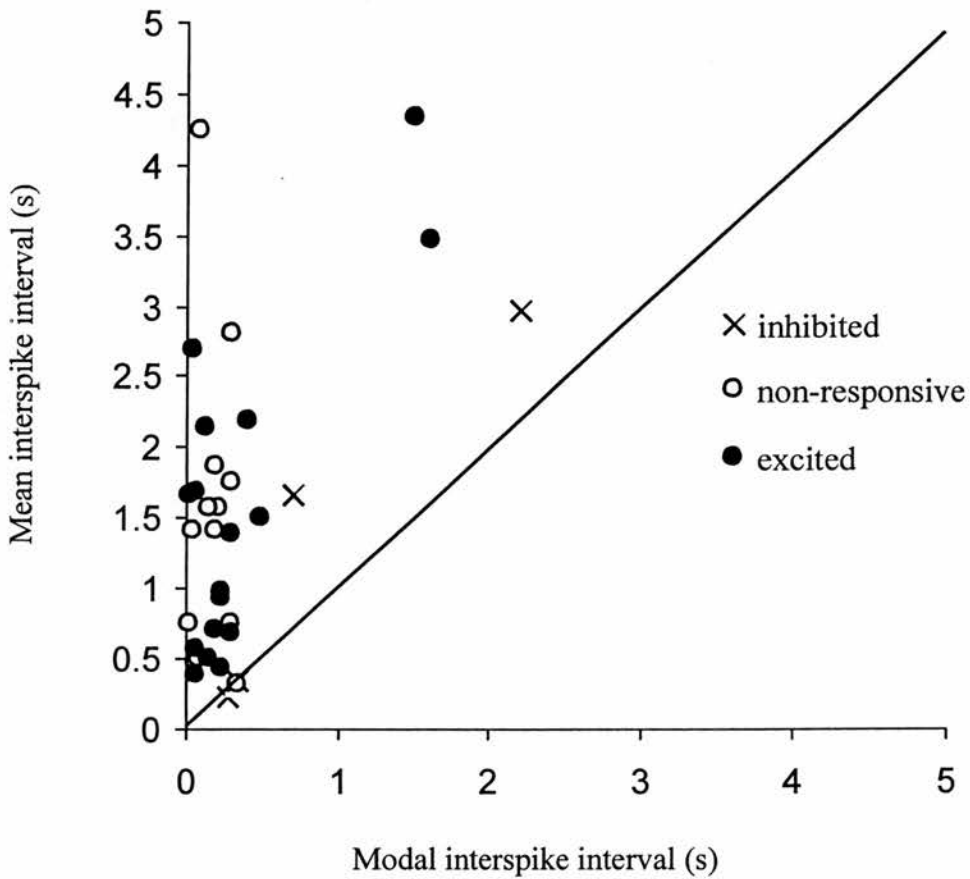


Fig. 2.1.3. Relationship between mean and modal of interspike interval of basal firing activity of arcuate neurones treated with L144,446. Neurones were divided into 3 groups according to their responses to L-144,446 (10^{-8} M, 2-min bath application). For each cell, mean and modal interspike intervals were analysed from its control period (10 min before the treatment).



The mean increase in firing rates of excited neurones were averaged and plotted (**fig. 2.1.2B**). The increase was clearly seen to begin a few minutes after the administration of L-144,446 – during the period of drug washout. The response thereafter increased progressively, to reach a peak approximately 1 spikes/s higher than the initial control level at about 20 min. The mean firing rate remained elevated above control levels for about 50 min after the administration.

The firing patterns of some arcuate neurones changed following exposure to L-144,446. Of 20 neurones that were excited by L-144,446, 15 neurones (75 %) showed an increase in regularity of their firing pattern. Two representative L-144,446-stimulated neurones showed changes of variation coefficient following the excitation compared to variation coefficient of control period (**fig. 2.1.4 and 2.1.5**). However, the overall decrease in variation coefficient of these L-144,446-stimulated neurones was not statistically significant (t-test, $P = 0.05$).

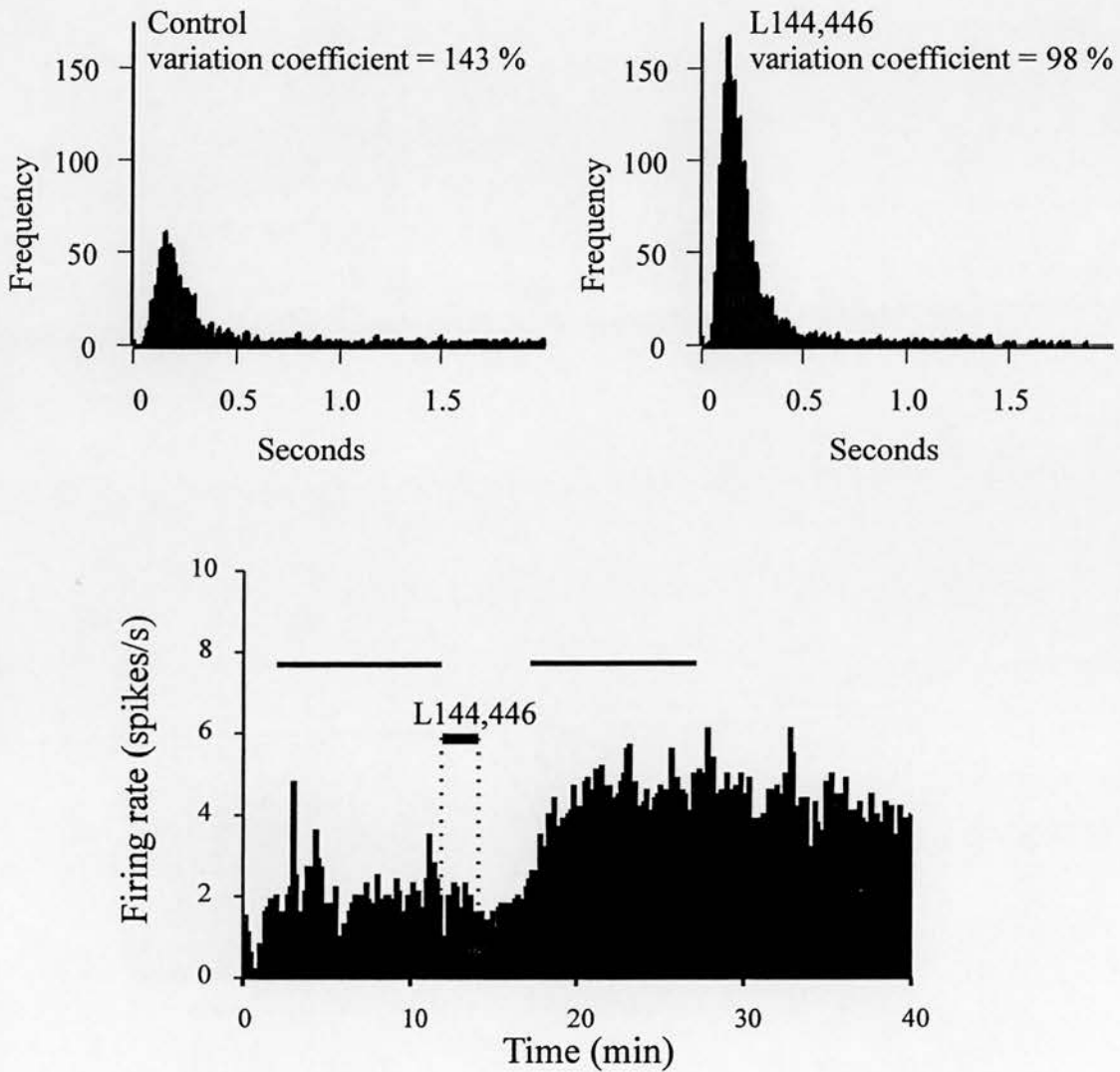


Fig. 2.1.4. Effects of L144,446 on regularity of discharge pattern of a representative arcuate neurone. Upper panel illustrates the ISI histogram compiled before (left) and after (right) bath application (2 min) of L-144,446 (10^{-8} M). The decrease in variation coefficient reflects an increase in regularity of its discharge pattern after L-144,446 treatment. Lower panel illustrates the effects of L-144,446 on the firing rate of this neurone. Two bars indicate the periods of control and L144,446 treatment ISI histograms.

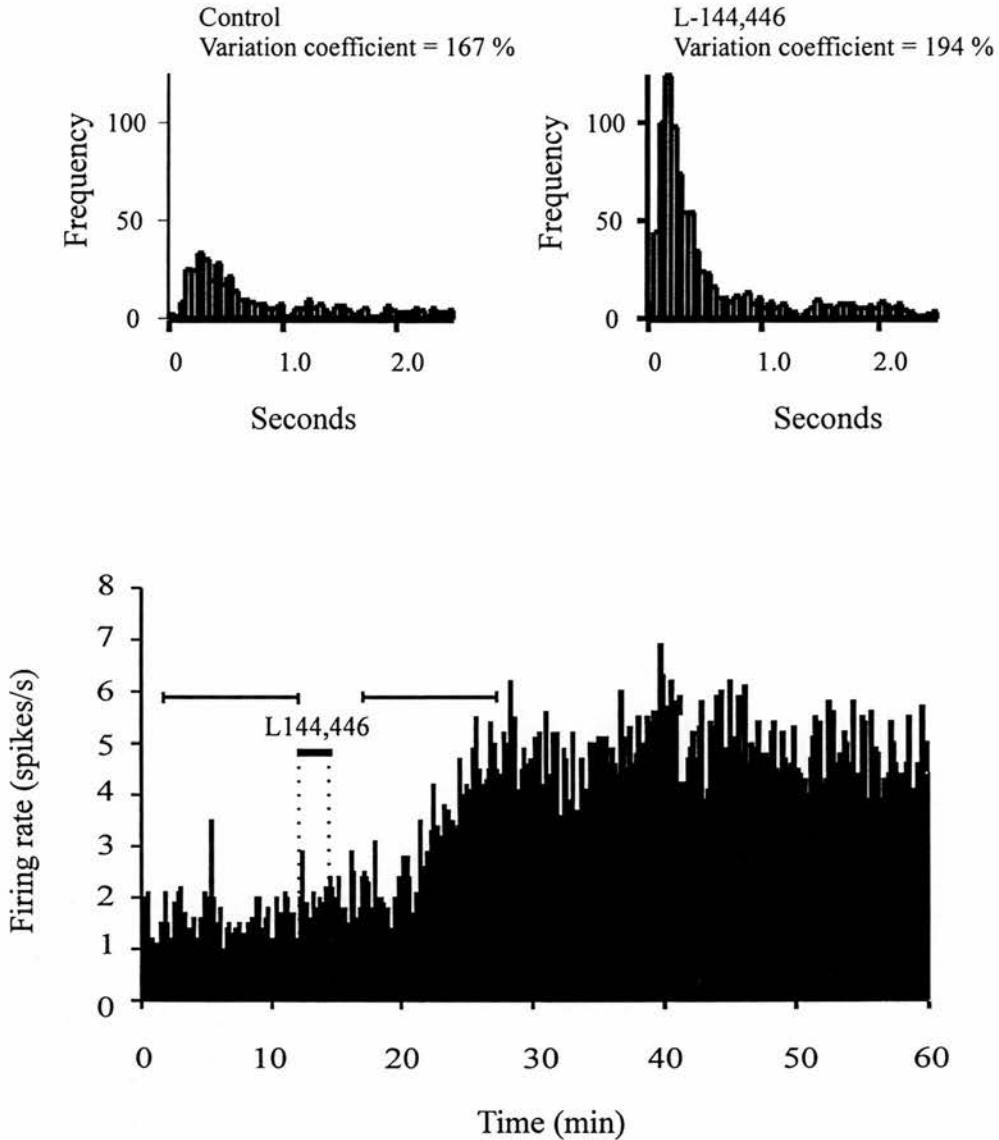


Fig. 2.1.5. Effects of L-144,446 on regularity of discharge pattern of a representative arcuate neurone. Upper panel illustrates the ISI histogram compiled before (left) and after (right) bath application (2 min) of L-144,446 (10^{-8} M). The increase in variation coefficient reflects a decrease in regularity of its discharge pattern after L-144,446 treatment. Lower panel illustrates the effects of L-144,446 on the firing rate of this neurone. Two bars indicate the periods of control and L-144,446 treatment ISI histograms.

2.3.5.3 Effects of GHRP-6 on arcuate neurones

The responses to GHRP-6 were examined in recordings from 47 arcuate neurones. Neurones were classified as excited, inhibited, or non-responsive in the same way as for the effects of L-144, 446. Figure 2.1.6 shows the mean change in firing rate in response to GHRP-6 of each neurone tested, plotted in order of magnitude. The figure also shows the mean change in firing rate due to NPY treatment of some neurones tested with GHRP-6. Effects of NPY will be shown later in this chapter. The population was divided into three groups. Eight of the tested neurones (15 %) were, by this test, significantly inhibited by the secretagogue. The average firing rate of this group decreased by 0.82 spikes/s (36 %, from 0.98 ± 0.20 spikes/s to 0.63 ± 0.17 spikes/s). Twenty-one neurones (45 %) did not show any significant change following administration of GHRP-6 ($P = 0.03$). Eighteen neurones (38 %) showed a significant increase in firing rate following GHRP-6 ($P < 0.03$). The average firing rate of this group increased by 145 %, from 0.84 ± 0.10 spikes/s to 2.05 ± 0.32 spikes/s.

The basal firing patterns of these three classified groups of the arcuate neurones tested with GHRP-6 were then compared. The modal interspike interval of the discharge was taken as the peak in interspike interval histogram of the 10-min period of recording before administration of the secretagogue. The modal interspike interval of neurones from these 3 groups was then plotted against mean firing rate (**fig. 2.1.7**). The excited neurones had mean firing rates which varied between 0.25 and 2.06 spikes/s with a mean \pm SEM of 0.85 ± 0.10 spikes/s. Most of the excited neurones had a uni-modal interspike interval distribution with an average mode of 0.46 ± 0.11 s (excluding one neurone which had a bi-modal interspike interval distribution). Non-responsive neurones had a mean firing rate of 0.92 ± 0.10 spikes/s ranging from 0.22 to 1.89 spikes/s, and an average modal interspike interval (averaged from 14 neurones with uni-modal interspike interval histograms) of 0.52 ± 0.12 s. The eight inhibited neurones showed a wide range of mean firing rate, from 0.22 to 1.60 spikes/s with a mean firing rate of 0.98 ± 0.20 spikes/s and an average modal interspike interval of 0.50 ± 0.09 s.

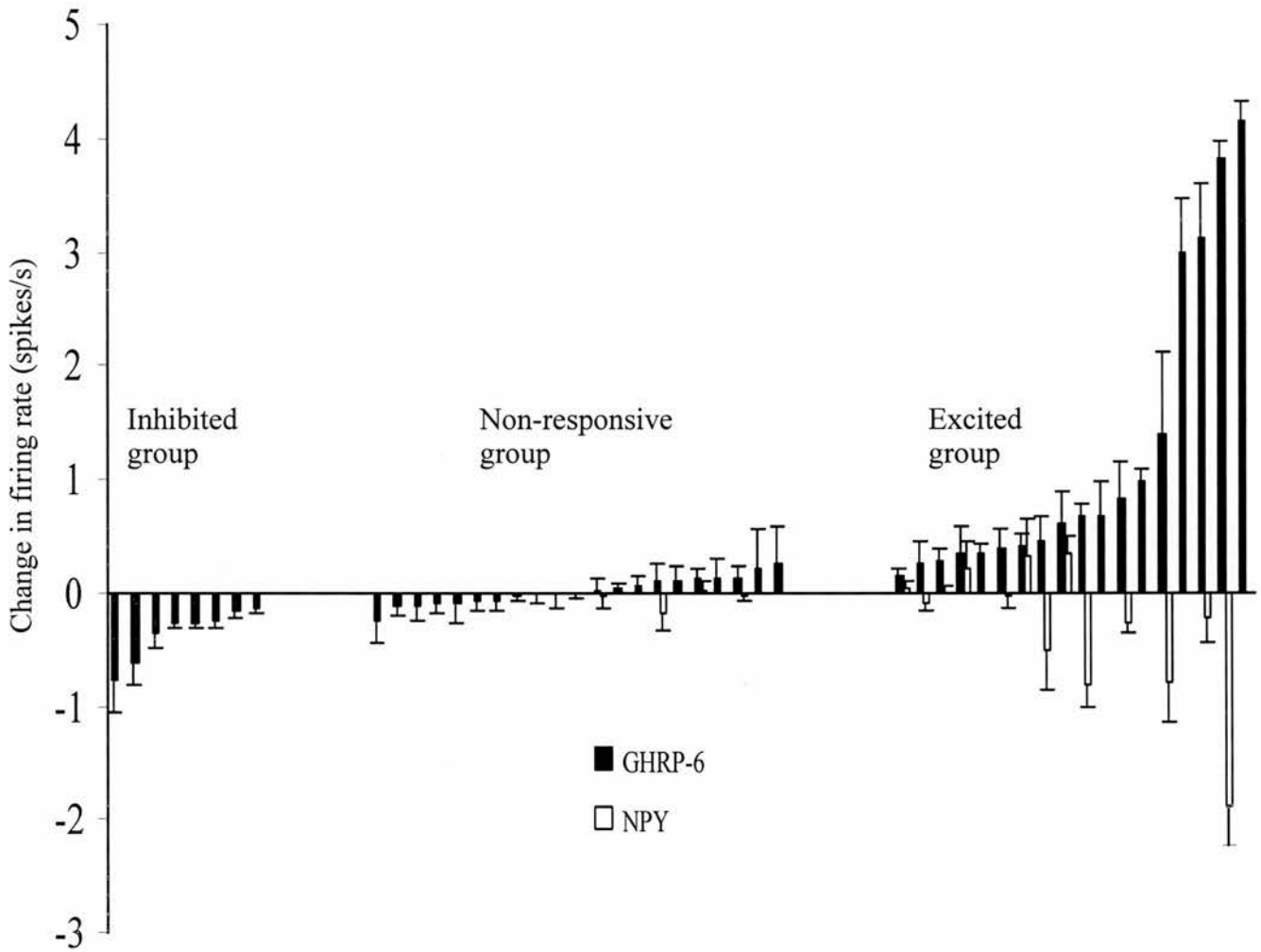


Fig. 2.1.6 . Ranking of changes in firing rate (mean \pm SD) of 47 arcuate neurones following GHRP-6. Mean firing rate of each individual neurone during the 10-min period after the GHRP-6 application (10^{-7} M, 2 min) was normalised by subtracting the mean firing rate of the control period (10-min period before the administration). Neurones were divided into 3 groups according to their responses. Changes in firing rate due to GHRP-6 treatment were statistically significant in the inhibited group (t-test, $p < 0.001$) and in the excited group ($p < 0.005$), but not in the non-responsive group. Each bar represents the change in firing rate of one individual neurone following GHRP-6 treatment. Some non-responsive and excited neurones were also tested with NPY (10^{-6} M) in the same recording. Responses to NPY were analysed the same way as for GHRP-6 responses (c.f. Fig.2.1.15).

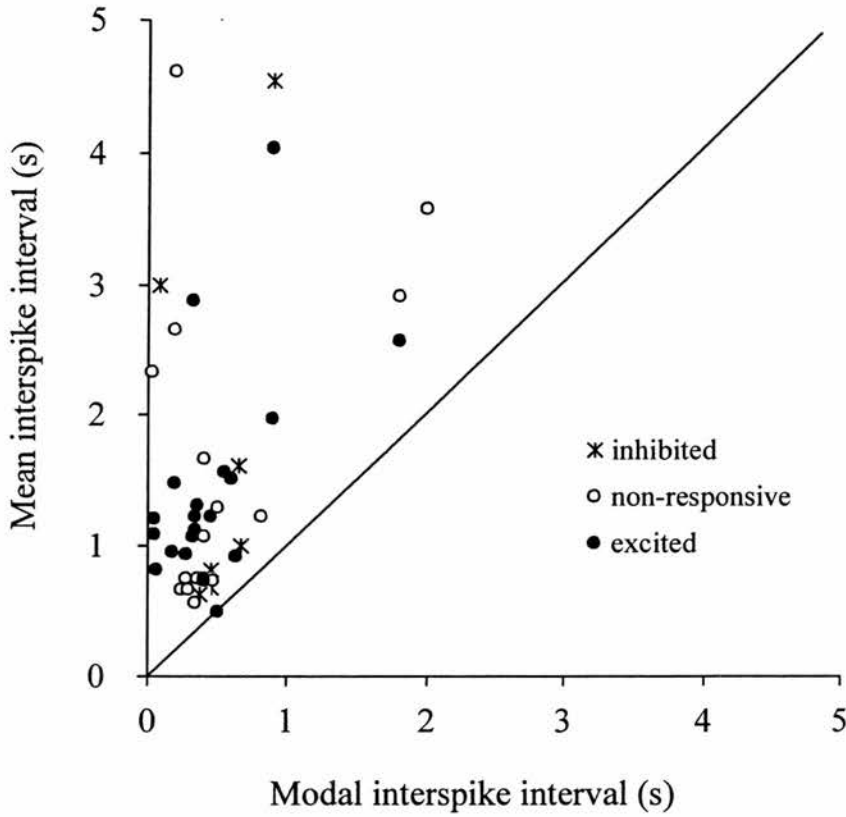


Fig. 2.1.7. Relationship between mean and modal interspike interval of basal firing activity of arcuate neurones treated with GHRP-6. Neurones were divided into 3 groups according to their responses to the GHRP-6 (10^{-7} M, 2-min bath application). For each cell, mean and modal interspike intervals were analysed from its control period (10 min before the treatment).

The relationship between modal interspike interval and mean firing rate of neurones tested with GHRP-6 reveals some characteristics of excited and inhibited groups. The excited group was found to be clustered in a range of modal interspike interval from 0.5 to 1.5 s. Most of them (14 from a total of 18) had a mean firing rate less than 1 spikes/s. All of the inhibited group had a modal interspike interval of less than 1 s. This inhibited group might be distinguished from those excited group, in that most of the inhibited neurones had mean firing rates higher than 1 spikes/s, whereas most of the excited neurones had a firing rate lower than 1 spike/s.

In addition, the analysis revealed that the excited group had non-significantly higher regularity in firing patterns than the non-responsive and inhibited groups (t-test, $p = 0.05$). Between these three groups, no significant difference in regularity in firing patterns was determined. The variation coefficients of the excited, non-responsive and inhibited groups were 115 %, 88 % and 83 % respectively.

The increase in firing rate of excited neurones stimulated by GHRP-6 (10^{-7} M) were seen in various patterns. Recordings of three representative arcuate neurones stimulated by GHRP-6 are shown in **fig. 2.1.8**. The mean increase in firing rates of excited neurones were averaged and plotted (**fig. 2.1.9**). The increase clearly began a few minutes after the administration of GHRP-6, during the period of drug washout. The response increased steeply, and seemed stable after reaching a peak in about 15 min, at a mean firing rate approximately 1.2 spikes/s higher than the initial control level. The mean firing rate maintained elevated above control levels for about 60 min after the administration.

Inhibitory effects of GHRP-6 were also observed for some arcuate neurones, and three arcuate neurones that were inhibited by GHRP-6 are illustrated in **fig. 2.1.10**. The mean firing rates of inhibited neurones are plotted in **fig 2.1.9**. The decrease started a few minutes after the administration of GHRP-6 and peaked in 10 min, at a mean firing rate about 0.5 spike/s lower than control levels. Thereafter, the mean firing rate gradually increased towards control levels. At 25 min after GHRP-6 administration, the firing rate still remained below the control level.

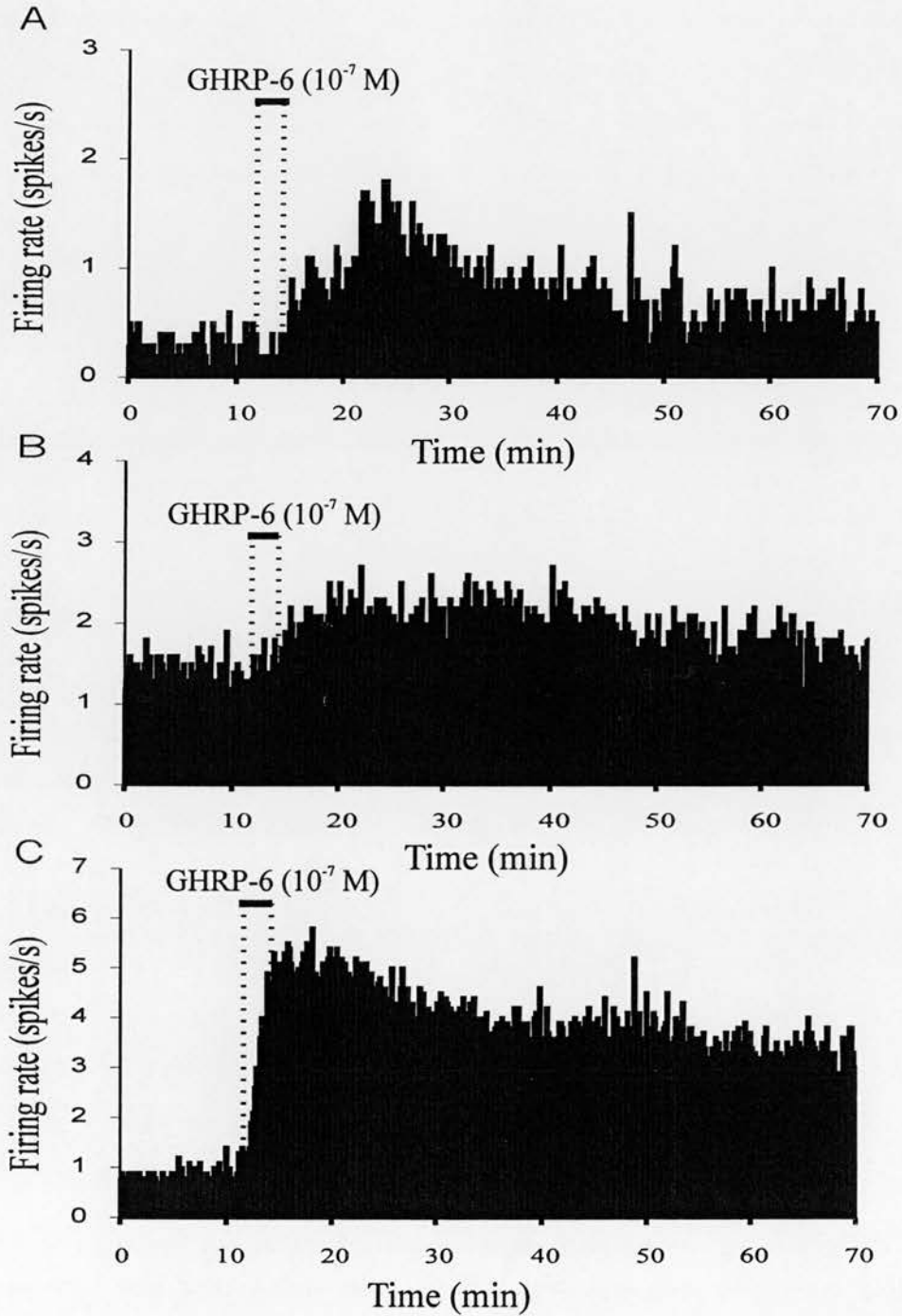


Fig. 2.1.8 . Rate-meter records showing GHRP-6 activation of arcuate nucleus neurones. Hypothalamic brain slices were superfused with GHRP-6 (10^{-7} M) for 2 min. The patterns of increase in firing rate varied among different GHRP-6-excited neurones. The stimulations were maintained for over 1 h before recordings were terminated. Periods of GHRP-6 administration are indicated with bars.

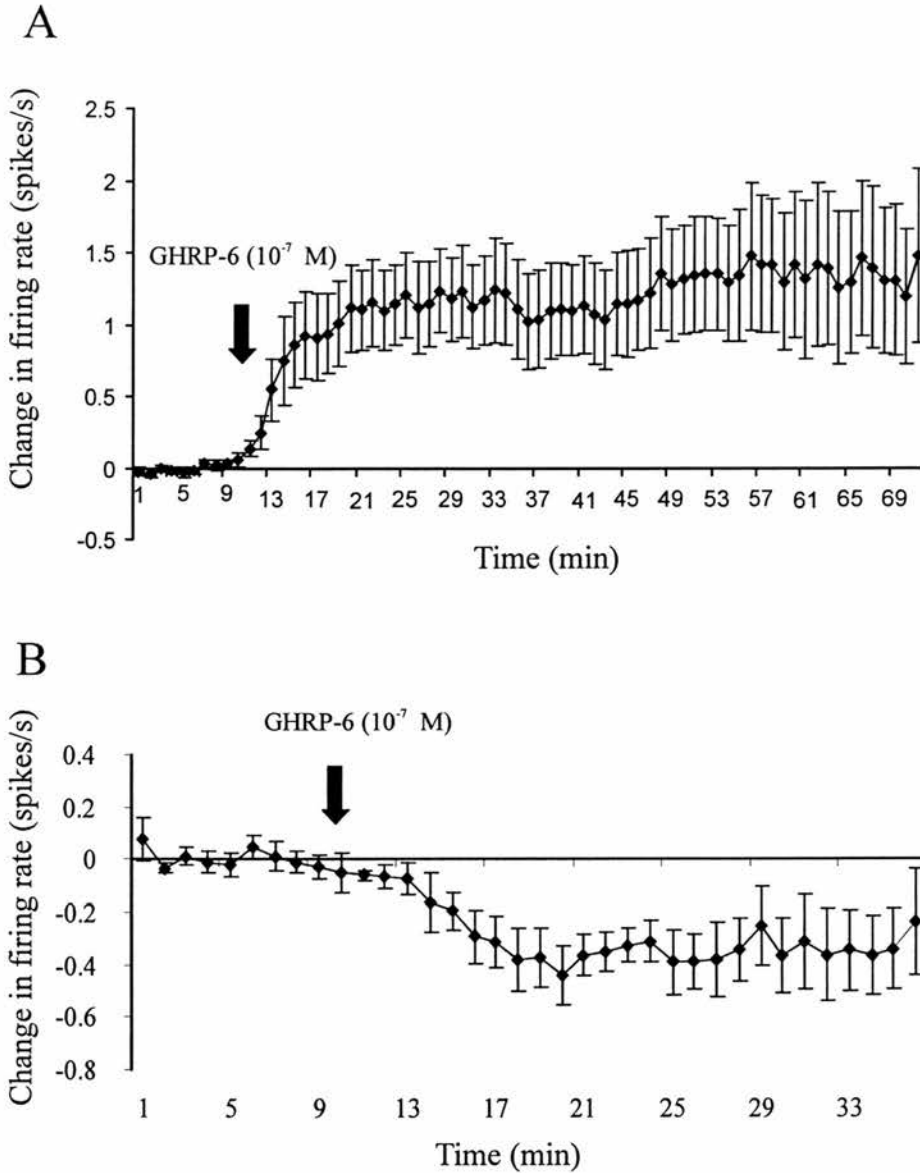


Fig. 2.1.9. Mean responses (mean \pm SEM spikes/s) of arcuate neurones that were excited (A) and inhibited (B) following GHRP-6 treatment. Arcuate neurones were divided into groups according to their responses to GHRP-6 (see **fig. 2.1.6**). In each group, mean firing rate of each individual neurone during the 10-min period after the GHRP-6 application (10^{-7} M, 2 min) was normalised by subtracting the mean firing rate of the control period (10-min period before the administration). Normalised mean firing rates of neurones were averaged minute-by-minute for both excited group ($n = 7-18$ per each point) and inhibited group ($n = 6-8$ per each point).

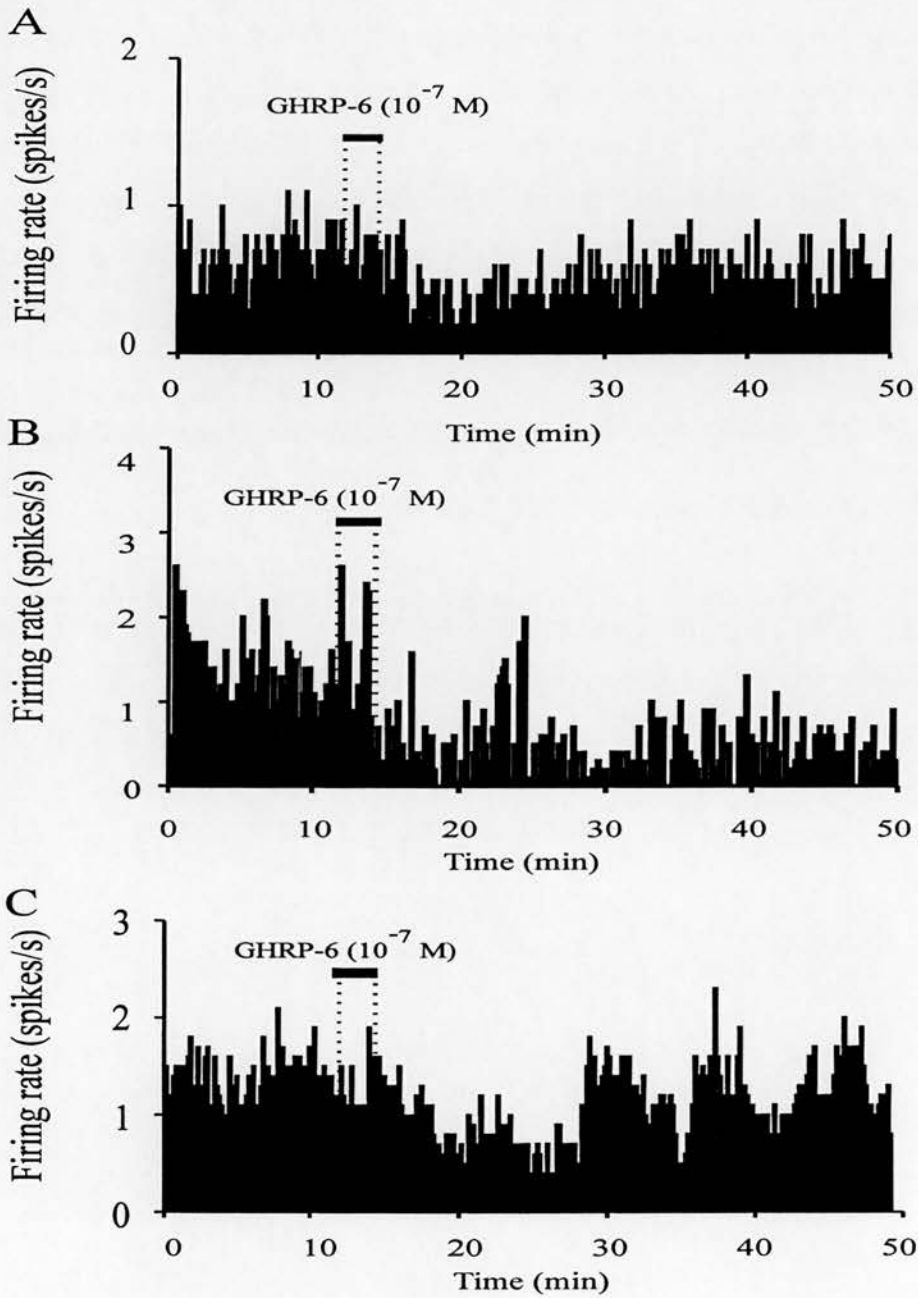


Fig. 2.1.10. Rate-meter records showing inhibitory effects of GHRP-6 on arcuate neurones. Hypothalamic brain slices were superfused with GHRP-6 (10^{-7} M) for 2 min. The inhibitions in firing rate were significant (t-test, $p < 0.001$). Periods of GHRP-6 treatments are indicated with bars.

GHRP-6 also changed the firing patterns of some arcuate neurones. Of 18 neurones that were excited by GHRP-6, 14 neurones (78 %) showed an increase in regularity of their firing pattern, while 4 neurones (22 %) showed a decrease in regularity of their firing pattern. Two representative GHRP-6-stimulated neurones showed effects of GHRP-6 on variation coefficient of their discharge patterns (**fig. 2.1.11 and 2.1.12**). The average variation coefficient of the GHRP-6-stimulated neurones reduced by 22 % from $113 \pm 11\%$ to $90 \pm 10\%$. However, the overall decrease in variation coefficient of these GHRP-6-stimulated group was not statistically significant (t-test, $P = 0.05$).

Comparison of responsiveness to GHRP-6 and L-144,446

The analysis of discharge patterns of L-144,446- and GHRP-6-stimulated arcuate neurones revealed close similarities. The proportions of arcuate neurones that were stimulated by both secretagogues were close (approximately 5/10 and 4/10 by L-144,446 and GHRP-6 respectively).

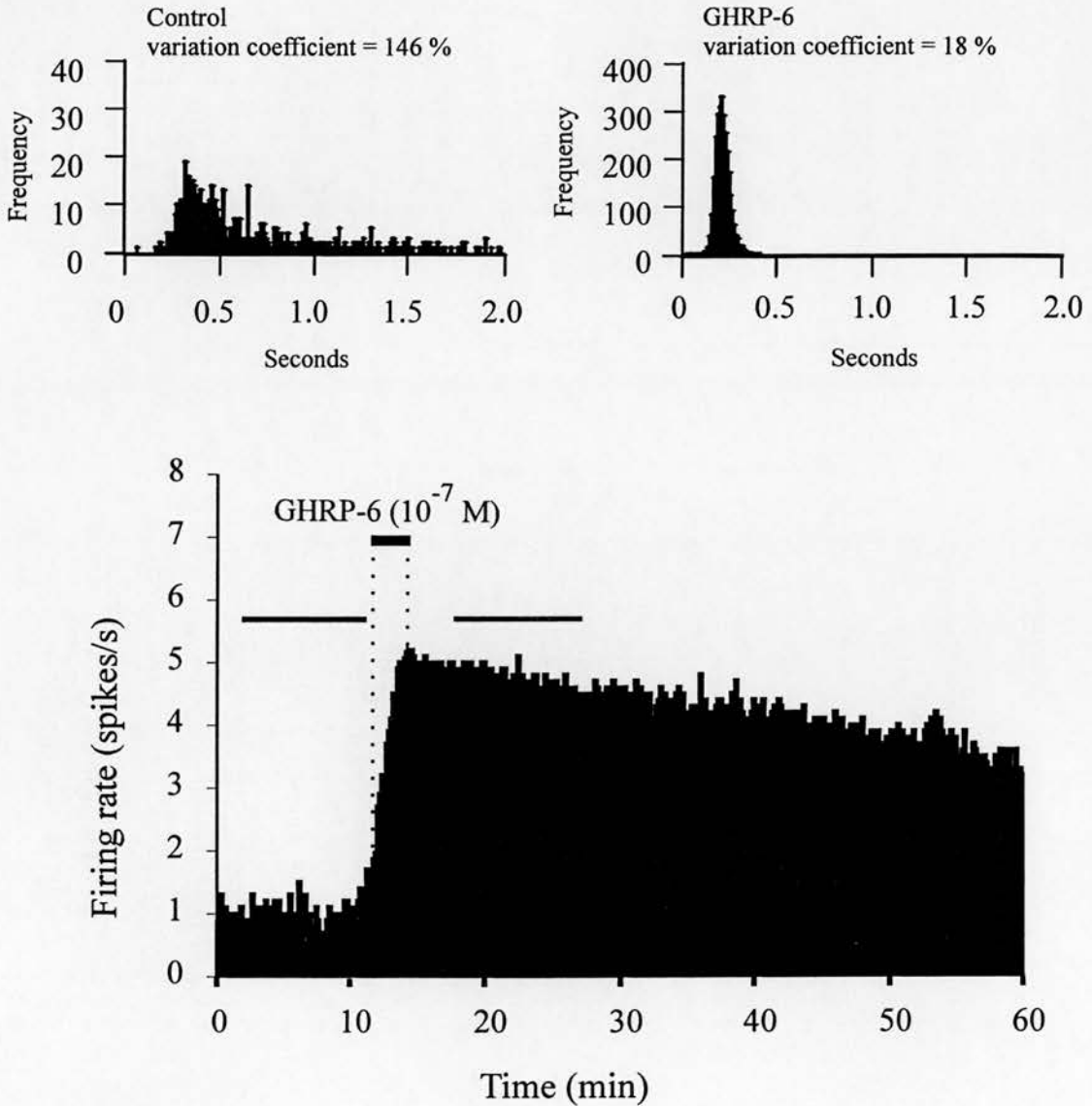


Fig. 2.1.11. Effects of GHRP-6 on regularity of discharge pattern of a representative arcuate neurone. Upper panel illustrates the ISI histogram compiled before (left) and after (right) bath application (2 min) of GHRP-6 (10^{-7} M). The decrease in variation coefficient reflects an increase in regularity of its discharge pattern after GHRP-6. Lower panel illustrates the effects of the GHRP-6 on the firing rate of this neurone. Two bars indicate the periods of control and GHRP-6 treatment ISI histograms.

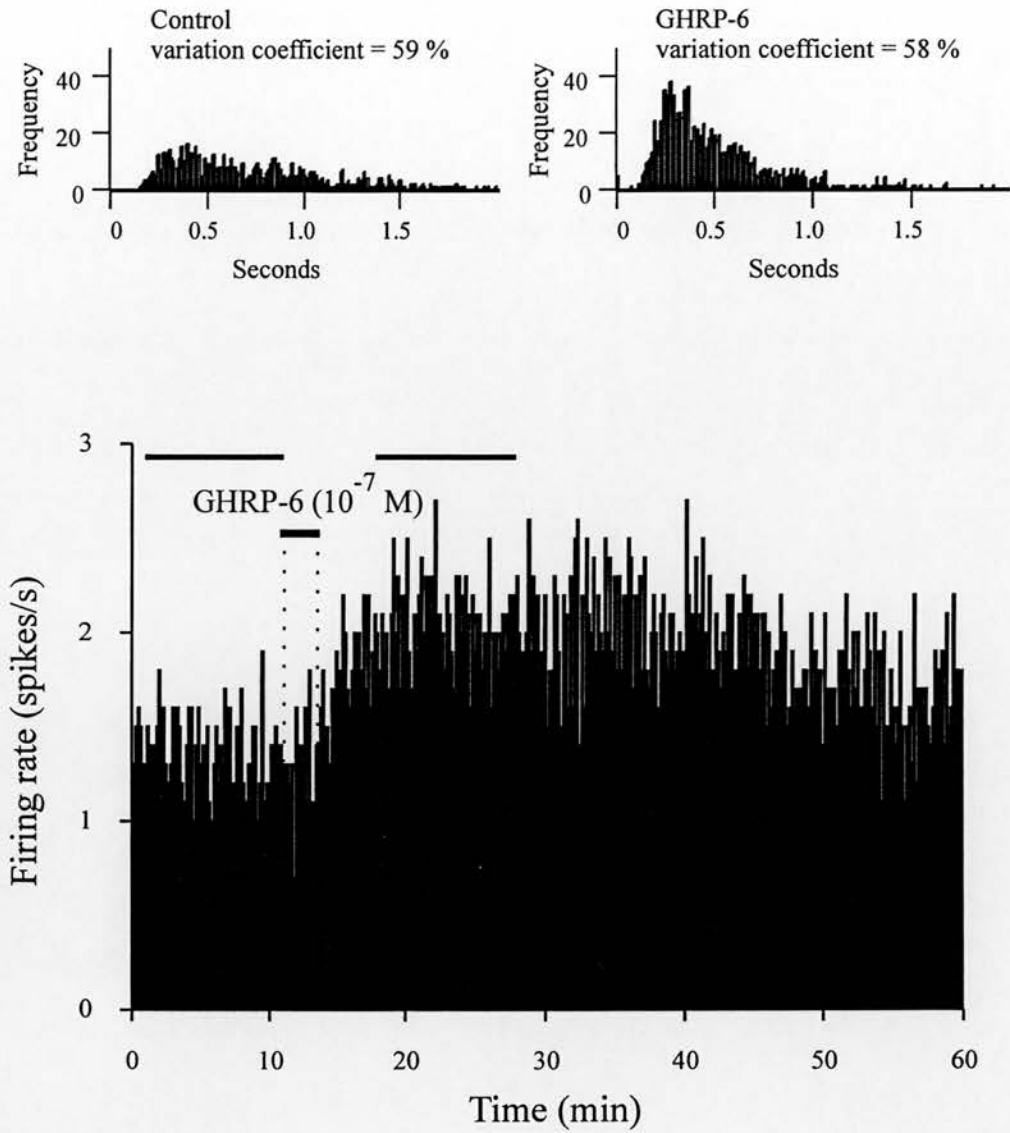


Fig. 2.1.12. Effects of GHRP-6 on regularity of the discharge patterns of a representative arcuate neurone. Upper panel illustrates the ISI histogram compiled before (left) and after (right) bath application (2 min) of GHRP-6 (10^{-7} M). The stable variation coefficient reflects no change in regularity of its discharge pattern after GHRP-6. Lower panel illustrates the effects of GHRP-6 on the firing rate of this neurone. Two bars indicate the periods of control and GHRP-6 treatment ISI histograms.

2.3.5.4 Effects of calcium removal on L-144,446 excitation in arcuate neurones

The dependence of the excitatory effects of L-144,446 on external calcium were examined in recordings of 30 arcuate neurones. Recordings started with perfusion of normal medium which contains 2.5 mM CaCl_2 in order to detect basal firing activity. Thereafter, the perfusate was switched to the modified medium with remaining 10 % Ca^{++} (see **materials of the chapter 2** for preparation of medium with reduced concentration of Ca^{++}). In these experiments, removal of external calcium resulted in a large increase in the electrical activity of the recorded neurones, and these cells were not tested further (**fig. 2.1.13A**). Twelve cells however showed a reduction in electrical activity to a low stable level. These cells were then tested, in the absence of external calcium, with exposure to L-144,446.

Of the 12 arcuate neurones tested under calcium removal condition, 5 neurones (42 %) were excited by L-144,446. In all of the 5 responsive cells, L-144,446 changed the firing pattern to be more irregular, with a multiple spike bursting pattern. A representative arcuate neurone which showed excitatory effect of L-144,446 during perfusion of Ca^{++} -free medium is shown in **fig. 2.1.13B**. Patterns of excitation were various among different neurones with different durations and response rates. Thus, the 10-min period of peak of activation of each neurones were analysed. At the peak of the responses, the mean firing rate of these 5 cells was elevated from 0 to 5.33 ± 1.5 spikes/s. At peak period, regularity markedly decreased with variation coefficient up to 356 %, whereas, in control medium, regularity increased with variation coefficient decreased from 115 to 90 % at the peak of GHRP-6 excitatory response.

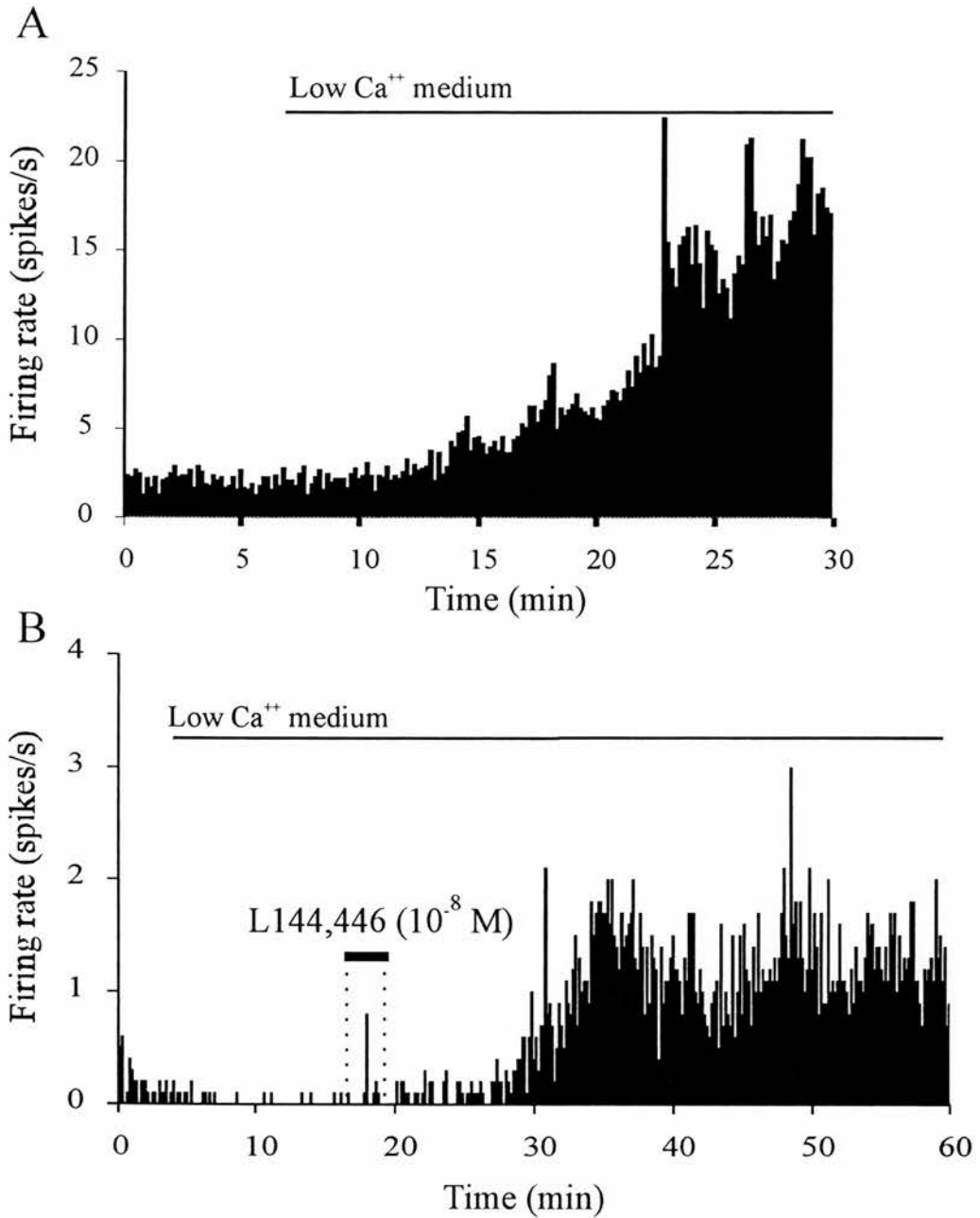


Fig. 2.1.13. (A) Effects of external calcium removal on electrical activity of the arcuate neurone. A neurone showed a large increase in firing activity after perfusate was switched to lower Ca⁺⁺ concentration medium. The perfusate Ca⁺⁺ concentration was reduced to 10% of that in normal medium by replacing with Mg⁺⁺. **(B) Calcium-independent effect of L144,446 on electrical activity of the arcuate neurone.** L-144,446 was applied after firing rate of the arcuate neurone was suppressed by the perfusion of low calcium medium. The periods of low calcium medium perfusion and L-144,446 application are indicated with bars.

Of the cells that did not respond to L-144,446, 4 cells were tested a second time with L-144,446 after return to normal medium. Three of these four cells failed to show any significant change in firing rate within 10 min after the exposure to the L-144,446 (**fig. 2.1.14A**), but the fourth cell showed a strong response (**fig. 2.1.14B**)

Thus at least some arcuate neurones respond to direct administration of L-144,446 in the absence of external calcium, indicating that this response is not dependent either upon calcium entry into the cells, or upon synaptic input arising from neurones in the slice but outside the arcuate nucleus.

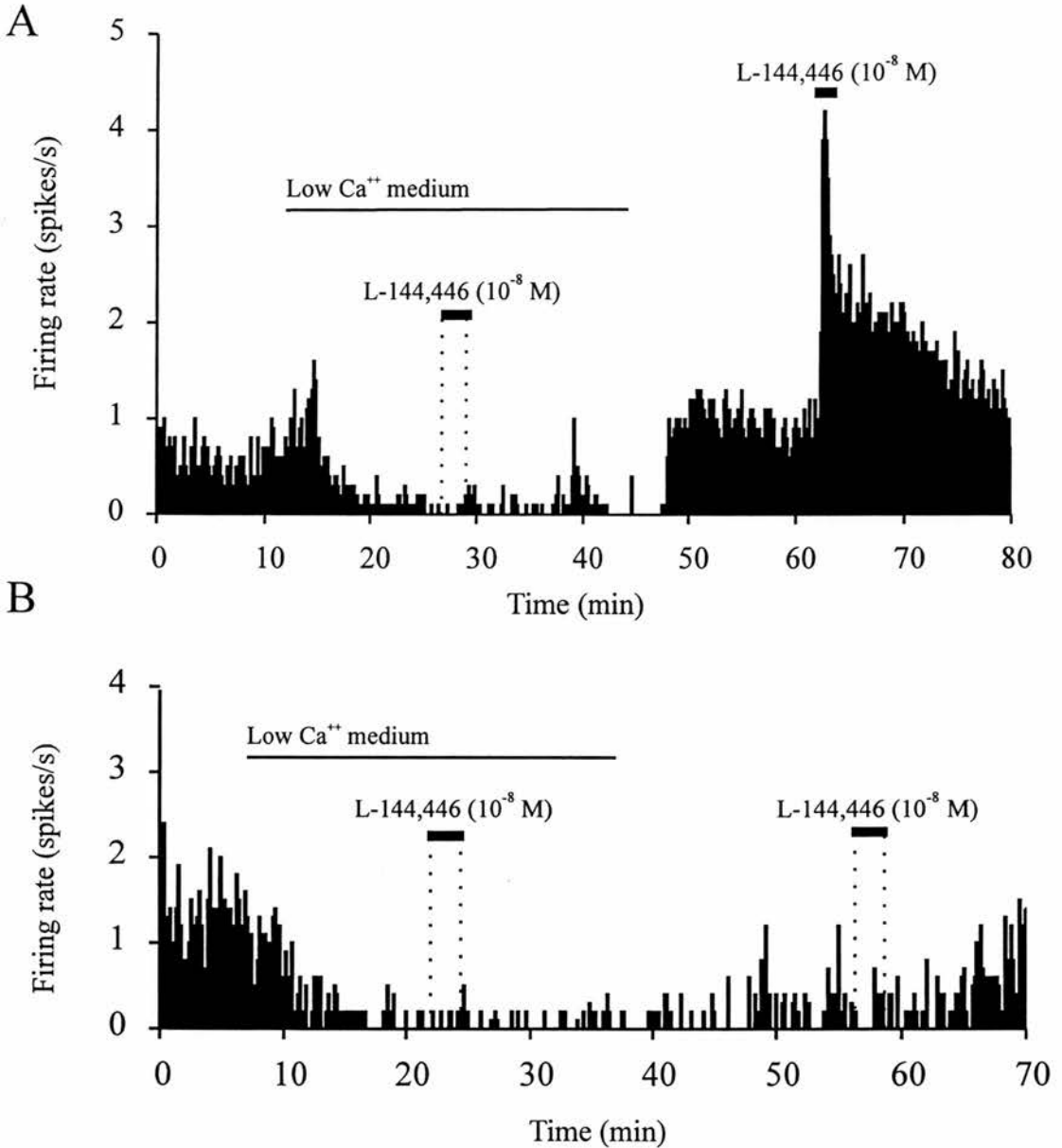


Fig.2.1.14. Dependence on external Ca⁺⁺ of the effects of L-144,446 on the electrical activity of arcuate neurones. Each arcuate neurone was challenged twice with L-144,446 (10⁻⁸ M, 2-min bath application); the first time during low Ca⁺⁺-medium perfusion and the second time during perfusion with normal medium. (A) L-144,446 failed to excitate the arcuate neurone during low Ca⁺⁺-medium perfusion but exhibited a rapid excitation when applied during normal medium perfusion. (B) In another arcuate neurone in which electrical activity was also suppressed by low Ca⁺⁺-medium perfusion, the L-144,446 treatment did not exhibit an excitatory effect either during low Ca⁺⁺ or normal Ca⁺⁺-medium perfusion periods. The periods of low Ca⁺⁺-medium perfusion and L-144,446 application are indicated with bars.

2.3.5.5 Effects of NPY on arcuate neurones

The effects of NPY on arcuate neurones were tested in extracellular recordings from 38 neurones. Initially, 16 arcuate neurones were tested with NPY at 10^{-7} M for 2 min. The response of each neurone to NPY administration was determined by comparison between its mean firing rates in a control period (measured over 10 min before drug administration), and after exposure to NPY (measured from 2 to 12 min following the treatment). The minute-by-minute firing rates in these two periods were compared statistically.

Five neurones (31.25 %) showed brief but significant inhibitory effects of NPY (data not shown). In these neurones, a slight decrease was rapidly seen, and inhibition was maximal at 15 min after the administration of NPY. The mean firing rate was reduced by approximately 0.5 spikes/s at the peak of inhibition.

Thereafter, the test concentration of NPY was increased to 10^{-6} M, keeping the perfusion period at 2 min. Twenty-two neurones were tested with NPY (10^{-6} M). Ranking of change in firing rate is shown in **fig. 2.1.15A**. Ten neurones (45 %) showed a significant inhibition in response to NPY ($P < 0.03$). The mean firing rates of inhibited neurones were plotted (**fig. 2.1.15B**). The mean firing rate decreased markedly as soon as the peptide reached the brain slice. Inhibition was maximal approximately 5 min after NPY administration. The mean firing rate of inhibited neurones, in the period from 2 to 12 min after exposure to NPY, was reduced by 0.70 spikes/s (35 %; from 1.99 ± 0.38 to 1.28 ± 0.34 spikes/s).

Fifteen arcuate neurones that were stimulated by GHRP-6 were also tested with NPY. Of these 15 neurones, 6 (40 %) were inhibited by NPY, 2 (13 %) were stimulated, and 8 (53 %) were non-responsive. Three representative GHRP-6-stimulated arcuate neurones that were inhibited by NPY are shown in **fig. 2.1.16**.

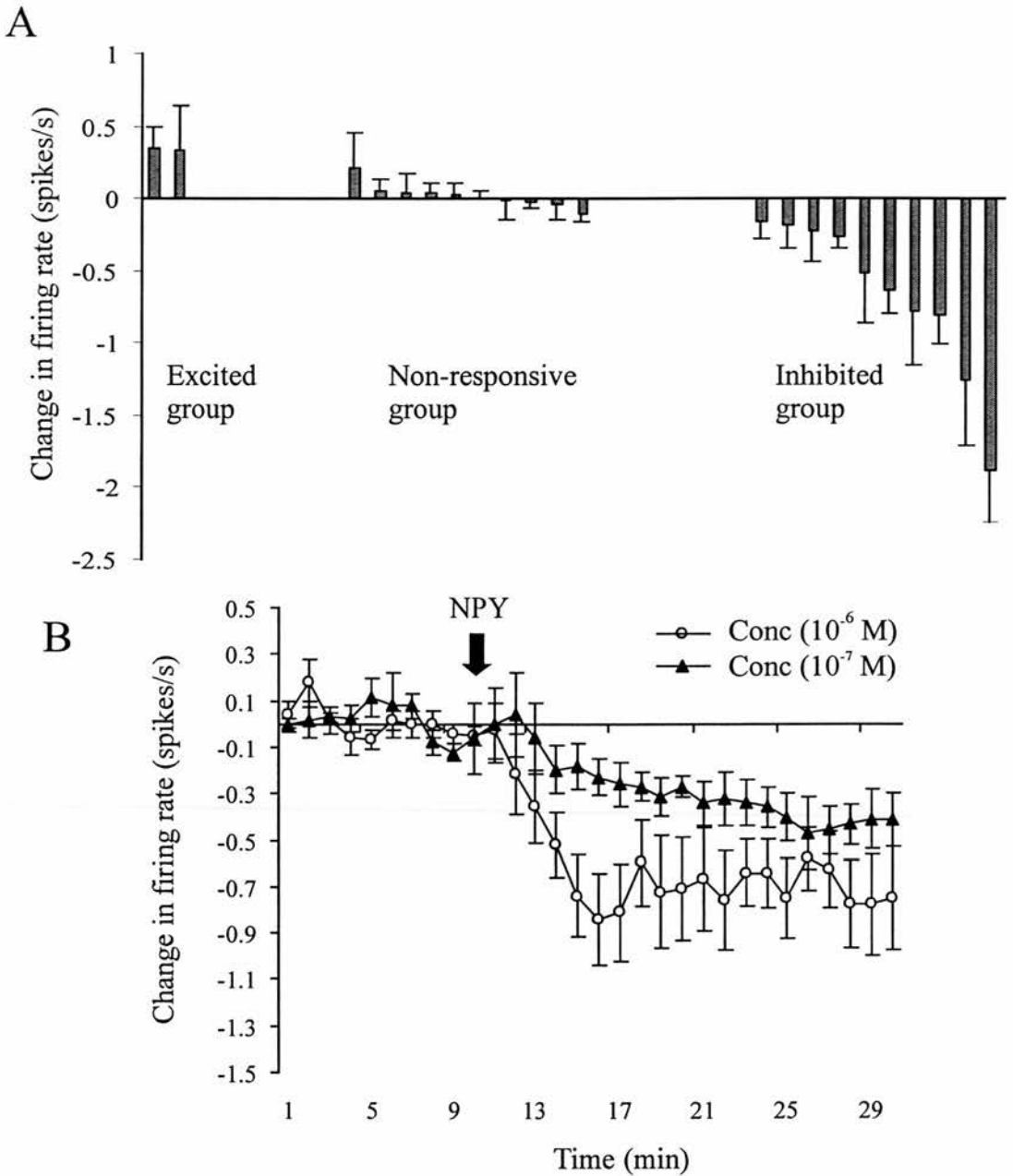


Fig. 2.1.15. (A) Ranking of changes in firing rate of 22 arcuate neurones following NPY treatment. Mean firing rate of each individual neurone during the 10-min period after the NPY application (10^{-6} M, 2 min) was normalised by subtracting the mean firing rate of control period (10-min period before the administration). Neurones were divided into 3 groups according to their responses. Changes in firing rate (\pm SEM spikes/s) after NPY treatment were statistically significant in the inhibited group and excited group (t-test, $p < 0.03$), but not in the non-responsive group. Each bar represents the change in firing rate of one individual neurone following NPY administration. **(B) Mean response of the inhibited group following NPY treatment.** Normalised mean firing rates of neurones inhibited by NPY at concentration either 10^{-6} M or 10^{-7} M were averaged minute-by-minute ($n = 9-10$ and $3-5$ per each point for concentration 10^{-6} M and 10^{-7} M respectively).

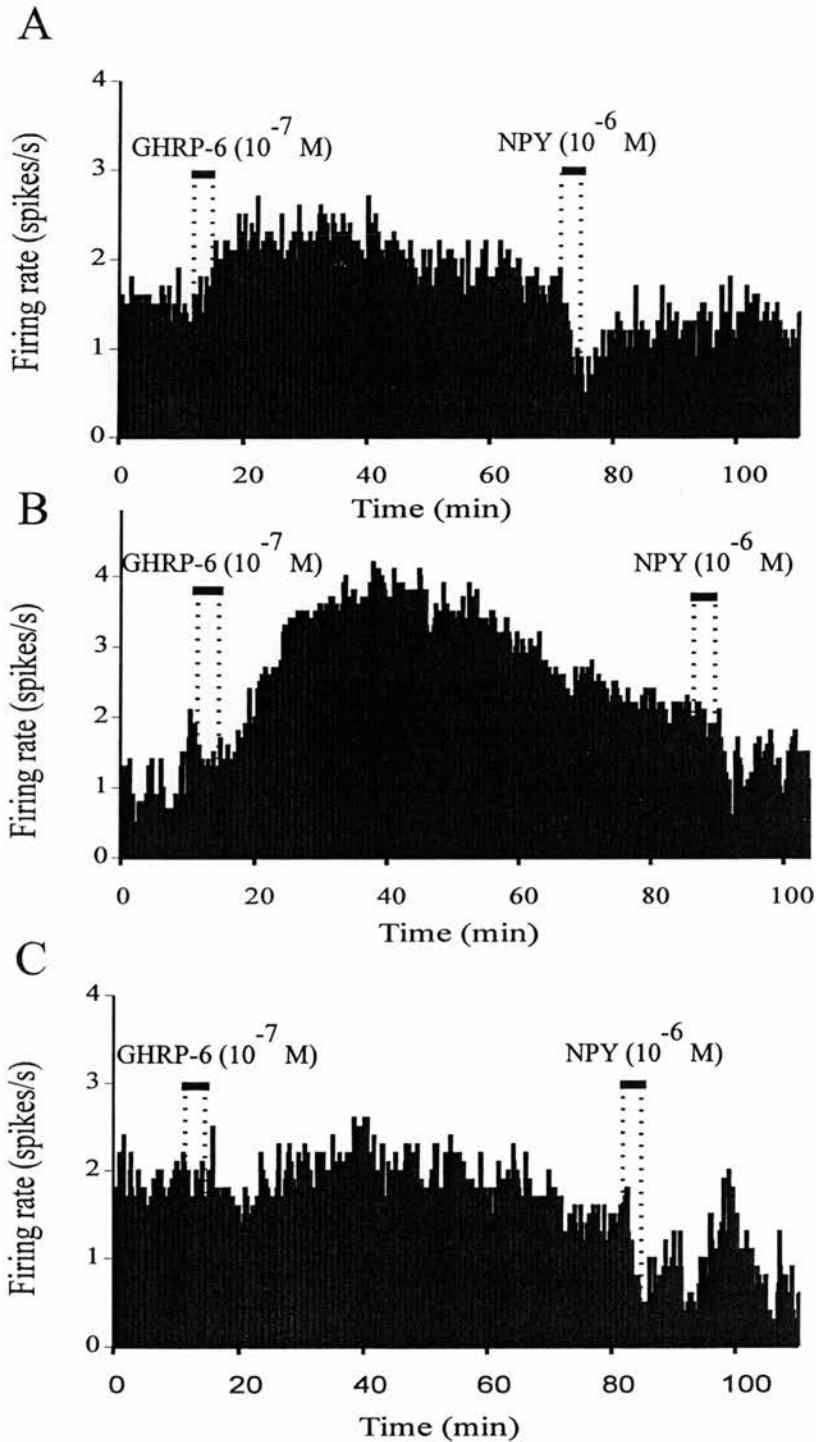


Fig. 2.1.16. Electrophysiological responses of GHRP-6-excited arcuate neurones to NPY. These neurones were determined to be GHRP-6-excited neurones (see fig. 2.1.6). Each neurone was tested twice; the first time with GHRP-6 (10^{-7} M, 2-min bath application) and the second time with NPY (10^{-6} M, 2-min bath application). In each case, the firing rate during the 10-min period after NPY administration was significantly inhibited compared to the firing rate during the 10-min period before NPY administration (t-test, $p < 0.001$). Periods of GHRP-6 and NPY treatments are indicated with bars.

2.3.5.6 Effects of somatostatin on arcuate neurones

The responses to somatostatin were examined in recordings obtained from 22 arcuate neurones. Each neurone was determined whether it responded to the somatostatin administration by comparison between its mean firing rates in a control period (measured over 10 min immediately before drug administration), and after exposure to somatostatin (measured from 2 to 12 min following the treatment). Initially, 10 arcuate neurones were tested with somatostatin at 10^{-8} M for 2 min. Four of these neurones were significantly inhibited, and 6 were non-responsive. Representative recordings of arcuate neurones that were significantly inhibited by somatostatin are shown in **fig. 2.1.17 (A)**. The mean firing rate of these arcuate neurones was reduced by 0.39 spikes/s (19 %; from 1.86 ± 0.87 to 1.46 ± 0.72 spikes/s) (**fig. 2.1.17 B**).

Ten arcuate neurones were tested with somatostatin at the higher concentration of 10^{-7} M, again for 2 min. Of these 10 neurones, 3 were inhibited, 1 was stimulated, and 6 were non-responsive. The average firing rate of inhibited neurones was a 17.50 % decrease (data not shown).

In addition, two arcuate neurones were tested with identical somatostatin administration, but under low calcium condition. Both of them were inhibited. At the peak of inhibition, the mean firing rate was decreased by 45.7 %. Thus, overall, 9 arcuate neurones (41 %) were shown to be significantly inhibited by somatostatin at concentration of 10^{-7} M or less.

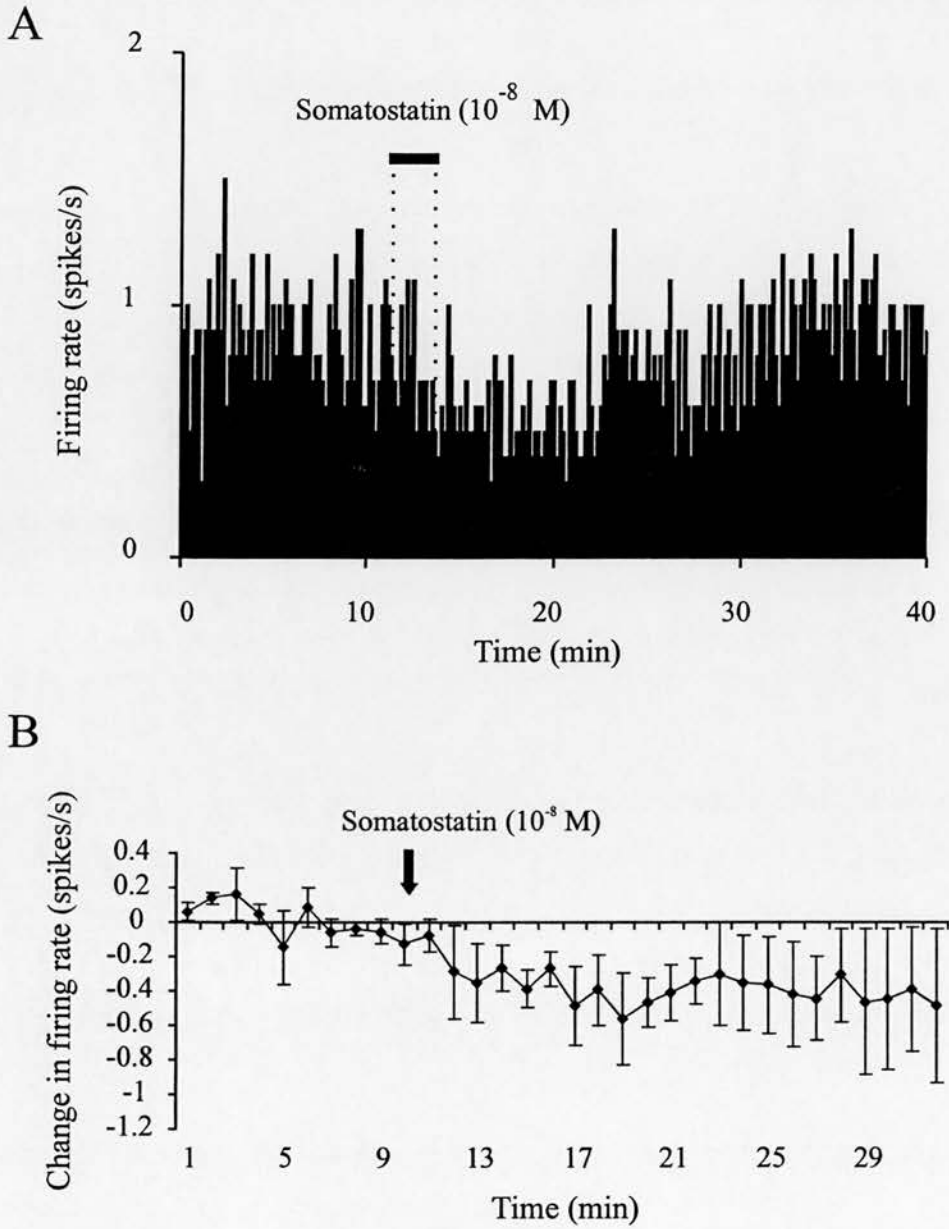


Fig. 2.1.17. Electrophysiological responses of arcuate neurones to somatostatin. (A) Rate-meter record showing inhibitory effect of somatostatin. The arcuate neurone was tested with somatostatin at 10^{-8} M for 2-min bath application. **(B) Mean response of arcuate neurones that were inhibited by somatostatin.** Neurones were determined to be inhibited according to their responses the same way as for NPY responses [see fig. 2.1.16 (A)]. Their normalised mean firing rates were averaged minute-by-minute ($n = 4$ per each point).

2.3.5.7 Responses of arcuate neurones to the absence of glucose

Responses of arcuate neurones to the absence of glucose were tested in extracellular recordings. At the start, firing activity of twenty arcuate neurones was recorded with perfusion of normal medium. Then, perfusion was switched from normal medium to glucose-free medium either for 4 min (10 neurones) or 5 min (10 neurones). After that, perfusion was switched back to normal medium.

Changes in firing rate from the period of 5 min immediately before glucose-free medium perfusion to the period of 5 min (3 to 8 min) after the start of glucose-free medium perfusion, were analysed. The minute-by-minute firing rates in these two periods were compared statistically.

No significant difference between perfusion of glucose-free medium for 4 or 5 min was made. Only 3 out of 20 neurones were significantly inhibited by more than 0.5 spikes/s (t-test, $p < 0.02$, data not shown). None of them was significantly excited by more than 0.5 spikes/s. Overall, no significant change in mean firing rate of arcuate neurones was obtained after perfusion of glucose-free medium (mean change of -0.07 ± 0.08 spikes/s, $n = 20$).

2.3.6 Electrophysiological studies of ventromedial hypothalamic neurones

2.3.6.1 Basal activity and activity patterns in the ventromedial hypothalamic nucleus

Stable extracellular recordings were made from 90 spontaneously active neurones in the ventromedial hypothalamic nucleus of the trimmed rat hypothalamic slice preparation. Each neurone was recorded from a different slice, and was tested with application of secretagogues alone or in combination with other drugs.

Like arcuate neurones, all recorded hypothalamic neurones were spontaneously active, and discharged with no conspicuous pattern – in particular no neurone fired phasically, and no neurone fired very regularly. The mean of variation coefficients was 106 % which confirms their highly irregular firing pattern. The mean spontaneous firing rate of these ventromedial hypothalamic neurones was 0.90 ± 0.06 spikes/s (mean \pm SEM; range 0.11 to 3.33 spikes/s). The discharge patterns of these neurones were also studied by analysing the distributions of interspike intervals in the same way as for arcuate neurones.

Most of the neurones analysed (90 %) had a clearly defined modal interspike interval. The average modal interspike interval in the control period was 0.43 ± 0.05 s, and only 13.70 % of them showed a modal interspike interval of less than 100 ms. Of the remainder, 86 % of these neurones showed a more dispersed interspike interval histogram with a predominant interspike interval greater than 100 ms. Firing pattern with bi-modal interspike intervals were also found in 10 % of them. The differences of mean firing rates and interspike intervals of neurones that responded and did not respond to GHRP-6 were analysed, and are described in section 2.3.1.2.

2.3.6.2 Responses of ventromedial hypothalamic neurones following administration of GHRP-6

Forty-seven ventromedial hypothalamic neurones were tested for their responses to GHRP-6. Each neurone was determined whether it responded to the GHRP-6 administration by comparison between its mean firing rates in a control period (measured over 10 min before the administration), and after exposure to GHRP-6 (measured from 5 to 15 min after administration). The minute-by-minute firing rates in these two periods were compared statistically. Two initial representative recordings of ventromedial hypothalamic neurones that were stimulated by two different concentrations of GHRP-6 are shown in **fig. 2.2.1 (A and B)**. Figure **A** shows a recording of a neurone, stimulated by GHRP-6 (10^{-6} M) for 2 min, that markedly increased its firing rate from 0.99 to 2.61 spikes/s, more than a 2-fold increase. Figure **B** shows a different neurone stimulated by a lower concentration (10^{-7} M) of GHRP-6 for 2 min, again showing an increase in firing rate from 0.88 to 1.79 spikes/s, approximately a

2-fold increase. After these pilot experiments involving tests of GHRP-6 at different concentrations, the concentration of 10^{-7} M for 2 min was selected to be the condition of the rest of the study.

The effects of GHRP-6 were examined on 47 neurones. To analyse the responses, and to classify neurones as excited, inhibited, or non-responsive, the firing activity over a 10-min control period were compared with the firing activity in the period 5-15 min after drug application, for all cells, as previously described in the studies of arcuate neurones. Figure 2.2.2 shows the mean change in firing rate of each neurone tested, plotted in order of magnitude. The population of ventromedial hypothalamic neurones was divided into three groups according to their responses to GHRP-6. Two of the tested neurones (4.26 %) were significantly inhibited by GHRP-6 ($P < 0.02$). Ten neurones (21.28 %) did not show any significant change following administration of GHRP-6 ($P = 0.05$). Thirty-five neurones (75 %) showed a significant increase in firing rate following GHRP-6 ($P < 0.02$). The mean firing rate of this group increased by 0.86 spike/s (113 %, from 0.76 ± 0.07 to 1.63 ± 0.16 spikes/s).

The basal firing patterns of these three classified groups of the ventromedial hypothalamic neurones were then compared. The modal interspike interval of the discharge was taken as the peak in interspike interval histogram of the 10-min period of recording before administration of GHRP-6. The modal interspike intervals of neurones from these 3 groups were then plotted against mean interspike interval (**fig. 2.2.3**). The excited neurones had mean firing rates which were evenly scattered, mostly between 0 and 1.5 spikes/s with a mean \pm SEM of 0.75 ± 0.07 spikes/s. Most of the excited neurones (32 of a total 35, 91 %) had uni-modal interspike interval distributions with an average mode of 0.35 ± 0.04 s, whereas the rest 3 of excited neurones had bi-modal interspike interval distributions. Non-responsive neurones had a mean firing rate of 0.86 ± 0.16 spikes/s and an average modal interspike interval (averaged from 9 of a total 10 neurones with uni-modal interspike interval) of 0.29 ± 0.07 s. The two inhibited neurones had mean firing rates of 0.66 and 1.06 spikes/s, with modal interspike intervals of 0.18 and 0.24 s respectively.

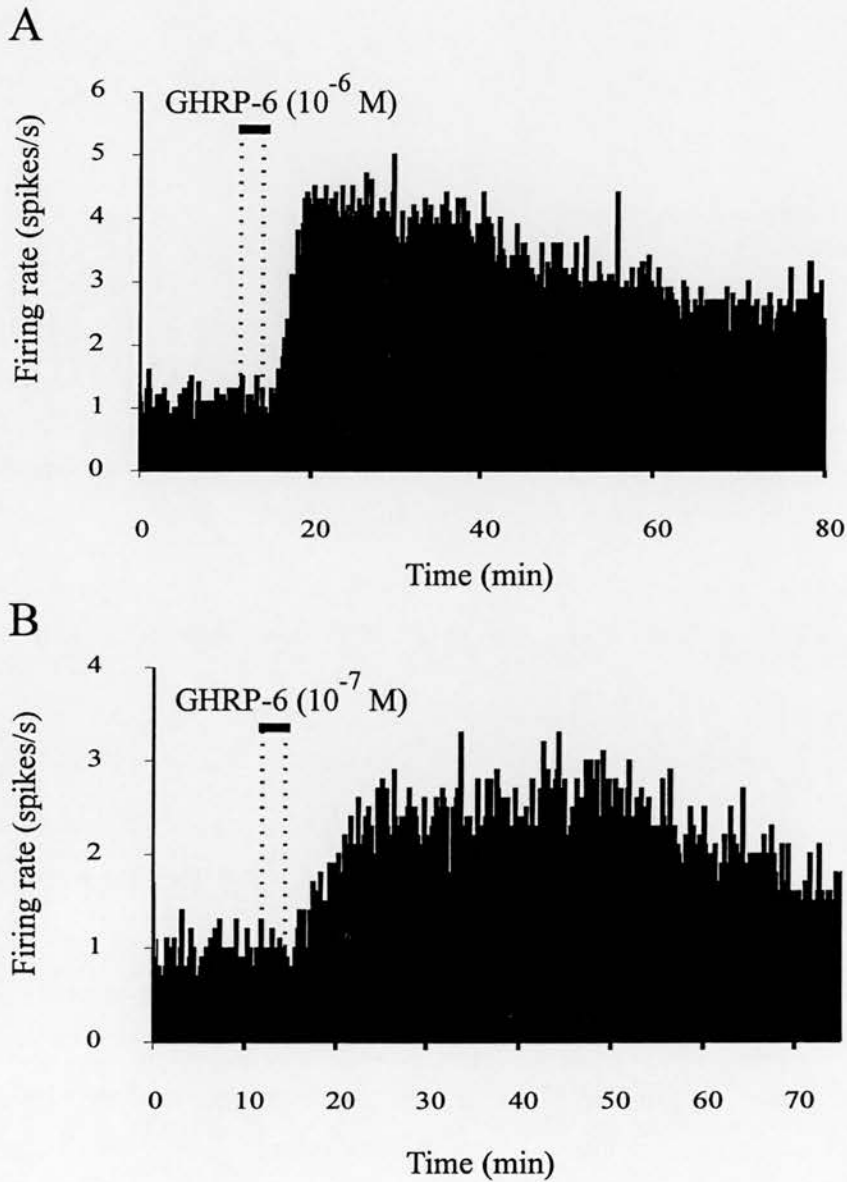


Fig. 2.2.1. Extracellular recordings of the firing rate of ventromedial hypothalamic neurones following perfusion of the hypothalamic slices with GHRP-6. (A) A neurone was stimulated with 10^{-6} M for 2 min. (B) Another neurone was stimulated with the same secretagogue at lower concentration (10^{-7} M) for 2 min. The firing rate was divided into 10-s intervals and is expressed as spikes/s. In each case, the firing rate during the 10-min period taken 5 to 15 min following GHRP-6 administration (averaged for 60x10-s intervals) was significantly different from the firing rate of control period taken 10 min before administration (averaged for 60x10-s intervals; $P < 0.001$, t-test).

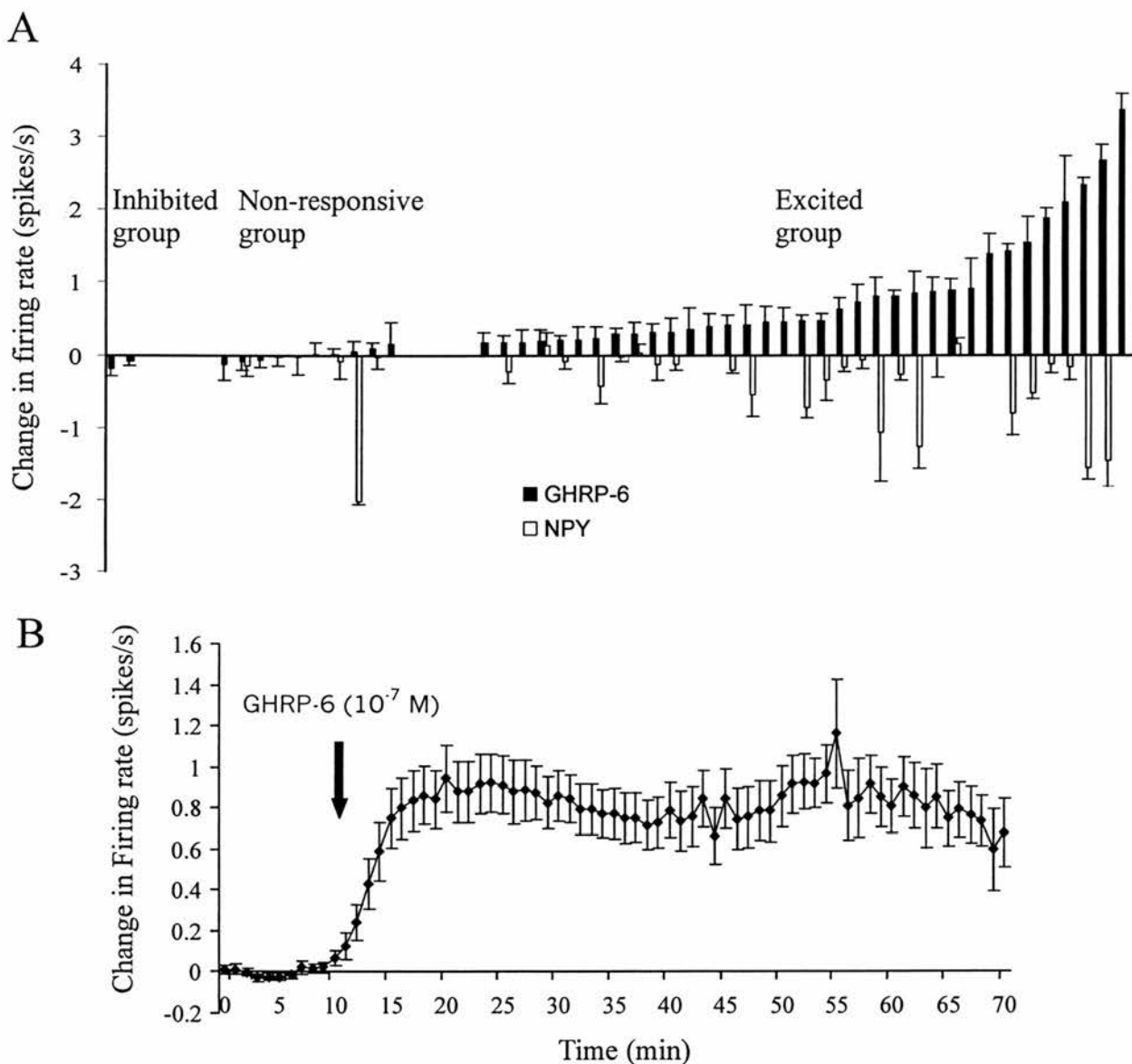


Fig. 2.2.2. (A) Ranking of changes in firing rate of 47 ventromedial hypothalamic neurones following GHRP-6 treatment. Mean firing rate of each individual neurone during the 10-min period after GHRP-6 application (10^{-7} M, 2-min) was normalised by subtracting the mean firing rate of the control period (10-min period before the application). Neurones were divided into 3 groups according to their responses. Changes in firing rate after GHRP-6 treatment were statistically significant in the inhibited and excited groups (t-test, $p < 0.02$), but not in the non-responsive groups. Each bar represents the change in firing rate (\pm SEM) of one individual neurone following GHRP-6 administration. Some non-responsive and excited neurones were also tested with NPY (10^{-6} M) in the same recording. Responses to NPY were analysed the same way as for GHRP-6 responses (c.f. Fig.). **(B) Mean response of the excited group following GHRP-6 treatment.** Normalised mean firing rates of the excited neurones were averaged minute-by-minute ($n = 8-33$ per point).

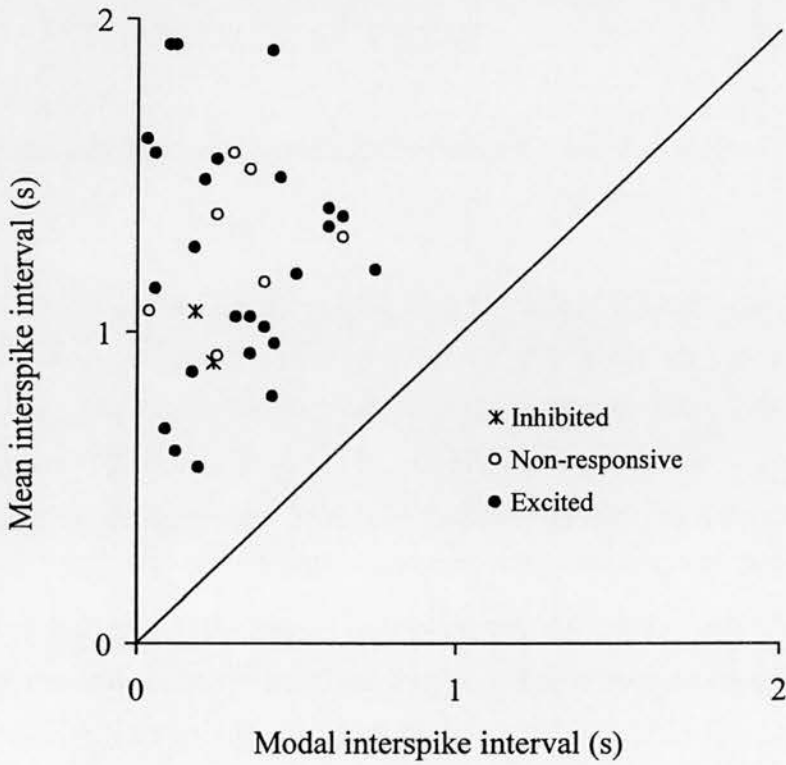


Fig. 2.2.3. Relationship between mean and modal interspike interval of basal firing activity of ventromedial hypothalamic neurones treated with GHRP-6. Neurones were divided into 3 groups according to their responses to GHRP-6 (10^{-7} M, 2-min bath application). For each cell, mean and modal interspike intervals were analysed from its control period (10 min before the treatment).

In summary, excited and inhibited ventromedial hypothalamic neurones were had similar mean firing rates. However, 14 of 15 neurones that showed a modal interspike interval of 0.4 s or higher, were excited by GHRP-6. This might indicate that neurones with a high modal interspike interval are likely to exhibit an excitatory response to the GHRP-6.

In addition, the analysis also showed no clear difference between the firing patterns of excited and non-responsive neurones in term of regularity. The excited groups had a non-significantly lower variation coefficient than that of non-responsive group (t-test, $p = 0.05$). The variation coefficients of the excited and non-responsive groups were $99 \pm 4 \%$ and $118 \pm 16 \%$ respectively.

The mean firing rates of excited neurones were plotted (**fig. 2.2.2**). The increase began as soon as the peptide reached the brain slice, and during the period of drug washout, the response increased progressively and peaked in approximately 10 min. At the peak, the mean firing rate was approximately 1 spikes/s higher than the initial control level. The response remained relatively stable above control levels for about 60 min after the administration.

The effects of GHRP-6 on the discharge patterns of ventromedial hypothalamic neurones were analysed. The average variation coefficient decreased from a control level of $99 \pm 4 \%$ to $91 \pm 6 \%$ following administration of GHRP-6 (7.50 % decrease). Of 35 neurones excited by GHRP-6, 22 neurones (63 %) showed a decrease in variation coefficient, which means they increased regularity of their firing pattern following the administration of GHRP-6. The remaining 13 excited neurones (37 %) showed an increase in variation coefficient. Figures 2.2.4 and 2.2.5 show recordings of two representative ventromedial hypothalamic neurones with increased and decreased regularities respectively by GHRP-6). However, the overall decrease in variation coefficient of these GHRP-6-stimulated group was not statistically significant (t-test, $P = 0.05$). This indicates that the regularity of firing pattern of the ventromedial hypothalamic neurones is not consistently affected by GHRP-6.

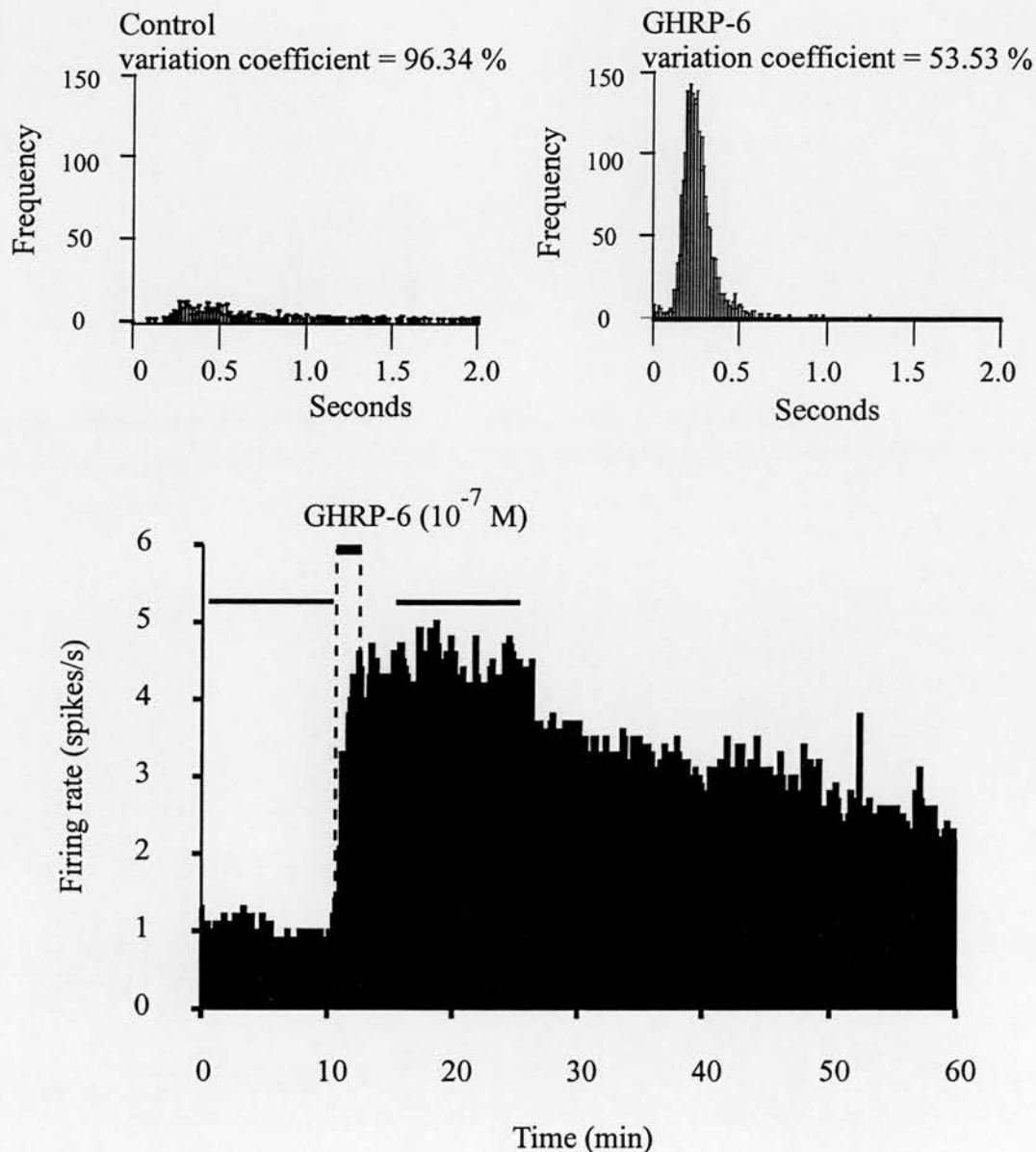


Fig. 2.2.4. Effects of GHRP-6 on regularity of discharge pattern of a representative ventromedial hypothalamic neurone. Upper panel illustrates the ISI histogram compiled before (left) and after (right) bath application (2 min) of GHRP-6 (10^{-7} M). The decrease in variation coefficient reflects an increase in regularity of its discharge pattern after GHRP-6 treatment. Lower panel illustrates the effects of GHRP-6 on firing rate of this neurone. Two bars indicate the periods of control and GHRP-6 treatment ISI histograms.

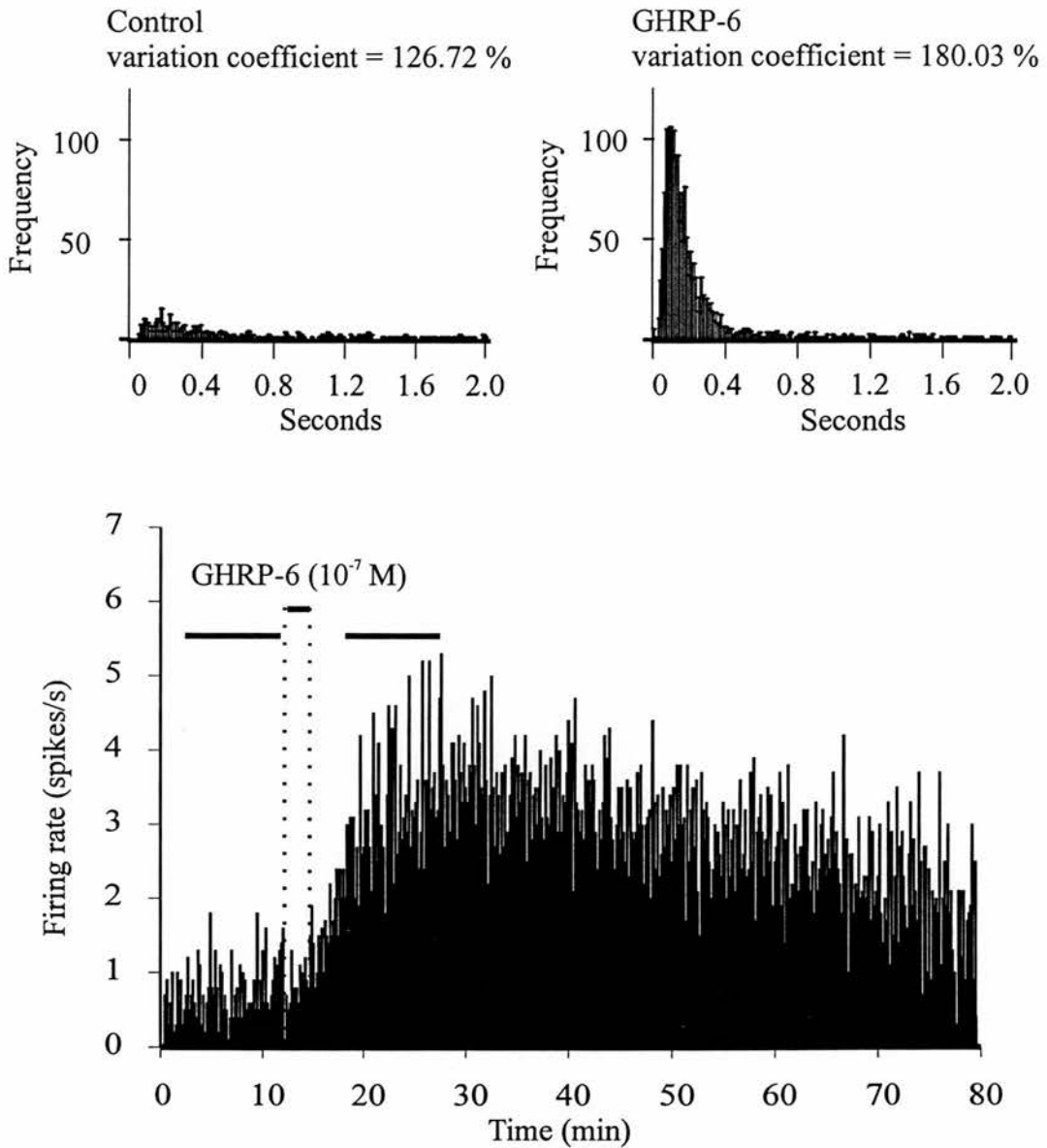


Fig. 2.2.5. Effects of GHRP-6 on regularity of discharge pattern of a representative ventromedial hypothalamic neurone. Upper panel illustrates the ISI histogram compiled before (left) and after (right) bath application (2 min) of GHRP-6 (10^{-7} M). The increase in variation coefficient reflects a decrease in regularity of its discharge pattern after GHRP-6 treatment. Lower panel illustrates the effects of the GHRP-6 on firing rate of this neurone. Two bars indicate the periods of control and GHRP-6 treatment ISI histograms.

2.3.6.3 Desensitisation to the effect of GHRP-6

Two ventromedial hypothalamic neurones that were stimulated by GHRP-6 were tested with a second challenge of the peptide. The first exposure caused rapid activation during the washout period (**fig. 2.2.6**). The increases in firing rate reached peaks and brief plateau periods, and gradually declined to control levels. After a washout period of longer than 100 min with no GHRP-6 in the perfusate, the neurones were challenged with a second exposure identical to the first exposure. The second exposure also increased firing rate, but with a noticeable attenuation. The mean increase in firing rate following the second exposure to GHRP-6 was only 79 % of that of the first exposure.

2.3.6.4 Effects of NPY on GHRP-6-stimulated ventromedial hypothalamic neurones

Thirty-nine ventromedial hypothalamic neurones were tested for their responses to NPY at 10^{-6} M for 2 min. Of the 39 neurones, 25 were neurones excited in response to GHRP-6. The response of each neurone to NPY was determined by comparison between its mean firing rate in a control period (measured over 10 min before drug administration), and after exposure to NPY (measured from 2 to 12 min after treatment).

Figure 2.1.7 A shows the mean change in firing rate of each neurone tested, plotted in order of magnitude. The population was divided into 3 groups. Twenty-five neurones (64 %) showed significant inhibitory effects of NPY, whereas 3 neurones (8 %) showed significant excitatory effects of NPY. The remaining eleven neurones tested did not show any significant change in firing rate (t-test, $P < 0.05$). Recordings of three representative GHRP-6-excited neurones that were inhibited by NPY are shown in **fig. 2.2.8**. The inhibitory effects ranged from about 10 % attenuation to almost complete abolition of firing activity.

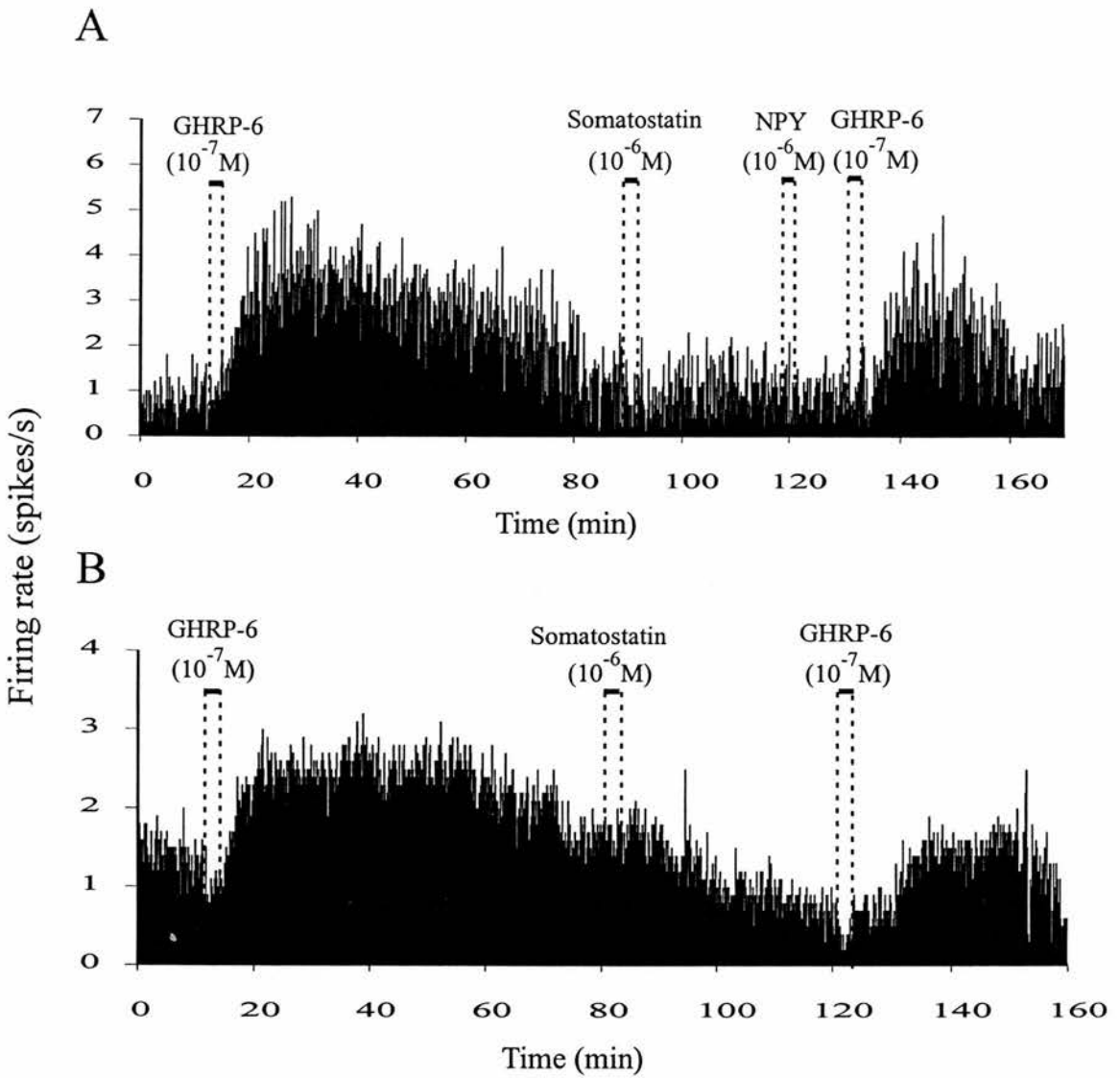


Fig. 2.2.6. Rate-meter records showing reduction of firing rate of ventromedial hypothalamic neurones in response to second challenge of GHRP-6. Each neurones were treated twice with GHRP-6 (10^{-7} M) with at least 100-min interval. Periods of treatments are indicated with bars.

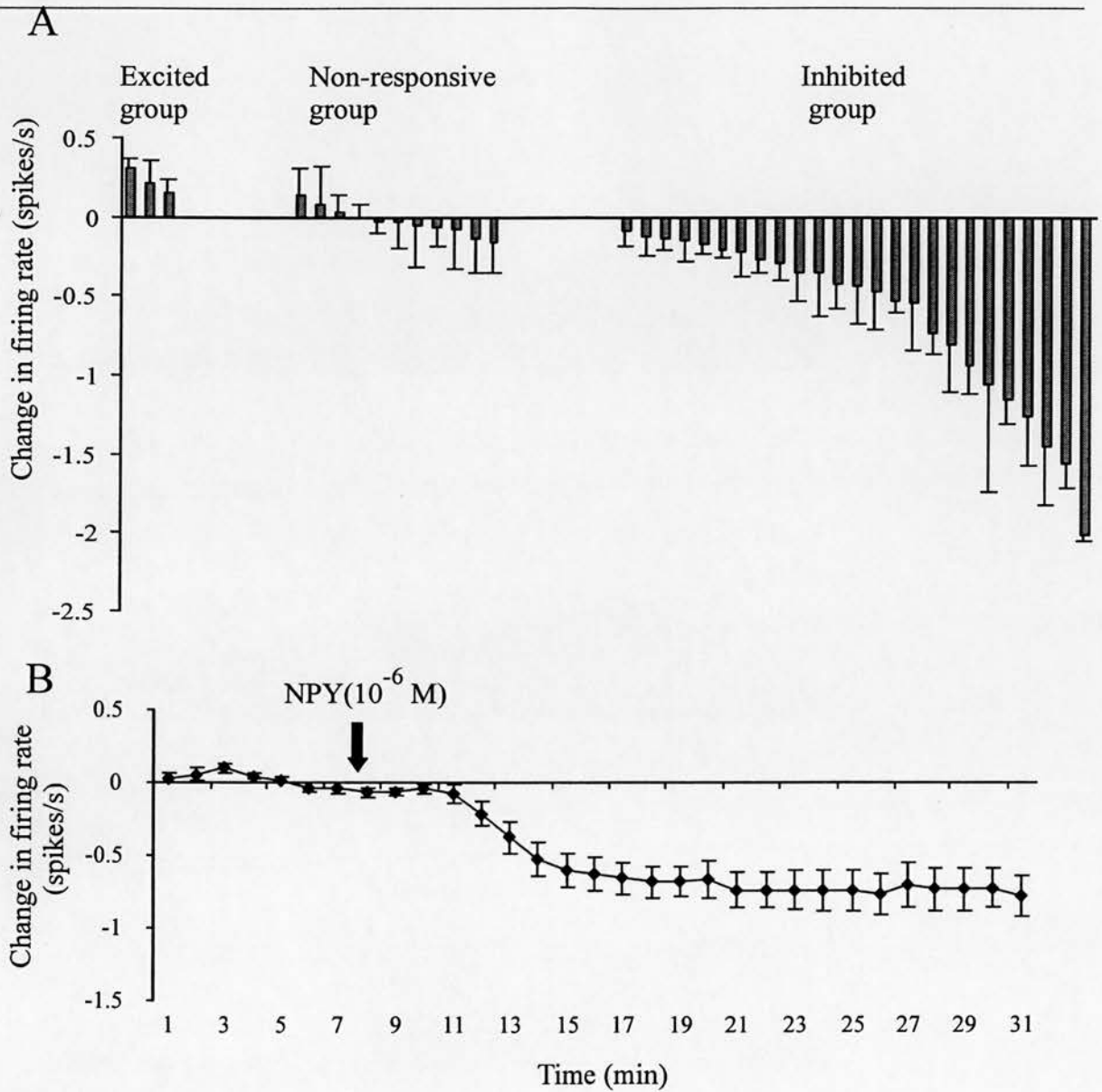


Fig. 2.2.7. (A) Ranking of changes in firing rate of 39 ventromedial hypothalamic neurones following NPY treatment. Mean firing rate of each individual neurone during the 10-min period after the NPY application (10^{-6} M, 2 min) was normalised by subtracting the mean firing rate of control period (10-min period before NPY application). Neurones were divided into 3 groups according to their responses. Changes in firing rate (\pm SEM spikes/s) after NPY treatment were statistically significant in the inhibited and excited groups (t-test, $p < 0.03$), but not in the non-responsive group. Each bar represents the change in firing rate of one individual neurone following NPY administration. **(B) Mean response of the inhibited group following NPY treatment.** Normalised mean firing rates of inhibited neurones were averaged minute-by-minute ($n = 23-25$ per each point).

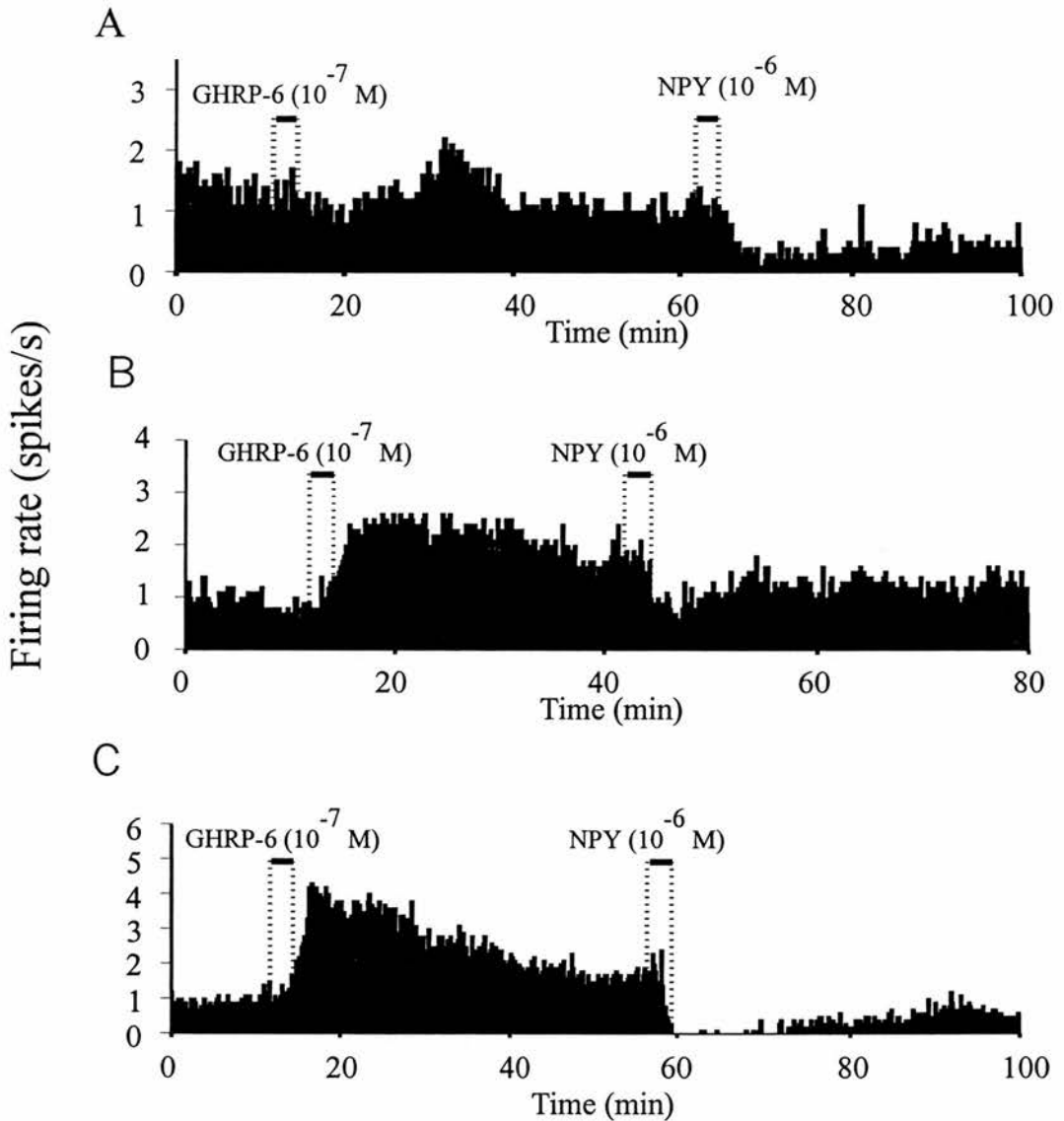


Fig. 2.2.8. Electrophysiological responses of GHRP-6-excited ventromedial hypothalamic neurons to NPY. These neurons were determined to be GHRP-6-excited neurons [see fig. 2.2.2 (A)]. Each neuron was tested twice; the first time with GHRP-6 (10^{-7} M, 2-min bath application) and the second time with NPY (10^{-6} M, 2-min bath application). In each case, the firing rate during the 10-min period after NPY application was significantly inhibited compared to the firing rate during the 10-min period before NPY application (t-test, $p < 0.001$). Periods of GHRP-6 and NPY applications are indicated with bars.

The mean firing rates of inhibited neurones were plotted (**fig. 2.2.7 B**). The decrease was maximal approximately 5 min after NPY administration. The mean firing rate of inhibited neurones reduced by 0.63 spikes/s (49 %, from 1.29 ± 0.13 to 0.66 ± 0.11 spikes/s).

Twenty-five neurones that were excited by GHRP-6 were also tested with NPY. Seventeen of these (68 %) were inhibited, 1 was excited, and 7 were non-responsive to NPY. Thus the majority of ventromedial hypothalamic neurones that were excited by GHRP-6 were inhibited by NPY.

2.3.6.5 Effects of a NPY Y1 receptor antagonist on GHRP-6- and NPY-responsive ventromedial hypothalamic neurones

Responses of NPY Y1 receptor antagonist, L-753044-001J003, were examined in recordings of six ventromedial hypothalamic neurones that responded to both administrations of GHRP-6 and NPY. After each neurone was stimulated and inhibited by GHRP-6 (10^{-7} M) and NPY (10^{-6} M) respectively, they were therefore treated with the antagonist for 5 min. Then the identical concentration and period of NPY was applied at 3 min after the pre-treatment of the antagonist started. In one cell, pre-treatment with the antagonist at 10^{-8} M did not block the inhibition by NPY (data not shown). A second ventromedial hypothalamic neurone was pre-treated with two concentrations of the antagonist (10^{-6} and 10^{-7} M). The higher concentration was found to effectively block the inhibitory effect of NPY, whereas the lower concentration did not (**fig. 2.2.9**). Thus, the antagonist concentration at 10^{-6} was selected for pre-treatment of two different ventromedial hypothalamic neurones. In one of these cells, the NPY Y1 antagonist completely blocked the NPY inhibitory effects, whereas in the other cell there was no clear effect.

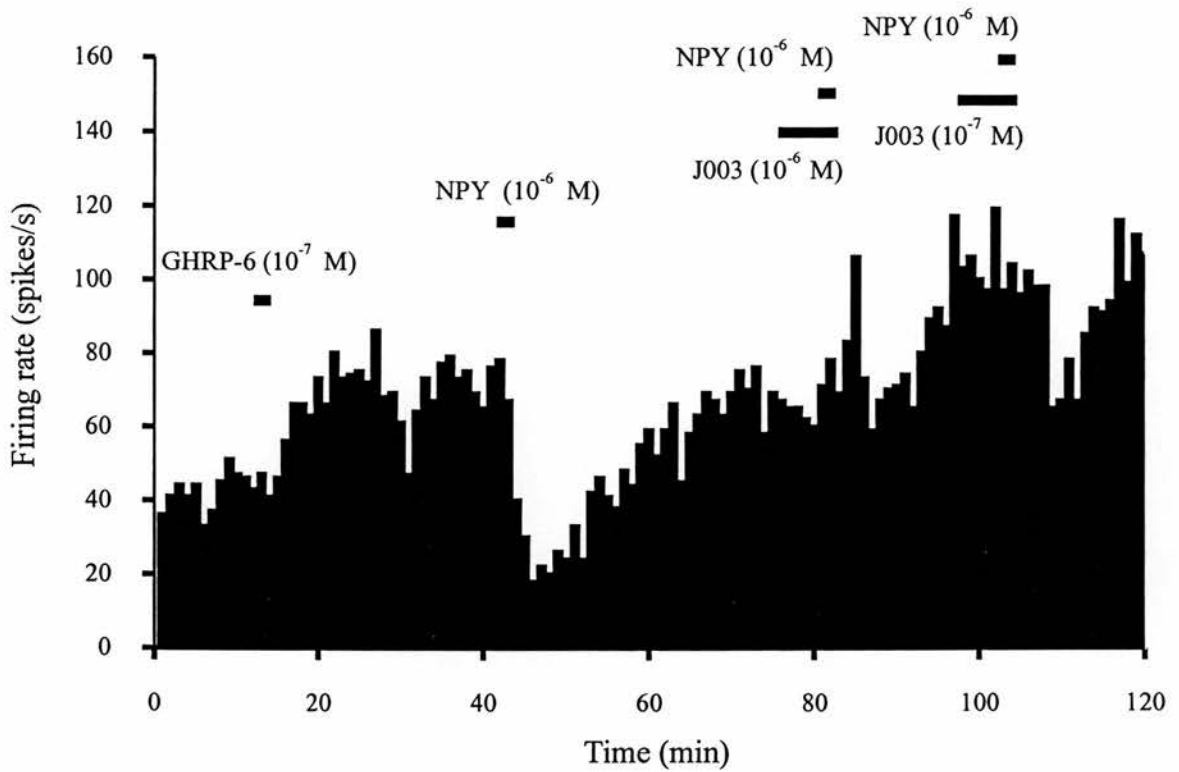


Fig. 2.2.9. Effects of NPY Y1 receptor antagonist (L-753044-001J003) on inhibitory effect of NPY on ventromedial hypothalamic neurone. The neurone was responsive to GHRP-6 (10^{-7} M) and NPY (10^{-6} M), and thereafter pretreated with two concentrations of the antagonist (10^{-6} M and 10^{-7} M) for 5 minutes. After the pretreatment, mixture of NPY and the antagonist at either 10^{-6} M and 10^{-7} M were followed for two minutes. Periods of peptides and antagonist treatments are indicated with bars.

2.3.6.6 Effects of somatostatin on ventromedial hypothalamic neurones

The responses to somatostatin were examined in recordings from 15 ventromedial hypothalamic neurones. The response of each neurone to somatostatin was determined by comparison between its mean firing rates in a control period (measured over 10 min before the administration), and after exposure to somatostatin (measured from 2 to 12 min following the treatment).

Nine ventromedial hypothalamic neurones were tested with somatostatin at 10^{-7} M for 2 min. Two neurones were inhibited, another 2 were stimulated, and 5 were non-responsive. The inhibitory effects of somatostatin were seen in reducing the mean firing rate of neurones from 0.72 to 0.56 spikes/s (21 % decrease).

Six other ventromedial hypothalamic neurones were tested with somatostatin at a higher concentration (10^{-6} M) for 2 min. Four of them were significantly inhibited and 2 were non-responsive. Recordings of representative neurones inhibited by somatostatin are shown in **fig. 2.2.10 A**. The mean firing rate of the four inhibited ventromedial hypothalamic neurones was reduced by 0.29 spikes/s (18%, from 1.63 ± 0.51 to 1.34 ± 0.57 spikes/s) (**fig. 2.2.10 B**).

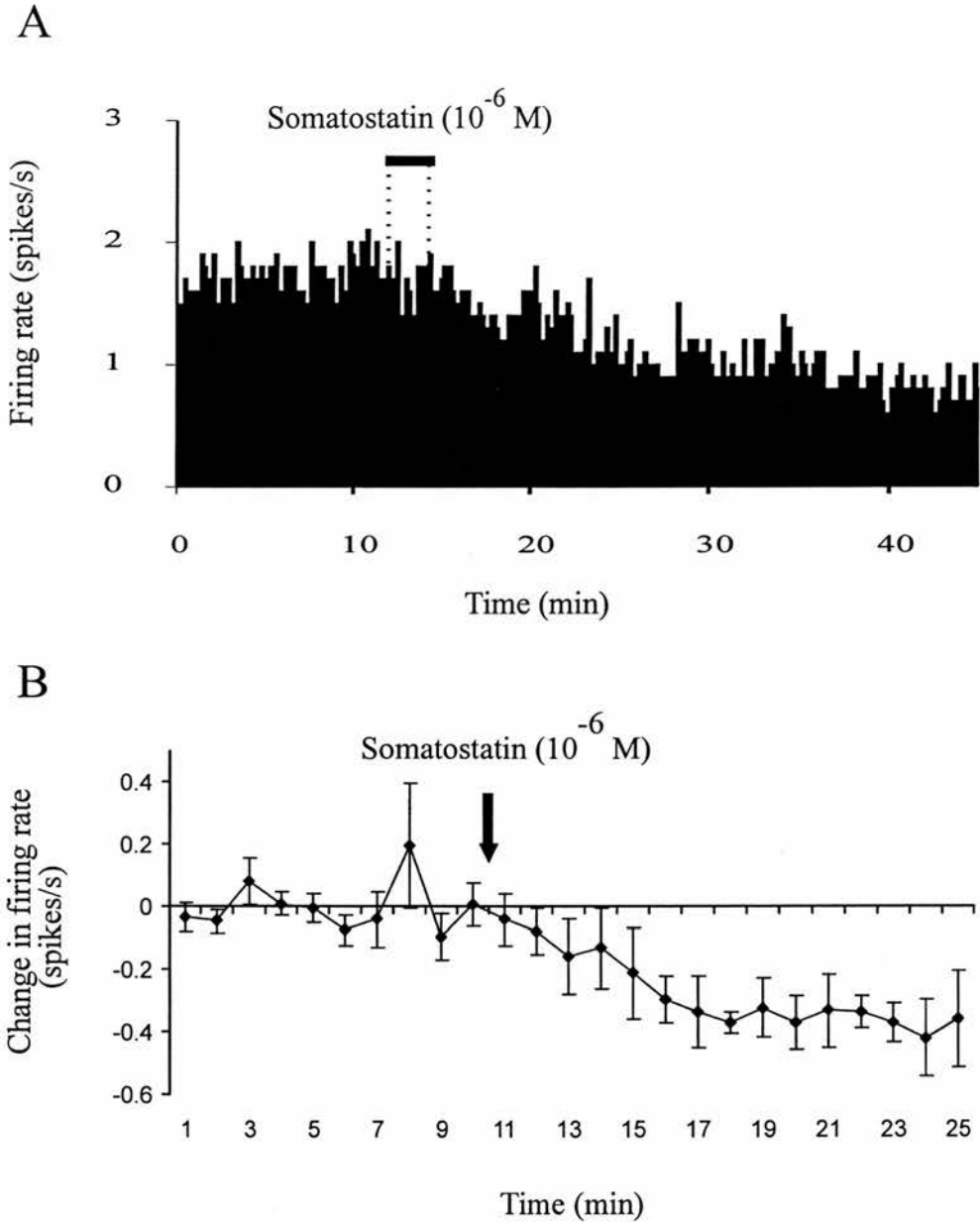


Fig. 2.2.10. (A) Electrophysiological response of a ventromedial hypothalamic neurone to somatostatin. A representative neurone was inhibited by bath application of somatostatin (10^{-6} M, 2 min). The firing rate during the 10-min period following somatostatin administration was significantly different from the firing rate before administration (t-test, $p < 0.01$). The period of somatostatin treatment is indicated with the bar. **(B) Mean response of inhibited neurones following somatostatin treatment.** Normalised mean firing rates of inhibited neurones were averaged minute-by-minute ($n = 4$ per each point).

2.3.6.7 Responses of ventromedial hypothalamic neurones to changes of glucose concentration

Responses to glucose concentration changes were examined in recordings obtained from 43 ventromedial hypothalamic neurones. Neurones were first detected by their spontaneous activity at 10 mM glucose in the medium. In the pilot experiment, fifteen of these neurones were tested with transformation of glucose concentration from 10 to 20 or 10 to 0 mM. After the transition from 10 to 20 mM, of 15 neurones, 1 was inhibited and 3 were excited. Whereas, after the transition from 10 to 0 mM, of 13 neurones, 3 were inhibited and 1 was excited. However, according to previous study, most neurones throughout the brain become silent at 0 mM glucose (Silver and Erecinska, 1994), so testing with glucose-free medium from the beginning of experiments was impracticable. In addition, examine with too low concentration below the physiological range might lead to neuronal activation by mechanisms that perhaps function to protect the brain during hypoglycemia, rather than through physiological glucoceptive mechanisms (Silver and Erecinska, 1994).

A subpopulation of neurones in the ventromedial hypothalamus became silent when the extracellular glucose concentration was decreased from 10 mM to 5 mM and become active again after raising the glucose concentration to 20 mM (Yang et al., 1999).

In some recordings, transition from 10 to 5 mM glucose was applied before the transition from 5 to 0 mM to avoid sudden extreme change. Changes in firing rate following transitions from 10 to 5, and 5 to 20 or 5 to 0 mM were analysed. Each neurone was determined whether it responded to the transition by comparison between its mean firing rates in a control period (measured over 5 min before the transition), and after transition (measured from 3 to 7 min following the transition). The minute-by-minute firing rates in these two periods were compared statistically using t-test.

A variety of responses of ventromedial hypothalamic neurones were seen. However, there was no typical pattern of response to interpret following the transition from 5 to 20 and from 5 to 0

mM. The most consistency was found in responses of neurones in the transition from 10 to 5 mM glucose.

In 20 neurones tested for transition from 10 to 5 mM glucose, 7 neurones (35 %) were significantly inhibited, 1 (5 %) was excited, and 12 (60 %) were non-responsive ($P < 0.03$). Two representative ventromedial hypothalamic neurones that were responsive by the transitions are shown in **fig. 2.2.11 (A and B)**. Figure A shows a neurone that was inhibited, whereas fig. B shows a different neurone excited by the transition from 10 to 5 mM glucose. The firing rates of inhibited neurones were averaged and plotted (**fig. 2.2.12**). The mean firing rate of inhibited neurones decreased by 0.27 spikes/s (28.4 ± 4.3 % inhibition, from 1.19 ± 0.37 to 0.92 ± 0.36 spikes/s).

Some neurones responded to transition from 5 to 20 mM glucose with a rapid burst of activity, followed by a return to normal activity. This kind of brief, transient responses, when firing rate was analysed, did not show any significant change in firing rate by the method chosen for analysis, and so these cells are included in the non-responsive group. However, it should be noted that while these neurones showed no sustained response, some did show a transient response.

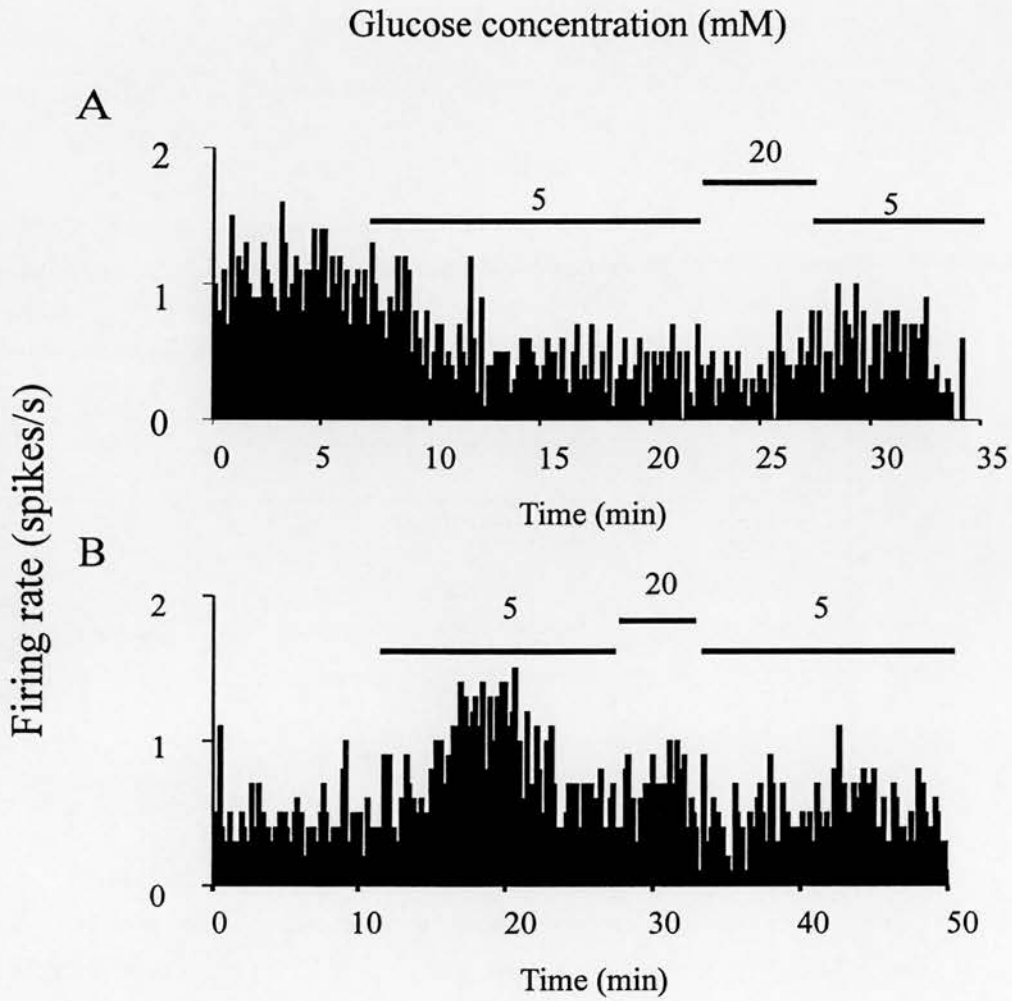


Fig. 2.2.11. Rate-meter records showing the effects of changes in glucose concentration on ventromedial hypothalamic neurones. Superfusate glucose concentration was switched from normal concentration of 10 to 5 and 20 mM while recording. The periods of treatments are indicated with bars.

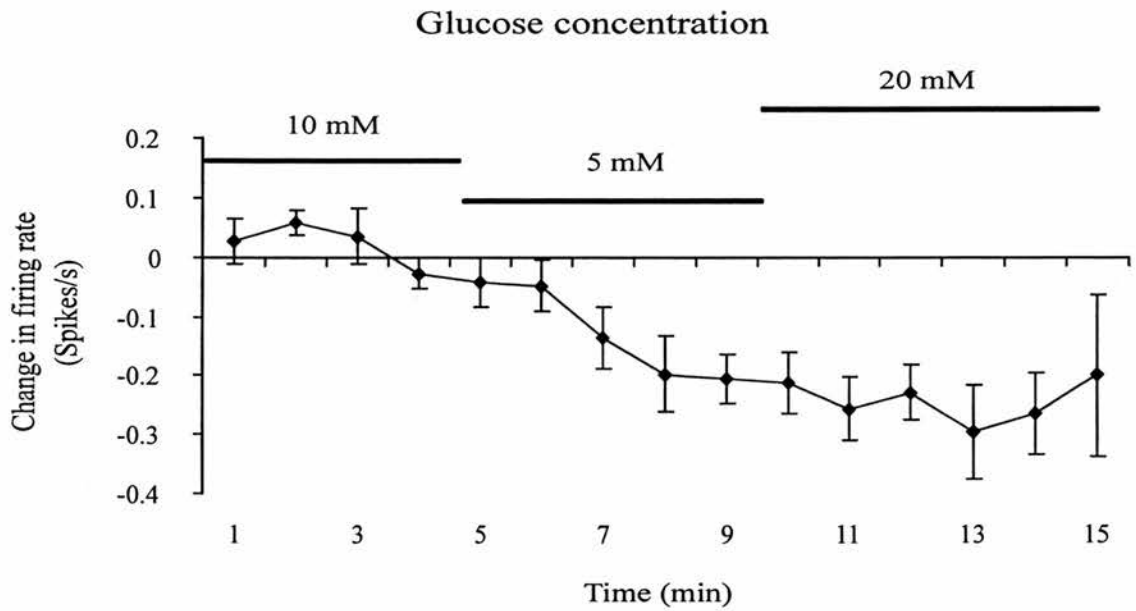


Fig. 2.2.12. Mean inhibitory response (\pm SEM) of ventromedial hypothalamic neurones to transition of superfusate glucose concentration from 10 to 5 mM.. Normalised mean firing rates of inhibited neurones were averaged minute-by-minute ($n = 8$ per each point). Periods of treatment are indicated with bars.

2.4 Discussion

This study reveals that arcuate neurones *in vitro* display spontaneous firing characteristics very similar to those described *in vivo*. In particular, the low spontaneous firing rate of approximately 1 spike/s is very similar to discharge rates of arcuate neurones *in vivo* in anaesthetised rats (Dickson et al., 1995b).

The effects of both the prototype secretagogue GHRP-6, and the non-peptide agonist L-144,446 on the electrical activity of hypothalamic neurones were compared in this study. At a concentration of 10^{-8} M, L-144,446 produced a similar level of increase in firing rate to GHRP-6 at a ten-fold higher concentration of 10^{-7} M. Their patterns of activation were found to be very similar in terms of latency and duration.

Both GHRP-6 and L-144,446 had very prolonged effects, maintaining excited firing rates above baseline levels for over an hour after washout. This slow onset and prolonged duration of response is very similar to the responses seen in anaesthetised rats following systemic administration of GHRP-6 (Dickson et al., 1995b). In particular, it is noticeable that both secretagogues stimulated arcuate neurones with the same latency period both in brain slice and in rat brain. Thus it appears that the latency and time course of the responses to secretagogue observed *in vivo* do not merely reflect the time course of penetration of these compounds into the brain, and the time course of their subsequent degradation or inactivation. Rather it would appear that *in vivo*, there is no observed delay in neuronal responsiveness beyond that seen *in vitro* following direct application. Moreover, the long duration of response does not require the continued presence of the secretagogue.

GHS-R is expressed in ventromedial hypothalamic neurones, and its expression is sensitive to GH (Bennett et al., 1997). GHS-R was found to be localised in GHRH-expressing ventromedial hypothalamic neurones (Tannenbaum et al., 1998a). However, it has not been consistently demonstrated that Fos expression is induced by GHS in the ventromedial hypothalamus *in vivo*. Nevertheless, in the present experiments, effects of NPY were seen in

greater number of ventromedial hypothalamic neurones than arcuate neurones. So far, both receptors for NPY and GHS were localised on ventromedial hypothalamic neurones but co-localisation of them has not been established. As most GHRP-6-excited neurones in the ventromedial hypothalamus were also inhibited by NPY, neurones in this nucleus seem likely to contain receptors for both peptides.

Lack of clear evidence of Fos expression induced by GHS in the ventromedial hypothalamus led to doubt as to whether the GHS receptors on neurones in this area are functionally coupled to electrical activity. In this study, the activation of electrical activity of ventromedial hypothalamic neurones by GHRP-6 provided clear evidence that these receptors are functional, and they seem likely to be coupled in a similar way to those in the arcuate nucleus. Thus, it is still unclear why Fos expression is not induced following GHS administration *in vivo*. One possibility is that some effects of GHS *in vivo* lead indirectly to inhibition of ventromedial hypothalamic neurones. In particular, it seems possible that systemic administration of GHRP-6 stimulates NPY-containing arcuate neurones that project their fibres to inhibit NPY-receptor-containing ventromedial hypothalamic neurones.

Sixty-eight percent of GHRP-6-excited ventromedial hypothalamic neurones, compared to 40 % of GHRP-6-excited arcuate neurones, were significantly inhibited by NPY. Clearly the responsiveness of neurones to NPY does not alone determine how they will respond when NPY neurones in the same or other brain regions are activated. The key questions are, *to which neurones do the NPY neurones that are activated by GH secretagogues project? and which factors are co-expressed by these neurones?*. Although some NPY neurones in the arcuate nucleus project to the paraventricular nucleus, few of these express Fos after GHS administration (Dickson and Luckman, 1997). Most of the GHS-responsive neurones in the arcuate nucleus are neurosecretory in that they project to the median eminence (Dickson et al., 1996), but these neurones may have other projections. Certainly there is a high density of NPY fibres in the ventromedial hypothalamic nucleus.

NPY neurones in the arcuate nucleus co-express many different factors, most conspicuously AGRP, which like NPY stimulates feeding and hence is presumed to have similar cellular

actions, and GABA, with potent inhibitory actions likely to supplement the inhibitory effects of NPY described here. Thus it is possible that systemic administration of GHRP-6 stimulates NPY-containing arcuate neurones that project to and inhibit NPY-receptor-containing ventromedial hypothalamic neurones.

Since inhibitory effects of somatostatin were also detected in ventromedial hypothalamic neurones *in vitro*, it is also possible that activation of somatostatin neurones by GHS may counteract the excitatory effects of GHS in the ventromedial hypothalamus *in vivo*. In the arcuate nucleus, Fos expression induced by GHRP-6 can be attenuated by exogenous administration of somatostatin (Dickson et al., 1997). In the mouse, though not in the rat, systemic injection of GHS induces Fos expression in the periventricular nucleus, probably in the neurosecretory somatostatin neurones. Periventricular somatostatin neurones express receptors for growth hormone, and their activation by GHS appears to be secondary to induction of growth hormone release by GHS. Exogenous growth hormone attenuates GHS-induced Fos expression in the arcuate nucleus in mice, and this effect is absent in transgenic mice that lack somatostatin SSTR2 receptors, hence it seems likely that following GHS activation of GHRH neurones, growth hormone is released and in feeds back upon periventricular somatostatin neurones that in turn inhibit GHRH neurones (Zheng et al). In rats treated chronically with i.c.v. GHRP-6 there is a sustained upregulation of somatostatin mRNA expression in the periventricular nucleus, consistent with activation of somatostatin release in the rat following GHS treatment (Bailey et al., 1999a).

Whether periventricular somatostatin neurones regulate ventromedial hypothalamic neurones is not known however. There are also somatostatin cells in the arcuate nucleus, and this population projects centrally rather than to the median eminence (Baker and Herkenham, 1995). However, these neurones do not express c-fos mRNA in response to systemic GHS (Dickson and Luckman, 1997), and somatostatin mRNA expression in the arcuate nucleus, unlike that in the periventricular nucleus, is unchanged following chronic i.c.v. infusion of GHS (Bailey et al., 1999a).

Most arcuate neurones that express Fos protein following GHRP-6 injection are likely to be neurosecretory neurones (Dickson et al., 1996). According to the effects of L-144,446 on arcuate neurones which showed similar characteristics to those that responded to GHRP-6, L-144,446 was also assumed to have effect on neurosecretory neurones as well. Two arcuate neurones that were activated by L-144,446 showed a clear bi-modal interspike interval pattern with the short modal interspike interval of 0.02 s for both neurones and long interval of 0.34 and 0.5 s respectively. These two neurones had similar firing patterns to that of arcuate neurones that was antidromically-identified as projecting to the ipsilateral paraventricular nucleus (Honda et al., 1999). This study also demonstrated that 20 % of arcuate neurones that were retrogradely-labelled from the paraventricular nucleus were Fos-positive following GHRP-6 injection. It shows the consistency of findings of characteristics of firing pattern of arcuate neurones.

Almost half of the arcuate neurones tested were inhibited by NPY. In addition, NPY Y1 or Y2 receptors were found to be localised in the arcuate nucleus (Cheng et al., 1998). Dual chromogenic and autoradiographic *in situ* hybridisation showed co-expression of GHS-R-mRNA with other peptides, including GHRH and NPY mRNA (Willesen et al., 1999). GHS-R mRNA was found to be expressed in 94 % of neurones expressing NPY, whereas only 20-25 % of GHRH mRNA-expressing neurones contained GHS-R mRNA. Thus most GHS-R expressing neurones might have other functions rather than the control of GH secretion. In particular, GHS-R expressing arcuate neurones that also contain NPY may play an important role in regulating food intake. It was demonstrated that secretagogue-induced food intake was blocked by NPY Y1 receptor antagonist (Lawrence et al., 2002), suggesting that the orexigenic effects of GHS may be mediated via NPY neurones in the arcuate nucleus.

The ventromedial hypothalamus is known to be sensitive to glucose (Yang et al., 1999), and responses of ventromedial hypothalamic neurones to glucose were also demonstrated in this *in vitro* study. Altogether, effects of GHRP-6, NPY and glucose were clear in ventromedial hypothalamic neurones. It was thought that neurones in this region responds to situation such as negative energy balance that leads to production of NPY in the arcuate nucleus and finally induces food intake in animal. However, lesions of the ventromedial hypothalamus of mice did

not block increased NPY mRNA expression in the arcuate nucleus to fasting (Bergen and Mobbs, 1996).

CHAPTER 3

FOS PROTEIN INDUCTION IN THE ARCUATE NUCLEUS AND THE VENTROMEDIAL HYPOTHALAMIC NUCLEUS

3.1 Introduction

The electrophysiological experiments described in Chapter 2 showed inhibitory effects of NPY on arcuate neurones and on ventromedial hypothalamic neurones. The co-localisation of GHS receptor and NPY mRNA in the arcuate nucleus of the rats (Willesen et al., 1999) means that arcuate neurones might be subject to inhibitory auto-regulation by NPY during exposure to GH secretagogues, and that GH secretagogues may result in NPY release in other brain areas. Thus although our *in vitro* study showed that the electrophysiological activity of ventromedial hypothalamic neurones was significantly activated by GH secretagogues, *in vivo* this excitatory action may be over-ridden by the inhibitory effects of NPY released from arcuate neurones.

The effects of NPY on these brain regions also reveal extensive actions of this orexigenic agent that has been regularly found in previous behavioural studies (Tanaka et al., 1994). Significant inhibitory effects of NPY on secretagogue-stimulated neurones *in vitro* suggests the presence NPY receptors in these areas, and suggest that the effects of these two orexigenic agents, NPY and GH secretagogues, converge on the same neurones at these sites. GH secretagogues are thought to activate NPY neurones in the arcuate nucleus (Dickson and Luckman, 1997), but the effects of these two orexigenic agents seem to oppose each other at the level of the single cell in both the arcuate nucleus and the ventromedial hypothalamus.

To understand the physiological significance of these observations, it is important to go beyond pharmacological classification of their effects and to look at the physiological role played by the different pathways. Is it possible that, *in vivo*, GH secretagogues do not activate ventromedial hypothalamic neurones because the direct action of secretagogues on ventromedial hypothalamic cells is opposed by NPY released from arcuate neurones? Does NPY released by arcuate neurones act in either the arcuate nucleus or in the ventromedial hypothalamus? Further investigation of the effects of the secretagogue GHRP-6, NPY and NPY Y1 receptor antagonist *in vivo*, using immunohistochemical techniques to map the expression of Fos protein as an index of neuronal activation should provide clarification as to how these brain areas respond to these compounds *in vivo*.

3.1.1 The hypothalamus: a site of homeostatic regulation

The hypothalamus has been a major focus of studies directed at investigating the mechanisms of control of energy balance in mammals. Many studies have confirmed that feeding regulation is a major function of the hypothalamus. The arcuate nucleus, the ventromedial hypothalamus and the lateral hypothalamus are all hypothalamic regions that exhibit a response to reduced glucose concentration (Funahashi et al., 1999). Additionally, the detection of Fos-like immunoreactivity in paraventricular and ventromedial hypothalamic nuclei of rats infused with glucose (4 mg/kg/min) supports the presence of a subpopulation of glucose-responsive neurons in these regions (Brogan et al., 2000). Double-immunostaining studies have revealed that the arcuate nucleus and ventromedial hypothalamus contain leptin receptor-like immunoreactive neurons that are innervated by orexin-like immunoreactive neurons (Funahashi et al., 2000). Both leptin and orexin have been recently proposed to play major physiological roles in feeding regulation; leptin inhibits feeding while orexin A and orexin B stimulate it. Furthermore, several nuclei in the hypothalamus express a transcript of fibroblast growth factor-5 (FGF-5), which is subjected to regulation by food deprivation (Li et al., 1999).

3.1.2 Brain regions involved in feeding behaviours

Many types of peptides regulate food intake. Among these, NPY is the most potent inducer of food intake. It is primarily synthesised in neurones of the arcuate nucleus that project to the paraventricular nucleus and dorsomedial nucleus of the hypothalamus (Dryden et al., 1996). Significant differences between concentrations of NPY in hyperphagic obese and lean normophagic Zucker rats are found in the arcuate nucleus, paraventricular nucleus and suprachiasmatic nuclei, whereas peripheral NPY is not modified (Beck et al., 1990a). It might be concluded that excessive food intake regulation is associated with central NPY-ergic dysregulation. The paraventricular nucleus is hypothesised to be the primary site of NPY action because the anticipated increase and decrease in NPY concentrations, in response to food deprivation followed by *ad libitum* food intake, occurred only in this site (Kalra et al., 1988). This idea was supported by radioimmunoassay studies of tissue NPY content, showing

that, as well as in the arcuate nucleus, increases of NPY content in the paraventricular nucleus, ventromedial hypothalamus and dorsomedial hypothalamus in obese Zucker rats were also observed (McKibbin et al., 1991). This evidence suggests that extensive interneural communication is involved in the coordination of diverse feeding regulation.

It has been proposed that increased NPY activity in the arcuate nucleus might be a result of the decreased circulating levels of insulin and glucose associated with a fast. Especially in the ventromedial hypothalamus, some neurones were found to be sensitive to glucose concentration changes (Yang et al., 1999). However, lesion of the ventromedial hypothalamus by gold thioglucose (GTG), which appears to act on glucose-responsive neurones, did not prevent the increase of NPY mRNA expression by fasting in the arcuate nucleus (Bergen and Mobbs, 1996). This suggests that GTG-sensitive ventromedial hypothalamic neurones play little part in the fasting-induced induction of NPY mRNA expression. By contrast, disruption of neural signaling in the ventromedial hypothalamus of rats by microinjection of the neurotoxin, colchicine, results in rapid and transient hyperphagia and body weight gain and a decrease in NPY content in the paraventricular nucleus (Kalra et al., 1997).

NPY Y1 receptors appear to mediate at least some of the effects of NPY on feeding. The Y1 receptor antagonist 1229U91 suppressed feeding significantly in rats induced to be hyperphagic by microinjection of colchicine in the ventromedial hypothalamus (Kalra et al., 1997). 1229U91, when co-administered with NPY, inhibited NPY-induced food intake in rats (Ishihara et al., 1998). The non-peptide Y1 receptor antagonist BIBP3226 also significantly reduced the intake of highly palatable diet and food intake stimulated by fasting (Kask et al., 1998). In addition, J115814, a potent and selective Y1 antagonist (i.v.) significantly suppressed feeding induced by NPY (i.c.v.) in satiated rats (Kanatani et al., 2001). Similar findings have also been observed in studies using G1264879A, a non-selective NPY-Y1 receptor antagonist (Danielsa et al., 2001). The role of Y1 receptors on feeding regulation was supported when antisense oligonucleotides to NPY-Y1 receptor, but not antisense to NPY, delivered to the ventromedial hypothalamus, suppressed feeding behaviour (Lopez-Valpuesta et al., 1996).

However, the Y1 receptor is not the only NPY receptor subtype that mediates effects of NPY on food intake. The first Y5 receptor-selective analogue of NPY, which was developed and found to have high affinity for the NPY Y5 receptor, also significantly stimulated feeding in rats (Cabrele et al., 2000). Pharmacological analysis showed that rat and human Y5 receptors have high affinity for the peptides that elicit feeding (Hu et al., 1996).

Zucker rats are a well-documented model of early-onset obesity, and have a hyperactive hypothalamic NPY system. Binding assay of NPY to hypothalamic homogenates of obese rats was reduced by 56 % compared with lean rats (McCarthy et al., 1991). This finding suggests that NPY receptors undergo down-regulation in response to the increased hypothalamic NPY activity. The Y5 receptor, a novel NPY receptor subtype, has been proposed to specifically mediate hyperphagia (Gerald et al., 1996) was reported to be selectively down-regulated in several hypothalamic brain regions especially in the arcuate nucleus of Zucker rats (Widdowson, 1997). In addition, restriction to 60 % of normal daily food intake over 10 days, or making obese by feeding male Wistar rats with high-calorie diet resulted in down and up-regulation of NPY Y5 receptor respectively (Widdowson et al., 1997). This study also found similar down and up-regulations of NPY Y2 receptors. The finding suggests that there is a regional increase in NPY release during negative energy balance, and a reduced regional release of NPY in positive energy balance. In studies using immunohistochemistry and *in situ* hybridisation, the number of Y1 receptor immunoreactive neurones and Y1 receptor mRNA level in the arcuate nucleus of rats decreased after food deprivation (Cheng et al., 1998). By contrast, fasting did not significantly change Y2 receptor mRNA expression in this area. A similar increase of Y1 receptor mRNA expression in the paraventricular and arcuate nuclei was observed in response to supplementing the drinking water with 10 % glucose for 72 h (Zammaretti et al., 2001). These results support the view that Y1 receptors in the arcuate nucleus may contribute to the feeding pattern induced by NPY in long-term changes in energy balance.

3.2 Aims

This study investigated the effects of NPY, Y1 receptor antagonist and the classical GH secretagogue GHRP-6 on Fos expression the hypothalamus *in vivo*. From the electrophysiological results, we predicted that NPY administration should block Fos activation in the arcuate nucleus in response to GHRP-6, and also predict that Y1 antagonist should potentiate Fos expression in response to GHRP-6 in both the arcuate nucleus and the ventromedial hypothalamic nucleus. Immunohistochemistry was used for these purposes.

3.3 Methods

3.3.1 Surgery

Adult male Sprague Dawley rats were maintained in a controlled environment with *ad libitum* access to food and water (see **Method 2.1.**). On the day of experiments, rats were anaesthetised with sodium pentobarbitone (sagatal, 0.5 ml/kg i.p.). This anaesthetic does not, by itself, induce Fos expression in any hypothalamic nuclei of rats (Takayama et al., 1994). An intracerebroventricular (i.c.v.) guide canula (23 gauge; Plastics One, USA) was positioned in the right lateral ventricle (co-ordinates: 1.6 mm lateral, 0.6 mm posterior to bregma, 4.5 mm below the surface of the skull). An intravenous (i.v.) canula was inserted into the right jugular vein. After the end of surgery, rats were maintained under anaesthesia for at least 2 h before the experiment.

3.3.2 Experimental Methodology

Rats were given an i.c.v. infusion of either vehicle (aCSF), NPY (4.68 nM in aCSF; Tocris, UK) or Y1 receptor antagonist (30.7 nM in aCSF; Merck) at a flow rate of 1.27 μ l/min for 45 min. Fifteen minutes after the start of the i.c.v. infusion, rats were given an i.v. injection of either vehicle (0.2 ml sterile saline) or GHRP-6 (50 μ g in 0.2 ml). Rats treated with aCSF (i.c.v.) and sterile saline (i.v.) were used as control rats. Ninety minutes after the i.v. injection, rats were deeply anaesthetised and perfused transcardially with chilled 0.1 M PB, followed by chilled fixative solution (4% paraformaldehyde in 0.1 M PB). Brains were removed and postfixed overnight in the same fixative solution at 4°C. They were then transferred to sucrose solution (30% sucrose in 0.1 M PB) for cryoprotection until they sank. Brains were frozen on dry ice and stored at -70°C until processed for immunohistochemical localisation of Fos protein. For-positive control rats, they were injected (i.p.) with 4.0 ml/kg of hypertonic saline solution (1.5 M NaCl)

3.3.3 Immunohistochemistry for Fos protein

Coronal sections (40 μm) were cut through the hypothalamus using a microtome. This entire process was performed in darkness in order to protect the fluorescent tracer from decay. Sections were washed in 0.1 M PB pH (7.4) for 5 min at room temperature (RT). Endogenous peroxidase was deactivated by incubation of the sections with methanol solution (20% methanol, 0.2% TritonX100 (Tx100) and 1% hydrogen peroxidase (H_2O_2 : in 0.1M phosphate buffer) for 15 min at RT. Sections were then washed twice with 0.2 M PBT (0.2 M PB and 0.2% Tx100) for 7.5 min each.

Before the Fos antibody incubation, sections were incubated with 1% normal sheep serum in PBT for 1 h at RT to prevent non-specific binding. Then they were incubated in Ab-2 Fos antibody (Rabbit polyclonal Ab-2 Fos, Oncogene Sciences; diluted 1:1000 in 1% normal sheep serum, 0.2% Tx100 and 0.1 M PB) for 36 h approximately at 4°C. Sections were washed with 0.1 M PBT (0.1 M PB and 0.2% Tx100) 6 times for 5 min each.

The antibody-antigen complex was localised with an incubation in secondary antibody (Biotinylated anti-rabbit immunoglobulin, Vector; diluted 1:100 and normal goat serum, Vector; diluted 3:100, in PBT) for 1 h at RT and followed by an incubation in Avidin DH (Biotinylated horseradish peroxidase, Vector; diluted 1:50 in PBT) for 1 h at RT. After that, sections were washed with PBT for (5 x 5 min each). Next, they were rinsed with 0.1 M sodium acetate buffer (pH 6.0) for 5 min.

Immunolabelling was visualised by an incubation in chromagen solution [nickel ammonium sulphate (0.25 mg/ml), diaminobenzidine (0.25 mg/ml), 4% glucose (w/v), 0.08% ammonium chloride (w/v) and 0.003% glucose oxidase (w/v)] for 10 min approximately (see **solution preparation**). Fos immunoreactive nuclei appeared as dark purple/black nuclei. The reaction was terminated with 0.1 M sodium acetate buffer for 5 min. Sections were then washed with H_2O for 5 min and finally rinsed with 0.1 M PB for 5 min. Sections were mounted onto gelatinised slides and air dried. They were dehydrated through a series of alcohols (50%, 70%,

90%, 95%, 100%, 100%). They were finally submerged in xylene for 5 min and transferred into another clean xylene for mounting (BDH).

3.3.4 Quantitative analysis

The slides were coded by covering identifying numbers with masking tape and assigned random numbers before quantification to prevent bias. The sections were viewed under a light microscope (x10 objective) and Fos positive nuclei were counted in the arcuate and ventromedial hypothalamic nucleus on both the left and right sides. Data are expressed as mean \pm SEM. T-test was used to compare the statistical differences between numbers of Fos positive neurones of different groups.

3.4 Materials

Stock solution of phosphate buffer (1 M PB)

Phosphate buffer (1 M) contains 11.5 % w/v disodium hydrogen orthophosphate 2-hydrate ($\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, AnalaR; BDH) and 2.72 % w/v sodium dihydrogen orthophosphate 1-hydrate ($\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, AnalaR; BDH). For a final volume of 1000 ml, these two chemicals were added to 900 ml ddH₂O and dissolved. The solution was adjusted to pH 7.4 with concentrated hydrochloric acid or sodium hydroxide. Finally, the volume was made up to 1000 ml with ddH₂O. This solution was later diluted to make up further diluted phosphate buffer solutions.

Heparinised phosphate buffer

Heparinised phosphate buffer was prepared by adding 129 mg heparin (Sigma HO777, 500,000 units) to 1000 ml of 0.1 M phosphate buffer.

4 % paraformaldehyde in 0.1 M phosphate buffer

4 % paraformaldehyde solution contains 1.15 % w/v $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 0.272 % w/v $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ and 4.0 % w/v paraformaldehyde (CH_2 ; Sigma). These salts were prepared in ddH_2O , and pH was adjusted to 7.3-7.4.

0.1M phosphate buffer + 0.2 % Triton X-100 (PBT)

Two ml of Triton X-100 is added to 1000 ml 0.1 M PB and thoroughly mixed on a stirrer.

1% normal sheep serum in 0.1 PBT

For 100 ml, 99 ml 0.1 M PBT was added with 1 ml normal sheep serum.

Hydrogen peroxide block in PB

Hydrogen peroxide solution was prepared in 0.1 M PB, containing 20 % methanol, 0.2 % Triton X-100 and 1 % H_2O_2 .

Primary antibody for Fos protein

Rabbit polyclonal anti Fos (Ab-2 Fos, Oncogene Sciences) was diluted 1: 1000 in buffer containing 1 % normal sheep serum, 0.2 % Triton X-100 and 0.1 M PB.

Secondary antibody

Secondary antibody for was prepared by using Vector stain elite kit rabbit IgG (Vector labs PK 6101). Secondary antibody mixture contained 1 % v/v Biotinylated anti-rabbit immunoglobulin, 3 % v/v normal goat serum and 0.1 M PBT.

Avidin-biotinylated horseradish peroxidase complex (ABC)

ABC solution was prepared by using ABC kit rabbit IgG's (Vector labs PK 6101). The solution was made in PBT and contained 2 % v/v avidin DH, 2 % v/v biotinylated horseradish peroxidase.

Solution for visualisation of the glucose oxidase-Nickel-DAB method.

Amounts of chemical for a total volume of 100 ml

50 ml of 0.2 M sodium acetate buffer

- 2.5 g nickel ammonium sulphate (BDH 101674A)
- 0.4 g glucose (Sigma G7528)
- 0.08 g ammonium chloride (BDH A4514)
- 25 mg in 1 ml diaminobenzidine (DAB) is added to 49 ml dH₂O
- 0.003 g glucose oxidase type VII-s (Sigma G7016), kept at -20 °C

Method

- A) Dissolve 2.5 g Nickel ammonium sulphate in 50 ml sodium acetate
- B) Add the glucose (0.4 g) and ammonium chloride (0.08 g) to the nickel

Solution

- C) Add the 50 ml DAB solution
- D) Add the glucose oxidase immediately before use

3.5 Results

3.5.1 Fos-like immunoreactivity in the arcuate nucleus induced by GHRP-6

Immunohistochemical visualisation of Fos-like immunoreactivity in rats given an i.v. injection of GHRP-6 (50 µg), showed dense nuclear staining of selective cell nuclei in the arcuate nucleus. Nuclear staining in the arcuate nucleus was similar to that observed in supraoptic nucleus of brain slices of rats treated with i.p. injection of hypertonic saline (1.5 M NaCl, 4 ml/kg) (**fig. 3.1A**). Patterns of neuronal activation demonstrated by Fos expression in the supraoptic nucleus after i.p. injection of hypertonic saline have been examined extensively in previous studies (Giovannelli et al., 1992).

Intravenous injection in rats of 50 µg GHRP-6 caused a staining of selective cell nuclei in the arcuate nucleus (**fig. 3.1C**). The treatment significantly increased Fos-like immunoreactivity in the arcuate nucleus to 13.9 ± 2.8 positive neurones per arcuate nucleus section, compared to 2.5 ± 0.5 in saline-treated rats (**fig. 3.1B**) (mean \pm SEM, $p < 0.01$, t-test). When expressed as percentage increase, compared to basal level, GHRP-6 significantly increased Fos-like immunoreactivity to 455 %.

3.5.2 Effects of NPY alone, and together with GHRP-6 on Fos-like immunoreactivity in the arcuate nucleus

Intracerebroventricular injection of NPY (4.68 nM) alone in rats did not induce a significant change in Fos-like immunoreactivity in the arcuate nucleus compared with aCSF infused rats (**fig. 3.1D**). Approximately 2 Fos-like positive neurones per arcuate nucleus section were seen after NPY injection, which was similar to the basal level of expression. In rats pre-treated with aCSF, treatment with GHRP-6 caused, as shown previously (Dickson et al., 1996), an increase in number of Fos-like immunoreactivity up to approximately 14 positive neurones per arcuate nucleus section. By contrast, in rats pre-treated with NPY, i.v. injection of GHRP-6 induced only about 6 Fos-like positive neurones per section in the arcuate nucleus (**fig. 3.1E**). Thus,

NPY pre-treatment significantly attenuated GHRP-6-induced Fos-like immunoreactivity by 60.7 % of that of saline-pre-treated rats ($p < 0.01$, t-test).

3.5.3 Effects of NPY Y1 receptor antagonist, L-753044-001J003, on Fos-like immunoreactivity in the arcuate nucleus

Intracerebroventricular injection of the NPY Y1 receptor antagonist (30.7 nM) in rats, by itself, did not significantly enhance Fos-like immunoreactivity in the arcuate nucleus as compared with saline-treated rats (data not shown). The average number of Fos-like positive neurones seen in the arcuate nucleus after the Y1 receptor i.c.v. injection and followed by i.v. injection of saline was 3.4 ± 0.32 (36.7 % over basal level). When given for pre-treatment before GHRP-6 treatment, the Y1 antagonist, produced a small, non-significant attenuation of the GHRP-6 induction of Fos-like immunoreactivity in the arcuate nucleus (by 25.1 %; data not shown).

In chapter 2, the distribution of secretagogue-responsive neurones throughout the arcuate nucleus was found to be uneven, when assessed electrophysiologically. Additionally, the density of spontaneously active neurones throughout the length of the arcuate nucleus detected extracellularly was also non-uniform. In this study, the effects of GHRP-6, NPY and Y1 receptor antagonist on Fos-like immunoreactivity in the arcuate nucleus were also analysed by focusing on specific parts of the arcuate nucleus. Levels of Fos-like immunoreactivity in the arcuate nucleus were analysed in 4 regions: from A to D (A: 1.8 – 2.3 mm, B: 2.3 – 2.8 mm, C: 2.8 – 3.3 mm, D: 3.3 – 4.16 mm posterior to bregma).

Figure 3.2A shows levels of Fos-like immunoreactivity induced by various treatments in areas A, B, C and D of the arcuate nucleus. The number of Fos-like immunoreactive neurones induced by GHRP-6 was greater in area B than in area A, and higher still in area C. Area D showed slightly fewer Fos-like immunoreactive neurones than area C. Similar patterns of variation in the density of Fos-like immunoreactive neurones were also observed in brain slices of rats pre-treated with either NPY or Y1 receptor antagonist and followed by treatment with

GHRP-6. The total average number of Fos-like immunoreactive neurones in the arcuate nucleus is shown in **fig. 3.2B**.

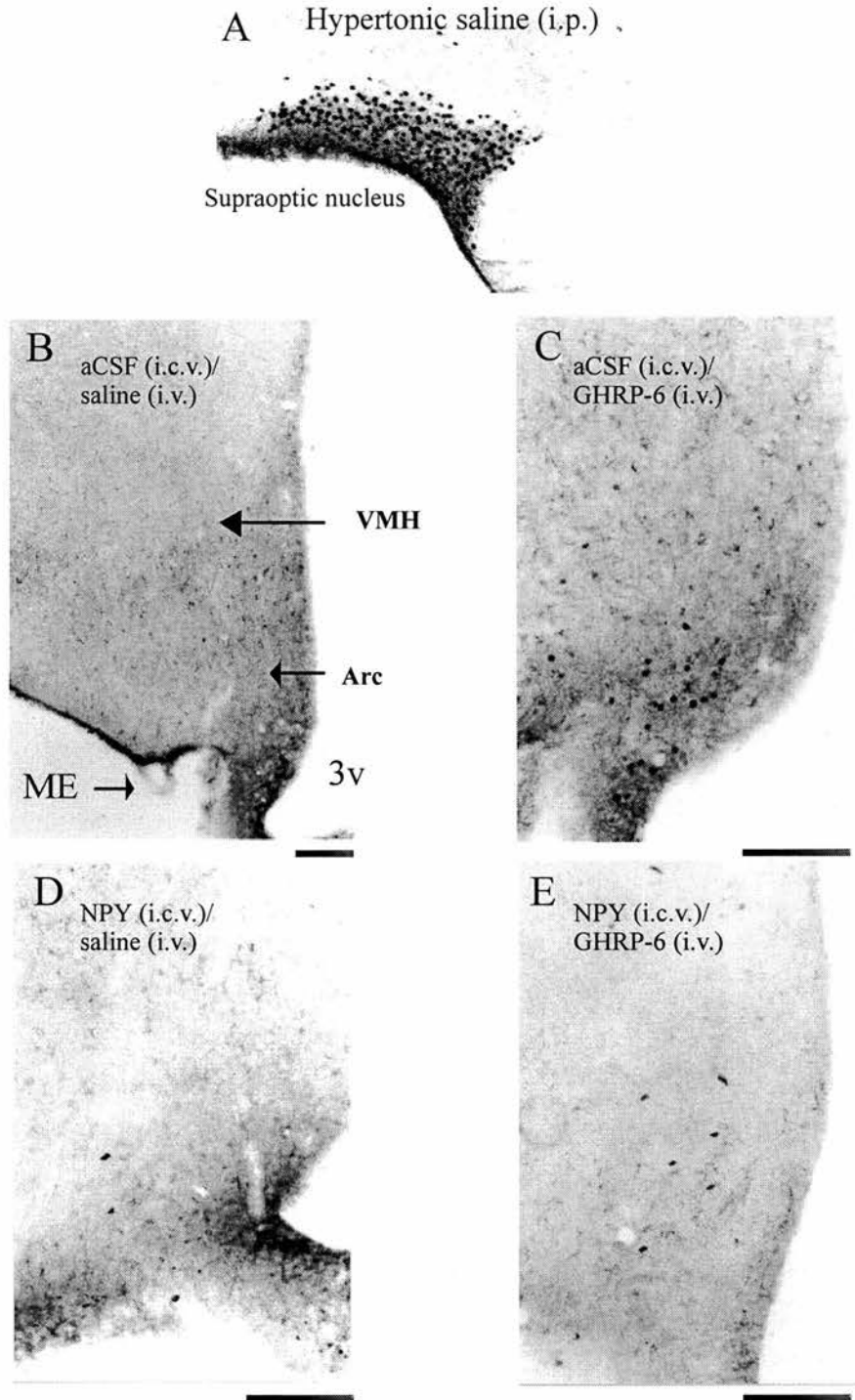


Fig. 3.1. Representative Photomicrographs showing Fos immunoreactivity observed in the arcuate nucleus of rats. Dense nuclear staining are seen in the supra optic nucleus of rat induced by hypertonic saline injection (1.5 M NaCl, 4 ml/kg, i.p.). Rats were pre-treated (i.c.v.) and injected (i.v.) as indicated. 3V: third ventricle, ME: median eminence. Scale bar = 1.0 mm.

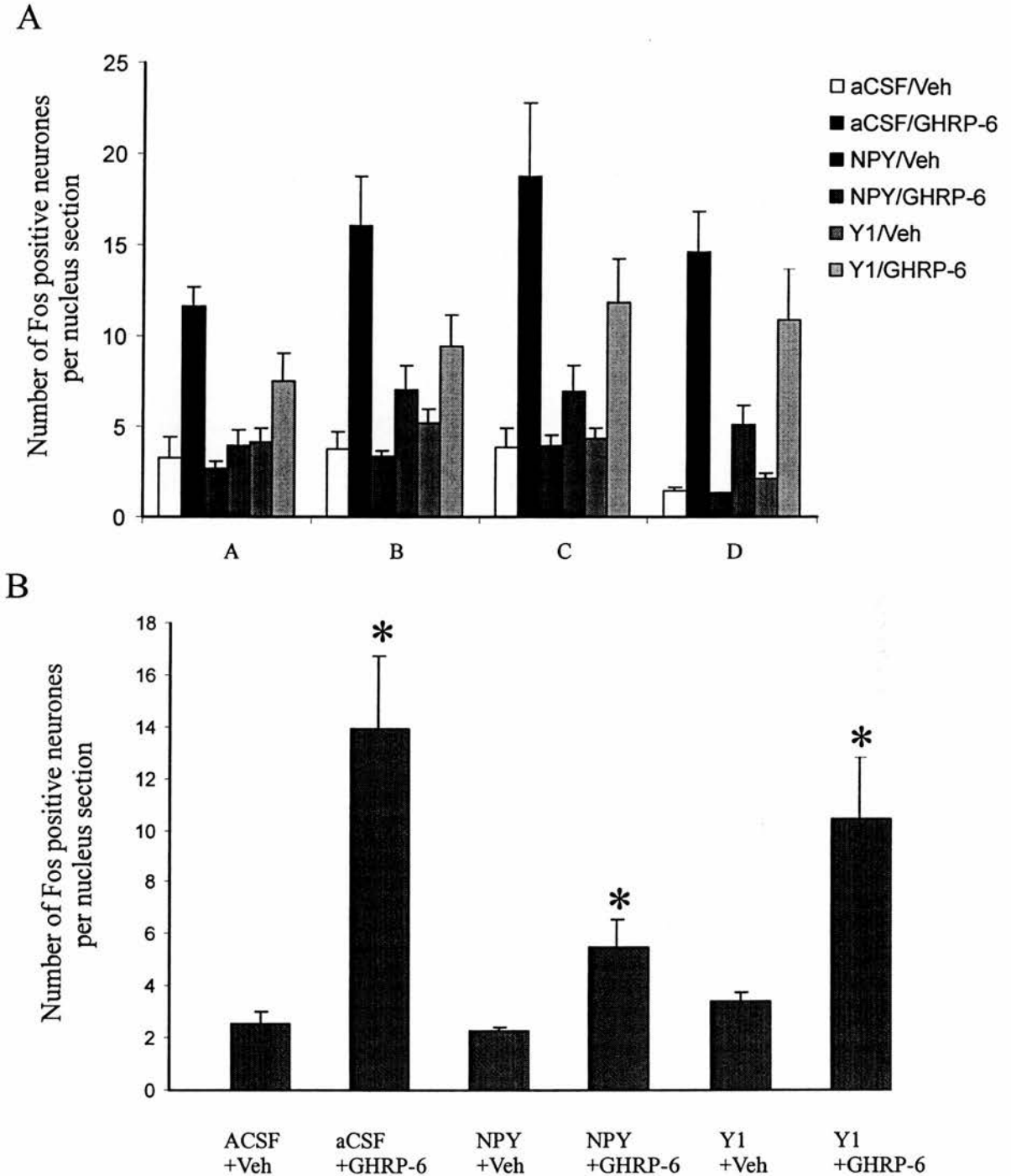


Fig. 3.2. Effects of GHRP-6 and NPY on the number of Fos positive neurons in the arcuate nucleus. (A) The total number of Fos positive nuclei per arcuate nucleus section is shown in 4 parts of the arcuate nucleus; A, B, C, and D (from the anterior to posterior). A: 1.8 - 2.3 mm, B: 2.3 - 2.8 mm, C: 2.8 - 3.3 mm, D: 3.3 - 4.16 mm posterior to bregma. (B) Bar graph shows the total number of Fos-positive nuclei per arcuate nucleus. Rats were pre-treated with i.c.v. injection of either aCSF or NPY (4.68 nM) and followed with i.v. injection (2 ml) of either vehicle (saline) or GHRP-6 (50 μ g). All data are the mean \pm SEM (n = 6 in each group). Statistical significance of differences compared with aCSF/Veh group: * = $p < 0.05$ (Post-hoc t-test)

3.5.4 Induction of Fos-like immunoreactivity in the ventromedial hypothalamic nucleus following i.c.v. injection of NPY Y1 receptor antagonist, L-753044-001J003

The ventromedial hypothalamic nucleus showed a basal level of Fos-like immunoreactivity of 3.99 ± 1.0 positive neurones/section (**fig. 3.1B**). Unlike in the arcuate nucleus, neither pre-treatment of NPY nor treatment with GHRP-6 (**fig. 3.3A1**), had any significant effect on Fos-like immunoreactivity in the ventromedial hypothalamic nucleus.

In rats pre-treated with the Y1 receptor antagonist and followed by saline, Fos-like immunoreactivity in the ventromedial hypothalamic nucleus was markedly increased (**fig. 3.3A2**). The average number of Fos-like immunoreactive neurones of this group was 16.7 ± 4.1 nuclei/nucleus section which was 318 % higher than that of rats pre-treated with aCSF and followed with saline treatment. However, when followed with injection of GHRP-6, the Y1 receptor antagonist did not sustain high level of Fos-like immunoreactivity (**fig. 3.3A3**). Approximately 6 Fos-like immunoreactive neurones/section were seen in rats pre-treated with the antagonist and followed with treatment of GHRP-6. It was 54.9 %, non-significantly, higher than that of control rats. The Fos-like immunoreactivity of this group was attenuated by 63.0 % compared to that of rats pre-treated with Y1 and followed with saline treatment. The total amount of Fos-like immunoreactive neurones induced by various treatments were shown in **fig. 3.3B**. Overall, a significant increase in Fos-like immunoreactivity in the ventromedial hypothalamic nucleus was seen only in rats pre-treated with Y1 receptor antagonist and followed by saline injection, but not by any other combination of treatment performed in this experiment (t-test, $p < 0.05$).

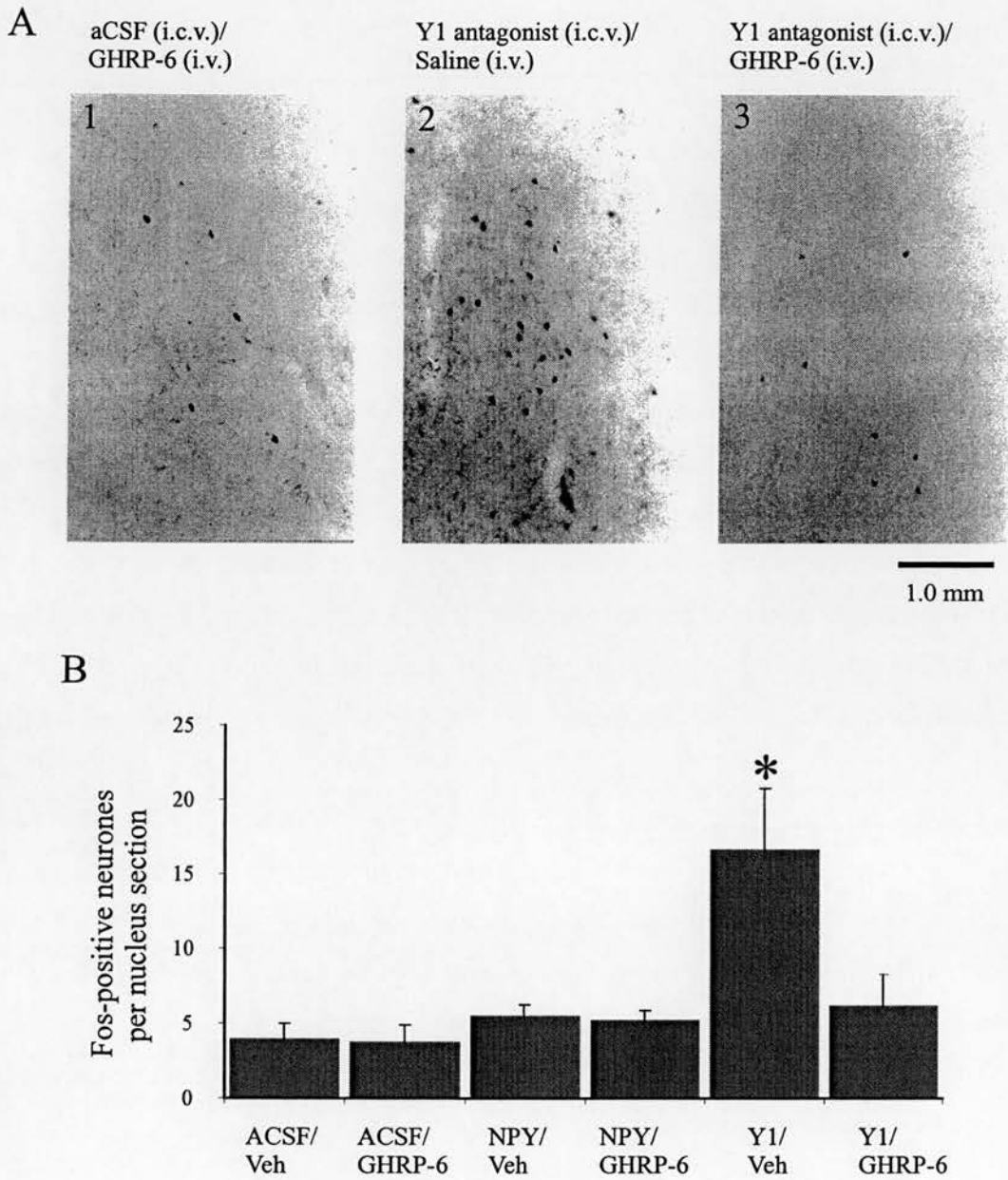


Fig. 3.3. Fos protein induction in the ventromedial hypothalamic nucleus. (A) Representative photomicrographs showing Fos immunoreactivity observed in the ventromedial hypothalamic nucleus of rats. (B) Number of Fos-positive neurones in the ventromedial hypothalamic nucleus. Rats were pre-treated (i.c.v.) and treated (i.v.) as indicated. All data are the mean \pm SEM ($n = 6$ in each group). Statistical significance of differences compared with aCSF/Veh group: $* = p < 0.05$ (Post-hoc t-test)

3.6 Discussion

The results demonstrate a significant induction of Fos-like immunoreactivity in the arcuate nucleus of rats injected (i.v.) with GHRP-6, consistent with previous reports (Dickson et al., 1996). Previously, i.c.v. injection of GHRP-6 was also found to induce Fos-like immunoreactivity in the arcuate nucleus (Dickson et al., 1995b). The distribution of Fos staining in these different studies was generally very similar to the data described here, and in particular, no other surrounding brain area was found to respond to GHRP-6, as measured by Fos induction. The present results also show that, in the arcuate nucleus, pre-treatment with NPY significantly attenuates the effects of GHRP-6 on Fos induction. This result is fully consistent with the electrophysiological study of chapter 2, which showed that NPY is predominantly inhibitory to arcuate neurones that are responsive to GHS. Thus the electrophysiological findings and the immunohistochemical findings when taken together, support the view that Fos is a reliable indicator of electrical activation in the arcuate nucleus.

Also as shown previously (Dickson et al., 1995b), administration of GHRP-6 did not induce significant Fos expression in the ventromedial hypothalamic nucleus. As shown in chapter 2, this brain region showed equally strong excitatory response to administration of both L-144,446 or GHRP-6 in brain slice extracellular recording. To explain this discrepancy, it was hypothesised that, *in vivo*, GHRP-6 administration leads to indirect inhibition of ventromedial hypothalamic neurones by stimulating NPY neurones in the arcuate nucleus that project to the ventromedial hypothalamic nucleus.

To investigate this hypothesis, the NPY Y1 receptor antagonist, L-753044-001J003, was applied to rats before treatment with either saline or GHRP-6. A marked increase in Fos-like immunoreactivity was seen in the ventromedial hypothalamic nucleus of rats followed the Y1 antagonist pre-treatment in saline-treated rats, and no increase was seen in the arcuate nucleus. This indicates the existence of NPY inhibitory inputs to the ventromedial hypothalamic nucleus that are tonically active *in vivo*, and demonstrate that neurones in this hypothalamic

region do possess the intracellular machinery necessary for the induction of expression of Fos protein.

In the arcuate nucleus, NPY Y1 receptor antagonist, when pre-treated to the rats that received saline treatment later, caused a 36.7 % and non-significant increase in Fos-like immunoreactivity compared to control group. This might suggest an existence of weak spontaneous NPY inhibition in this brain region. Though, it remains unclear whether such NPY inhibition is regulated by NPY neurones in the arcuate nucleus itself as autoregulation or by presynaptic neurones from other brain regions.

In comparison, treatment with GHRP-6 in Y1 antagonist pre-treated rats caused a large increase (315.8 % increase over control level) in Fos-like immunoreactivity in the arcuate nucleus, but only a small increase (35.4 % increase) in the ventromedial hypothalamic nucleus. This indicates that treatment with GHRP-6 has an inhibitory effect on neurones in the ventromedial hypothalamic nucleus *in vivo*, in contrast to the direct excitatory effect of GHS observed in *in vitro* electrophysiological experiment (chapter 2).

CHAPTER 4
NPY mRNA EXPRESSION IN THE ARCUATE NUCLEUS IN
RESPONSE TO FOOD RESTRICTION AND CHRONIC
CENTRAL ADMINISTRATION OF GHRP-6

4.1 Introduction

Since ghrelin was identified, its effects on feeding have been widely studied. Both i.c.v. and i.p. administration of ghrelin increased feeding in freely feeding rats (Wren et al., 2000). The lowest doses of ghrelin that produced a significant stimulation of feeding were 1 nM and 30 pM after i.p. injection and microinjection to the arcuate nucleus of rats respectively (Wren et al., 2001). These doses were considered to be very low, as the plasma ghrelin concentration after i.p. injection of 1 nM ghrelin was not significantly different from that occurring after a 24-h fast. Stimulated feeding by injection of ghrelin once a day for 7 days was associated with a significant increase in body weight by days 6 and 7 (Wren et al., 2001). This tends to suggest reduced energy expenditure in ghrelin-treated rats.

There is evidence that the orexigenic effect of ghrelin is mediated via NPY. Co-localisation of the GHS receptor and NPY mRNA has been reported in the rat arcuate nucleus (Willesen et al., 1999) and ghrelin-induced feeding is abolished by i.c.v. administration of anti-NPY IgG 4 h before ghrelin administration (Nakazato et al., 2001). In the same study, ghrelin-induced feeding was also suppressed by treatment with anti-AGRP IgG. In another study, treatment with a single i.c.v. injection of ghrelin (500 ng/rat) increased NPY mRNA expression in the arcuate nucleus by 160 % relative to vehicle-treated rats (Shintani et al., 2001).

The previous chapters have shown that NPY is a potent inhibitor of electrical activity in the ventromedial hypothalamic nucleus, which is a satiety centre, and have explored the hypothesis that GH secretagogues activate an inhibitory NPY input from the arcuate nucleus to this region.

The evidence that acute administration of GH secretagogues activates NPY neurones in the arcuate nucleus is strong, but chronic central infusion of GH secretagogues does not lead to a persistent activation of NPY mRNA expression in this region (Bailey et al., 1999a). Central administration of ghrelin for 3 days significantly increased the expression of both NPY and agouti-related peptide (AGRP) mRNA expression in the rat arcuate nucleus (Kamegai et al.,

2001b), but no increase has been seen with longer infusions of GH secretagogues (Bailey et al., 1999a), although changes in appetite and weight gain persist.

Chronic infusions of GH secretagogues also lead to maintained increases in GH secretion and growth rate, through actions both at the pituitary and on GHRH neurones (see **Introduction**). However, chronic GH secretagogue treatment does not produce a sustained increase in GHRH mRNA expression (Bailey et al., 1999a). Instead, paradoxically, it produces a sustained increase in somatostatin mRNA expression in the periventricular nucleus. The likely explanation is that the enhancement of GH secretion, following GHS action at the pituitary and on GHRH neurones, leads to enhanced activity of somatostatin neurones as a result of GH feedback, which in turn results in somatostatinergic inhibition of GHRH neurones (see **Introduction**), and this opposes the excitatory drive by GH secretagogues. Thus, in rats treated with GH secretagogues there are normal levels of GHRH mRNA despite hyperactivation of somatostatin neurones, reflecting continued excitation of GHRH neurones by GH secretagogues.

The absence of a sustained change in NPY mRNA expression may have a similar explanation. If activation of NPY neurones increases appetite, rats eat more, and hence satiety signals increase. If satiety signals in turn inhibit NPY neurones, then we may expect to see little overall change in NPY expression.

In this chapter we test this hypothesis. We treated rats chronically with i.c.v. GHRP-6, and observed food intake, weight gain, and measured NPY mRNA expression in the arcuate nucleus. Half of the rats were given free access to food; half were put on a restricted diet. These rats were not allowed therefore to eat more than the control group during GHRP-6 infusion. We expected to find no change in NPY mRNA expression in the ad-lib fed GHRP-6-treated rats. We expected also to find an increase in NPY mRNA expression in the food restricted groups, reflecting hunger due to the food restriction. In rats, food restriction during lactation, when demand for stored energy reserves is high, results in markedly increased NPY mRNA expression in the hypothalamus (Wilding et al., 1997). Conversely, NPY mRNA

expression in the arcuate nucleus is reduced in rats fed with high-fat diet (Stricker-Krongrad et al., 1998).

4.2 Aims

This study was aimed to investigate sensitivity of arcuate neurones to food restriction and chronic treatment of GHRP-6 in level of NPY mRNA expression. The aim was also to examine whether there is additive effect of food restriction and chronic treatment of GHRP-6 in rats maintained under combination of both factors. In these rats, we expected to find a larger increase in NPY mRNA expression in the GHRP-6-treated food restricted group, reflecting an increase in appetite induced by the secretagogue.

4.3 Methods

4.3.1 Animals and surgical implantation

Adult male Sprague Dawley rats (250-350 g) were maintained in a controlled environment (12 h light: 12 h dark, 21 – 23 °C) with free access to food and water. All surgical procedures were carried out under a Home Office Project and Personal License. Experiment was performed during the light cycle. Rats were anaesthetised with halothane (5 % halothane in a O₂/NO₂ mixture) and placed in a stereotaxic frame. An osmotic minipump (ALZA Corporation, USA) filled with either 0.2 ml of GHRP-6 (1 µg/µl) or an equal volume of aCSF as vehicle, was attached with a catheter and a guide cannula. Each rat was given either GHRP-6 or vehicle via the osmotic minipump pump. The pump was placed under the body skin of the rat. Either GHRP-6 or vehicle filled inside the pump was delivered through the catheter and the guide cannula to the right lateral ventricle (co-ordinates: 3.0 mm posterior, 2 mm lateral of bregma, 0.1 mm from the surface of the skull) at 1.0 µl/h for 7 days. The needle was held in place with dental acrylic bonded to stainless steel screws anchored to the skull. After the implantation, rats were housed and maintained individually in stainless steel cages.

Rats implanted with GHRP-6 were divided into two groups: GHRP-6/*ad libitum* and GHRP-6/food restricted (n = 7 each). Rats implanted with aCSF were also divided into vehicle/*ad libitum* and vehicle/food restricted (n = 6 each). The group of vehicle/*ad libitum* fed rats was taken as the control group. During the 7-day period of recovery, GHRP-6/food restricted and vehicle/food restricted groups were given free access to water but limited access to pelleted food (from 9.00 to 11.00 am each day). The groups of GHRP-6/*ad libitum* and vehicle/*ad libitum* were given food and water freely 24 h a day. All rats were handled daily to minimize stressed-related effects while measuring body weights and the amounts of consumed food and water.

On the day of the experiment, rats had received a 7-day central infusion of GHRP-6 or aCSF. They were quickly killed by using a guillotine, and brains were immediately removed, frozen on dry ice and stored at $-70\text{ }^{\circ}\text{C}$ until *in situ* hybridisation was performed. Coronal brain sections through the hypothalamus were cut at $-16\text{ }^{\circ}\text{C}$ on a cryostat at $15\text{ }\mu\text{m}$ thickness. Sections were collected on RNAase-free gelatinised slides and stored in desiccated slide boxes at $-70\text{ }^{\circ}\text{C}$ before undergoing *in situ* hybridisation. Every third section through the hypothalamus was collected and counterstained with toluidine blue and coverslipped with permount. These sections were later used as marker sections to confirm the location of areas of interest on the section after *in situ* hybridisation.

4.3.2 Probe preparation

An oligonucleotide probe (NPY, MWG, Germany) was synthesised to correspond to previously tested sequences. The probe sequences were complementary to the nucleotides spanning the rat NPY (30-mer) coding region:, bases 5'-GGA GTA GTA TCT GGC CAT GTC CTC TGC TGG-3' with GC content of 56.7 %. The lyophilised probes were reconstituted using sterile double distilled water to a stock concentration of $100\text{ pmol}/\mu\text{l}$. On the day of labelling, the stock solution was further diluted to give a working solution of $10\text{ pmol}/\mu\text{l}$. The probes were thereafter 3' end-labelled using terminal deoxynucleotidyl transferase (Roche Diagnostics, Germany) and ^{35}S dATP (ICN, France) to a specific activity of $832115\text{ cpm}/\text{pmol}$ in the final hybridisation mixture. They were finally purified and counted using a β -counter.

4.3.3 *In situ* hybridisation

Selected slides of hypothalamic sections were left to warm up to room temperature. They were then fixed in 4 % paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) for 10 min and washed twice with 0.1 M phosphate buffer saline (5 min each). After that, they were acetylated with a solution containing 0.25 % acetic anhydride, 0.1 M triethanolamine (TEA) and 0.9 % NaCl to reduce non-specific DNA binding to charged particles, and rinsed twice with sterile double

distilled water (2 min each). Sections were dehydrated through a series of ethanol solutions (70 %, 95 % and 100 %), delipidated with 100 % chloroform (CHCl_3) to improve probe access, followed by rehydration in ethanol solutions (100 % and 95 %). Slides were then air-dried thoroughly.

Dried sections were hybridised in the following buffer: for 1.0 ml, 100 μl working solution of probe, 450 μl hybridisation buffer (see **solution preparation**), 450 μl formamide and 10 μl dithiothreitol (DTT). For a pair of brain sections on one slide, 45 μl of hybridisation mixture was required. Nescofilm coverslips were used to cover the sections to ensure that the mixture was evenly distributed across the tissue and that no air bubbles were trapped. Hybridisation was carried out in a moist chamber for overnight at 37 °C. Hybridised slides were then dipped briefly in 3 washes of 1x standard sodium citrate (SSC: 0.0075 M NaCl and 15.0 M tri-sodium citrate) at room temperature to remove the nescofilm and to wash out excess unhybridised probes. Next, they were washed with 1x SSC at 55°C in a water bath for 4 x 15 min, then with 1x SSC at room temperature twice (30 min each). Finally, slides were rinsed briefly twice with double distilled water at room temperature, and left to dry in an incubator.

The following processes were performed in darkness. Dried slides were arranged onto plastic holders and dipped in autoradiographic emulsion that was freshly prepared by diluting emulsion (Kodak, G5 Ilford, UK) with warm water (ratio 3:1). They were dried overnight, then kept in sealed plastic boxes and stored at 4 °C for 3 weeks.

Autoradiographic silver grains of hypothalamic sections of test slides were roughly checked whether the reaction period was optimum. When satisfied, the rest of the slides were placed in racks. They were dipped in developer solution for 5 minutes, double distilled water for 2 s, fixer I and II for 5 min each, and finally washed with double distilled twice for 5 min each. Then slides were air-dried thoroughly.

Sections were counterstained by running through the haematoxylin and eosin solutions (see **material for solution preparation and method**). They were then mounted with DPX and coverslipped.

4.4 Quantitative analysis

Hybridisation signals were quantified by using a computer image analysis programme, NIH image. Sampling was done bilaterally in the arcuate nucleus with the x5 and x20 objectives. Relative amount of NPY mRNA in the arcuate nucleus was assessed by the silver grain density of autoradiographic emulsion dipped slides. Autoradiographic grains were viewed under light microscope attached to a high performance CCD video camera (Cohu). Signal was displayed in dark-field image. Silver grains in the arcuate nucleus and the background, and unit area of measurements, were quantified and digitised by NIH Image analysis system version 1.58 on an Apple McIntosh computer. The NIH-Image public domain image processing software package was developed by Wayne Rashband. Using the x5 objective, a cell was considered to be positive when it contained more than 3 times the background number of silver grains. The average number of silver grain per cell was measured using the x20 objective, for each cell. The value of silver grain density per unit area was obtained by subtracting the mean silver grain area of the background measurements from each specific nucleus measurement, and divided by the area measurements. A total of 5 positive NPY mRNA-containing cells was counted for each side of the arcuate nucleus. The mean average silver grain per unit area was calculated for each arcuate nucleus. Mean silver grains per unit area was then calculated for each area of the arcuate nucleus (A, B, C, D and A-D is whole arcuate nucleus), and finally the mean value per rat and group means were calculated. Group means for all areas and groups were compared statistically using Two-way ANOVA (Student-Newman Keuls Method).

4.5 Materials and solution preparation

Gelatine coated slides

Slides (super premium twin-frost; BDH 406/0181/04) were washed in chromic acid overnight. The next day, the slides were rinsed in cold running tap water for approximately 2 h until the slides were completely rinsed. The slides were then passed through three changes of a ddH₂O and once in DEPC-treated water. After that, the slides were immersed in 80 % alcohol [400 ml

absolute (100 %) ethanol + 100 ml DEPC ddH₂O] and dried in the 60°C oven. Once the slides were dry, they were removed from the oven, and allowed to cool to room temperature. Next, the slides were subbed in chrome-alum/gelatine solution and drain on paper towelling. They were subbed in gelatine again after they were dry.

Solution preparation

Chromic acid

For 2000 ml final volume, 200 g potassium dichromate was added to 2000 ml. Concentrated sulphuric acid (20 ml) was slowly added to the flask whilst it was immersed in cold water (to cool exothermic reaction).

Diethyl pyrocarbonate treated water (DEPC-treated water)

One ml of diethyl pyrocarbonate (Sigma) was added to 1000 ml of ddH₂O in a bottled. The bottled was shaken vigorously for 2 min, allowed to stand for 1 h, and then autoclaved.

Chrome alum gelatine subbing solution

A beaker containing 800 ml ddH₂O was heated in a microwave to 70°C. Gelatine powder (2.25 g) was added to the heated water on a magnetic stirrer. After this dissolved and the solution had cooled down, 0.23 g chromic potassium sulphate was added on stirrer.

Tissue fixation solutions

1.0 M phosphate buffer saline (PBS, pH 7.2-7.4)

Amount and final concentration of chemicals for a final volume of 1000 ml

115.0 g	Disodium hydrogen orthophosphate 2-hydrate	11.5 % w/v
27.2	Sodium dihydrogen orthophosphate 1-hydrate	2.72 % w/v

These two chemicals were dissolved in 800 ml DEPC-treated ddH₂O and then made up to 1000 ml. The solution was adjusted to pH 7.2-7.4 with 1 M NaOH. More dilute solutions were made by diluting this PBS stock with DEPC-treated ddH₂O

4 % paraformaldehyde (PFA) in 0.1 M PBS (pH 7.4)

Firstly, 8 % of PFA was made by adding PFA to 80°C ddH₂O. The solution was then adjusted to pH 7.4 with 1 M NaOH. Finally an equal volume of 0.2 M PBS was added to make a final solution with 4 % PFA in 0.1 M PBS. The solution was chilled in a fridge before use.

Triethanolamine (TEA)/acetic anhydride solution

Triethanolamine solution was made in ddH₂O, containing 1.48 % v/v TEA, 0.25 % v/v acetic anhydride and 0.42 % v/v concentrated hydrochloric acid or 0.9 % w/v NaCl.

Probe labelling method

The following components were put into a sterile Eppendorf tube.

27 µl	sterile ddH ₂ O
10 µl	5xTdT tailing buffer
5 µl	2.5 mM Cobalt chloride (CoCl ₂)
5 µl	³⁵ S dATP
2 µl	probe (working dilution 10 pmol/ µl)

Then 1 µl TdT enzyme (25 units/ µl) was added to start the reaction. The mixture was mixed well using a Gilson pipette. The Eppendorf was put into a polystyrene holder and allowed to float in the water bath (37°C) for 1 h. After 1 h, the Eppendorf was removed from the water bath and cooled on ice to stop the reaction.

Hybridisation buffer

Hybridisation buffer contained:

For 50 ml		
5 M NaCl (292 mg/ml)	12 ml	RT
Tris 7.6 (149 mg/ml)	1 ml	RT
EDTA 250 mM (232.6 mg/ml)	400 µl (dissolved in hot water)	RT
Dextran sulphate 25 %	5 ml	4°C
NaPPI 5 % (50 mg/ml)	500 µl	RT
Yeast tRNA (25 mg/ml)	200 µl	-20°C

Yeast total RNA (20 mg/ml)	250 μ l (boiling water 10 min)	-20°C
Salmon testes DNA (10 mg/ml)	1 ml	4°C
Poly (A) (15 mg/ml)	330 μ l	-20°C
Formamide (50 %)	20 ml	RT
Denhardt's solution*	1 ml	
*Denhardt's solution was made with:		
BSA 1.6 % (200 mg/ml)	4°C	
Ficoll 6 % (100 mg/ml)	4°C	
PVP 6 % (100 mg/ml)	RT	

Preparation of hybridisation buffer components

5 M Sodium chloride

For a final volume of 50 ml, 14.6 g NaCl was added to 45 ml sterile ddH₂O in sterile falcon tube and dissolved. The volume was made up to 50 with sterile ddH₂O. Then, the solution was added with 50 μ l DEPC, and shaken vigorously. The solution was left for 1 h and then autoclaved. The solution was aliquoted into sterile 2-ml Eppendorfs and stored at -20°C.

Tris pH 7.6

For a final volume of 20 ml, 20 ml sterile ddH₂O was put into a sterile falcon tube. It was added with 2.98 g Trizma base. The solution was adjusted to pH 7.6, aliquoted into sterile 1-ml Eppendorfs, and stored at room temperature.

Denhardt's solution

For a final volume of 50 ml, 0.5 g ficoll and 0.5 polyvinylpyrrolidone were added to 40 ml DEPC-treated water in a sterile falcon tube and mixed well. Then 1 g BSA was added and mixed gently. The solution was made up to 50 ml with DEPC-treated water, and filtered through a 0.2 μ m Acrodisk filter. The solution was dispensed into 1 ml aliquots in 2-ml sterile Eppendorfs and stored at -20°C.

EDTA 250 mM

EDTA (23.26 g) was added to 250 ml of sterile ddH₂O. The solution was adjusted to pH 8.0 with NaOH, and autoclaved in 250 ml Duran. It was dispensed into 400 µl aliquots.

Dextran sulphate

Dextran sulphate (12.5 g) was added to 15 ml of DEPC-treated water. The solution was mixed and warmed at 50 °C for about 4 h. Then, it was made up to 25 ml with DEPC-treated water and stored at 4°C.

Sodium pyrophosphate (NaPPi)

NaPPi (0.5 g) was dissolved with 10 ml sterile ddH₂O, and aliquoted into sterile Eppendorfs (400 µl each). The aliquots were stored at room temperature.

Yeast tRNA

Yeast tRNA (100 mg) was put into a sterile 50 ml falcon tube. Then, 4 ml DEPC-treated water was added and mixed well. The solution was aliquoted into sterile 200-µl Eppendorfs and frozen at -20°C.

Yeast total RNA

Yeast total RNA (60 mg) was dissolved in 3 ml of DEPC-treated water, and aliquoted into sterile 250-µl Eppendorfs. The aliquots were stored at -20°C. When needed for making up the hybridisation buffer, a 250-µl aliquot was boiled for 10 min before hybridisation buffer was made.

Salmon testes DNA

Salmon testes DNA (50 mg) was added to 5 ml DEPC-treated water in tube, and agitated for 2 h. Mixture was sheared by passing through a sterile 19-gauge needle for eight times. The tube

was placed into a beaker of boiling water for 5 min, and cooled to room temperature. After that, it was aliquoted (1 ml each), and stored at 4°C.

Poly (A)

Fifteen mg of poly (A) was added to 1 ml DEPC-treated water and aliquoted into 330- μ l Eppendorfs.

Formamide (50 %)

Twenty-five ml of 100 % stock was used per 50 ml hybridisation buffer.

1.0 M Dithiothreitol (DTT)

DTT (154 mg) was added to 1 ml of DEPC-treated water. The solution was left for 30 min and filtered through an acrodisk. It was aliquoted into sterile 50- μ l Eppendorfs, and stored at -20°C. DTT was not added to the hybridisation buffer until the day of use.

Post hybridisation washes

20x standard sodium citrate (SSC) stock solution

Amount and final concentration of chemicals for 1000 ml

175.4 g	Sodium chloride	17.54 % w/v
88.2 g	tri-sodium citrate	8.82 % w/v

This stock solution was used for making diluted SSC solution by diluting with ddH₂O.

Counterstaining

Haematoxylin and eosin solutions

Harris' Haematoxylin:

Amount of chemicals for approximately 230 ml

1 g	Haematoxylin
10 ml	absolute alcohol
20 g	ammonium or potassium alum (aluminium sulphate)
200 ml	distilled water
0.5 g	mercuric oxide
8 ml	glacial acetic acid

Method

- A) Dissolve haematoxylin in the alcohol and add to the alum (previously dissolved in hot water)
- B) Bring quickly to the boil and add the mercuric oxide, when the solution will turn dark purple
- C) Cool rapidly under the tap, filter before use
- D) Add glacial acetic to sharpen the nuclear staining

Make up in the fume cupboard, using a flask of ample size on account of the frothing that takes place on the addition of the mercuric oxide

1 % Eosin

Dissolve 1 g eosin in 100 ml distilled water

Scott's Tap Water Substitute-STWS

For 1000 ml, dissolve 20 g magnesium sulphate and 3.5 g sodium bicarbonate (sodium hydrogen carbonate) in 1000 distilled water.

5 % Potassium Alum

For 100 ml, dissolve 5 g aluminium potassium sulphate or potassium aluminium sulphate in 100 ml distilled water.

1 % Acid Alcohol

For approximately 1000 ml, add 700 ml 74OP or IMS alcohol with 300 ml distilled water. Finally, add 10 ml hydrochloric acid (conc).

Haematoxylin and Eosin Staining

Method

Run slides through the following solution.

Dewax in xylene 20-30 min

Rehydrate through alcohols

Absolute alcohol

90 % alcohol

70 % alcohol

(lithium carbonate in 70 % if needed to remove Bouin's)

Wash in running water

Dip in acid alcohol (if needed to differentiate)

Wash in running water

STWS 3 min

Water 3 min

Eosin (filter before use) 2 min

(check staining under microscope)

Dip in running water

Potassium alum 2 min

Wash in running water

Dehydrate through alcohols

70 % alcohol

90 % alcohol

95 % alcohol

absolute alcohol

absolute alcohol

Clear in xylene

Coverslip using DPX mountant

4.6 Results

4.6.1 Effect of chronic administration of GHRP-6 on food and water intake

The effect of central administration of GHRP-6 was examined in rats implanted with chronic i.c.v. cannula and minipump. Two groups of rats were given food and water *ad libitum*, and two groups were given food for only 2h per day, (food restriction group). For each feeding regime, rats were centrally infused with either GHRP-6 (1 $\mu\text{g}/\mu\text{l}/\text{h}$) or 0.9 % saline.

All rats given food restriction ate only a few grams on the first day after surgery (data not shown), probably because they were naïve to the condition that food was going to be removed after 2-h. On this first day after surgery, all rats received vehicle i.c.v. By day 2, the food intake of all rats increased up to approximately 6 and 8 g in saline and GHRP-6 groups respectively. Thus, analysis of the effects of GHRP-6 on food and water intake and body weight of rats were analysed from day 2 of the experiment.

In *ad libitum* fed rats, rats given saline showed no consistent variation in food intake throughout the 6 days of the experiment. However, rats given GHRP-6 showed a higher food intake on day 5 and day 6 than vehicle-treated rats (**fig. 4.1A**). On day 5, the GHRP-6 group showed an average food intake at 32.7 g, compared to 25.2 g consumed by control rats. Statistical analysis showed a significant effect of GHRP-6 treatment on food intake on day 5 and day 6 of the experiment (one way repeated measures ANOVA; $p = 0.05$). When the amount of food intake was analysed as cumulative food intake over 6 days, the GHRP-6 group ate 17.7 g more than the control group (t-test, $p < 0.05$) (**fig. 4.1C**)

In food restricted groups, food intake was similar on day 2 in saline and GHRP-6 groups (**fig. 4.1B**). Thereafter, average food intake gradually increased up to day 6 in both groups. Statistical analysis showed no significant differences between saline and GHRP-6 groups on any day of the experiment (Two-way ANOVA, $p = 0.05$). Overall, increase in food intake in both saline and GHRP-6 groups was dependent on duration of food restriction, but not on

GHRP-6 treatment. When analysed as cumulative food intake over 6 days, the GHRP-6 group ate 4.5 g more than the saline group (not significantly different; **fig. 4.1D**)

Effects of feeding regime (*ad libitum* and food restriction) and effects of treatment (saline and GHRP-6), on food intake, were determined statistically. Two-way ANOVA revealed that food restriction, but not type of treatment, had a significant influence on amount of food intake ($p < 0.05$, Student-Newman-Keuls Method). This was reflected by relatively equal amount of food intake seen in saline and GHRP-6 groups of each day of both feeding regimes.

The water intake in the above rats was also measured. Like food intake, water intake of rats on a restricted diet was almost completely absent on day 1, and then had returned to average level on day 2 with about 33 ml of water intake in rats of both feeding regimes. Thereafter, the water intake of rats fed *ad libitum* in saline group during the first 5 days was relatively stable, but increased on day 6 after surgery (**fig. 4.2A**). In the GHRP-6 group, water intake was significantly increased on day 4 and day 5, compared to saline group (One-way repeated measures ANOVA, $p = 0.05$). The cumulative water intake over 6 days was 14.1 % significantly higher in GHRP-6 treated rats than in the saline-treated rats (t-test, $p < 0.05$; **fig. 4.2C**)

In food-restricted rats given either saline or GHRP-6, water intake slightly decreased from day 2 to day 4 but was rather stable thereafter. In food-restricted rats, GHRP-6 group showed non-significantly higher water intake than the saline group on day 2 and day 3, but after that both groups showed relatively equal volume of water intake (Two-way ANOVA, $p = 0.05$). No significant difference between saline and GHRP-6 groups of rats maintained on a feeding restricted diet was seen, even when expressed as cumulative water intake over 6 days (t-test, $p = 0.05$; **fig. 4.2D**).

However, statistic analysis revealed that the feeding regime had significant influence on water intake, whereas GHRP-6 treatment did not significantly influence water intake (Two-way ANOVA, $p < 0.05$, Student-Newman-Keuls Method).

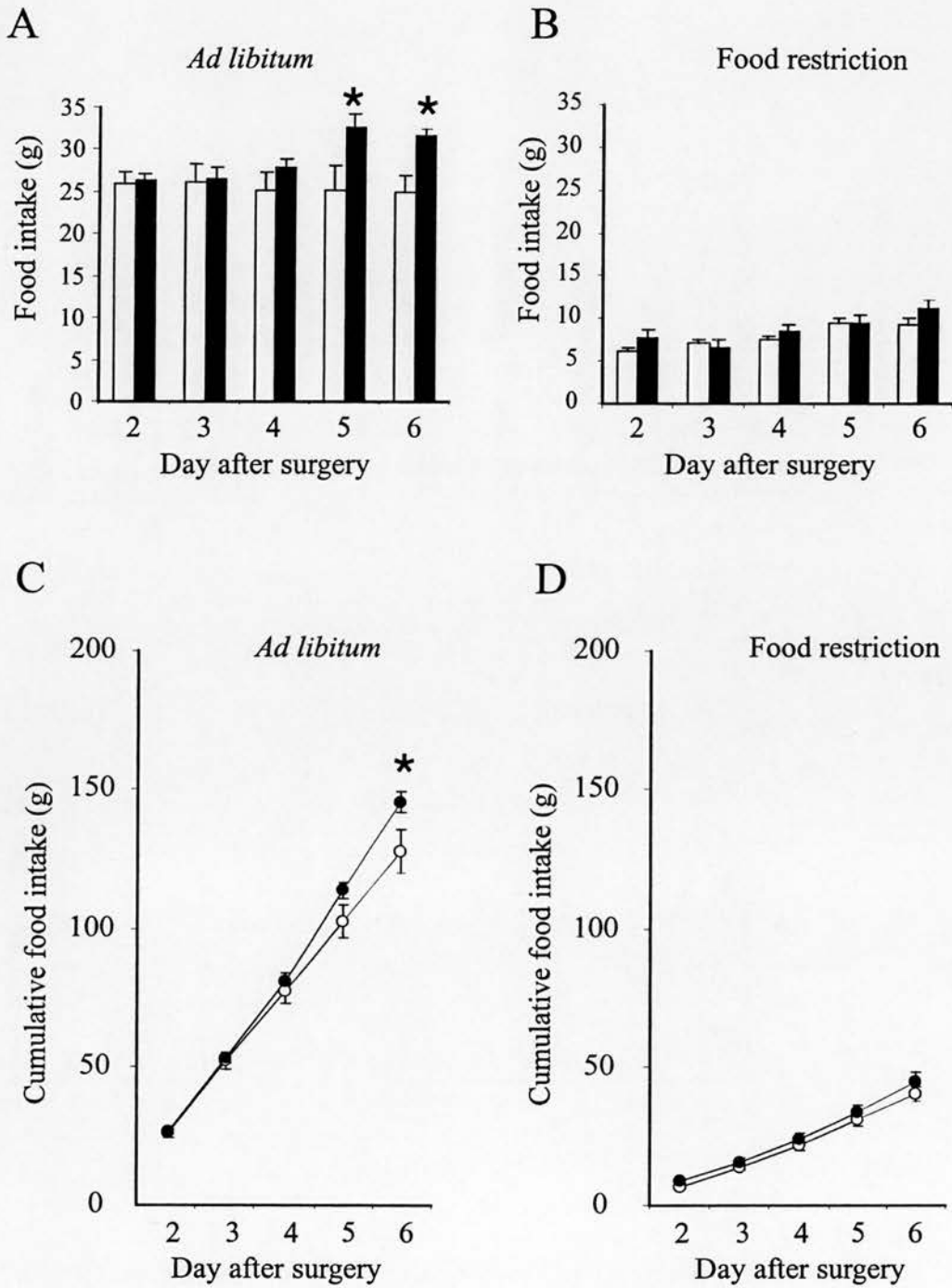


Fig. 4.1. Effect of i.c.v. injection of GHRP-6 or saline on food intake. Effects of saline (0.9% saline: white, $n = 6$) and GHRP-6 ($1 \mu\text{g}/\mu\text{l}/\text{h}$: black, $n = 7$) on food intake of rats that were fed *ad libitum* (A) and maintained on a restricted feeding diet (B) were illustrated. Data were also expressed as cumulative food intake (C: *ad libitum* and D: restricted feeding diet). Error bars are SEM. Statistical significance of differences between groups: $* = p < 0.05$, (for food intake: Repeated measure, one-way ANOVA, Student-Newman-Keuls Method and for cumulative: t-test).

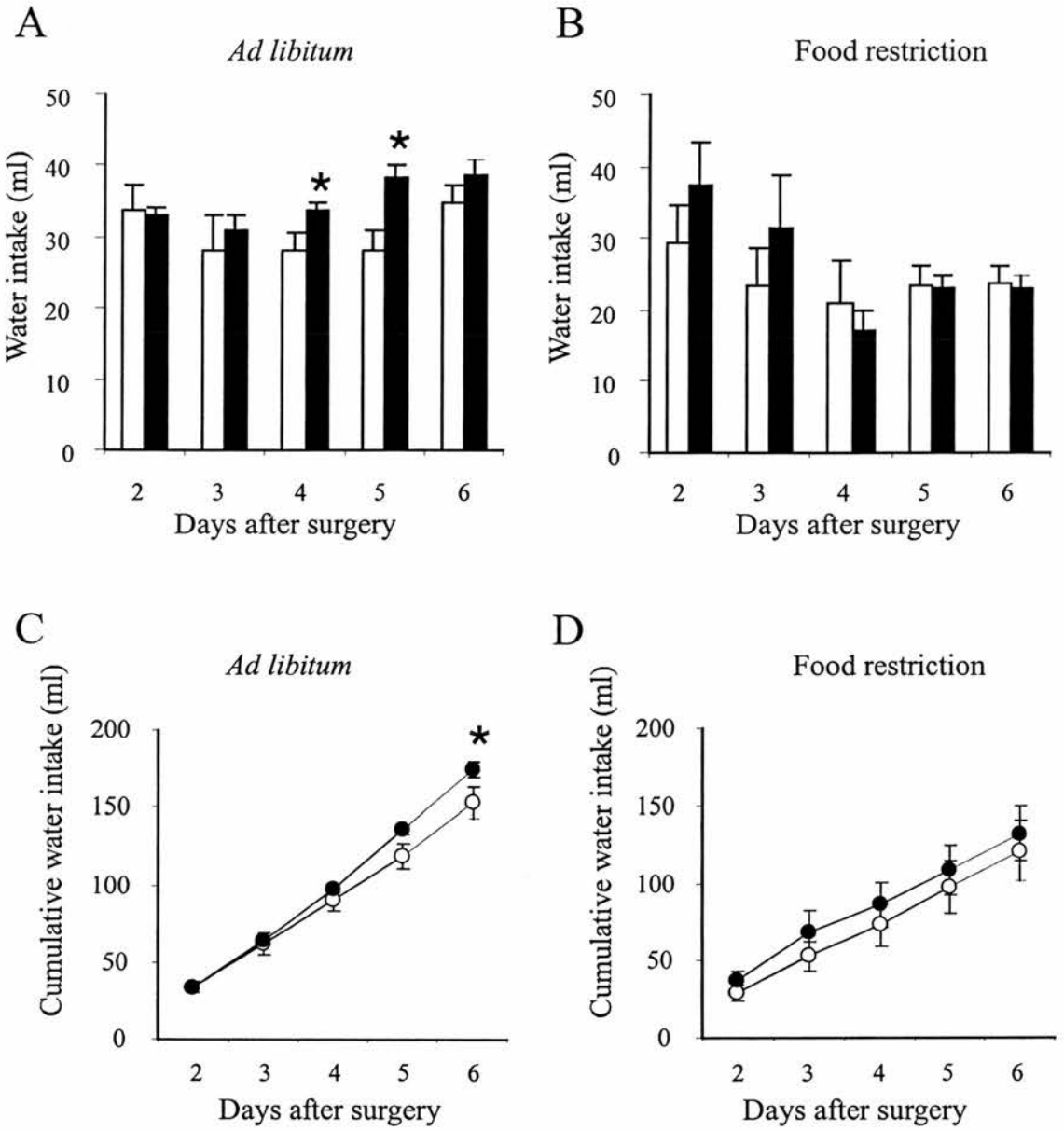


Fig. 4.2. Effect of i.c.v. injection of GHRP-6 or saline on water intake. Effects of saline (0.9 % saline: white, n = 6) and GHRP-6 (1 $\mu\text{g}/\mu\text{l}/\text{h}$: black, n = 7) on water intake of rats that were fed *ad libitum* (A) and maintained on a restricted feeding diet (B) were illustrated. Data were also expressed as cumulative water intake (C: ad libitum and D: restricted feeding diet). Error bars are SEM. Statistical significance of differences between groups: * = $p < 0.05$, (Repeated measures, one-way ANOVA, Student-Newman-Keuls Method for daily water intake and t-test for cumulative water intake).

4.6.2 Effect of chronic administration of GHRP-6 on rat body weight

In rats receiving food *ad libitum*, i.c.v. infusion of GHRP-6 (1 mg/ml/h) caused an approximately 3-fold greater daily weight gain measured on day 2 after surgery, compared to rats given i.c.v. saline infusion : saline group showed daily weight gain about 3 g while GHRP-6 showed about 11 g (**fig. 4.3A**). Thereafter, the daily weight gain of GHRP-6 treated rats was higher on each day than in the saline group. Data were also expressed as cumulative body weight gain over 6 days of experiment (**fig. 4.3C**). When expressed as cumulative body weight gain, the GHRP-6 group showed significant differences in cumulative body weight change relative to the saline group over 5 and 6 days (t-tests, $p < 0.05$).

In rats maintained on a restricted feeding diet, both saline and GHRP-6 groups showed a large decrease in body weight on day 2 (**fig. 4.3B**). Rats in the saline group lost about 20 g, whereas GHRP-6-treated rats lost about 25 g. However, from day 3 to day 6, the magnitude of daily weight loss was reduced to about 5 to 10 g per day for both saline and GHRP-6 groups. Overall, rats given either saline or GHRP-6 showed similar patterns of daily weight change. The cumulative weight loss of the GHRP-6 group was significantly less (18.9 %) than that of saline group over 6 days (t-test, $p < 0.05$) (**fig. 4.3D**).

Altogether, statistical analysis of the body weight of rats on a restricted feeding diet revealed that daily weight change was dependent on length of time after the start of food restriction, but not on type of treatment (Two-way ANOVA, $p < 0.05$, Student-Newman-Keuls Method).

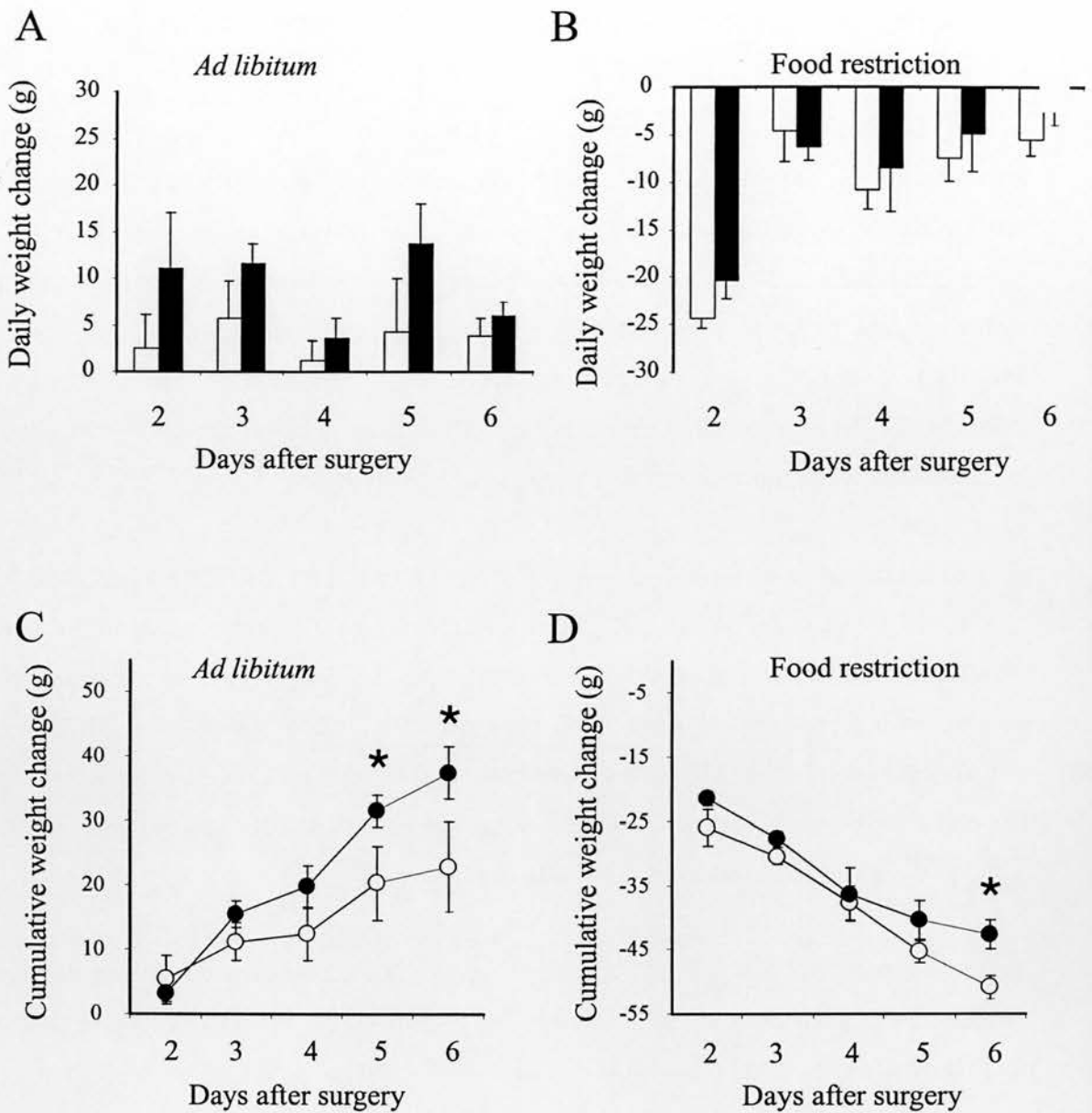


Fig.4.3 . Effect of i.c.v. injection of GHRP-6 or saline on body weight change. Effects of saline (0.9 % saline: white, n = 6) and GHRP-6 (1 $\mu\text{g}/\mu\text{l}/\text{h}$: black, n = 7) on body weight change of rats that were fed *ad libitum* (A) and maintained on a restricted feeding diet (B) were illustrated. Data were also expressed as cumulative weight change (C: *ad libitum* and D: restricted feeding diet). Error bars are SEM. Statistical significance of differences between groups: * = $p < 0.05$, (Repeated measures, one-way ANOVA for daily weight change and t-test for cumulative weight change).

4.6.3 Effects of food restriction and chronic administration of GHRP-6 on expression of NPY mRNA in the arcuate nucleus

Light microscopic examination of coronal section taken through the rat brain, revealed autoradiographic NPY mRNA hybridisation signal in hypothalamic brain slices (**fig. 4.4**). The number of neurones labelled with silver grains in 4 areas located rostro-caudally from area A to D in the arcuate nucleus was counted and analysed. Basal expression of NPY transcripts within the arcuate nucleus was examined in rats fed *ad libitum* and receiving chronic vehicle infusion. In these rats, the average number of NPY mRNA hybridising neurones in the arcuate nucleus varied from about 32.4 ± 45.8 /arcuate nucleus/section (area A) to 270.7 ± 82.2 /arcuate nucleus/section (area C), with an average of 149 ± 52.3 /arcuate nucleus/section for all 4 areas (**fig. 4.5A**).

In rats fed *ad libitum* and receiving chronic infusion of GHRP-6, the arcuate nucleus showed a relatively even distribution of NPY mRNA expression throughout the arcuate nucleus. The average number of positive neurones varied from 83 ± 65.1 to 181.0 ± 98.3 with an average of 172.5 ± 71.9 for all 4 areas. The NPY mRNA expression of this group was not significantly different from the control group (t-test, $p > 0.05$).

Intensely labelled neurones were substantially detected in the arcuate nucleus of rats maintained on a restricted feeding diet. In rats receiving chronic infusion of vehicle, the number of NPY mRNA hybridising neurones was 405.2 ± 139.3 in area B. Overall, the total average number of positive neurones of this group was 350.6 ± 77.0 , 96.1 % higher than in control group. The arcuate nucleus of rats maintained on the same feeding regime but receiving chronic infusion of GHRP-6 showed the average number of positive neurones for all 4 regions of 234.0 ± 39.3 which was non-significantly higher than those in both groups of rats fed *ad libitum*, but lower than that of rats treated with chronic saline and feeding restricted diet (Two-way ANOVA, $p > 0.05$).

Data of NPY mRNA expression in the arcuate nucleus of rats maintained on either feeding regimes and combined with treatment of either vehicle or GHRP-6 were analysed statistically. Two-way ANOVA test revealed that food restriction had a significant influence in inducing NPY mRNA expression, where as *ad libitum* feeding regime and GHRP-6 chronic administration did not (Two-way ANOVA, Student-Newman-Keuls Method, $p < 0.05$).

Data were also expressed as total silver grains per arcuate nucleus (**fig. 4.5B**). Both groups of rats maintained on a feeding restricted diet showed high number of total silver grains than both groups of rats fed *ad libitum*. The average number of positive neurones of all 4 areas of the arcuate nucleus of 4 groups is shown in **fig. 4.5C**.

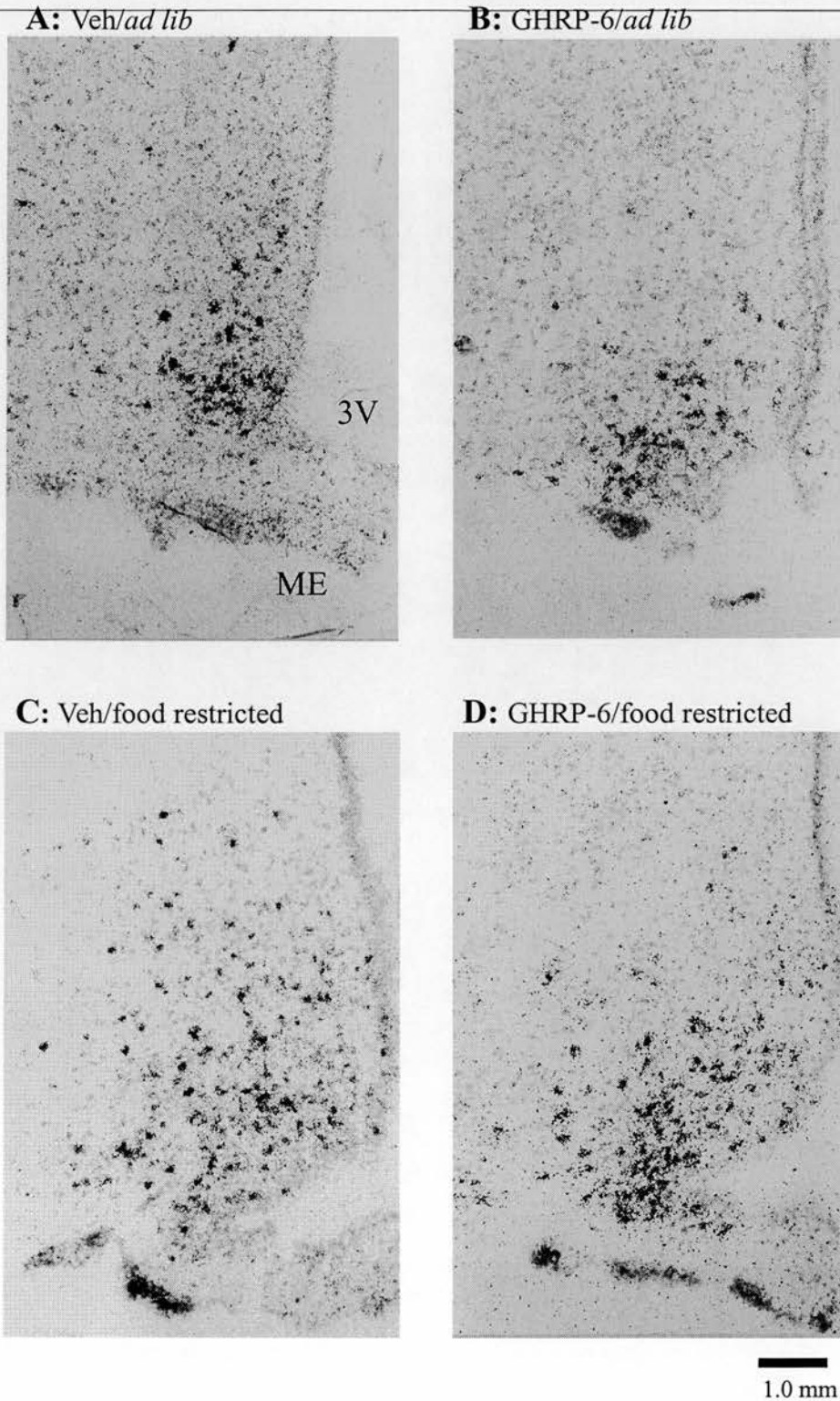


Fig. 4.4. Effect of feeding regimes and chronic i.c.v. administration of GHRP-6 on NPY mRNA expression in the arcuate nucleus of rats. Rats were fed and chronically infused as indicated for 7 days. NPY mRNA levels in the arcuate nucleus were determined by *in situ* hybridisation. Representative sections of the arcuate nucleus showing expression of NPY mRNAs are visualised using x10 objective. 3V: third ventricle, ME: median eminence

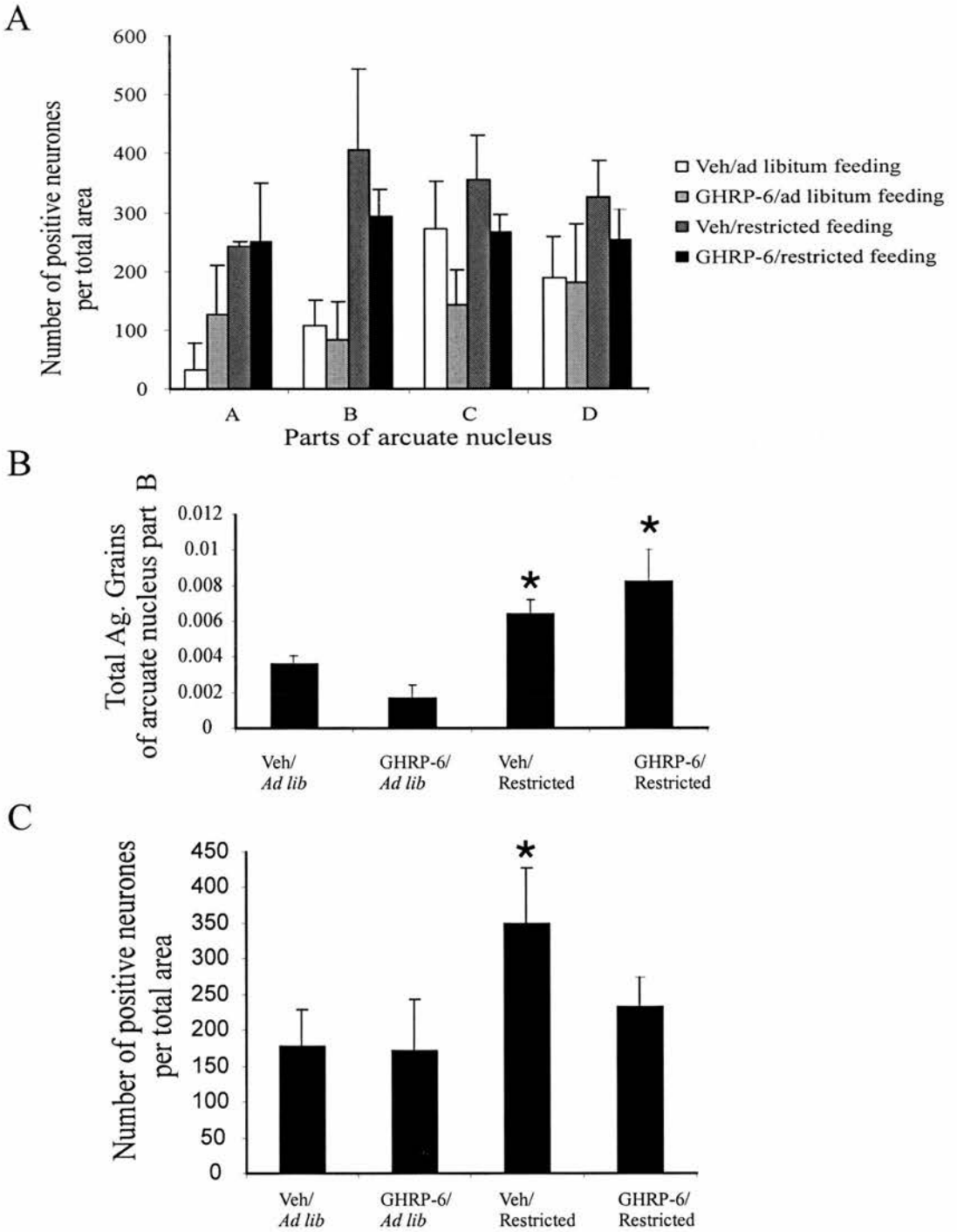


Fig. 4.5. The number of arcuate nucleus neurones expressing NPY mRNA, as detected by in situ hybridisation. (A) Bar graph shows the total number of positive neurones per total area of arcuate nucleus. Rats were chronically infused (i.c.v.) with either saline (0.9 % saline) or GHRP-6 (1 $\mu\text{g}/\mu\text{l}/\text{h}$), and maintained either on *ad libitum* or a restricted feeding diet for 7 days. The total number of positive neurones per arcuate nucleus is shown in 4 parts of the arcuate nucleus; A, B, C, and D (from the anterior to posterior). A: 1.8 - 2.3 mm, B: 2.3 - 2.8 mm, C: 2.8 - 3.3 mm, D: 3.3 - 4.16 mm posterior to bregma. (B) The total amount of silver grains of part B of the arcuate nucleus. (C) The average number of positive neurones per total area of arcuate nucleus. All data are the mean \pm SEM (n = 3-7 in each group). Statistical significance of differences compared with veh/*ad lib* group: * = $p < 0.05$ (Two-way ANOVA)

4.7 Discussion

Orexigenic effects of GHSs on food intake in rats have been well-documented, and it has been hypothesised that these orexigenic effects are mediated via NPY pathway in the hypothalamus. Our previous results of the electrophysiological experiments (chapter 2) showed convergence between the actions of secretagogues and NPY in the arcuate nucleus and in the ventromedial hypothalamic nucleus. Furthermore, our immunohistochemistry studies (chapter 3) revealed an interaction between GHRP-6 and NPY in the arcuate nucleus. In previous studies, induction of *c-fos* mRNA expression in NPY neurones of the arcuate nucleus has been described following administration of GHRP-6 (Dickson and Luckman, 1997).

In this experiment, significant effects of chronic administration of GHRP-6 on cumulative food and water intake were observed in rats fed *ad libitum*, compared to rats receiving chronic administration of vehicle. However, as shown previously (Bailey et al), chronic treatment with GHRP-6 had no significant effect on NPY mRNA expression in the arcuate nucleus. This does not seem to be because our techniques were too insensitive to measure a change in NPY mRNA expression because, again as reported previously, food-restricted rats showed a large and significant increase in NPY mRNA expression.

It appeared possible that in rats given GHRP-6, the secretagogue caused an increase in food intake through activation of NPY neurones, but that the increased food intake in turn resulted in an increased inhibition of NPY cells reflecting stronger satiety signals, possibly through increased leptin secretion. We therefore looked at the effects of GHRP-6 infusion in conditions where the rats were not able to increase their food intake. If the orexigenic effects of GHRP-6 are mediated by NPY neurones, we might expect in these circumstances to reveal a stimulatory effect of GHRP-6 on NPY mRNA expression.

In food-restricted rats, as planned, GHRP-6 produced little increase in food intake compared with vehicle-infused rats, and both GHRP-6 and vehicle-infused rats lost weight progressively during the course of treatment. NPY mRNA expression was higher in both food-restricted

groups than in the *ad libitum* fed groups, but the level of expression in the food restricted rats treated with GHRP-6 was lower than in the food-restricted rats treated with vehicle. The difference between groups was not significant, but clearly there was no increase in NPY mRNA expression in response to GHRP-6.

At face value, these data suggest that, while increased hunger associated with food restriction is associated with increased NPY mRNA expression in the arcuate nucleus, the increase in food intake that follows chronic GHRP-6 treatment is **not** a consequence of increased activity in NPY neurones.

Acutely, GHRP-6 does induce activity in NPY neurones of the arcuate nucleus. However, GHRP-6 had no acute effect on food intake in these experiments. It should be remembered that not all NPY neurones in the arcuate nucleus are necessarily involved in feeding. Only a subset of these NPY neurones which project to the paraventricular nucleus have a clearly defined role in feeding regulation, and few of these appear to be sensitive to GHRP-6. Most of the arcuate neurones that are sensitive to GHRP-6 project to the median eminence, and their function remains largely unknown.

It seems possible therefore that the effects of chronic GHRP-6 on food intake may be unrelated to the actions of GHRP-6 in the arcuate nucleus. Is it possible that they are instead related to the actions of GHRP-6 in the ventromedial hypothalamus? At this site, GHRP-6 has no effect when given acutely *in vivo*, although the cells there express functional GHS receptors. I have speculated that the reason why no acute response is seen in the ventromedial hypothalamus *in vivo* may be because NPY released from arcuate neurones counteracts the stimulatory effects of GHRP-6. However, studies of mRNA expression suggest that during chronic GHRP-6 treatment, the activation of NPY cells may be only transient. If the effects of GHS in the ventromedial hypothalamus are sustained while the effects of NPY are transient, then this might explain a delayed effect of chronic treatment on food intake, as observed in the present experiments. However, a late activation of ventromedial hypothalamic neurones would be expected to reduce feeding rather than to stimulate feeding.

Clearly, there are many other pathways in the hypothalamus that are involved in the control of feeding, and these may be indirectly affected by changes in the activity of either arcuate neurones or ventromedial hypothalamic neurones. Future studies may need to focus on changes in expression of orexins, AGRP, CART, CRF, α -MSH or β -endorphin to help resolve the pathways involved.

CHAPTER 5
GENERAL DISCUSSION

The arcuate nucleus has been a major focus for numerous studies to clarify the mechanisms of regulation of GH secretion. It has been consistently found to mediate effects of the class of novel synthetic compounds termed GHSs. Many neurones in this brain area were previously demonstrated to be NPY neurones (Willesen et al., 1999), and NPY synthesised in the arcuate nucleus is associated with the regulation of feeding (Beck et al., 1990b), which is one recently discovered action of GHS. Although some of the basic elements of this control system have been identified, little is known about the mechanisms by which GHSs exert their effects on feeding and GH secretion.

In this study, effects of secretagogues L-144,446 and GHRP-6 on electrophysiological activity of arcuate neurones was studied using hypothalamic brain slices for extracellular recordings *in vitro*. Significant excitatory effects of these secretagogues were observed, though not every recording was tested whether these effects were direct but atleast some of them were shown to be direct effects. In these experiments, the electrical activity of arcuate neurones was also shown to be inhibited by both NPY and somatostatin. Interestingly, there was large proportion of arcuate neurones that responded to both GHS and NPY. Thus, these results suggest the co-expression of receptors for GHSs, NPY and somatostatin in the arcuate nucleus.

The effects of these compounds were also tested on neurones in the ventromedial hypothalamic nucleus. This area was previously demonstrated to show dense expression of GHS-R mRNA in the rat, though in the mouse the expression in the ventromedial hypothalamic nucleus is not as dense as in the arcuate nucleus (Mitchell et al., 2001). Whereas systemic or central application of GHS results in dense Fos expression in the arcuate nucleus of both rats and mice, similar induction of dense Fos expression is not seen in the ventromedial hypothalamic nucleus. Surprisingly, therefore, when tested *in vitro*, responses of ventromedial hypothalamic neurones to GHRP-6 were very similar to those recorded in arcuate neurones. This finding confirmed the existence of functional GHS receptors in the ventromedial hypothalamic nucleus, and indicated that the GHS receptors in the ventromedial hypothalamic nucleus are coupled to electrical excitation in a similar way to GHS receptors on neurones of the arcuate nucleus.

According to significant response of ventromedial hypothalamic neurones to GHRP-6 *in vitro*, but apparently not *in vivo*, it was hypothesised that these neurones were affected by inhibitory inputs from nearby areas. Here, we showed that ventromedial hypothalamic neurones were also inhibited by bath application of NPY or somatostatin *in vitro*. Since the arcuate nucleus is a major source of NPY synthesis, we speculated that NPY or somatostatin was involved in preventing ventromedial hypothalamic neurones from neuronal activation by GHSs *in vivo*.

Thereafter, the effects of GHRP-6 and NPY on neuronal activity of the arcuate nucleus and the ventromedial hypothalamic nucleus were examined by using immunohistochemistry to detect Fos induction as an indicator of cellular activation. As expected, arcuate neurones were significantly excited by systemic administration of GHRP-6. Consistent with the *in vitro* electrophysiological study, this excitation was significantly attenuated by central administration of NPY. Previously, GHRP-6 and a non-peptide GH secretagogue, MP-0677, were demonstrated to induce Fos protein in the arcuate nucleus of rats, and these effects were also attenuated by central administration of somatostatin (Dickson et al., 1997).

Also in this immunohistochemical study, Fos induction was observed in the ventromedial hypothalamic nucleus after central administration of NPY Y1 receptor antagonist, L-753044-001J003. Importantly, the ability to induce Fos expression in the ventromedial hypothalamic nucleus shows that the intracellular pathways responsible for regulation of *c-fos* are present in these neurones, hence the absence of Fos expression at this site after GHS does not simply reflect an inability of these cells to express Fos. Furthermore, these results demonstrated that *in vivo* there appears to be spontaneous or tonic NPY inhibition of ventromedial hypothalamic neurones, and our electrophysiological studies showed that this was mediated at least in part by Y1 receptors. However, in rats that were given a Y1 antagonist centrally and followed with systemic injection of GHRP-6, no significant Fos induction was observed in this area. Thus it appears that following GHRP-6 injections, there is active inhibition of neurones in the ventromedial hypothalamic nucleus, and this inhibition is not wholly through Y1 receptors. It is likely therefore that NPY is not the only inhibitory compound whose release in the ventromedial hypothalamic nucleus is activated by GHRP-6, or that the Y1 receptor is not the

only receptor through which NPY can inhibit neurones in the ventromedial hypothalamic nucleus.

Since inhibitory effects of somatostatin were also detected in ventromedial hypothalamic neurones *in vitro*, it is also possible that activation of somatostatin neurones by GHS may counteract the excitatory effects of GHS in the ventromedial hypothalamus *in vivo*. In the arcuate nucleus, Fos expression induced by GHRP-6 is attenuated by exogenous administration of somatostatin (Dickson et al., 1997). In the mouse, though not in the rat, systemic injection of GHS induces Fos expression in the periventricular nucleus, probably in the neurosecretory somatostatin neurones. By using a double-label *in situ* hybridisation, somatostatin neurones in the periventricular nucleus of the hypothalamus were reported to co-express the GH receptor mRNA (Burton et al., 1992). Thus periventricular somatostatin neurones may be activated as a result of the growth hormone release that follows administration of GHS. Exogenous growth hormone attenuates GHS-induced Fos expression in the arcuate nucleus in mice, and this effect is absent in transgenic mice that lack somatostatin SSTR2 receptors. Thus it seems likely that, after GHS activation of GHRH neurones, growth hormone is released and feeds back upon periventricular somatostatin neurones that in turn inhibit GHRH neurones (Zheng et al., 1997). In rats treated chronically with i.c.v. GHRP-6 there is a sustained up-regulation of somatostatin mRNA expression in the periventricular nucleus, consistent with activation of somatostatin release in the rat following GHS treatment (Bailey et al., 1999a).

Whether periventricular somatostatin neurones regulate ventromedial hypothalamic neurones is not known. There are also somatostatin cells in the arcuate nucleus, and this population projects centrally rather than to the median eminence (Baker and Herkenham, 1995). In previous studies, GHS-R mRNA expression was localised in 30 ± 6 % of arcuate neurones that express somatostatin mRNA (Willesen et al., 1999). However the arcuate somatostatin neurones do not express Fos in response to systemic GHS (Dickson and Luckman, 1997), and somatostatin mRNA expression in the arcuate nucleus, unlike that in the periventricular nucleus, is unchanged following chronic i.c.v. infusion of GHS (Bailey et al., 1999a).

In addition, it has been reported that a subpopulation of NPY-producing neurones in the arcuate nucleus co-produces GABA (Horvath et al., 1997). Microinjection of either NPY or muscimol, a GABA_A receptor agonist, into the paraventricular nucleus stimulated food intake, and co-injection of these compounds elicited a significantly higher response than that evoked by either individual treatment (Pu et al., 1999). These findings suggest complementary actions of co-released NPY and GABA in stimulation of feeding. Thus, it is possible that activation of arcuate NPY neurones results in the co-release of NPY and GABA, and hence blocking NPY actions alone does not eliminate their inhibitory effects on the ventromedial hypothalamic nucleus.

According to accumulating evidence of NPY involvement in food intake, it was hypothesised that NPY neurones in the arcuate nucleus are sensitive to food deprivation or prolonged treatment of GHS. To investigate this question, we measured NPY mRNA expression in the arcuate nucleus of rats after chronic central administration of GHRP-6. Previous studies have shown no change in expression of NPY mRNA under these conditions, which seems surprising given the acute effects of GHS on NPY neurones, and given the effects of chronic GHS on food intake which seems therefore likely to be mediated by activation of NPY neurones. However it seemed possible that GHS produced an increased excitation of NPY neurones, leading to increased food intake, leading to increased satiety signals, leading in turn to inhibition of NPY neurones. We therefore looked at the effects of chronic GHS in conditions where the treated rats were not allowed to increase their food intake. The results of this experiment showed that as shown previously, chronic GHRP-6 had no significant effect on NPY mRNA expression in *ad lib* fed rats, despite producing an increase in food intake and body weight. Furthermore, food restriction produced, as expected, a significant increase in NPY mRNA expression, demonstrating our ability to measure an increase NPY mRNA expression by our techniques. However, in food restricted rats, chronic treatment with GHRP-6 produced no further increase in NPY expression above vehicle controls. Thus the increased food intake in rats treated chronically with GHS is not accompanied by increased expression of NPY mRNA, even in conditions where the rats are unable to satisfy an increased hunger drive by eating.

Although NPY cells that project from the arcuate nucleus to the paraventricular nucleus have been implicated in the control of feeding, previous studies have shown that few of these neurones are activated by GHS. Instead, the majority of neurones activated by GHS appear to project to the median eminence and not to the paraventricular nucleus. These neurones express NPY, but exactly what their functional role is remains to be determined.

In summary, *in vitro*, GHS activates neurones in the arcuate nucleus and the ventromedial hypothalamic nucleus whereas somatostatin and NPY inhibit these neurones. In both brain regions, many of neurones that were activated by GHS were also inhibited by NPY. I present a hypothesis to explain why GHS activates arcuate neurones but not ventromedial hypothalamic neurones *in vivo*. The immunohistochemistry study that applied the NPY Y1 receptor antagonist, revealed that ventromedial hypothalamic neurones were prevented from excitatory effect of GHS by NPY. The immunohistochemistry results also suggest that neurones in the ventromedial hypothalamic nucleus were affected by NPY release more than arcuate neurones. As well as, systemic treatment with GHRP-6 resulted in inhibition on ventromedial hypothalamic neurones. Finally, we discovered that NPY neurones in the arcuate nucleus increased NPY mRNA expression in response to food restriction but not in response to chronic treatment of GHRP-6, despite the orexigenic effects of GHRP-6.

Further studies

The expression of NPY mRNA was affected by long term fasting, but not by chronic central treatment of GHRP-6. Though, orexigenic effect of chronic administration of the secretagogue was significant. This suggests that there might be different brain regions that mediate hunger's effects and activate food intake. This raises the question about which central pathways that respond to hunger induced by chronic GHS. AGRP is among peptides speculated to play a role. AGRP mRNA level in the arcuate nucleus was previously demonstrated to be increased in parallel with NPY mRNA by central infusion of ghrelin every 12 h for 72 h (Kamegai et al., 2001b). However, it is yet to be identified whether the expression of AGRP mRNA is altered by a 7-day chronic administration of GHS. To understand the mechanism, *in situ* hybridisation is needed for this purpose. Additionally, detection of up- and down-regulation of receptors for

AGRP and NPY in hypothalamic brain regions in response to chronic administration of GHS might be able explain the mechanism.

In addition, muscimol, a GABA receptor agonist, was previously found to amplified NPY-induced food intake in sated rats (Pu et al., 1999). It was reported that a sub-population of NPY-producing neurones in the arcuate nucleus co-produces GABA (Horvath et al., 1997). This GABA pathway might as well responds to chronic administration of GHS or negative energy balance. For further studies, the expression of GABA mRNA in the arcuate nucleus might be examined.

Orexin-containing neurones in the lateral hypothalamus were also significantly activated by central administration of GHRP-6 (Lawrence et al., 2002). The expression of orexin mRNA in this brain area is also a subject to be investigated to clarify the central response to chronic treatment of GHS or food restriction.

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