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Pharmacological and Molecular Characterization of Endogenous Opioid Peptide-Induced Feeding Responses in Rats.

by

Robert Silva

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

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Abstract

PHARMACOLOGICAL AND MOLECULAR CHARACTERIZATION OF THE ENDOGENOUS OPIOID PEPTIDE-INDUCED FEEDING RESPONSES IN RATS

by

Robert Silva

Advisor: Professor Richard Bodnar

Central administration of the endogenous opioid peptides, β-endorphin and dynorphin potently stimulates ingestive behaviors. A series of four studies were conducted to elucidate the pharmacological mechanisms mediating these ingestive effects. The results of the first study demonstrated that β-endorphin-induced feeding was significantly attenuated by pretreatment with either general (NTX), μ (βFNA), δ (naltrindole) or κ_1 (NorBNI) opioid receptor antagonists. In addition, β-endorphin-induced feeding was significantly reduced following pretreatment with antisense targeting either exons 1, 3, or 4 of the MOR-1 gene, and exon1 of the DOR-1 gene. In contrast, antisense directed against any exons of either the KOR-1 or KOR-3/ORL-1 clones were ineffective, thereby suggesting that the primary opioid receptor mediating β-endorphin-induced feeding is the μ opioid receptor. Using a similar technique, the second study revealed that dynorphin-induced intake was also significantly reduced by pretreatment with either general-, κ_1-, δ- and μ- opioid antagonists. In contrast to β-endorphin-induced feeding, dynorphin-induced feeding was significantly reduced by antisense targeting either exons 1 and 2, but not 3 of the KOR-1 gene, exons 1 and 2, but not 3 of the KOR-3/ORL-1 gene, exon1, but not 2 or 3 of the DOR-1 gene, and exon1, but not 2, 3 or 4 of the MOR-1 gene, thereby suggesting that dynorphin-induced feeding is mediated primarily by the κ_1 opioid

receptor. The results from the third study revealed that feeding elicited by morphine and its active metabolite, morphine-6β-glucuronide (M6G) can be distinguished from each other using antisense targeting individual G-protein receptor subunits. Antisense targeting only the $G_i\alpha_2$ subunit significantly reduced morphine-induced feeding whereas antisense targeting either the $G_i\alpha_1$, $G_i\alpha_3$, or $G_z\alpha$ subunits significantly reduced M6G-induced feeding. The final study revealed that feeding elicited by β-endorphin and dynorphin were both significantly reduced by pretreatment with antisense targeting the $G_i\alpha_1$ subunit. In addition, dynorphin-induced feeding was also significantly reduced by pretreatment with antisense targeting the $G_o\alpha$ subunit. The common sensitivity β-endorphin and dynorphin-induced feeding suggests that this neuronal network may represent a final common pathway for a distinct class of opioid agonists.

I would like to thank my parents for their support and committment to my education.

A special thanks to my mentor and friend, Dr. Richard Bodnar whose knowledge and guidance continue to inspire my scientific endeavors.

To Tamara, whose patience, love, and support made this work possible.

Table of Contents

Page

List of Tables

List of Figures

List of Abbreviations

AS ODN: antisense oligodeoxnucleotide

βEND: β-endorphin, endogenous opioid peptide

βFNA: β-funaltrexamine, general μ receptor antagonist

DALCE: D-Ala^2, Leu^5,Cys^6-enkephalin, selective δ_1 receptor antagonist

DAMGO: D-Ala^2, met-Phe^4,Gly(ol)^5-enkephalin, selective μ receptor agonist

DOR-1: δ opioid receptor clone

DPDPE: D-Pen^2, D-Pen^5-enkephalin, selective δ_1 receptor agonist

GPRC: G-protein receptor complex

i.c.v.: intracerebroventricular

KOR-1: κ opioid receptor clone

KOR-3: κ_3 opioid receptor clone

MA: mercaptoacetate, lipoprivic antimetabolic agent

MS ODN: missense oligodeoxynucleotide

M6G: morphine-6β-glucuronide

MOR-1: μ opioid receptor clone

NalBzOH: naloxone benzoylhydrazone, selective κ_3 receptor agonist

NS ODN: nonsense oligodeoxynucleotide

NTII: naltrindole-5'-isothiocyanate, selective δ_2 receptor antagonist

NTX: naltrexone, general opioid antagonist

NorBNI: nor-binaltorphamine, selective κ receptor antagonist

NRM: nucleus raphe magnus

NRGC: nucleus reticularis gigantocellularis

NTS: nucleus tractus solitarius

ORL-1: orphan opioid receptor clone

OFQ/N: orphanin/nociceptin, endogenous opioid peptide

PVN: paraventricular nucleus of the hypothalamus

PAG: periaqueductal gray

POMC: proopiomelanocortin, endogenous opioid peptide precursor

2DG: 2-deoxy-D-glucose, antimetabolic agent

VMH: ventral medial nucleus of the hypothalamus

VTA: ventral tegmental area

CHAPTER 1. INTRODUCTION

The role of the endogenous opioid system in the modulation of ingestive behavior has been a source of intense study over the past quarter-century. Interactions between opioid peptides and agonists with their putative receptors induce feeding under a variety of environmental and homeostatic conditions (Gosnell and Levine, 1996). Following early studies of stereospecific binding properties of opioid compounds (Portoghese, 1966), subsequent biochemical assays revealed individual binding sites for opioid compounds (Pert and Snyder, 1973; Simon et al., 1973; Terenius, 1973) at multiple receptors (Martin et al., 1976). The pharmacological characterization of multiple opioid receptor subtypes evolved in parallel with the development of selective opioid receptor subtype agonists and antagonists. A variety of studies employing selective opioid agonists for μ (morphine, M6G & DAMGO), δ_1 (DPDPE), δ_2 (deltorphin II), κ_1 (U50,488H), and κ_3 (NalBzOH) receptors as well as selective opioid antagonists for mu (βFNA), μ_1 (naloxonazine), δ_1 (DALCE), δ_2 (NTII), and κ_1 (NorBNI) receptors have allowed for the investigation of opioid mediation of ingestive behaviors under different conditions. Selective agonists for all opioid receptor subtypes typically stimulate food intake, whereas selective antagonists for each of the opioid receptor subtypes typically reduce food intake and body weight. It should be noted that opioid agonists stimulate food intake over a short time course, and typically do not alter body weight. This ingestive response is distinguished from hyperphagia which is comprised of increased intake and increased weight gain. Also, the extent to which opioid compounds act to induce feeding is not limited to the pharmacological availability of these compounds at their putative receptors. The pharmacological actions of opioids induce a cascade of signaling events

involving different neurotransmitter systems which modulate complex factors involved in food intake including hedonic, metabolic and motivational mechanisms that contribute to the central control and maintenance of food intake (see review: Levine et al., 1985). However, the dose ranges employed in these pharmacological studies do not reflect the physiological levels in particular parts of the central nervous system during ingestion. Thus, opioid-mediated food intake is dependent upon both the pharmacological and physiological of opioid compounds for the full expression of opioid-induced feeding responses.

In addition to the early work involving exogenously-administered synthetic opiate drugs, the discovery by Hughes and co-workers (1975) of endogenous peptidergic ligands which interacted with opioid receptors, led to the pharmacological characterization of a number of biologically-active opioid peptides. Three original opioid peptides were derived from the cleavage of three precursor hormones, namely, pro-opiomelanocortin (POMC), pro-enkephalin and pro-dynorphin (see review: Akil et al., 1984). From these precursor molecules, a number of opioid peptides were isolated and found to induce feeding following direct central microinjection including β-endorphin (βEND) (Grandison and Guidotti, 1977; McKay et al., 1981), enkephalin analogues (Jackson and Sewell, 1985a) and dynorphin (Morley et al., 1982; Morley and Levine, 1983).

Biochemical βEND binding occurs at multiple opioid receptors (μ, δ, κ and proposed ϵ: Chang et al., 1979; Schulz et al., 1979; Akil et al., 1980). βEND stimulates food intake following microinjection into the hypothalamic ventromedial (Grandison and Guidotti, 1977) and paraventricular (Leibowitz and Hor, 1982) nuclei and nucleus accumbens (Majeed et al., 1986). Basal levels of pituitary and plasma βEND are elevated in genetically obese mice and

rats (Margules et al., 1978). Although these studies suggest a role for βEND in the mediation of ingestive behavior, they do not specify which opioid receptors participate in this feeding response. Opioid antagonist analyses of βEND feeding have been limited to goldfish, and reductions were observed following pretreatment with general (naloxone) and μ-selective (βFNA), but not by κ (NorBNI) or δ (7-binzidilidendenaltrexone and naltriben)-opioid receptor antagonists (DePedro et al., 1995, 1996).

Similarly, feeding is elicited following ventricular administration of dynorphin A_{1-17} and dynorphin A_{1-13} which are blocked by general opioid antagonist pretreatment (Gosnell et al., 1986a; Morley and Levine, 1981, 1983a; Morley et al., 1982; Walker et al., 1980). Although dynorphin-induced feeding has been postulated to result from direct interaction with κ opioid receptors (see reviews: Levine et al., 1985; Gosnell and Levine, 1996), only one selective antagonist study (Mann et al., 1988) demonstrated that dynorphin-induced feeding is unaffected by pretreatment with the selective μ_1 antagonist, naloxonazine.

The use of selective opioid receptor subtype antagonists is problematic as a unitary measure of pharmacological characterization of opioid agonists for the reason that antagonists are typically active at multiple opioid receptor subtypes. That is, a selective opioid receptor subtype antagonist often blocks the action of other selective opioid receptor subtype agonists. The inherent limitations in the exclusive use and subsequent interpretation of selective antagonist data suggest that further converging data are necessary for full characterization of opioid agonist effects.

Recent advances in the field of molecular biology have led to the cloning of the opioid receptor classes into MOR-1, DOR-1 and KOR-1 genes (see reviews: Uhl et al., 1994; Rossi

and Pasternak, 1997). These findings have established the usefulness of the antisense oligodeoxynucleotide (AS ODN) technique. The AS ODN technique utilizes short (18-25 nucleotide bases) probes complementary to specific, unique regions of mRNA from a targeted gene. Antisense probes apparently mediate their effects by hybridizing with mRNA transcripts and interfering with transcription and/or translation of the targeted gene to yield reductions in protein levels (see review: Myers and Dean, 2000). The selectivity and specificity of AS ODN probes far exceeds that of available antagonists. The AS ODN technique allows for the functional characterization of opioid receptors by correlating the molecular biology of the cloned receptors with in vivo opioid receptor pharmacology (see review: Pasternak and Standifer, 1995).

Initial studies using analgesic assays validated the efficacy of this technique by demonstrating that the administration of AS ODN probes directed against regions of the DOR-1 gene selectively reduced analgesia elicited by selective δ_1 (DPDPE) and δ_2 (deltorphin) opioid agonists, whereas AS ODN probes directed against specific regions of either MOR-1 or KOR-1 genes respectively reduced analgesia elicited by μ (morphine/DAMGO) or κ_1 (U50,488H) opioid agonists (Chien et al., 1994; Rossi et al., 1997b; Rossi and Pasternak, 1997). In addition, this technique has been especially useful in examining the differences between morphine- and M6G-induced feeding. Although μ antagonism blocks both morphine- and M6G-induced feeding, a detailed mapping study of the four coding exons of the MOR-1 gene indicated that AS ODN probes directed against either coding exons 1 or 4 of the MOR-1 gene blocked morphine-induced feeding, yet had no effect on M6G-induced feeding (Leventhal et al., 1998b). In contrast, AS ODN probes

directed against either coding exons 2 or 3 of the MOR-1 gene blocked M6G-induced feeding, yet were ineffective against morphine-induced feeding. These findings are nearly identical to the pattern of MOR-1 AS ODN effects upon morphine- and M6G-induced analgesia (Rossi et al., 1995a; Rossi and Pasternak, 1997; Rossi et al., 1995b).

Opioid receptors mediate their cellular signaling effects through the putative activation of guanine nucleotide binding proteins (G-proteins) to which they are coupled (Childers, 1988; Pasternak and Standifer, 1995; Reisine and Bell, 1993; Standifer et al.,1996; Uhl et al., 1994). G-proteins are composed of three distinct subunits (α, β, γ), which couple the receptors to their effectors. The α-subunit has been identified as the pharmacologically-relevant subunit due to its intrinsic GTPase activity, although the β and γ subunits can also modulate the activity of various effectors (see review: Standifer and Pasternak, 1997). The α-subunit also seems to establish the identity of G-protein-mediated opioid receptors. For example, whereas pertussis toxin (PTX) irreversibly inactivates a number of G-proteins, including $G_i\alpha$ and $G_o\alpha$, but not $G_s\alpha$, cholera toxin (CTX) selectively blocks the $G_s\alpha$ subtype (Gilman, 1987; Goode and Raffa, 1997; Hildebrandt et al., 1983; Katada and Ui, 1981; Stryer and Bourne, 1986). PTX pretreatment selectively blocks the analgesic responses to opioids including morphine, DAMGO, and sufentanil (Bodnar et al., 1990; Goode and Raffa, 1997). Therefore, the sensitivity to PTX-inactivation establishes a relationship between μ-mediated antinociception and inhibitory coupling to $G_i\alpha$ proteins.

The family of G-protein receptor complexes (GPRC) can be subdivided according to the identity of the α-subunit. μ-opioid receptors couple to multiple G-proteins to modulate the inhibition of cAMP (see reviews: Standifer and Pasternak, 1997; Roerig, 1998). Many

different G-proteins have been implicated in μ-opioid-mediated functions. Specifically, administration of antisera raised against $G_i\alpha_2$ significantly reduced the antinociceptive activity of DAMGO, morphine, β-endorphin, DPDPE, and Deltorphin II , whereas pre-treatment with antisera against $G_{x/z}\alpha$ attenuated DAMGO, β-endorphin, and morphine analgesia in mice (Sanchez-Blazquez et al., 1993; Garzon et al., 1994). Identical results were reported using AS ODN probes targeted against $G_i\alpha_2$, and $G_{x/z}\alpha$ subunits (Sanchez-Blazquez et al., 1995; Raffa et al., 1996).These results suggest that μ-mediated antinociception is linked to activation of $G_i\alpha_2$ and $G_{x/z}\alpha$ α-subunits. In contrast, antisera raised against $G_s\alpha$ enhanced both morphine and β-endorphin analgesia in mice (Sanchez-Blazquez and Garzon, 1992). Thus, the identity of G-protein α-subunits establishes a specificity profile among G-protein receptor complexes that allows for their classification.

Due to the success of the AS ODN technique in evaluating the functional effects of opioid receptor genes and G-protein activation in agonist-induced analgesia and feeding paradigms, this technique will be employed to investigate the pharmacological properties of feeding induced by the endogenous opioid peptides βEND and dynorphin A_{1-17}. Specifically, the present proposal attempts to elucidate the opioid receptor subtypes involved in the mediation of βEND (Experiment 1) and dynorphin A_{1-17} (Experiment 2)-induced feeding by utilizing selective opioid receptor subtype antagonists as well as AS ODN probes directed against the individual coding exons of the MOR-1, DOR-1, KOR-1 and KOR-3 opioid receptor genes. In addition, the present dissertationl will also attempt to classify the individual G-protein sensitivity profiles for feeding induced by the μ agonists morphine and M6G (Experiment 3) as well as for βEND and dynorphin-induced feeding (Experiment 4) using

AS ODN probes directed against individual G-protein α-subunits. Therefore, the Specific Aims of the dissertation are as follows:

1. **The first experiment will evaluate alterations in food intake in rats elicited by the endogenous opioid peptide, β-endorphin following pretreatment with equimolar doses of either general (NTX), μ- (βFNA), δ- (naltrindole) and κ- (NorBNI)-opioid receptor antagonists. The effects of ventricularly administered AS ODN probes directed against each of the coding exons of the MOR-1, DOR-1, KOR-1 and KOR-3 opioid receptor genes, as well as a control missense probe, upon β-endorphin-induced feeding will also be evaluated.**
2. **The second experiment will evaluate alterations in food intake in rats elicited by the endogenous opioid peptide, Dynorphin A_{1-17} following pretreatment with equimolar doses of either general (NTX), μ- (βFNA), δ- (naltrindole) and κ- (NorBNI)-opioid receptor antagonists. The effects of ventricularly administered AS ODN probes directed against each of the coding exons of the MOR-1, DOR-1, KOR-1 and KOR-3 opioid receptor genes, as well as a control missense probe, upon dynorphin-induced feeding will also be evaluated.**
3. **The third experiment will evaluate the effects of ventricularly administered AS ODN probes directed against either the $G_i\alpha_1$, $G_i\alpha_2$, $G_i\alpha_3$, $G_s\alpha$, $G_o\alpha$, $G_{z/2}\alpha$, or $G_q\alpha$ G-protein α-subunits as well as a control nonsense probe upon food intake induced by either morphine or**

morphine-6β-glucoronide in rats.

4. **The fourth experiment will evaluate the effects of ventricularly administered AS ODN probes directed against either the $G_i\alpha_1$, $G_i\alpha_2$, $G_i\alpha_3$, $G_s\alpha$, $G_o\alpha$, $G_{v/z}\alpha$, or $G_q\alpha$ G-protein α-subunits as well as a control nonsense probe upon food intake induced by either β-endorphin or dynorphin A_{1-17} in rats.**

The following sections provide background information regarding: I) Opioid Peptides, II) Opioid Receptor Subtypes, III) Opioid Receptor Genes, IV) the AS ODN technique, V) G-protein Receptor Complexes VI) Opioid Peptides and Ingestive Behavior.

I. Opioid Peptides.

Opioid peptides are derived from one of four gene precursor peptides: proopioimelanocortin (POMC), proenkephalin, prodynorphin and pro-orphanin/pro-nociceptin. The first three of these peptides are characterized by an opioid-active core sequence of Tyr-Gly-Gly-Phe-, while the latter peptide precursor varies from this typical opioid motif (see review: Sherman et al., 1989; Mansour et al., 1995; Meunier et al., 1995; Reinscheid et al., 1995). Biochemical analyses revealed that the first three of these parent peptides (POMC, proenkephalin, prodynorphin) result from three distinct genes (see review: Akil et al., 1984). The latter peptide (pro-orphanin) was not derived from any previously known precursors and may comprise a new family of opioid peptides (Alt et al., 1998). Each of these large parent peptides undergo enzymatic cleavage to yield many smaller biologically-active peptides that can be divided into four major families based on their respective precursors.

A. Proopioimalanocortin (POMC).

The POMC precursor, through post-translational processing yields ACTH, α, β, and γ forms of melanocyte stimulating hormone (MSH) and β-lipotropin (βLPH) (Eipper and Mains, 1978; Mains et al., 1977; Roberts et al., 1979). In turn, the C-terminal fragment of βLPH contains a 31 amino acid peptide named β-endorphin (βEND). However, of all POMC cleavage products, only βEND possesses opioid activity (Mains et al., 1977). The first major cell group of neuronal βEND is in the hypothalamic arcuate nucleus (Watson et al., 1978), which projects rostrally to innervate the preoptic area, septum and bed nucleus of the stria terminalis, laterally to innervate other hypothalamic nuclei, temporal cortex and the amygdala, and caudally to innervate the periventricular thalamus, periaqueductal gray (PAG), nucleus raphe magnus, nucleus reticularis gigantocellularis, locus coeruleus, nucleus of the vagus, and the lateral reticular nucleus. The second major β-endorphin cell group is located in the caudal portion of the nucleus tractus solitarius and projects laterally to innervate the lateral reticular nucleus (Khachaturian et al., 1985).

Several binding studies have suggested that although βEND binds to at all three opioid receptor subtypes (μ, δ, κ and proposed ϵ receptors), it binds with the greatest affinity at both μ and δ receptors in vitro (Akil et al., 1980; Lord et al., 1977; Schulz et al., 1979). More recently, Alt and co-workers (1998) established that βEND binding was nearly identical to that of the selective μ agonist DAMGO in competition binding experiments, thereby confirming that βEND is a full agonist at the pharmacologically-characterized μ opioid receptor. This study also confirmed earlier experiments which demonstrated that both met- and leu-enkephalin were potent agonists of μ receptors in primary cultures of rat zona

glomerulosa cells (Kapas et al., 1995).

B. Prodynorphin.

Cleavage of prodynorphin yields three major peptide products, each containing a leu-enkephalin core: α- and β-neoendorphin, dynorphin A_{1-17}, and dynorphin B (Fischili et al., 1982; Goldstein et al., 1979, 1981; Kakidani et al., 1982; Kangawa et al., 1981). Further, enzymatic cleavage of dynorphin A_{1-17} yields several biologically-active C-terminal cleavage products including dynorphin A_{1-8} as well as several other intermediate length peptides (Seizinger et al., 1981; Suda et al., 1982). Immunoreactive dynorphin perikarya are distributed in several telencephalic (cerebral cortex, striatum, amygdala and hippocampus), diencephalic (supraoptic, paraventricular (PVN), and arcuate nucleus of the hypothalamus), mesencephalic (PAG), and brainstem (parabrachial and spinal trigeminal nucleus, NTS, lateral reticular nucleus) structures as well as in the dorsal and ventral horns of the spinal cord (Khachaturian et al., 1982; Robson et al., 1983; Watson et al., 1982). Dynorphin-containing cells in the parabrachial nucleus, PVN, as well as dorsal and lateral hypothalamus send long projections to the spinal cord, whereas dynorphinergic cells in the dorsal parabrachial nucleus send direct projections to the central nucleus of the amygdala (Code and Fallon, 1986). In addition, most dynorphin perikarya in the PVN of the hypothalamus co-exist with vasopressin in the magnocellular nuclei (Watson et al., 1982).

Several studies have established the selectivity of dynorphin for the κ opioid receptor in vitro (Chavkin and Goldstein, 1981; Chavkin et al., 1982; James et al., 1982a, 1982b). The activity of dynorphin at κ opioid receptors was first established by Huidobro-Toro and co-workers (1981) in which dynorphin activity at the guinea pig ileum was effectively blocked

by pretreatment with the putative κ opioid receptor antagonist ketocyclazocine. Further, competition binding analysis using dynorphin and the irreversible opioid antagonist β-chlornaltrexamine (CNA) revealed that pretreatment with high concentrations of dynorphin resulted in the selective protection of κ, but not μ or δ opioid binding sites, thereby implicating highly selective κ receptor binding by dynorphin (James et al., 1982a). Thus, there is substantial evidence to suggest that dynorphin is the endogenous ligand of the pharmacologically described κ opioid receptor.

C. Proenkephalin.

Post-translational processing of proenkephalin yields several biologically active opioid peptides including four copies of methionine-enkephalin, as well as individual species of leucine-enkephalin, met-enkephalin-Arg^6-Phe^7, and met-enkephalin-Arg^6-Gly^7-Leu^8 (MERGL) (Comb et al., 1982; Kimura et al., 1980). The biological activity of proenkephalin cleavage products, met- and leu-enkephalin, was first established by Hughes and co-workers (1975) following purification of this peptide from brain tissue. Immunoreactive enkephalinergic cells are found in telencephalic structures such as the cerebral cortex, olfactory tubercle, amygdala, bed nucleus of the stria terminalis and the preoptic area (Khachaturian et al., 1983). In the diencephalon, structures such as the hypothalamus and the periventricular and lateral geniculate nuclei of the thalamus as well as midbrain structures such as superior and inferior colliculi, PAG and interpeduncular nucleus also contain immunoreactive enkephalin perikarya (Hokfelt et al., 1977; Watson et al., 1977). In the pons and medulla, perikarya are found in the raphe nuclei, nuclei reticularis gigantocellularis and paragigantocellularis, nucleus tractus solatarius, the lateral reticular nuclei, spinal trigeminal

nucleus and the spinal cord dorsal gray (Khachaturian et al., 1983, 1985, Sar et al., 1978).

D. Proorphanin/Pronociceptin.

Orphanin/nociceptin (OFQ/N) is a recently isolated heptadecapeptide that bears structural similarity to dynorphin A_{1-17} (Meunier et al., 1995; Reinscheid et al., 1995). Isolation of cDNA encoding the larger precursor peptide, proorphanin/pronociceptin revealed that this prohormone contained one copy of the 17 amino acid peptide, OFQ/N (Meunier, 1995). The presence of highly conserved cleavage sites located both upstream and downstream from OFQ/N on the precursor peptide suggests that proorphanin/pronociceptin may yield additional peptides as a result of enzymatic cleavage (Meunier et al., 1995; Reinscheid et al., 1995). Unlike classic opioid peptides, OFQ/N does not contain a Tyr-Gly-Gly-Phe- -terminal core sequence, but rather has a Phe-Gly-Gly-Phe motif. Further, unlike traditional opioid peptides, it binds with very low affinity to classical opioid receptor subtypes. Immunohistochemical and autoradiographic analyses have identified proorphanin/pronociceptin gene expression in the amygdala, subthalamic nuclei, hypothalamus, substantia nigra, thalamus, central gray, central tegmental field, nucleus of the lateral lemniscus, superior olive and spinal trigeminal nucleus of the brainstem (Houtani et al., 1996; Nothacker et al., 1996). Immunochemical analysis revealed that neuronal OFQ/N is localized in the sensory trigeminal complex, raphe nuclei, locus coeruleus, PAG, amygdala, hypothalamus, superficial dorsal horn, bed nucleus of the stria terminalis, medial preoptic area, lateral septum and median eminence (Henderson and McKnight, 1997; Riedl et al., 1996; Schulz et al., 1996).

E. Endomorphins.

Endomorphins are the most recent opioidergic peptides to be isolated from brain tissue. Zadina and co-workers (1997) identified two distinct peptides whose N-terminal sequence differs significantly from classic opioid peptides: endomorphin-1 (Tyr-Pro-Trp-Phe-NH_2) and endomorphin-2 (Tyr-Pro-Phe-Phe-NH_2). Based on preliminary immunochemical assays, endomorphin-1 has been localized to neuroanatomical regions including the thalamus, hypothalamus, cortex and striatum (Zadina et al., 1997). Endomorphin-2-like immunoreactivity has been localized in the medulla, as well as dorsal root and dorsal root ganglia of the spinal cord (Martin-Schild et al., 1997). However, a peptide precursor has not yet been identified.

II) Opioid Receptor Subtypes.

Early biochemical assays revealed individual structure-activity relationships involving opioidergic compounds (Portoghese, 1966, 1970). Despite these initial findings, no clear receptor mechanism emerged which classified the pharmacological mechanism of these opioid agents. Prior to 1973, attempts to biochemically label opioid receptors using ligand binding were unsuccessful until three independent laboratories (Pert and Snyder, 1973; Simon et al., 1973; Terenius, 1973) demonstrated opioid binding sites using radiolabeled opioid ligands. Following these seminal discoveries, Martin and co-workers (1976) identified naloxone-reversible effects of three different opioid agonists morphine, ketocyclazocine and SKF 10047 in chronic spinal dogs. The three different agonist-induced behavioral effects of these agonists were distinguishable phenotypically as well as by the lack of cross-tolerance observed in this study. These findings led to the proposal of three distinct receptors each selectively sensitive to binding by their respective agonists: μ (morphine), κ

(ketocyclazocine), and σ (SKF 10047). It should be noted that the lack of naloxone-reversible effects by σ agonists has subsequently led to the characterization of the σ receptor as a non-opioid receptor (Vaupel et al., 1983; Zukin et al., 1984). Following the full characterization of enkephalin peptides, Lord and co-workers (1977) confirmed, using an [^{3}H]-leu-enkephalin assay, that enkephalinergic peptides were putative ligands of a novel opioid receptor termed the δ receptor (Lord et al., 1977). Since these seminal studies, a large body of evidence has emerged to support the concept of opioid receptor multiplicity (see reviews: Pasternak and Wood, 1986; Pasternak, 1993; Zukin and Zukin, 1981). Indeed, the development of more specific opioid agonists and antagonists has provided both pharmacological and biochemical evidence to suggest that μ (Pasternak and Wood, 1986), δ (Jiang et al., 1991; Mattia et al., 1991), and κ (Rothman et al., 1990) opioid receptors can be divided further into sub-classes of their respective opioid receptor subtype.

A. μ opioid receptor.

Anatomical distribution of μ receptors is diffuse in the forebrain, midbrain and hindbrain. Neuroanatomical regions displaying the most dense distribution of μ receptors include the olfactory bulb, nucleus accumbens, caudate-putamen, thalamus, hippocampus, amygdala, superior and inferior colliculi, NTS, spinal trigeminal nucleus, diagonal band of Broca, bed nucleus of the stria terminalis, medial preoptic area, PAG, dorsal and median raphe, NRM, LC, parabrachial nucleus, pontine and medullary reticular nuclei, nucleus ambiguous, nucleus gracilis and cuneatus, dorsal motor nucleus of vagus, laminae I and II of the spinal dorsal horn, and dorsal root ganglia (Ding et al., 1996; Mansour et al., 1988, 1994). Pharmacological characterization of the μ receptor has been accomplished by the use of

highly selective μ receptor agonists (i.e., DAMGO: Handa et al., 1981), as well as selective antagonists (i.e., βFNA: Portoghese et al., 1980; Takemori et al., 1981, and Cys^2-Tyr^3-Orn^5-Pen^7, CTOP: Gulya et al., 1986). Both pre-synaptic and post-synaptic localization of μ receptors has been reported (Elde et al., 1995).

The development of highly-selective μ receptor antagonists led to the further sub-classification of the μ opioid receptor. Whereas the μ_1 opioid receptor subtype binds most enkephalins and βEND with similar very high affinities, the μ_2 opioid receptor subtype selectively binds morphine-like compounds more potently than enkephalins (see review: Pasternak and Wood, 1986). Using both in vitro and in vivo biochemical assays, both naloxazone and naloxonazine were shown to act as irreversible and selective antagonists of the proposed μ_1 opioid receptor subtype (Hahn et al., 1982; Ling et al., 1986; Pasternak and Hahn, 1980; Pick et al., 1991). In addition, CTOP and βFNA act as respective reversible and irreversible antagonists at both μ_1 and μ_2 opioid receptor subtypes (Portoghese et al., 1980). Therefore, an important tool for distinguishing potential agonist effects between these two sites is to compare the individual antagonist effects of β-FNA and naloxonazine following agonist administration. Moreover, autoradiographic studies revealed similar, but not identical distributions of μ_1 and μ_2 opioid receptors (Goodman and Pasternak, 1985; Moskowitz and Goodman, 1985). Importantly, these two receptor subtypes have also been distinguished from each other in analgesic assays. CXBK mice, which display a selective loss of μ_1, but not μ_2 receptors were found to be less sensitive to analgesia induced by supraspinal or systemic administration of morphine without showing changes in spinal analgesic sensitivity, thereby indicating a supraspinal mechanism for the μ_1 receptor (Bodnar et al., 1988; Paul et al., 1989;

Rossi et al., 1993). The sensitivity of CXBK mice to intrathecal morphine suggests that the μ_2 receptor appears to play a role in the spinal mediation of morphine-induce analgesia.

B. κ opioid receptors.

Based on the seminal work completed by Martin and co-workers (1976), the κ opioid receptor was characterized by the agonist actions of ketocyclazocine at this receptor binding site. Subsequent work with dynorphin-based peptides also revealed putative activation of the κ opioid receptor by these peptides (Chavkin and Goldstein, 1981; Huidobro-Toro et al., 1981; Wuster et al., 1981). In general, κ receptor binding is most dense in the caudate-putamen, nucleus accumbens, amygdala, hypothalamus, neural lobe of the pituitary, median eminence, and NTS. Moderate binding is observed in the PAG, raphe nuclei, spinal trigeminal nucleus and dorsal horn (Mansour et al., 1988).

The availability of selective κ agonists such as U50,488 and U69,593 and κ-selective antagonists such as norbinaltorphamine (NorBNI), led to the establishment of three pharmacologically distinct κ opioid receptor subtypes. The κ_1 receptor subtype is classified based on the selective actions of the agonists, U50,488H and U69,593 as well as the antagonist NorBNI (Portoghese et al., 1987; VonVoigtlander et al., 1983). Zukin and co-workers (1988) used an [^{3}H]ethylketocyclazocine competition binding paradigm to establish the binding properties of various κ-selective agonists in both guinea pig and rat brain tissue preparations. Whereas the guinea pig brain preparations revealed a single population high-affinity ([^{3}H]EKC) of κ binding sites, the rat brain preparations contained two distinct populations of both high-affinity and low-affinity κ binding sites. Further, this study revealed that the κ agonist U69,593 selectively displaced the high-affinity site in both guinea pig and

rat preparations, but was ineffective at the rat low-affinity site, whereas the κ agonists U50,488H, EKC, bremazocine, cyclazocine and dynorphin competed for both sites, albeit with different potencies. These results provided strong evidence suggesting the existence of pharmacologically distinct κ_1 (U69,593-sensitive) and κ_2 (U69,593-insensitive) opioid receptor subtypes. In addition, subsequent studies using a tritiated preparation of the κ agonist, naloxone benzoylhydrazone (*NalBzOH*) provided evidence in support of the existence of a novel, U50,488H-insensitive κ_3 opioid receptor subtype (Clark et al., 1989; Gistrak et al., 1989; Paul et al., 1990). Thus, full characterization of the κ opioid receptor has provided strong evidence for the existence of three pharmacologically-distinct κ receptor subtypes.

C. δ opioid receptors

Cellular localization of δ opioid receptor mRNA revealed that the distribution of δ receptors differs from μ or κ receptor distributions, with expression identified in the olfactory bulb, allo- and neocortex, caudate-putamen, nucleus accumbens, olfactory tubercle, ventromedial hypothalamus, hippocampus, amygdala, red nucleus, pontine nuclei, reticulotegmental nucleus, motor and spinal trigeminal nuclei, linear nucleus of the medulla, lateral reticular nucleus, spinal cord, and dorsal root ganglia (Mansour et al., 1994). Initial characterization of the δ opioid receptor was based on pharmacological assays which utilized leu-enkephalin and enkephalin analogues such as D-Ser^2,Leu-enkephalin-Thr^6 (DSLET) and D-Ala^2, D-Leu^5-enkephalin (DADL) as general δ opioid receptor agonists (Lord et al., 1977; Mosberg et al., 1983), as well as the general δ receptor antagonists ICI174864 (Cotton et al., 1984) and naltrindole (Portoghese et al., 1988).

Further classification of the δ opioid receptor occurred as a result of the development of novel δ receptor agonists and antagonists. The δ_1 receptor has been pharmacologically characterized by the agonist D-Pen^2, D-Pen^5-enkephalin (DPDPE: Mosberg et al., 1983) as well as long term actions of the antagonist D-Ala^2,Leu^5,Cys^6-enkephalin (DALCE) (Bowen et al., 1987; Jiang et al., 1990). The δ_2 receptor subtype has been characterized by the agonist actions of D-Ser^2, Leu^5-enkephalin-Thr^6 (DSLET) and D-Ala^2,Glu^4-deltorphin (Stewart et al., 1994; Jiang et al., 1991) as well as by the antagonist actions of naltrindole 5'-isothiocyanate (NTII) and the naltrindole analogue, Naltriben (NTB) (Portoghese et al., 1990; Stewart et al., 1994). As was previously established for the μ receptor, analgesic assays identified distinct cross-tolerance differences as well as differential antagonist sensitivities among the δ receptor agonists D-Ala^2-deltorphin II and DPDPE (Jiang et al., 1991; Mattia et al., 1992). These data provide strong support for the existence of multiple δ opioid receptor subtypes.

III) Opioid Receptor Genes.

Many of the selective agonists and antagonists utilized in pharmacological research exhibit a high degree of selectivity and specificity. However, there are several experimental pitfalls associated with the exclusive use of selective agonists and antagonists. First, under certain conditions, the selectivity of some agonists and antagonists has been challenged. For example, although NorBNI has been characterized as a selective and reversible κ_1-opioid receptor antagonist (Portoghese et al., 1987), it also displays long durations of action (Horan et al., 1992) and blocks analgesia elicited by μ, δ and κ-opioid agonists following chronic antagonist administration (Spanagel et al., 1994). Similarly, the δ-selective opioid antagonist naltrindole can also exert antagonist actions at μ-receptors (Portoghese et al., 1988). In

addition, large variations in molecular weight displayed by different antagonists necessitate the use of equimolar dose ranges when interpreting antagonist data. The inherent limitations in the exclusive use of agonists and antagonists weakens their potential experimental utility. The characterization of opioid receptor pharmacology has been aided by the cloning of the traditional opioid receptor subtypes. The isolation of the cDNA sequences encoding the opioid receptors has yielded several opioid receptor genes confirming earlier pharmacological and biochemical data which suggested the existence of these receptor subtypes.

In 1992, two independent laboratories successfully developed a cloning strategy which yielded a cDNA sequence encoding the mouse δ opioid receptor gene (DOR-1), and thereby provided the first molecular characterization of opioid receptors (Evans et al., 1992; Kieffer et al., 1992). Based on the identified amino acid sequence of the DOR-1 gene, several groups successfully cloned the cDNA sequences encoding the μ- (MOR-1), κ- (KOR-1) and κ_3 (KOR-3/ORL-1) opioid receptors (see reviews: Kieffer, 1995; Uhl et al., 1994). Each of the opioid receptor genes was found to be a G-protein receptor complex (GPRC) containing seven transmembrane-spanning regions and shared high amino acid sequence homology (65-70%) with one another, primarily in their transmembrane hydrophobic regions and intracellular loops (Reisine and Bell, 1993). Originally, the DOR-1, KOR-1, and KOR-3/ORL-1 genes were found to each contain three coding exons, whereas the MOR-1 gene contained four coding exons. However, subsequent molecular cloning has established many more coding exons contained within these genes (Pan et al., 1999).

A. MOR-1 gene

Based on the sequence previously identified for the DOR-1 gene isolated from mouse,

Chen and colleagues (1993a, 1993b) created a DOR-1 probe via the PCR assay and subsequently isolated a novel cDNA which was later characterized as the rat μ opioid receptor gene (MOR-1). Using similar techniques involving the DOR-1 gene sequence, other groups isolated MOR-1 cDNA sequences from different types of rat brain tissue such as the olfactory bulb (Thompson et al., 1993). Subsequent studies also identified the mouse (Kaufman et al., 1995) and human (Wang et al., 1994) counterparts of the originally characterized rat DOR-1 gene. In vitro analyses revealed that the MOR-1 clone displayed high affinity for μ-selective opioid agonists such as βEND ($Ki \leq 10^{-9}$ nM) as well as μ-selective antagonists such as βFNA and naloxonazine ($Ki \leq 10^{-8}$ nM), while displaying a much lower affinity for δ- (DPDPE) ($Ki \geq 10^{-6}$ nM) and κ- (U50,488H) ($Ki \geq 10^{-6}$) selective ligands (Chen et al., 1993a; Raynor et al., 1993; Wang et al., 1993). Like DOR-1, recombinant analysis of the MOR-1 clone revealed agonist-induced inhibition of cAMP, suggesting its coupling to adenylyl cyclase (Piros et al., 1995). In situ hybridization and immunhistochemical localization have identified the MOR-1 receptor gene in the nucleus accumbens, caudate-putamen, diagonal band of Broca, globus pallidus, bed nucleus of the stria terminalis, most thalamic nuclei, medial and cortical amygdala, mammillary nuclei, presubiculum, interpeduncular nucleus, median raphe, NRM, parabrachial nucleus, locus coruleus, nucleus ambiguous, NTS and spinal cord (Arvidsson et al., 1995b; Mansour et al., 1995). These findings correlate well with neuroanatomical localization of endogenous μ opioid receptors using traditional autoradiographic techniques.

B. KOR-1 gene

While searching for somatostatin receptor genes in mice, Yasuda and colleagues

(1993) identified a gene found to encode the mouse κ opioid receptor. Based on the originally-identified DOR-1 gene, Chen and colleagues (1993b) isolated a KOR-1 gene in the rat genome. A number of other laboratories also isolated the KOR-1 gene in rat (Meng et al., 1993; Minami et al., 1993) and later in (Simonin et al., 1995; Zhu et al., 1995) cell lines. In vitro binding studies demonstrated that the KOR-1 receptor clone retained a very high affinity for dynorphin A ($Ki=10^{-10}$ nM), and selective κ opioid receptor agonists such as U50,488H ($Ki \leq 10^{-8}$ nM) as well as κ-selective antagonists such as NorBNI ($Ki \leq 10^{-10}$), yet little or no affinity for enkephalins, δ- and μ-selective ligands ($Ki \geq 10^{-6}$) (Raynor et al., 1993; Yasuda et al., 1993). Like DOR-1 and MOR-1, the KOR-1 receptor clone mediates agonist-induced inhibition of cAMP formation indicating the inhibition of adenylyl cyclase (Lai et al., 1995). In situ hybridization has identified KOR-1 receptor mRNA in the nucleus accumbens, VTA, caudate-putamen, olfactory tubercle, bed nucleus of the stria terminalis, medial preoptic area, PVN, supraoptic, dorsomedial and VMH, amygdala, midline thalamic nuclei, PAG, raphe nuclei, locus coruleus, spinal trigeminal nucleus and NTS. The mRNA localization technique used in this study confirmed previous autoradiographic κ-sites using κ-selective ligands and identified previously-unrecognized κ opioid receptor sites (Mansour et al., 1995).

C. DOR-1 gene

Two independent laboratories successfully cloned and isolated the δ opioid receptor from mouse cDNA (Evans et al., 1992; Kieffer et al., 1992). Both laboratories isolated this receptor by constructing a cDNA library from a NG108-15 cell line known to express high levels of the δ opioid receptor. To express the δ receptor in mammalian cells, the cDNA library was transfected into COS cells, which do not express opioid receptors. High-affinity

labeling of transfected cells using radiolabeled enkephalin peptides was used to identify the receptor-expressing cells. The isolated cDNA encoded a 372 amino acid protein whose primary structure resembled the pharmacologically-described endogenous δ opioid receptor. Subsequent work revealed counterpart genes in other species such as the rat (Fukuda et al., 1993) and human (Knapp et al., 1994) which were both highly homologous to the original DOR-1 gene isolated from mouse.

The pharmacological characterization of the DOR-1 gene product was very similar to that of the native δ opioid receptor (Kieffer et al., 1992). The DOR-1 cloned receptor displayed high affinity for the enkephalin peptides leu- and met-enkephalin (Ki $<10^{-8}$ nM), and δ-selective agonists and antagonists such as naltrindole (Ki $\leq 10^{-10}$ nM) (Kieffer et al., 1992; Raynor et al., 1993; Yasuda et al., 1993). In addition, further characterization of the DOR-1 clone revealed that these receptors display signal transduction properties identical to those of native δ receptors. Law and co-workers (1994) used a recombinant technique to express DOR-1 receptor clones in CHO cell lines and observed agonist-induced inhibition of cAMP, suggesting the coupling of the cloned receptor to the adenylyl cyclase effector system. Both in situ hybridization and immunohistochemical techniques allowed for the neuroanatomical localization of opioid receptor clones. Both of these techniques have identified the DOR-1 receptor gene in the anterior olfactory nucleus, neorcortex, caudate-putamen, nucleus accumbens, olfactory tubercle, diagonal band of Broca, globus pallidus, ventral pallidum, septal nuclei, amygdala, VMH, pontine nuclei, brainstem and spinal cord (Arvidsson et al., 1995a; Mansour et al., 1995). These distributions closely correlated with previously identified distributions of native δ opioid receptors (Simonin et al., 1994).

D. KOR-3/ORL-1 gene

In addition to the three classic types of opioid receptors, a previously unrecognized KOR-3/ORL-1 opioid receptor was the fourth member of the opioid receptor family to be cloned in mouse, rat and human by hybridization screening at low stringency with classic opioid receptor subtype cDNA probes (Bunzow et al., 1994; Fukada et al., 1994; Keith et al., 1994; Mollereu et al., 1994; Pan et al., 1994, 1995; Wang et al., 1994; Wick et al., 1994). The KOR-3/ORL-1 gene encoded a protein with a primary structure very similar to those of previously-identified opioid receptors (63-65% amino acid sequence homology) (see review: Meunier, 1995). In 1995, two laboratories independently identified the endogenous ligand for this receptor named nociceptin/orphaninFQ (OFQ/N) (Meunier et al., 1995; Reinscheid et al., 1995). Despite displaying no affinity for traditional opioid receptors, the KOR-3/ORL-1 receptor clone binds OFQ/N with high affinity and mediates agonist-induced inhibition of adenylyl cyclase activity (Meunier et al., 1995; Reinscheid et al., 1995). Although potent opioid agonists such as etorphine, cyclazocine and nalorphine each inhibited adenylyl cyclase activity at the KOR-3/ORL-1 receptor, morphine, DAMGO, U50,488H and DPDPE were completely inactive (Pan et al., 1995). Thus, unlike classic opioid receptors, the KOR-3/ORL-1 receptor clone has very low affinity for traditional opioid compounds despite OFQ/N's structural similarities to dynorphin A (Meunier et al., 1995). *In situ* hybridization and immunohistochemical localization have identified high concentrations of KOR-3/ORL-1 receptor mRNA in the PVN, VMH, amygdala, pyriform cortex, thalamus, hippocampus, PAG and spinal cord (Anton et al., 1996; Fukada et al., 1994; Chen et al., 1994; Wick et al., 1994).

The successful cloning of individual opioid receptors has allowed for a detailed

examination of opioid receptor pharmacology in vitro, and opioid receptor localization. Based on these advances, the roles of the individual opioid receptors in the mediation of opioid-mediated behaviors can be assessed. Specifically, the receptor pharmacology of opioid-induced feeding can be explored using selective opioid receptor antagonists.

E. General Opioid Antagonism and Ingestive Behavior

The use of general and selective opioid antagonists in the study of ingestive behavior suggests that the endogenous opioid system modulates feeding under a variety of complex challenge situations. The advantage of this manipulation is that selective blockade of opioid receptors by antagonist treatment allows for the monitoring of behavioral changes in the absence of receptor activity. However, selective opioid agonist treatment is additive to basal endogenous opioid functions and thus may hinder interpretation of behavioral data. Thus, systemic naloxone decreased feeding in rats under spontaneous (Jalowiec et al., 1981) and nocturnal (Brands et al., 1979) conditions. In addition, this treatment also effectively blocked feeding induced by deprivation (Brands et al., 1979), 2DG-induced glucoprivation, insulin glucoprivation, and both stress- and hypothalamic stimulation-induced feeding (Lowy et al., 1980; Morley and Levine, 1980; Carr and Simon, 1983; Jenck et al., 1986). Opioid-mediated feeding paradigms also revealed that pretreatment with naloxone reduced feeding induced by exposure to a high-fat diet (Cooper et al., 1985; Islam and Bodnar, 1990), thus suggesting that opioids also mediate food intake by modulation of dietary fat intake. Pretreatment with naloxone has also been shown to effectively reduce palatable intake induced by sucrose (Siviy and Reid, 1983), saccharin (Cooper, 1983) and maltose dextrin (Beczkowska et al., 1993). Also, chronic naloxone administration decreases food intake and body weight in rats (Mann

et al., 1988b; Marks-Kaufman et al., 1984; Olson et al., 1985).

The use of selective opioid antagonists has firmly implicated opioid receptors in the mediation of ingestive behaviors. For example, central injections of the μ-selective opioid antagonist βFNA, was shown to decrease food intake induced by deprivation, and the μ-selective agonist DAMGO (Levine et al., 1991). Moreover, μ antagonism with βFNA also decreased feeding under lipoprivic (Stein et al., 2000) and glucoprivic (Arjune et al., 1990; Beczkowska et al., 1993) conditions. Antagonism of the κ opioid receptor by pretreatment with the κ-selective opioid antagonist NorBNI also potently blocks nocturnal- and 2DG-induced feeding as well as sucrose and high-fat intake (Arjune and Bodnar, 1990; Beczkowska et al., 1993; Stein et al., 2000). In contrast, pretreatment with δ-selective opioid antagonists has yielded conflicting results. Feeding induced by the δ opioid receptor agonist DPDPE was blocked by pretreatment with the δ_2-selective antagonist NTII, but was unaffected by pretreatment with the δ_1-selective antagonist DALCE (Yu et al., 1997). However, feeding induced by a different δ-selective agonist, deltorphin was blocked by both NTII and DALCE (Yu et al., 1997). Therefore, the role of the δ opioid receptor in the mediation of food intake is consistent with a modulatory, rather than direct role for this receptor in ingestive behavior.

IV) The AS ODN Technique

Advances in molecular cloning techniques confirmed prior biochemical and pharmacological evidence indicating the binding properties and localization of opioid receptor subtypes. Moreover, these methods have led to the discovery of a novel opioid receptor (KOR-3/ORL-1) and the isolation of its endogenous ligand OFQ/N. However, the cloning of

different opioid receptor subtypes does not completely account for the pharmacologically-characterized subtypes of μ, δ and κ opioid receptors. There are two principal methods typically used to investigate opioid receptor genes in vivo: 1) the knockout and 2) knockdown approaches. The knockout technique involves a gene deletion, or the irreversible removal of a given gene from the genome. Despite the usefulness of this approach, plieotropic effects, the potential compensatory expression of genes other than the knockout gene, can hinder the assessment of the functional relevance of the deleted gene (Wahlestedt, 1994). In addition, this technique is quite labor-intensive and costly. Most importantly, the prototypical species for which the knockout technique is employed, is the mouse, a species which remains very difficult to assess using ingestive paradigms. The antisense knockdown technique involves the temporary elimination of the expression of a gene by administration of an antisense oligodeoxynucleotide (AS ODN). There are many advantages of this technique over the knockout model: 1) this technique avoids compensatory plieotropic effects because the reduction in protein levels is temporary, 2) it is relatively inexpensive and easy to administer, 3) this technique is reversible and thereby allows for the direct evaluation of experimental variables both in the presence and absence of the protein in question, 4) the use of missense probes serve as an experimental control condition and establish the efficacy of the AS ODN conditions. Finally, this technique can be performed in the rat, which is the prototypical animal model used to study ingestive behavior. In addition to the knockdown approach using antisense, a number of other knockdown techniques such as ribozyme- (Leavitt et al., 2000) and dsRNA- (Vaz Gomez and Wahlestedt, 2000) mediated knockdown have also been advanced. In fact, the ribozyme knockdown technique has recently been evaluated as a

potential therapy for HIV disease (Duarte et al., 1997).

The recent advances in molecular biology and gene sequencing has established the use of the AS ODN technique. The technique involves the use of short (18-25 bases) stretches of synthetic nucleic acids which are complementary to regions of cellular mRNA from a specific gene. Antisense oligonucleotides are thought to utilize classic Watson-Crick base-pairing to hybridize with complementary targeted mRNA to reduce the synthesis of the desired protein. The conceptual basis for this technique was first proposed in 1967 (Belikova et al., 1967), but the necessary technology was not available until 1978 when Zamecnik and Stephenson were able to successfully apply this strategy, and demonstrated the sequence-specific inhibition of Rous sarcoma virus expression in vitro. While the pharmacodynamics of cellular transport of AS ODN probes remain somewhat controversial, several laboratories have reported that AS ODN probes cross the lipid bilayer through pinocytosis, and are subsequently released into the cytoplasm (see review: Juliano et al., 1999). Once in the cytoplasm, AS ODN probes have been shown to rapidly enter and accumulate in the cell nucleus (Akhtar and Juliano, 1992; Fisher et al., 1993; Leonetti et al., 1991). In vitro studies have suggested that AS ODN probes are taken up into neuronal or immune cell lines, and once in the cell, they can remain intact for up to several days (see review: Pasternak and Standifer, 1995).

AS ODN probes putatively act to disrupt protein synthesis by a variety of mechanisms: 1) translational blockade, in which hybridization of AS ODN probes to complementary mRNA can cause steric blockade of the ribosomal subunit thereby inhibiting translation; 2) splicing arrest, in which binding of AS ODN probes may interfere with

appropriate mRNA splice sites; and 3) RNase-H degradation of mRNA, in which formation of the DNA-RNA hybridization duplex created by AS ODN binding to target mRNA, may serve as the substrate for a cellular enzyme (RNase-H) that degrades DNA-RNA duplexes (Myers and Dean, 2000). It is important to note that AS ODN-mediated protein disruption may occur via one or more of the aforementioned mechanisms as well as via an unidentified mechanism. Nevertheless, AS ODN probes can selectively downregulate the mRNA encoding the protein of interest. The selectivity of the antisense technique can be demonstrated using a missense probe, which differs from the desired AS ODN probe by the sequence reversal of two pairs of bases. The use of a MS or nonsense ODN is an important experimental control condition because MS ODN probes are typically inactive, and thus may represent the basal state (Myers and Dean, 2000; Wahlestedt, 1994). Importantly, antisense paradigms utilizing MS ODN probes have consistently demonstrated that this sequence reversal of as few as two pairs of bases, renders the probe inactive (see review: Pasternak and Standifer, 1995). A number of experiments have used the antisense approach to successfully downregulate NMDA, dopamine, muscarinic cholinergic, $GABA_B$, and neuropeptide Y receptors in vitro (Holopainen and Wojcik, 1993; Wahlestedt et al., 1993). However, the efficacy of the using the AS ODN technique in vivo depends on the ability of the protein in question to clearly mediate a given function in an observable manner. Indeed, the opioid system is an excellent candidate with which to study the effects of antisense because opioid receptor function has been firmly implicated in analgesic and ingestive behavioral phenomena. Consequently, successful employment of the AS ODN technique in both analgesic and ingestive paradigms has firmly established the efficacy of this technique.

Initial opioid receptor antisense studies confirmed the efficacy of this technique by providing converging molecular evidence both in vivo and in vitro. Standifer and co-workers (1994, 1995) demonstrated downregulation of mouse DOR-1 receptor following administration of DOR-1 AS ODN probes in NG108-15 cells. Binding of the δ receptor agonist, DPDPE was significantly reduced in vitro by 40-50% and in vivo by 25-30%. A missense control probe (MS ODN) was completely ineffective, thus confirming the specificity of the antisense condition. Importantly, the efficacy of the antisense paradigm is based on the ability of AS ODN probes to selectively reduce cellular protein concentrations, however, several investigations using mRNA and protein immunoblotting techniques have revealed that AS ODN probes typically reduce receptor levels by approximately 40-50% (see review: Pasternak and Standifer, 1995). .Thus, behavioral or biological systems that are pharmacologically insensitive to this level of receptor loss, are not good candidates for in vivo analysis of AS ODN effects. Despite the modest loss of receptors as a result of AS ODN treatment, this technique has proven to be very effective in the examination of opioid-mediated behavioral phenomena including analgesia and ingestive behavior.

A. AS ODN Probes and the MOR-1 Gene

Initial antisense studies suggested the MOR-1 gene played an important role in opioid mediated systems. First, in vitro analysis revealed that AS ODN probes directed against each of the four coding exons of the MOR-1 probe effectively downregulated MOR-1 receptor mRNA, thus suggesting that AS ODN targeting any region of the MOR-1 gene resulted in receptor disruption (Rossi et al., 1995a; Standifer et al., 1994). Subsequent antisense mapping of the MOR-1 gene revealed differential sensitivity patterns using two very potent μ-opioid

receptor agonists: morphine and its active metabolite, M6G (Rossi et al., 1995a). Centrally-administered AS ODN probes directed against coding exons 1 or 4 of the MOR-1 gene effectively blocked supraspinal analgesia elicited by morphine, but were ineffective against M6G-induced analgesia. In contrast, AS ODN probes targeting coding exons 2 or 3 of the MOR-1 gene effectively blocked M6G-induced analgesia, yet were completely ineffective against morphine-induced analgesia. This was surprising given that M6G is a morphine metabolite and displays a 100-fold more potent profile when administered centrally on both thermal (Abbott and Palmour, 1988; Pasternak et al., 1987; Shimomura et al., 1971; Sullivan et al., 1989) and visceral (Frances et al., 1992) nociceptive tests despite labeling μ receptors with slightly less affinity than morphine (Paul et al., 1989). The divergent MOR-1 AS ODN sensitivity profiles of morphine and M6G suggest that the pharmacological mechanisms subserving each of these μ agonists may be different. Further, the pattern of MOR-1 AS ODN sensitivity observed for analgesia induced by the μ agonist, DAMGO, was identical to that observed for morphine: AS ODN probes directed against either coding exons 1 or 4 effectively blocked DAMGO-induced analgesia (Rossi et al., 1995b).

The role of the MOR-1 gene in the mediation of food intake was also extensively studied. Leventhal and co-workers (1996) examined the effects of AS ODN probes on spontaneous food intake and body weight. AS ODN probes directed against all four coding exons of the MOR-1 gene significantly reduced body weight and spontaneous food intake. These findings suggested that the MOR-1 gene itself encodes the receptor mediating spontaneous food intake. Similar to findings involving analgesic assays, AS ODN probes directed against either coding exons 1 or 4 of the MOR-1 gene effectively blocked feeding

induced by the μ agonists morphine and DAMGO, yet had no effect on M6G-induced feeding, whereas AS ODN probes directed against either coding exons 2 or 3, but not 1 or 4 of the MOR-1 gene effectively blocked M6G-induced feeding, and were ineffective against feeding induced by morphine or DAMGO (Leventhal et al., 1997, 1998b). The AS ODN profile of μ-opioid receptor agonist-mediated feeding provides further converging evidence suggesting that distinct receptor mechanisms may account for the differential AS ODN sensitivity profiles observed for the different μ receptor agonists. The common pharmacological actions displayed by morphine or DAMGO on one hand, and M6G on the other hand, may result from the activation of two different receptors, and provides strong evidence for the existence of a novel M6G receptor. Such a receptor could conceivably result from alternative splicing of the MOR-1 gene (see reviews: Pasternak and Standifer, 1995; Rossi and Pasternak, 1997). It should be noted that feeding induced by morphine, M6G and DAMGO are each potently blocked by pretreatment with the μ-selective antagonist βFNA (Leventhal et al., 1997, 1998b; Levine et al., 1991). Therefore, the AS ODN technique provides strong converging evidence to suggest that the MOR-1 gene encodes the μ opioid receptor responsible for the ingestive effects induced by the aforementioned μ-selective agonists.

In addition to the MOR-1 gene's involvement in the μ-agonist-mediated food intake, AS ODN probes were also effective in reducing intake induced by regulatory challenges. AS ODN probes directed against all four coding exons of the MOR-1 gene significantly reduced glucoprivic-induced feeding, with more potent reductions (81-93%) observed following pretreatment with AS ODN probes directed against either coding exons 1 or 2 (Burdick et al.,

1998). Also, feeding induced by the fatty acid oxidation inhibitor, mercaptoacetate (MA) was effectively reduced by pretreatment with AS ODN probes directed against either coding exons 1, 2 or 3, but not coding exon 4 of the MOR-1 gene (Stein et al., 2000). Further, pretreatment with the μ-selective antagonist βFNA, potently blocked both glucoprivic- (2DG) and lipoprivic- (MA) feeding, whereas the μ_1-selective antagonist was ineffective (Burdick et al., 1998; Stein et al., 2000). This antagonist sensitivity pattern suggests that feeding induced under both glucoprivic and lipoprivic conditions is mediated by low-affinity μ_2 receptors. The potency of antagonist and AS ODN effects upon 2DG- and MA-induced firmly establish the μ opioid receptor in the mediation of feeding induced by a number of regulatory conditions.

B. AS ODN Probes and the KOR-1 Gene

Initial studies demonstrated that the KOR-1 gene encodes a receptor which is linked to the pharmacologically-described κ_1 opioid receptor (Adams et al., 1994). Pretreatment of rats with AS ODN probes directed against the KOR-1 gene selectively blocked κ-mediated analgesia elicited by U50,488H. The KOR-1 AS ODN treatment was ineffective against μ- or δ opioid agonist-induced analgesia. The KOR-1 gene is also implicated in feeding induced by 2DG. Burdick and co-workers (1998) established the sensitivity of 2DG-induced feeding to AS ODN probes directed against coding exon 2, but not coding exons 1 or 3 of the KOR-1 clone. Further, the KOR-1 gene was also implicated in MA-induced feeding. AS ODN probes directed against coding exon 3, but not coding exons 1 or 2, effectively blocked MA-induced feeding (Stein et al., 2000). Importantly, antisense mapping of μ-agonist induced feeding induced by M6G was unaffected by pretreatment with AS ODN probes directed against all

three coding exons of the KOR-1 gene, yet feeding induced by the κ_1-selective agonist U50488H is potently blocked by pretreatment with AS ODN probes directed against coding exon 3, but not coding exons 1 or 2 of the KOR-1 gene (Leventhal et al., 1998b). Also, pretreatment with the κ-selective antagonist NorBNI potently blocked feeding induced by 2DG and MA (Arjune and Bodnar, 1990; Stein et al., 2000). Taken together, both antagonist and AS ODN data clearly suggest that the KOR-1 gene plays a critical role in κ-mediated analgesia as well as feeding induced by MA and 2DG. It is important to note that the differential sensitivities of the two latter conditions (MA and 2DG) to AS ODN probes targeting KOR-1 suggest that each of these effects are mediated by different regions of the gene and may represent reliance on KOR-1 splice variants (see review: Pasternak and Standifer, 1995).

C. AS ODN Probes and the DOR-1 Gene

Initial behavioral analysis of AS ODN effects demonstrated that intrathecal administration of an AS ODN probe directed against the DOR-1 gene in mice over the course of 5 days decreased spinal analgesia induced by DPDPE and deltorphin while this treatment failed to alter μ- (DAMGO) or κ_1- (U50,488H) mediated analgesia (Standifer et al., 1994). Importantly, analgesia induced by both of these δ receptor agonists was recovered and returned to pretreatment levels following cessation of AS ODN treatment. Similar findings were reported which indicated the selective loss of δ-mediated analgesia following DOR-1 AS ODN treatment (Lai et al., 1994; Tseng et al., 1994). These findings firmly established the utility of the AS ODN technique and identified the relationship between opioid receptor genes and opioid mediated actions in vivo. Standifer and co-workers (1994) also

demonstrated that AS ODN probes directed against each coding exon of the DOR-1 gene significantly reduced δ-agonist binding in NG108-15 cells, which suggested that individual coding exons of the opioid receptor genes could be examined for their functional activity in opioid-mediated behaviors. That is, a given opioid receptor gene could be "mapped" to identify the functional role of individual coding exons of the opioid receptor genes.

The establishment of an effective antisense mapping strategy led to the further characterization of the DOR-1 gene and its role in δ-mediated analgesia. Antisense probes directed against each of the three coding exons of the DOR-1 gene significantly reduced spinal analgesia induced by the δ_1-opioid receptor agonist DPDPE or the δ_2-agonist deltorphin (Rossi et al., 1997b). Central administration of AS ODN probes directed against each of the three coding exons of the DOR-1 gene blocked deltorphin-induced analgesia, whereas supraspinal DPDPE-induced analgesia was reduced by AS ODN probes targeting only coding exon 3, but not coding exons 1 or 2 of the DOR-1 gene (Rossi et al., 1997b). The ability of AS ODN probes directed against all three coding exons of the DOR-1 to reduce deltorphin-induced analgesia at both the spinal and supraspinal levels suggests that DOR-1 mediates the analgesic actions of deltorphin. Pharmacological characterization revealed that deltorphin is a direct agonist of the δ_2-opioid receptor (Stewart et al., 1994; Jiang et al., 1991), and therefore suggests that the DOR-1 gene encodes the δ_2 opioid receptor. Although these findings suggest that the DOR-1 gene encodes the δ_2 receptor, individual δ opioid receptor subtypes have not yet been cloned (see review: Rossi and Pasternak, 1997). Several studies have examined the role of the DOR-1 gene using ingestive behavior paradigms (Burdick et al., 1998; Leventhal et al., 1998b; Stein et al., 2000). Overall, these studies

suggested that a variety of regulatory challenge conditions including morphine-6β-glucuronide (M6G)-, 2-deoxy-glucose (2DG)- and mercaptoacetate (MA)-induced feeding, were insensitive to pretreatment with AS ODN probes targeting each of the three coding exons of the DOR-1 gene. However, feeding induced by the δ_2-selective agonist, deltorphin II was significantly reduced by pretreatment with an AS ODN probe directed against coding exon 3, but not coding exons 1 or 2 of the DOR-1 gene (Leventhal et al., 1998b). Thus, AS ODN studies suggest a minimal role for the DOR-1 opioid receptor gene in the mediation of ingestive behavior.

D. AS ODN Probes and the KOR-3/ORL-1 Gene

Antisense mapping studies have demonstrated a close association between the KOR-3/ORL-1 gene and the pharmacological described κ_3 opioid receptor (see review: Pasternak and Standifer, 1995; Pasternak et al., 1995). Pan and co-workers (1995) delivered AS ODN probes directed against a region of the second extracellular loop of the KOR-3/ORL-1 receptor and demonstrated a selective blockade of κ_3 analgesia elicited by the selective κ_3 opioid agonist NalBzOH that was ineffective against analgesia elicited by either μ, δ, or κ_1 agonists. Further, this study demonstrated that a highly-selective monoclonal antibody directed against the endogenous κ_3 opioid receptor recognizes the KOR-3/ORL-1 gene expressed in COS-7 cells. Antisense mapping of the KOR-3/ORL-1 gene revealed that analgesia elicited by NalBzOH was blocked by AS ODN probes directed against either coding exons 2 or 3, but not coding exon 1 of the KOR-3/ORL-1 gene (Pan et al., 1995). More recently, the putative endogenous ligand for the KOR-3/ORL-1 receptor clone, OFQ/N (Meunier et al., 1995; Reinscheid et al., 1995) was assessed in an AS ODN mapping study

(Rossi et al., 1997a, 1998). OFQ/N mediated analgesia was significantly attenuated by AS ODN probes directed against either coding exons 2 or 3 of the KOR-3/ORL-1 gene, whereas both AS ODN probes targeting coding exon 1 and a mismatch control sequence were ineffective (Rossi et al., 1998). This AS ODN sensitivity pattern was identical to that observed for analgesia elicited by the selective κ_1 receptor agonist, NalBzOH, suggesting that the second and third coding exons of the KOR-3/ORL-1 gene are important mediators of the analgesic actions of NalBzOH and OFQ/N.

The KOR-3/ORL-1 gene has also been implicated in opioid mediated homeostatic functions such as the control of food intake. Antisense mapping of the KOR-3/ORL-1 gene revealed that OFQ/N-induced food intake was effectively blocked by pretreatment with AS ODN probes directed against all three coding exons of the KOR-3/ORL-1 gene, whereas feeding induced by M6G was unaffected by this treatment (Leventhal et al., 1998a, 1998b). These findings suggested that the receptor responsible for OFQ/N-induced feeding is encoded by the KOR-3/ORL-1 gene. Further, feeding induced by pretreatment with 2DG (glucoprivic) was significantly reduced by AS ODN probes directed against each of the coding exons of the KOR-3/ORL-1 gene (Burdick et al., 1998). In addition, pretreatment with AS ODN probes directed against either coding exons 1 or 2, but not coding exon 3 of the KOR-3/ORL-1 gene effectively blocked feeding induced by mercaptoacetate (lipoprivic) (Stein et al., 2000). These studies provide converging evidence to suggest the involvement of the KOR-3/ORL-1 gene (κ_3 opioid receptor) in the mediation of food intake induced by a variety of homeostatic challenge and agonist situations.

The success of the AS ODN technique, together with the development of selective

opioid receptor antagonists, have provided converging evidence to support the concept of opioid receptor multiplicity. Further, these techniques have elucidated important processes involved in opioid receptor activation and microstructure. As a result, the detailed pharmacology of opioid receptors has revealed that they belong to a family of receptors that are mediated by the activation of G-proteins.

V) G-protein receptor complexes

Opioid receptors belong to a superfamily of seven-transmembrane domain, heterotrimeric G-protein receptor complexes (GPRCs), which share a common mechanism of action upon activation. These receptor complexes are composed of three distinct subunits termed α, β and γ, which are coupled to receptors. The characteristic feature of G-protein receptor complexes is their intrinsic GTPase activity which drives the series of enzymatic regulatory reactions responsible for signal transduction. The α-subunit retains catalytic activity because of its direct binding interaction with the guanine nucleotide species, GTP and GDP. G-protein receptor complexes contain a guanine-nucleotide binding α-subunit which is tightly associated with a β and γ subunit (Taussig and Gilman, 1995).

A. GPRC Activation

The activation of a GPRC involves a series of enzymatic reactions which proceed in the following cycle: 1) in the basal state, the G-protein receptor complex is an $\alpha\beta\gamma$ heterodimer with GDP bound to the α-subunit (release of GDP in the absence of ligand binding proceeds at a very minimal rate ($k \leq 0.1$ min^{-1}), 2) stereospecific agonist binding induces a conformational change of the receptor protein complex which results in dramatic acceleration of the rate of GDP dissociation from the α-subunit, which, in turn, results in

instantaneous binding of cellular GTP to the empty guanine nucleotide binding pocket, 3) binding of GTP induces the α-subunit to undergo a conformational change resulting in its dissociation from the β-γ dimer. At this point, both the GTP-bound α-subunit and the dissociated β-γ dimer interact with individual effector enzymes to mediate their individual effects. 4) The intrinsic GTPase activity of the α-subunit results in cleavage of the terminal phosphate group yielding the basal state GDP-bound α-subunit. The GTPase-mediated exchange of GTP for GDP induces the re-association of the α and βγ-dimer subunits resulting in deactivation and a return to the basal state (see reviews: Freissmuth et al., 1999; Roerig, 1998).

B. GPRC a-subunit Classification

The activation of the α-subunit represents the principal effector mechanism mediating GPRC signal transduction. To date, at least 20 different α-subunits have been identified which display significant functional diversity (Freissmuth et al., 1999). The principal distinction among α-subunits involves the nature of the second messenger cascade which they activate. G-proteins regulate the activities of several effector proteins including various isoforms of adenylyl cyclase, phospholipase C and cGMP phosphodiesterase (Ross, 1989). The $G_s\alpha$ and $G_i\alpha$ subunits both directly regulate the activity of adenylyl cyclase. The $G_i\alpha$-family of subunits ($G_i\alpha_1$, $G_i\alpha_2$, $G_i\alpha_3$, $G_o\alpha$, $G_{xz}\alpha$) is responsible for inhibitory regulation of adenylyl cyclase, and was classified based on this group's sensitivity to disruption by pertussis toxin (PTX) via ADP ribosylation. In contrast, $G_s\alpha$ subunits are responsible for stimulation of adenylyl cyclase and are characterized by sensitivity to ADP ribosylation by cholera toxin (CTX) (Taussig and Gilman, 1995). These toxins disrupt the GTPase activity

of the normal G-protein activity cycle and can be used to identify distinct effector pathways involved in a given neuronal response network. Unfortunately, these toxins often affect multiple systems and thereby often cause non-specific molecular and behavioral effects which obscure experimental findings particularly related to such homeostatic behaviors as intake and weight (Bodnar et al., 1990; Chance et al., 1989). Finally, the $G_q\alpha$ subunit activates the phospholipase C effector pathway upon stimulation. The complex interaction between opioid receptors and their G-proteins/effector systems has provided extensive insights into opioid-mediated behavioral phenomena. Standifer and colleagues (1996) examined the effects of inhibition of G-protein-α-subunits on opioid receptor-mediated analgesia using antisense probes directed against individual types of α subunits and reported distinctions among different types of opioid-mediated analgesia. Thus, characterization of opioid receptor activation of G-proteins can be effectively utilized to identify the molecular processes involved in opioid-mediated behavioral phenomena.

VI) Opioid Peptides and Ingestive Behavior

The role of the opioid system in the modulation of ingestive behavior was first suggested by observations that chronic morphine treatment increased water intake in rats (Flowers et al., 1929). Further, metabolic rate was found to increase in dogs following pretreatment with morphine, suggesting that opioids may regulate both energy expenditure and food intake (Barbour et al., 1930).

A. β-endorphin and Ingestive Behavior

The role of endogenous opioid peptides in the mediation of behavior was first established by observations that direct βEND microinjections into either the lateral ventricle,

PVN or the VMH increased food intake in rats (Grandison and Guidotti, 1977; Leibowitz and Hor, 1982; McKay et al., 1981). Further, βEND-induced feeding was also observed following direct microinjections into the nucleus accumbens (Majeed et al., 1986). More recently, DePedro and co-workers (1996) established that pretreatment with βEND induced a feeding response in goldfish. Further, they found that this response could be abolished by pretreatment with either the μ opioid receptor antagonist βFNA or the μ_1 receptor antagonist naloxonazine, thereby suggesting that this feeding response is mediated in part by the activation of μ opioid receptors. Indications of βEND involvement in the mediation of ingestive behavior also arise from reports linking physiological βEND and obesity. Margules and co-workers (1978) identified elevations in pituitary βEND concentrations in genetically obese mice (ob/ob) and rats (fa/fa). Subsequent analysis demonstrated significant decreases in hypothalamic βEND following fasting in rats (Gambert et al., 1980). Additional evidence for the role of βEND in ingestive behavior involves reductions in CSF βEND concentrations in humans with the clinically diagnosed eating disorders anorexia nervosa and bulimia nervosa (Demitrack et al., 1993; Brewerton et al., 1992). Despite the potency of the βEND-induced feeding effect, there are few insights into the mechanism of action for this effect. Opioid antagonist analyses of βEND feeding have been limited to goldfish and report reductions following pretreatment with general (naloxone) and μ-selective (βFNA), but not κ (NorBNI) or δ (naltriben)- opioid receptor antagonists (DePedro et al., 1995, 1996). Therefore, full characterization of the mechanisms by which βEND acts to stimulate feeding has not been reported.

B. Dynorphin A and Ingestive Behavior

Several studies have identified that feeding elicited following ventricular administration of dynorphin A_{1-17} and dynorphin A_{1-13} is effectively blocked by general opioid antagonist pretreatment (Gosnell et al., 1986a; Morley and Levine, 1981, 1983a, 1983b; Morley et al., 1982; Walker et al., 1980). Further, dynorphin-induced feeding is also observed following intracerebral administration into either the VMH, PVN, VTA, median raphe and the nucleus accumbens (Gosnell et al., 1986b; Hamilton and Bozarth, 1988; Klitenick and Wirtshafter, 1995; Majeed et al., 1986). In contrast, antibodies directed against dynorphin peptide fragments effectively block feeding induced by electrical stimulation of the lateral hypothalamus (Carr and Bak, 1990). Quantification paradigms have demonstrated that dynorphin concentrations in the CNS are increased by 2DG-induced glucoprivation, chronic food restriction, streptozotocin-induced diabetes, and exposure to a palatable diet (Aravich et al., 1993; Berman et al., 1994, 1995, 1997; Welch et al., 1996). This would suggest that dynorphin plays a central role in modulation of intake under a number of different regulatory challenge situations. Although the ingestive effects mediated by dynorphin have been attributed by its direct interaction with κ-opioid receptors (see reviews: Levine et al., 1985; Gosnell and Levine, 1996), only one study (Mann et al., 1988a) has formally addressed this issue by demonstrating that dynorphin-induced feeding is unaffected by pretreatment with the selective μ_1 antagonist, naloxonazine. Therefore, full characterization of dynorphin-induced feeding has not been established.

C. Other Opioid Peptides and Ingestive Behavior

Despite attempts to characterize feeding induced by endogenous enkephalinergic peptides, such as Met-enkephalin, a relative paucity of data exists in this area. An inherent

limitation associated with this type of paradigm involves the activity of endopeptidases and other regulatory enzymes (see review: Orlowski, 1983), which rapidly degrade their substrate peptides in vivo. Specifically, inhibition of the met- and leu-enkephalin endopeptidase, endopeptidase 24.15, significantly increased the magnitude of enkephalin-induced analgesia (Kest et al., 1992). Therefore, a number of studies have established the role of enkephalinergic peptides in feeding behavior by utilizing a number of enkephalin analogues which have been modified to prevent degradation by these enzymes in vivo. Indeed, a number of enkephalin analogues have been identified which produce feeding in non-deprived animals. D-Ala^2-D-Leu^5-enkephalin (DADLE) has been shown to increase spontaneous intake following direct microinjection into the VMH (Tepperman and Hirst, 1983), PVN (Gosnell et al., 1986b), lateral ventricle (Jackson and Sewell, 1985a) and nucleus accumbens (Majeed et al., 1986). Similarly, (D-Ala^2)-met-enkephalinamide (DALA) induced feeding when administered into the paraventricular, dorsomedial and lateral hypothalamus, septum and amygdala (Stanley et al., 1989). Feeding studies utilizing other enkephalin analogues such as DPDPE and Deltorphin demonstrate that feeding responses induced by enkephalinergic peptides are both effectively blocked by general (naltrexone) and selective δ (DALCE, NTII) opioid receptor antagonists (Ragnauth et al., 2000; Yu et al., 1997). These findings suggest that enkephalins mediate their ingestive effects by direct interactions with the pharmacologically described δ opioid receptor.

OFQ/N, like other opioid peptides, stimulates naloxone-reversible food intake following direct microinjection into the lateral ventricles, hypothalamus and nucleus accumbens (Leventhal et al., 1998a; Pomonis et al., 1996; Stratford et al., 1997). The

sensitivity of OFQ/N-induced feeding to AS ODN probes targeting all three coding exons of the KOR-3/ORL-1 gene suggests that the receptor responsible for these ingestive effects is encoded by this gene (Leventhal et al., 1998a). In addition, the recently identified opioid peptides, endomorphin-1 and endomorphin-2 (Zadina et al., 1997) also stimulate food intake when administered centrally in mice (Asakawa et al., 1998).

D. Summary of Opioid Peptides and Ingestive Behaviors

The role of the endogenous opioid peptides in the mediation of behavioral phenomena is well established. Specifically, βEND and dynorphin have been shown to play a substantial role in the mediation of ingestive behavior under a variety of homeostatic conditions. Central administration of these peptides induces a potent feeding response in rats. However, despite the emerging evidence suggesting a role for these peptides in ingestive behavior, the opioid receptor subtypes responsible for these effects have not been fully characterized. Specifically, the receptor pharmacology of both βEND- and dynorphin-induced feeding has not been formally assessed. In addition, the opioid receptor-mediated signal transduction mechanisms responsible for βEND- and dynorphin-induced feeding are also poorly understood. Thus, the central focus of this dissertation is to delineate the receptor pharmacology and signal transduction pathways responsible for feeding induced by βEND and dynorphin as described in the following section.

VII. Rationale

The four specific aims of this dissertation are:

1. **To evaluate alterations in food intake elicited by the endogenous opioid peptide, β-endorphin following pretreatment with equimolar doses of**

either general (NTX), μ- (βFNA), δ- (naltrindole) and κ- (NorBNI)-opioid receptor antagonists. The effects of ventricularly administered AS ODN probes directed against each of the coding exons of the MOR-1, DOR-1, KOR-1 and KOR-3 opioid receptor genes, as well as a control missense probe upon β-endorphin-induced feeding will also be evaluated.

2. **To evaluate alterations in food intake elicited by the endogenous opioid peptide, Dynorphin A_{1-17} following pretreatment with equimolar doses of either general (NTX), μ- (βFNA), δ- (naltrindole) and κ- (NorBNI)-opioid receptor antagonists. The effects of ventricularly administered AS ODN probes directed against each of the coding exons of the MOR-1, DOR-1, KOR-1 and KOR-3 opioid receptor genes, as well as a control missense probe upon dynorphin-induced feeding will also be evaluated.**
3. **To evaluate the effects of ventricularly administered AS ODN probes directed against either the $G_i\alpha_1$, $G_i\alpha_2$, $G_i\alpha_3$, $G_s\alpha$, $G_o\alpha$, $G_{x/z}\alpha$, or $G_q\alpha$ G-protein α-subunits as well as a control nonsense probe upon food intake induced by morphine and morphine-6β-glucuronide in rats.**
4. **To evaluate the effects of ventricularly administered AS ODN probes directed against either the $G_i\alpha_1$, $G_i\alpha_2$, $G_i\alpha_3$, $G_s\alpha$, $G_o\alpha$, $G_{x/z}\alpha$, or $G_q\alpha$ G-protein α-subunits as well as a control nonsense probe upon food intake induced by β-endorphin and dynorphin A_{1-17} in rats.**

<u>Specific Aim One:</u> β-Endorphin-induced Feeding: Pharmacological Characterization Using Selective Antagonists and Antisense Probes.

The endogenous opioid peptide βEND has previously demonstrated potent orexogenic properties when administered centrally (Grandison and Guidotti, 1977; McKay et al., 1981). However, previous antagonist studies of βEND-induced feeding have been limited to goldfish, and find reductions in βEND-endorphin induced feeding following pretreatment with general (naloxone), μ-selective (βFNA) and δ-selective (naltriben) antagonists (DePedro et al., 1995, 1996).

The fundamental limitation of the exclusive use of the antagonist technique is the need to maintain equimolar doses of antagonists. Despite the usefulness of selective opioid antagonists in differentiating between different opioid receptor subtypes, their selectivity is quite limited. Moreover, the lack of available selective antagonists for some opioid receptor subtypes further weakens their experimental utility. The selectivity of antisense probes far exceeds that observed for antagonists due to the sequence-specific nature of the antisense mechanism of action. Importantly, another major advantage of the antisense technique over the use of selective antagonists is that several experimental controls such as the verification of protein down-regulation and the use of missense or nonsense probes, can be used to confirm the efficacy of the technique. Also, the antisense technique can be used to identify subtleties in receptor gene sequences that may account for the existence of receptor splice variants. Clearly, selective antagonists are inherently insensitive to this aspect of opioid receptor pharmacology, and thus offer a far more limited interpretation of pharmacological data. Therefore, based upon both the high degree of selectivity and the availability of multiple control conditions, the antisense technique is superior to the exclusive use of currently available opioid receptor antagonists. However, this dissertation employs both antagonists

and antisense procedures since the antisense technique is still quite new and used in a limited fashion. In contrast, the large data set collected about antagonists will enable us to examine similarities in our studies relative to previous studies.

Previous studies investigating μ-opioid agonist (morphine, M6G)-induced feeding employed a treatment paradigm in which AS ODN probes directed against individual coding exons of the MOR-1 gene. In this 6-day protocol, AS ODN probes (10 μg) were microinjected on days 1, 3 and 5, and assessed for agonist-induced feeding on day 6 (24 h following last AS ODN injection). This treatment paradigm was selected based on the turnover and synthesis rate of opioid receptors (~ 72 h) and therefore maximizes the effects of the AS ODN probes. Also, the 10 μg AS ODN dose was selected because a higher dose (25 μg) produced non-specific reductions in agonist-induced analgesia in that the AS ODN effects persisted well after the termination of AS ODN treatment (7 days) (Rossi et al., 1997b).

Detailed mapping studies of the MOR-1 gene have revealed that opioid agonists display differential sensitivities to AS ODN probes targeting individual coding exons of opioid receptor genes. For example, pretreatment with AS ODN probes directed against coding exons 1 or 4 blocked DAMGO-induced feeding, while probes targeting coding exons 2 or 3 were inactive. In contrast, AS ODN probes targeting either coding exons 2 or 3 of the MOR-1 gene effectively attenuated M6G-induced feeding while probes targeting either coding exons 1 or 4 were inactive (Leventhal et al., 1997, 1998b). Thus, these μ-selective agonists displayed differential sensitivities only distinguishable by the use of the AS ODN approach, since feeding induced by other μ-agonists such as DAMGO, as well as M6G are

both potently blocked by βFNA (Leventhal et l., 1997, 1998b). The present study will employ two techniques to determine which opioid receptor subtypes participate in βEND-induced feeding in rats: general and selective opioid antagonists, and AS ODN probes directed against opioid receptor genes. Potential reductions in βEND-induced feeding will be assessed by the use of equimolar doses of selective opioid receptor subtype antagonists. A second AS ODN technique will attempt to establish the relationship of the cloned opioid receptors to opioid actions using sequences complementary to specific regions of opioid receptor gene mRNA (Pasternak and Standifer, 1995) and thereby provide converging evidence for feeding responses elicited by other opioid agonists (Leventhal et al., 1997, 1998a, 1998b). Cumulative food intake is used in this paradigm because animals typically ingest the largest amount of food during the initial period after agonist administration, and show very inconsistent intakes in each hour thereafter. Since each time point is evaluated separately, we will be able to observe durations of antagonist and antisense effects across the cumulative time course. Cumulative food intake will be measured following 1, 2 and 4 h following either control or agonist treatments in this and subsequent studies. This time course was chosen based upon the previously demonstrated peak feeding effects of the opioid agonists employed (see review: Gosnell and Levine, 1996). The 4 h time course was also chosen in order to maximize the available testing time during the 12 h light cycle, and to minimize circadian effects upon feeding which are more likely to occur when the testing phase overlaps with light onset or offset.

The **first specific aim** of this dissertation is to examine the effects of general and selective opioid receptor antagonists as well as AS ODN probes directed against the MOR-1,

DOR-1, KOR-1 and KOR-3/ORL-1 opioid receptor genes upon βEND-induced feeding in rats.

1. Since the βEND peptide is presumed to induce its effects by direct activation of the μ receptor, it is hypothesized that the μ-selective opioid receptor antagonist βFNA will significantly and potently block βEND-induced feeding at a very low dose, whereas the delta and κ-selective opioid receptor antagonists naltrindole and NorBNI respectively should only minimally alter βEND-induced feeding.

2. If βEND-induced feeding is mediated by μ opioid receptors, then AS ODN probes directed against individual coding exons of the MOR-1 gene should effectively block βEND-induced feeding, whereas AS ODN probes directed against either the DOR-1, KOR-1 or KOR-2/ORL-1 genes should be largely ineffective.

3. Pretreatment with an MS ODN (coding exon 1) probe, identical to the MOR-1 (coding exon 1) AS ODN probe except for the sequence reversal of two pairs of bases, should fail to reduce βEND-induced intake.

Specific Aim Two: Pharmacological Characterization of the Dynorphin A_{1-17} -induced Feeding Response in Rats.

Pretreatment with dynorphin A_{1-17} induced naloxone-reversible feeding behavior in rats (Gosnell et al., 1986a; Morley and Levine, 1981, 1983b; Morley et al., 1982; Walker et al., 1980). In addition, dynorphin levels are increased by 2DG glucoprivation, chronic food restriction, streptozotocin-induced diabetes, and exposure to a palatable diet (Aravich et al., 1993; Berman et al., 1994, 1995, 1997; Welch et al., 1996). Thus, the role of dynorphin in feeding behavior is well established under a variety of conditions. However, the receptor

mechanism for dynorphin's ingestive effects has not been previously examined. To date, only a single study utilizing the selective μ-opioid antagonist naloxonazine assessed the pharmacology of dynorphin-induced feeding. Therefore, the present study will employ two techniques to determine which opioid receptor subtypes participate in dynorphin A_{1-17}-induced feeding in rats: general and selective opioid antagonists, and AS ODN probes directed against opioid receptor genes. Potential reductions in dynorphin-induced feeding will be assessed by the use of equimolar doses of selective opioid receptor subtype antagonists. A second AS ODN technique will attempt to establish the relationship of the cloned opioid receptors to opioid actions using sequences complementary to specific regions of opioid receptor gene mRNA (Pasternak and Standifer, 1995) and thereby provide converging evidence for feeding responses elicited by other opioid agonists (Leventhal et al., 1997, 1998a, 1998b).

The **second specific aim** of this dissertation is to examine the effects of general and selective opioid receptor antagonists as well as AS ODN probes directed against the MOR-1, DOR-1, KOR-1 and KOR-3/ORL-1 opioid receptor genes upon dynorphin A_{1-17}-induced feeding in rats.

1. Since the dynorphin A_{1-17} peptide is presumed to induce its effects by direct activation of the κ opioid receptor (e.g. Chavkin et al., 1982), it is hypothesized that the κ-selective opioid receptor antagonist NorBNI will significantly potently block dynorphin-induced feeding, whereas the μ and δ-selective opioid receptor antagonists βFNA and naltrindole, respectively should only minimally alter dynorphin-induced feeding.

2. If dynorphin A_{1-17}-induced feeding is mediated by κ opioid receptors, then AS

ODN probes directed against individual coding exons of the KOR-1 and possibly the KOR-3/ORL-1 gene should effectively block dynorphin-induced feeding. whereas AS ODN probes directed against either the DOR-1 or KOR-1genes should ineffective.

3. Pretreatment with an MS ODN (coding exon 1) probe, identical to the KOR-1 (coding exon 1) AS ODN probe except for the sequence reversal of two pairs of bases. should fail to reduce dynorphin-induced intake.

Specific Aim Three: Morphine and Morphine-6β-glucuronide-induced Feeding: Distinguishing Signal Transduction Profiles Using G-protein α-subunit Antisense Probes.

Morphine and other opioid agonists such as butorphanol and levorphanol potently stimulate a feeding response (Levine et al., 1994, Levine and Morley, 1983; Sanger and McCarthy, 1980). Morphine-6β-glucuronide is a morphine metabolite which is produced by the glucuronidization of morphine at the three and six positions (Jaffe and Martin, 1985) which also significantly and dose-dependently increases spontaneous food intake (Leventhal et al., 1998b) and analgesia (Abbott and Palmour, 1988; Pasternak et al., 1987). The μ opioid receptor has been implicated in both morphine- and M6G-induced feeding since each is blocked by the selective μ-antagonists βFNA and naloxonazine (Leventhal et al., 1998b; Mann et al., 1988). In contrast, both morphine- and M6G-induced feeding are unaffected by either selective κ_1, δ_1 or δ_2 opioid receptor subtype antagonists. Further, antisense mapping studies using AS ODN probes targeting individual coding exons of the MOR-1 gene reported that AS ODN probes directed against either coding exons 2 or 3, but not 1 or 4 of the MOR-1 gene effectively blocked feeding elicited by M6G, whereas AS ODN probes targeting exon

1 but not coding exon 2 of the MOR-1 gene blocked morphine-induced feeding (Leventhal et al., 1998b). A similar pattern emerged in analgesic assays (see review: Rossi and Pasternak, 1997), suggesting that M6G may act through the functional activation of a novel and pharmacologically distinct receptor.

Opioids mediate their effects by the putative activation of G-protein coupled receptor complexes (see review: Roerig, 1998). Analgesic assays using AS ODN probes directed against α-subunits of G-protein receptors established that morphine- and M6G-induced analgesia display differential G-protein α-subunit sensitivity profiles. Standifer and co-workers (1996) demonstrated that supraspinal analgesia induced by M6G, but not morphine was significantly reduced in mice by pretreatment with AS ODN probes targeting the $G_i\alpha_1$, $G_s\alpha$ and $G_{xz}\alpha$ G-protein α-subunits. In contrast, morphine-induced analgesia was significantly reduced by pretreatment with AS ODN probes targeting the $G_i\alpha_2$, $G_o\alpha$ and $G_s\alpha$ G-protein α-subunits. The distinct effector pathway sensitivities displayed by morphine and M6G in these analgesia studies provide further converging evidence for existence of a novel μ-opioid receptor.

To determine whether the distinctions between feeding responses induced by morphine and M6G involve different G-protein α-subunits, the **third specific aim** of this dissertation will evaluate the effects of ventricularly-administered AS ODN probes directed against either the $G_i\alpha_1$, $G_i\alpha_2$, $G_i\alpha_3$, $G_s\alpha$, $G_o\alpha$, $G_{xz}\alpha$, or $G_q\alpha$ subunits as well as a control nonsense probe upon spontaneous food intake induced by morphine and M6G in rats. Also, since AS ODN probes directed against MOR-1 clone decreased spontaneous intake and body weight (Leventhal et al., 1996), and since knockdown techniques have not been

systematically evaluated for G-protein-mediated effects upon spontaneous food intake and body weight per se, these variables will be assessed prior to and following administration of each G-protein AS ODN probe.

1. If the receptor mechanism responsible for morphine-induced feeding is the pharmacologically defined μ-opioid receptor, then pretreatment with AS ODN probes directed against either the $G_i\alpha_2$, $G_o\alpha$ or $G_s\alpha$ G-protein α-subunits should effectively block morphine-induced feeding, thus confirming previous analgesic data.

1a. Antisense probes directed against the $G_i\alpha_1$, $G_i\alpha_3$, $G_q\alpha$, or $G_{z}\alpha$ G-protein α-subunits should fail to significantly alter morphine-induced feeding.

2. If the receptor mechanism responsible for M6G-induced feeding is the same receptor responsible for M6G-induced analgesia, then pretreatment with AS ODN probes directed against either the $G_i\alpha_1$, $G_s\alpha$ or $G_{z}\alpha$ G-protein α-subunits should effectively block M6G-induced feeding.

2a. Antisense probes directed against the $G_i\alpha_2$, $G_i\alpha_2$, $G_q\alpha$ or $G_o\alpha$ G-protein α-subunits should fail to significantly alter M6G-induced feeding.

3. A random sequence nonsense probe (NS ODN) should fail to significantly alter either morphine- or M6G-induced feeding.

4. Administration of AS ODN or NS ODN probes should fail to significantly alter cumulative food intake.

5. Administration of AS ODN or NS ODN probes should fail to significantly alter body weight.

Specific Aim Four: G-protein Sensitivity Profile of Endogenous Opioid-induced

Feeding: Functional Characterization of β-endorphin and Dynorphin $_{1-17}$ Using G-protein α-subunit Antisense Probes in Rats.

Naloxone-reversible food intake is observed following central administration of both βEND (Grandison and Guidotti, 1977; Liebowitz and Hor, 1982; Majeed et al., 1986) and dynorphin A_{1-17} (Gosnell et al., 1986a; Morley and Levine, 1981,1983a, 1983b; Morley et al., 1982; Walker et al., 1980). Investigations of βEND-induced feeding have suggested that this peptide exerts its functional effects via the putative activation of the μ opioid receptor. DePedro and co-workers (1996) established that βEND-induced feeding in goldfish was effectively blocked by the μ-selective opioid receptor antagonist βFNA. In addition several binding studies have reported preferential binding of βEND to μ-opioid receptors in vitro (Chang et al., 1979; Schulz et al., 1979; Akil et al., 1980). Dynorphin A $_{1-17}$-induced feeding has been postulated to result from selective activation of κ-opioid receptors (see reviews: Levine et al., 1985; Gosnell and Levine, 1996), despite limited experimental support. Biochemical assays have offered the strongest evidence to support κ opioid receptor binding (e.g. Chavkin et al., 1982).

Functional characterization of the effector mechanisms involved in opioid agonist-induced feeding has yielded interesting findings. Supraspinal analgesia induced by the putative μ agonists morphine, DAMGO and M6G have been characterized using AS ODN probes or antisera directed against G-protein α-subunits (Standifer et al., 1996; Raffa et al., 1994; Rossi et al., 1995a; Sanchez-Blazquez and Garzon, 1992; Sanchez-Blazquez et al., 1993). These studies suggest that individual μ agonists display differential G-protein sensitivity profiles which can be compared to determine parallel effector pathways. For

example, a recent study reported that the μ agonists morphine, heroin, methadone and buprenorphine each displayed different patterns of G-protein activation in inducing μ-opioid receptor mediated supraspinal analgesia (Sanchez-Blazquez et al., 2001).

The signal transduction mechanisms responsible opioid-mediated feeding induced by βEND and dynorphin A $_{1-17}$ remain largely uncharacterized. Initial analgesic assays reported that βEND-induced spinal analgesia is significantly blocked by antisera raised against either $G_i\alpha_2$ or $G_{x/z}\alpha$ subunits, while βEND-induced analgesia is potentiated by pretreatment with antisera raised against the $G_s\alpha$ subunit (Sanchez-Blazquez et al., 1993). Importantly, Sanchez-Blazquez and Garzon (1992) reported that βEND-induced analgesia is also effectively blocked by pretreatment with pertussis toxin (PTX), which selectively inactivates G_i /G_o transducer proteins (see review: Gilman, 1987). The ability of PTX to selectively block analgesic responses induced by other opioids including morphine, DAMGO and sufentanil (Bodnar et al., 1990; Goode and Raffa, 1997) suggests that opioid-mediated effects utilize a G_i /G_o effector pathway.

The signal transduction characteristics of both βEND and dynorphin-induced feeding have not been formally evaluated. Therefore, the **fourth specific aim** of this dissertation will attempt to determine which G-protein α subunits mediate the effects of both βEND- and dynorphin A $_{1-17}$-induced feeding in rats. This study will evaluate the effects of ventricularly-administered AS ODN probes directed against either the $G_i\alpha_1$, $G_i\alpha_2$, $G_i\alpha_3$, $G_s\alpha$, $G_o\alpha$, $G_{x/z}\alpha$, or $G_q\alpha$ subunits as well as a control nonsense probe, upon spontaneous food intake induced by either βEND or dynorphin A $_{1-17}$.

CHAPTER 2. GENERAL METHODS

I. Subjects, surgery and cannula verification. Adult male Sprague-Dawley rats, 90-120 days of age, were purchased from Charles River Laboratories, Kingston, NY and were individually housed in suspended wire mesh cages and maintained on a 12-h light: 12-h dark cycle with water and food available *ad libitum*. Each animal was pretreated with chlorpromazine (3 mg/kg, i.p.) and anesthetized with Ketamine HCl (100 mg/kg, i.m.). A stainless steel guide cannula (22-gauge, Plastics One, Roanoke, VA) was implanted stereotaxically (Kopf Instruments, Tujunga, CA) into the left lateral ventricle using the following coordinates: incisor bar (+5 mm), 0.5 mm anterior to the bregma suture, 1.3 mm lateral to the sagittal suture and 3.6 mm from the surface of the skull. All animals were allowed at least 2 weeks to recover from stereotaxic surgery before behavioral testing began. Rats weighed between 300 and 350 g before surgery, and weighed 400 to 500 g after completion of testing. Following behavioral testing, all animals were killed with an overdose of anesthetic, and cannula placements were verified visually.

II. Drugs. Selective opioid receptor antagonists, agonists and AS ODN probes were employed to fully characterize the pharmacology of endogenous opioid-induced feeding.

A. Opioid antagonists and agonists. βFNA (Research Biochemicals Intl., Natick, MA), an irreversible selective μ-receptor antagonist (Portoghese et al., 1980), NorBNI (Research Biochemicals Intl., Natick, MA), a short acting κ-antagonist (Portoghese et al., 1987), Naltrindole (Research Biochemicals Intl., Natick, MA) a short-acting δ antagonist (Portroghese et al., 1990) and NTX (Research Biochemicals Intl., Natick, MA), a general

opioid antagonist (Sawynok et al., 1979), were each dissolved in 0.9% normal saline.

Morphine (Pennick Laboratories, NJ), M6G (Research Technology Branch of the National Institute of Drug Abuse, Rockville, MD), β-Endorphin (Peninsula Laboratories, Belmont, CA), and dynorphin A_{1-17} (Peninsula Laboratories, Belmont, CA) were each dissolved in 0.9% normal saline. The half-life of β-END and dynorphin in cerebrospinal fluid was previously determined to be approximately 317 minutes (Max et al., 1985) and 150 minutes (Muller et al., 1996) respectively, which is well within the time course under study.

All drugs were administered i.c.v. in 5 µl volumes over 30 s through a stainless steel internal cannula (28-gauge, Plastics One, Roanoke, VA) which was attached to a Hamilton microsyringe by polyethylene tubing. Following each injection, the internal cannula was removed and immediately replaced with a stainless steel dummy cannula (28-gauge, Plastics One) to prevent any effusion, and to insure cannula patency between microinjection conditions. βFNA was administered 24 h prior to agonist administration to allow for full development of irreversible antagonist effects (Portoghese et al., 1980; Arjune et al., 1990), while NorBNI was administered 30 min prior to opioid agonist administration (Portroghese et al., 1990). Naltrexone and naltrindole were administered 1 h prior to agonist administration (Portoghese et al., 1990). The time interval between injections within each condition was based upon the respective peak and selective actions of the opioid antagonists. All opioid agonists were administered just prior to the start of behavioral testing.

B. AS ODN probes. All phosphodiester oligodeoxynucleotides (Midland Certified

Reagent Company. Midland. TX) were dissolved in 0.9% normal saline at a concentration of 5 μg/μl and purified (see review: Rossi and Pasternak. 1997). Traditional phosphodiester oligodeoxynucleotide antisense probes were used in these studies in order to minimize the potential toxic and nonspecific effects often observed following the in vivo administration of modified (i.e. phosphorothioate) oligodeoxynucleotides (Chiasson et al., 1994; Crooke. 1993). Opioid receptor gene AS ODN probes used in Specific Aims One and Two were directed against the individual coding exons of either the MOR-1, DOR-1. KOR-1. or KOR-3/ORL-1 opioid receptor genes. **Table 1** summarizes the sequences of the AS ODN probes (19-22 bases in length) that were directed against four regions of the MOR-1 clone. three regions of the KOR-3/ORL-1 clone. three regions of the KOR-1 clone. the three regions of the DOR-1 clone and mismatches for the MOR-1 and KOR-3/ORL-1 clones. All AS ODN probes used in Specific Aims One and Two were administered at a dose of 10 μg based upon their previously determined effectiveness in feeding studies (Leventhal et al., 1997. 1998a.1998b) without producing nonspecific effects (see review: Rossi and Pasternak. 1997). Antisense probe sequences were designed based upon the predicted sequences of opioid receptor clones. A number of other morphological characteristics of opioid receptor gene sequences such as coding region localization. GC content and T_m calculations were used to identify the optimal sense regions of the opioid receptor genes in order to design the appropriate antisense probes (e.g. Pan et al., 1995). The localization of sequences with high GC content and T_m values is important in the design of antisense probes since the stability of antisense hybridization is significantly greater in these regions based on the unique hydrogen-bonding

Table 1. Sequences of antisense oligodeoxynucleotides.

Probe	**Sequence (5' - 3')**
A. MOR-1 Opioid Receptor Clone:	
Coding exon 1 AS	CGC CCC AGC CTC TTC CTC T
Coding exon 1 MS	CGC CCC **GA**C CTC TTC C**CT** T
Coding exon 2 AS	TTG GTG GCA GTC TTC ATT TTG G
Coding exon 3 AS	TGA GCA GGT TCT CCC AGT ACC A
Coding exon 4 AS	GGG CAA TGG AGC AGT TTC TG
B. DOR-1 Opioid Receptor Clone:	
Coding exon 1 AS	TGT CCG TCT CCA CCG TGC
Coding exon 2 AS	ATC AAG TAC TTG GCG CTC TG
Coding exon 3 AS	AAC ACG CAG ATC TTG GTC AC
C. KOR-1 Opioid Receptor Clone:	
Coding exon 1 AS	GCT GCT GAT CCT CTG AGC CCA
Coding exon 2 AS	CCA AAG CAT CTG CCA AAG CCA
Coding exon 3 AS	GGC GCA GGA TCA TCA GGG TGT
D. KOR-3/ORL-1 Opioid Receptor Clone:	
Coding exon 1 AS	GGG GCA GGA AAG AGG GAC TCC
Coding exon 2 AS	GAC GAG GCA GTT CCC CAG GA
Coding exon 3 AS	GGG CTG TGC AGA AGC CGA GA

Note: Bold characters denote differences between AS and MS ODNs.

characteristics of these oligodeoxynucleotide bases. Further, the specific antisense probes used in these studies were chosen because of their previously-demonstrated effectiveness in specifically and selectively reducing analgesia induced by their respective opioid receptor subtype agonists (Chien, et al., 1994; Pan et al., 1995; Rossi et al., 1994, 1995a, 1995b, 1997b). Analysis of GenBank revealed that each of the AS ODN sequences were specific to the specified regions of the MOR-1, DOR-1, KOR-1 and KOR-3/ORL-1 clones, and are not present in other opioid receptor or other cDNA sequences.

G-protein AS ODN probes used in Specific Aims Three and Four were specific for the G-protein α-subunits for which they were targeted. **Table 2** summarizes the sequences of each of the base sequences of the AS ODN probes (15-20 bases in length) that were directed against either the $G_i\alpha_1$, $G_i\alpha_2$, $G_i\alpha_3$, $G_s\alpha$, $G_o\alpha$, $G_{xz}\alpha$, or $G_q\alpha$ subunits as well as a nonsense ODN probe consisting of a random base sequence. These sequences were chosen because of their previously-demonstrated effectiveness in specifically and selectively reducing analgesia induced by either morphine, M6G and other μ-opioid receptor agonists as well as their ability to specifically downregulate their specific G-protein α-subunit (Rossi and Pasternak, 1997; Standifer et al., 1996). All sequences were subject to a GenBank search, were found to be unique to their respective G protein α-subunit, and were based on rat sequences with the exception of $G_{xz}\alpha$ (human). All AS ODN probes as well as MS ODN and NS ODN probes were dissolved in 0.9% saline at a concentration of 5 μg/μl and administered i.c.v. in 5 μl volumes over 30 s through a stainless steel internal cannula (28-gauge) which was attached to a Hamilton syringe by polyethylene tubing. G protein AS ODN probe infusions were delivered as a single

Table 2. Base sequences of antisense oligonucleotide probes

Target protein	Sequence (5' - 3')
$G_i\alpha_1$	AGA CCA CTG CTT TGT A
$G_i\alpha_2$	CTT GTC GAT CAT CTT AGA
$G_i\alpha_3$	AAG TTG CGG TCG ATC AT
$G_o\alpha$	CGC CTT GCT CCG CTC
$G_s\alpha$	TTG TTG GCC TCA CGC TG
$G_q\alpha$	GCT TGA GCT CCC GGC GGG CG
$G_{z/z}\alpha$	GGG CCA GTA GCC CAA TGG G
Nonsense	GGG GGA AGT AGG TCT TGG

injection, 24 h prior to the agonist treatment. All AS ODN probes used in Specific Aims Three and Four were administered at a dose of 25 μg based upon their previously demonstrated effectiveness in specifically and selectively reducing analgesia induced by either morphine or M6G as well as their ability to specifically downregulate their specific G-protein α-subunit (Rossi et al., 1995a; Standifer et al., 1996) . Opioid receptor clone AS ODN probes were delivered in three infusions administered at 48-h intervals. These time courses of treatment allow for both the downregulation of de novo receptor protein synthesis as well as the turnover of existing receptors (see reviews: Pasternak and Standifer, 1995; Rossi and Pasternak, 1997).

III. General procedures. To assess agonist-induced changes in spontaneous food intake, all animals were underwent behavioral testing in the home cage between 2-8 h following the onset of the light cycle to minimize circadian effects on food intake. All animals were adapted to at least four days of baseline testing to eliminate any novelty-induced feeding responses elicited by placement of food pellets on the floor of the cage, as well as to ensure stability of baseline spontaneous food intake. Intake values were measured by the weight of the food pellets in grams (g) and adjusted for spillage that was collected on paper towels placed below the suspended wire mesh cage. Following baseline, cumulative intakes were assessed after 1, 2 and 4 h following administration of the agonist. Cumulative food intake was measured following 1, 2 and 4 h following either control or agonist treatments in this and subsequent studies. This time course was chosen based upon the previously demonstrated peak feeding effects of the agonists employed (see review: Gosnell and Levine, 1996). The 4 h time course was also chosen in order to

maximize the available testing time during the 12 h light cycle and to minimize circadian effects upon feeding which are more likely to occur when the testing phase overlaps with light onset or offset.

IV. Statistical Analyses. To determine significant agonist effects in each experiment, separate one-way analyses of variance were performed on cumulative food intakes after 1, 2, and 4 h. Time was not considered a separate variable because intakes are typically larger in the first hour following agonist administration and decline thereafter. Tukey comparisons ($P<0.05$) were used to determine individual significant agonist effects relative to vehicle treatment. Dunnett comparisons ($P<0.05$) were used to determine individual significant antagonist or AS ODN effects relative to its corresponding agonist-induced feeding condition.

To determine significant antagonist or AS ODN effects upon βEND-induced feeding in Experiment 1, difference scores were determined for each condition in each animal by subtracting a corresponding vehicle intake from the experimental score. Separate one-way analyses of variance were then performed on these food intake difference scores after 1, 2, and 4 h. Dunnett's comparisons ($P<0.05$) were used to determine individual significant antagonist or AS ODN effects relative to its corresponding βEND-induced feeding condition.

In Experiment 3, separate one-way analyses of variance were performed on body weight at 1, 2, and 3 days following vehicle injection for each of the different AS ODN treatments. Separate one-way analyses of variance were performed on spontaneous (24 h) food intake at 1 and 2 days following vehicle injection for each of the different AS ODN

treatment conditions. Individual significant effects of both weight and overall intake were identified using Tukey comparisons ($P<0.05$).

In Experiment 4, a two-way analysis of variance was performed to determine whether the two opioid agonists elicited equi-effective feeding relative to control levels at each intake point with control and agonist conditions as one variable, and the two agonist treatments as the second variable. In assessing G-protein AS ODN effects upon agonist-induced feeding in Experiments 3 and 4, separate one-way analyses of variance were performed for each intake point for control, agonist alone, and each G-protein AS ODN condition upon agonist-induced feeding 24 and 48 h thereafter.

CHAPTER 3: SPECIFIC AIM ONE.

β-Endorphin-induced Feeding: Pharmacological Characterization Using Selective Antagonists and Antisense Probes.

Introduction

The role of the endogenous opioid peptides and their receptors in modulating ingestive behavior has been a source of intense study over the past quarter-century (see review: Gosnell and Levine, 1996). In addition to the stimulation of feeding by opiate drugs acting at μ (e.g. morphine: Sanger and McCarthy, 1980) and κ (e.g. ketocyclazocine and butorphanol: Morley et al., 1982; Levine and Morley, 1983) receptors, feeding was observed following direct microinjections of opioid peptides themselves, including βEND (Grandison and Guidotti, 1977), as well as dynorphin (Morley and Levine, 1981). Subsequent investigations using selective opioid receptor subtype agonists and antagonists (see reviews: Gosnell and Levine, 1996; Bodnar, 1996), as well as AS ODN probes directed against opioid receptor genes (Leventhal et al., 1997, 1998b) have since identified the specific receptor mechanisms through which μ and κ opioid agonists mediate their feeding effects. However, full characterization of the mechanisms by which naturally occurring opioid peptides act to stimulate feeding has not been adequately examined in the rat, the most common species used for opioid-induced feeding studies.

Although these studies suggest a role for βEND in the mediation of ingestive behavior, they fail to elucidate specifically which opioid receptors participate in this response. Initial investigations of βEND-induced feeding have been limited to goldfish and have reported reductions induced by general (naloxone) and μ-selective (βFNA), but not by κ

(NorBNI) or δ (naltriben) opioid receptor antagonists (De Pedro et al., 1995,1996).

The present study utilized centrally administered general and selective opioid antagonists, as well as AS ODN probes directed against opioid receptor genes in order to examine the specific role of opioid receptors in the pharmacological profile of βEND-induced feeding. Using equimolar doses (5-40 nmol) of general (NTX), μ (βFNA), δ (naltrindole) and κ (NorBNI) opioid antagonists as well as AS ODN probes directed against specific coding exons of the MOR-1, DOR-1, KOR-1 and KOR-3/ORL-1 opioid receptor clones, this study sought to provide converging evidence elucidating the pharmacological mechanism of βEND-induced feeding. The findings of the present study have been recently published (Silva et al., 2001a).

Methods

Drugs: βEND (0.25 - 10 μg, Peninsula Laboratories, Belmont, CA), NTX (5-40 nmol, Sigma Chemical Co., St Louis, MO), βFNA (0.5-40 nmol, Research Biochemicals Inc., Natick, MA), naltrindole (5-40 nmol, Research Biochemicals Inc.) and NorBNI (5-40 nmol, Research Biochemicals Inc.) were used in this study.

Antisense probes: All phosphodiester oligodeoxynucleotides (Midland Certified Reagent Company, Midland, TX) were purified in our (G.W.P., G.C.R.) laboratory. Each AS ODN probe was directed against the individual coding exons of either the MOR-1, DOR-1, KOR-1 or KOR-3/ORL-1 opioid receptor genes (**Table 1**). All AS ODN probes were administered at 10 μg doses dissolved in 5 μl volumes of 0.9% normal saline based upon their previously-determined effectiveness in feeding studies (Leventhal et al., 1997, 1998a, 1998b).

Procedures: Following baseline determinations, the first group of twelve animals were assessed for food intake after 1, 2 and 4 h following microinjection of βEND at doses of 0, 0.25, 1, 5 and 10 μg in counterbalanced order at weekly intervals. Following the determination that a 10 μg dose of βEND produced the most consistent feeding response (see Results), the test phase of the antagonist studies had subgroups of animals pretreated with equimolar doses (0.5-40 nmol) of either the general (NTX, n=8), δ-selective (naltrindole, n=8), μ-selective (βFNA, n=9) or κ-selective (NorBNI, n=9) opioid receptor antagonists. βEND-induced food intake was subsequently assessed after 1, 2 and 4 h following microinjection of βEND (10 μg) as previously described. In the antisense conditions, separate subgroups of animals received microinjections of AS ODN probes (10 μg) directed against either coding exons 1, 2, 3 or 4 of the MOR-1 gene (n=8/condition), coding exons 1, 2 or 3 of the DOR-1 gene (n=8/condition), coding exons 1, 2 or 3 of the KOR-1 gene (n=7/condition), coding exons of the KOR-3/ORL-1 gene (n=7/condition) or a MS ODN probe directed against coding exon 1 of the MOR-1 gene (n=6) which differed from its corresponding AS ODN probe by the sequence reversal of two pairs of bases (**Table 1**). During each 6-day testing phase, rats received microinjections of their particular AS ODN probes on days 1, 3 and 5 as previously described (Leventhal et al., 1997). Twenty-four h following the last AS or MS ODN treatment (day 6), all animals received βEND (10 μg), and food intake was assessed after 1, 2 and 4 h.

Results

βEND-Induced Feeding Dose-Response Curve

βEND produced a dose-dependent and time-dependent increase in food intake

after 1 [$F(4,52) = 7.48$, $p<0.0001$], 2 ($F = 8.98$, $p<0.0001$), and 4 ($F = 18.06$, $p<0.0001$) h, with only the highest (10 μg) dose significantly increasing intake over the entire 4-h time course (**Figure 1**). Since the highest dose produced the greatest magnitude of effects upon feeding responses across all test intervals, this i.c.v. dose was used in all subsequent experiments.

NTX and βEND-Induced Feeding

βEND-induced feeding was significantly altered following pretreatment with NTX after 1 [$F(4,79) = 16.45$, $p<0.0001$], 2 ($F = 19.47$, $p<0.0001$), and 4 ($F = 29.53$, $p<0.0001$) h. βEND-induced feeding was significantly reduced by the two highest (20- and 40-nmol) NTX doses, and was transiently potentiated by the lowest (5-nmol) NTX dose after 1 h (**Figure 2**), indicating opioid receptor mediation of βEND-induced feeding.

βFNA and βEND-Induced Feeding

βEND-induced feeding was significantly reduced following pretreatment with βFNA after 1 [$F(6,98) = 5.81$, $p<0.0001$], 2 ($F = 9.49$, $p<0.0001$), and 4 ($F = 14.15$, $p<0.0001$) h. βEND-induced feeding was significantly reduced by each of the βFNA pretreatment doses: 0.5 (4 h), 2.5 (1-4 h), 5 (1, 4 h), 20 (4 h), and 40 (2-4 h) nmol (**Figure 3**), indicating μ-opioid receptor mediation of βEND-induced feeding.

Naltrindole and βEND-Induced Feeding

βEND-induced feeding was significantly altered following pretreatment with naltrindole after 1 [$F(4,79) = 10.83$, $p<0.0001$], 2 ($F = 14.17$, $p<0.0001$) and 4 ($F = 24.85$, $p<0.0001$) h. βEND-induced feeding was significantly reduced by the two highest (20- and 40-nmol) naltrindole doses after 4 h, and was transiently (1 h) potentiated by the

Figure 1. Alterations (mean, ±SEM) in food intake (g) following i.c.v. administration of the endogenous opioid peptide, β-END (0.5-10 μg). Significant differences in food intake were observed following βEND (n=12) relative to vehicle treatment after 1 ($F(4,52)= 7.48$, $p<.0001$), 2 ($F= 8.98$, $p<.0001$) and 4 ($F= 18.06$, $p<.0001$) h. The y-axis in this and subsequent figures represents cumulative food intake values. The asterisks (*) in this and subsequent figures indicate significant increases in β-END-induced feeding relative to corresponding vehicle control values (Dunnett comparisons, $P<0.05$). Please note that control intake was minimal during this testing period (2-8 h into the light cycle) in rats adapted to the testing paradigm such that the symbols often encompass the small SEM's in this and subsequent protocols. Baseline intake values are represented as Control (open circles) in this and subsequent figures.

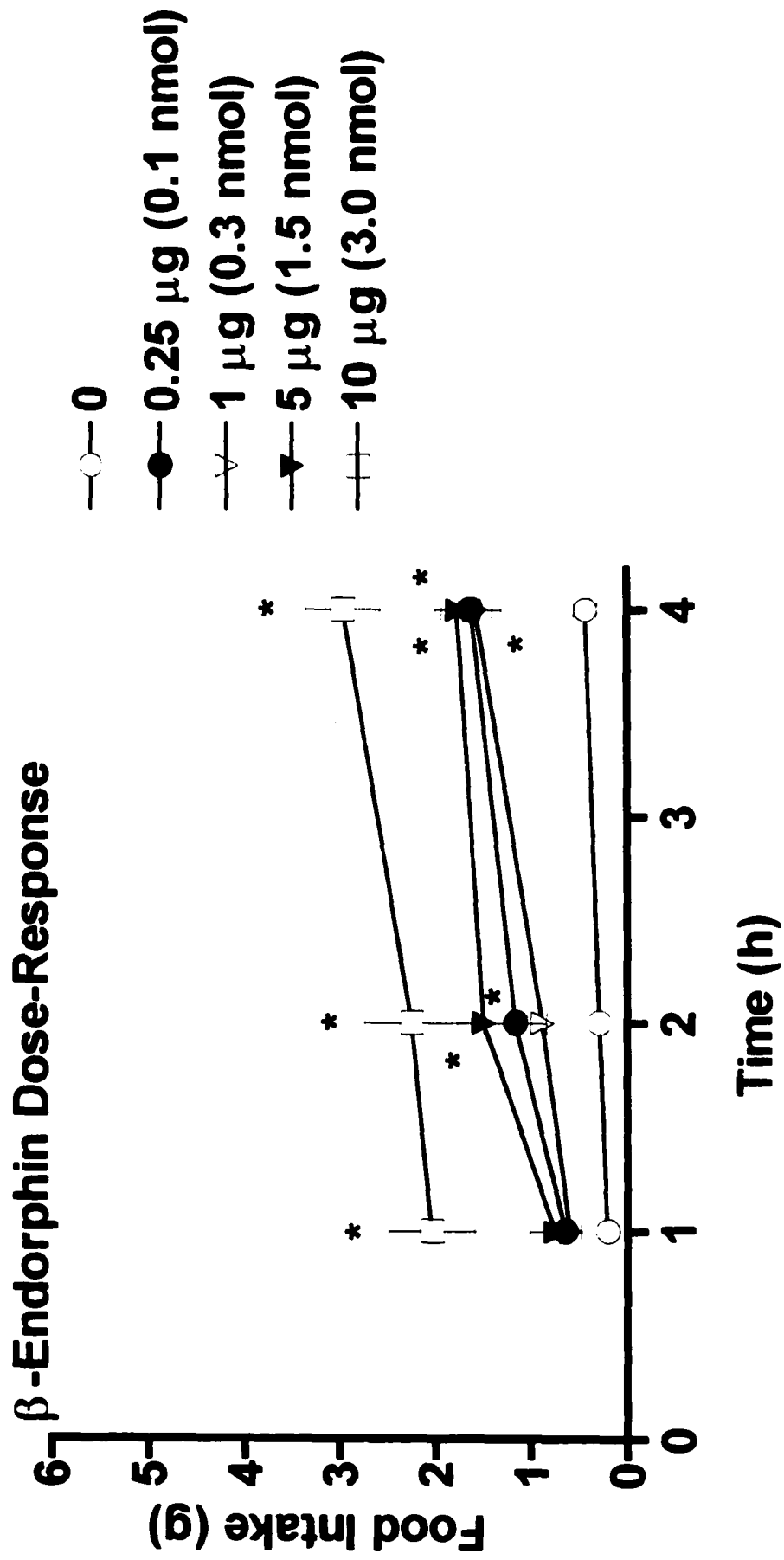


Figure 2. Alterations (mean, ±SEM) in food intake (g) following i.c.v. administration of either vehicle alone, β-END alone or β-END following ventricular pretreatment (1 h) with the general opioid receptor antagonist, NTX at doses of either 5, 20 or 40 nmol (n=8/condition). Significant differences in food intake were observed following all βEND treatment conditions relative to vehicle after 1 ($F(4,79)= 16.45$, $p<.0001$), 2 ($F= 19.47$, $p<.0001$) and 4 ($F= 29.53$, $p<.0001$) h. Difference-score analyses also displayed significant effects after 1 ($F(3,36) =21.03$, $p<.0001$), 2 ($F=10.95$, $p<.0001$), and 4 ($F=7.95$, $p<.0003$) h. The asterisks (*) in this figure indicate significant increases in agonist-induced feeding relative to corresponding vehicle control values (Dunnett comparisons, $P<0.05$) The crosses (+) in this figure indicate significant decreases in agonist-induced feeding following either antagonist or AS ODN pretreatment relative to corresponding agonist-induced feeding values alone (Dunnett comparisons, $p<0.05$).

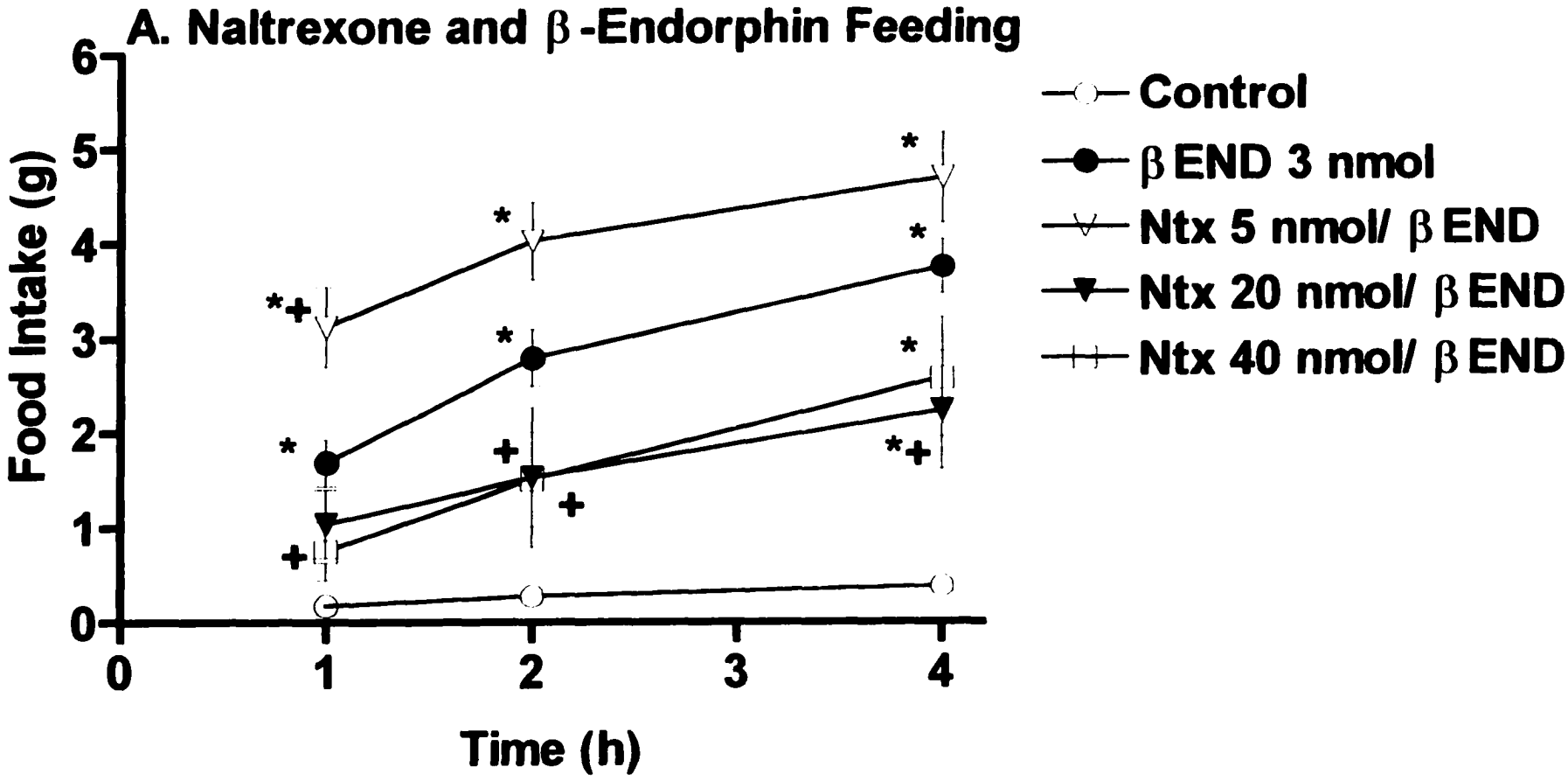
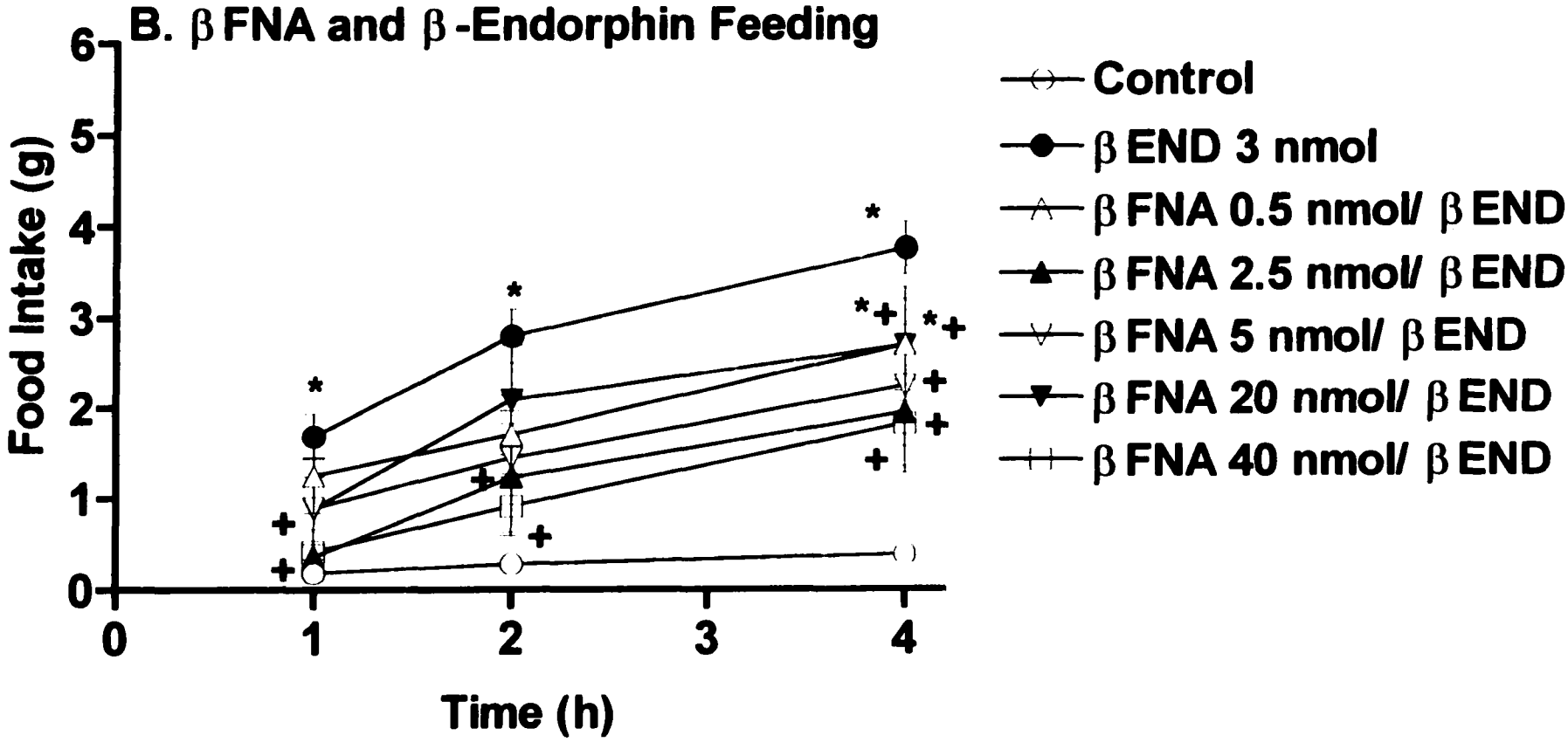


Figure 3. Alterations (g, ±SEM) in food intake following i.c.v. administration of either vehicle alone, β-END alone or β-END following ventricular pretreatment (24 h) with the μ-opioid receptor antagonist, β-FNA at doses of either 0.5, 2.5, 5, 20 or 40 nmol (n=9/condition). Significant differences in food intake were observed following βEND treatment conditions relative to vehicle after 1 ($F(6,98)= 5.81$, $p<.0001$), 2 ($F= 9.49$, $p<.0001$) and 4 ($F= 14.15$, $p<.0001$) h. Difference-score analyses also displayed significant effects after 1 ($F(5,45) =2.32$, $p<.05$), 2 ($F=2.51$, $p<.044$), and 4 ($F=5.05$, $p<.0009$) h. The asterisks (*) in this figure indicate significant increases in agonist-induced feeding relative to corresponding vehicle control values (Dunnett comparisons, $P<0.05$) The crosses (+) in this figure indicate significant decreases in agonist-induced feeding following either antagonist or AS ODN pretreatment relative to corresponding agonist-induced feeding values alone (Dunnett comparisons, $p<0.05$).



lowest 5-nmol naltrindole dose (**Figure 4**), suggesting δ-opioid receptor mediation of βEND-induced feeding.

NorBNI and βEND-Induced Feeding

βEND-induced feeding was significantly altered following pretreatment with NorBNI after 1 [$F(4,81)=8.09$, $p<0.0001$], 2 ($F=13.22$, $p<0.0001$), and 4 ($F = 22.85$, $p<0.0001$) h. βEND-induced feeding was significantly reduced by the two highest (20- and 40-nmol) NorBNI doses after 2 and 4 h (**Figure 5**), suggesting κ_1-opioid receptor mediation of βEND-induced feeding.

MOR-1 AS ODN Probes and βEND-Induced Feeding

βEND-induced feeding was significantly reduced following pretreatment with MOR-1 AS ODN probes after 2 [$F(6,81) = 8.21$, $p<0.0001$] and 4 ($F = 13.92$, $p<0.0001$) h. βEND-induced feeding was significantly reduced by AS ODN probes directed against coding exons 1 (4 h), 3 (2, 4 h), and 4 (2, 4 h) of the MOR-1 gene (**Figure 6**). Pretreatment with either an AS ODN probe directed against coding exon 2, or a control MS ODN probe failed to significantly alter βEND-induced feeding. These data indicate that βEND-induced feeding is dependent upon the integrity of coding exons 1, 3, and 4 of the MOR-1 gene for the full expression of this ingestive response.

DOR-1 AS ODN Probes and βEND-Induced Feeding

βEND-induced feeding was significantly reduced following pretreatment with DOR-1 AS ODN probes only after 1 [$F(4,59) = 11.88$, $p<0.0001$] h (**Figure 7**). βEND-induced feeding was significantly although transiently (1 h) reduced by the AS ODN

Figure 4. Alterations (mean, ±SEM) in food intake (g) following i.c.v. administration of either vehicle alone, β-END alone or β-END following ventricular pretreatment (1 h) with the δ-opioid receptor antagonist, naltrindole (n=8/condition). Significant differences in food intake were observed following βEND and naltrindole treatment conditions relative to vehicle after 1 ($F(4,79)= 10.83$, $p<.0001$), 2 ($F= 14.17$, $p<.0001$) and 4 ($F= 24.85$, $p<.0001$) h. Difference-score analyses also displayed significant effects after 1 ($F(3,30) =5.66$, $p<.003$), 2 ($F=4.69$, $p<.008$), and 4 ($F=7.62$, $p<.0006$) h. The asterisks (*) in this figure indicate significant increases in agonist-induced feeding relative to corresponding vehicle control values (Dunnett comparisons, $P<0.05$) The crosses (+) in this figure indicate significant decreases in agonist-induced feeding following either antagonist or AS ODN pretreatment relative to corresponding agonist-induced feeding values alone (Dunnett comparisons, $p<0.05$). probe directed against coding exon 1 of the DOR-1 gene; no other significant alterations were observed. These data indicate the relatively limited actions of AS ODN probes targeting different coding exons of the DOR-1 gene in the mediation of βEND-induced feeding.

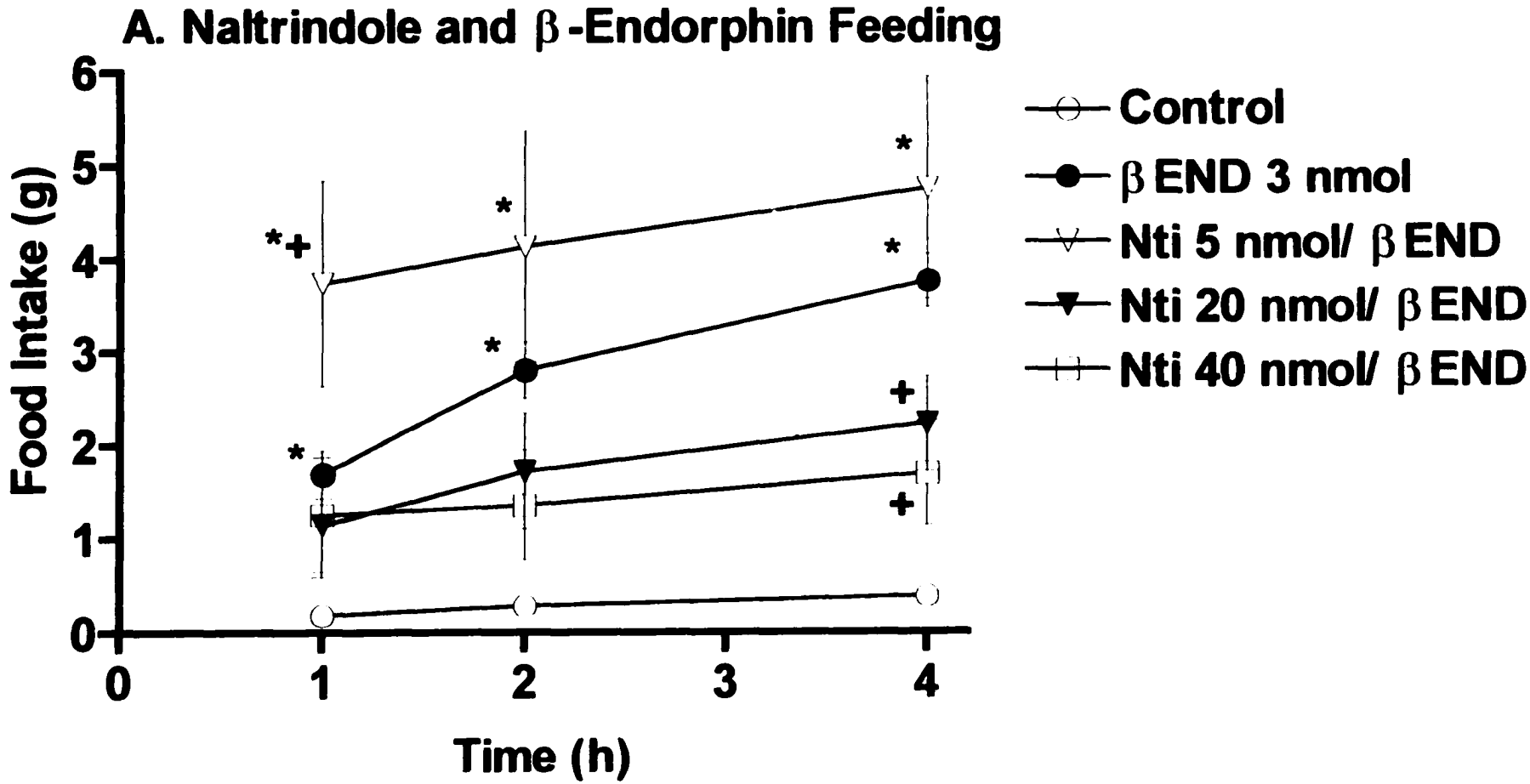


Figure 5. Alterations (mean, ±SEM) in food intake (g) following i.c.v. administration of either vehicle alone, β-END alone or β-END following ventricular pretreatment with the κ_1-opioid receptor antagonist, NorBNI at doses of either 5, 20 or 40 nmol (n=9/condition). Significant differences in food intake were observed following βEND and NorBNI treatment conditions relative to vehicle after 1 ($F(4,81)= 8.09$, $p<.0001$), 2 ($F= 13.22$, $p<.0001$) and 4 ($F= 22.85$, $p<.0001$) h. Difference-score analyses also displayed significant effects after 1 ($F(3,30) =3.85$, $p<.019$), 2 ($F=5.76$, $p<.003$), and 4 ($F=4.48$, $p<.016$) h. The asterisks (*) in this figure indicate significant increases in agonist-induced feeding relative to corresponding vehicle control values (Dunnett comparisons, $P<0.05$) The crosses (+) in this figure indicate significant decreases in agonist-induced feeding following either antagonist or AS ODN pretreatment relative to corresponding agonist-induced feeding values alone (Dunnett comparisons, $p<0.05$).

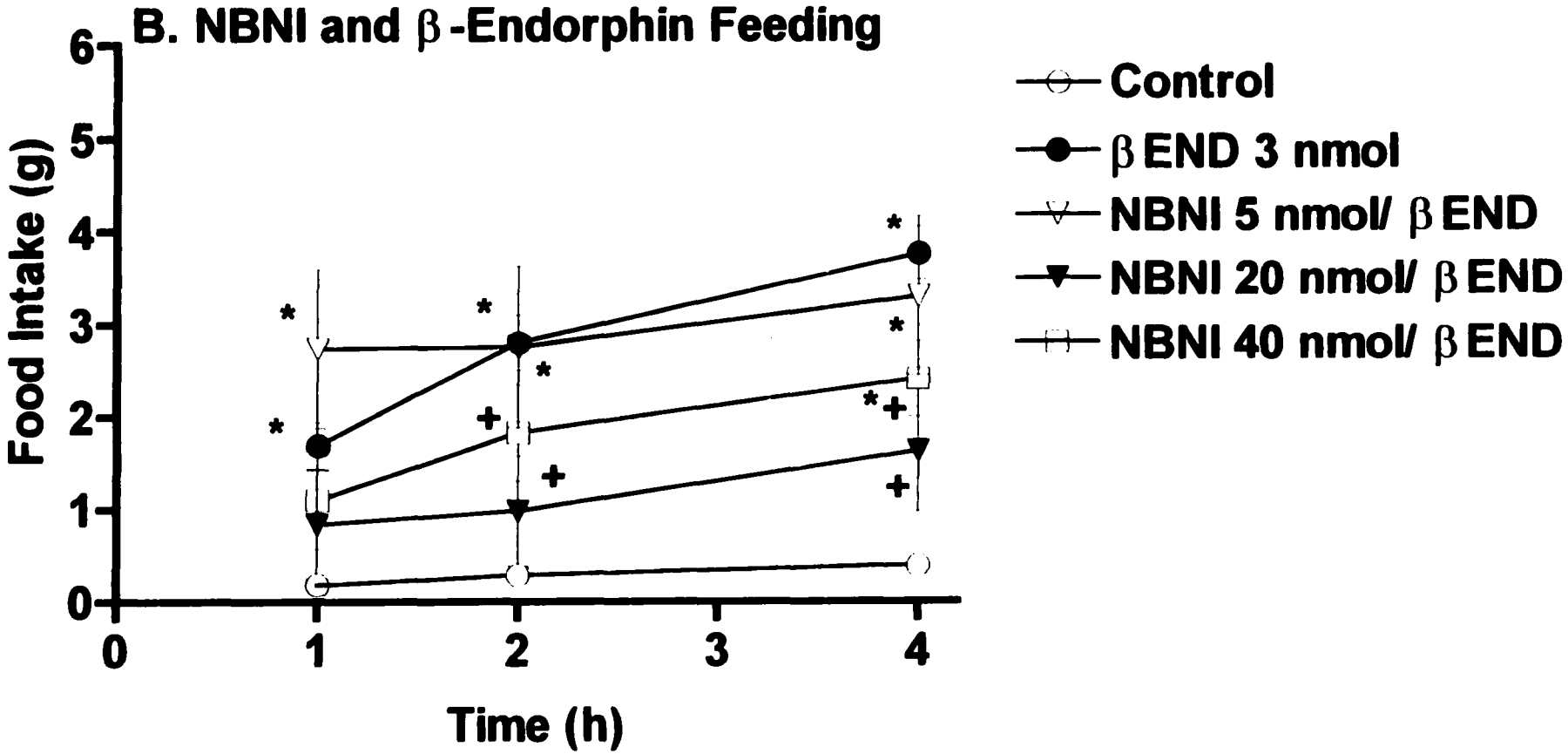


Figure 6. Alterations (mean, ±SEM) in food intake (g) following i.c.v. administration of either vehicle alone, β-END alone or ventricular pretreatment over three days (Days 1, 3 and 5) with AS or MS ODN probes directed against specific coding exons of the MOR-1 opioid receptor clone with β-END administration occurring 24 h later (Day 6) (n=8/condition). Significant differences in food intake were observed following βEND and MOR-1 AS ODN treatment conditions relative to vehicle after 1 ($F(6,81)= 6.27$, $p<.0001$), 2 ($F= 8.21$, $p<.0001$) and 4 ($F= 13.92$, $p<.0001$) h. Difference-score analyses also displayed significant effects after 1 ($F(5,120) =8.52$, $p<.0001$), 2 ($F=7.34$, $p<.0001$), and 4 ($F=20.08$, $p<.0001$) h. The asterisks (*) in this figure indicate significant increases in agonist-induced feeding relative to corresponding vehicle control values (Dunnett comparisons, $P<0.05$) The crosses (+) in this figure indicate significant decreases in agonist-induced feeding following either antagonist or AS ODN pretreatment relative to corresponding agonist-induced feeding values alone (Dunnett comparisons, $p<0.05$).

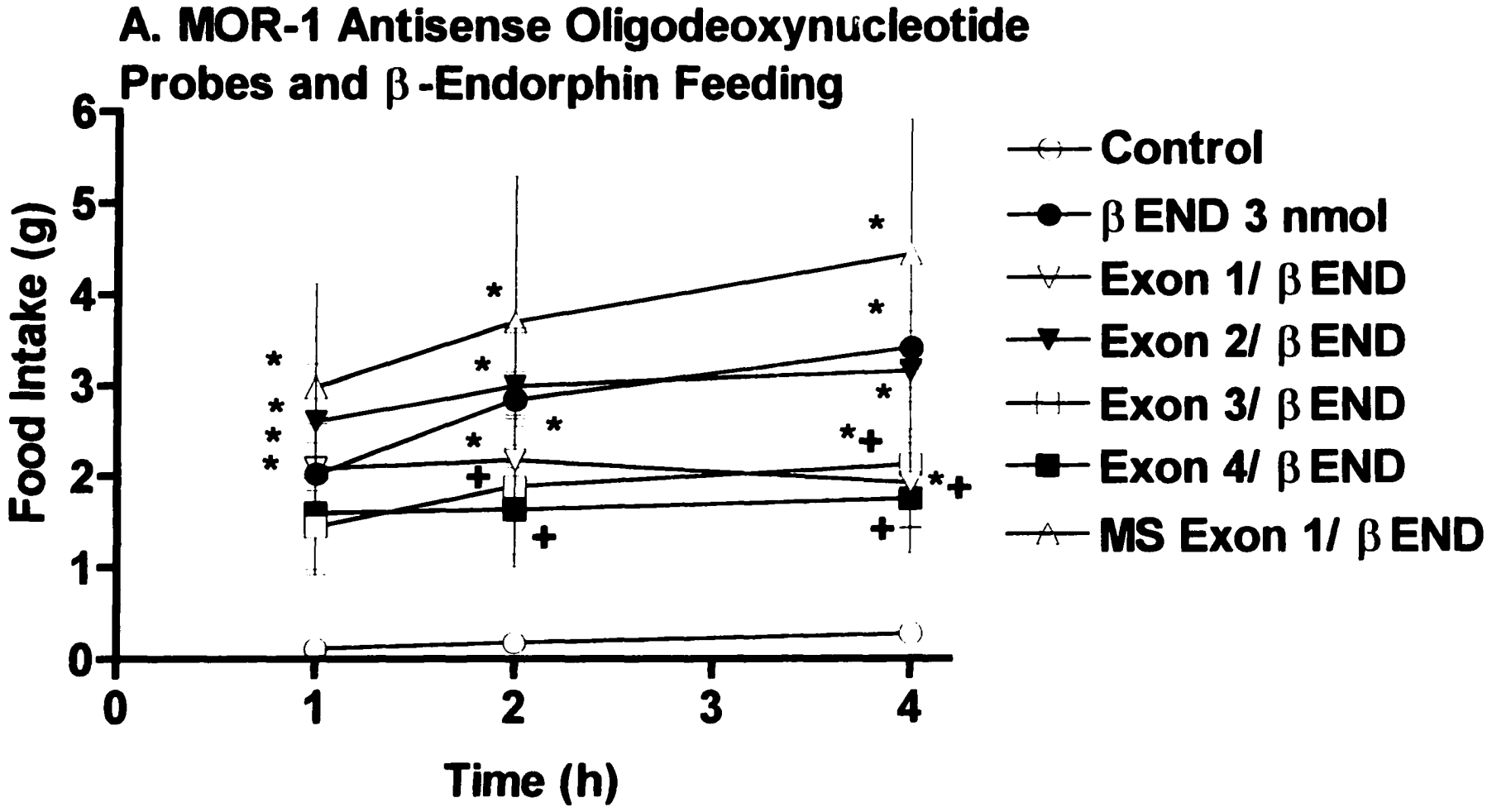
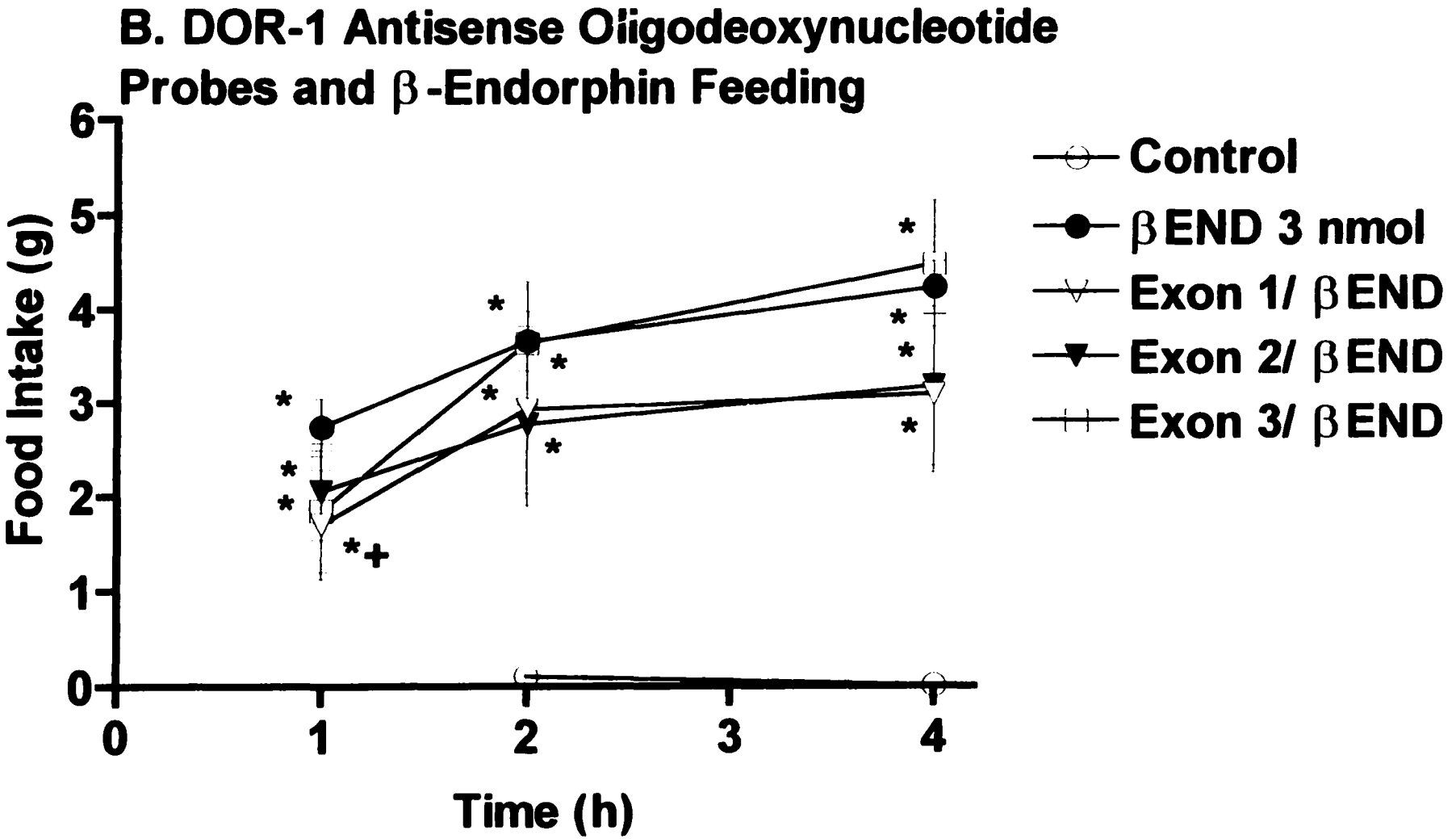


Figure 7. Alterations (mean, ±SEM) in food intake (g) following i.c.v. administration of either vehicle alone, β-END alone or ventricular pretreatment over three days (Days 1, 3 and 5) with AS or MS ODN probes directed against specific coding exons of the DOR-1 opioid receptor clone with β-END administration occurring 24 h later (Day 6) (n=8/condition). Significant differences in food intake were observed following βEND and DOR-1 AS ODN treatment conditions relative to vehicle after 1 ($F(4,59)= 11.88$, $p<.0001$), 2 ($F= 14.81$, $p<.0001$) and 4 ($F= 22.35$, $p<.0001$) h. Difference-score analyses also displayed significant effects after 1 ($F(3,57) =3.57$, $p<.019$), 2 ($F=2.59$, n.s.), and 4 ($F=5.37$, $p<.0025$) h. The asterisks (*) in this figure indicate significant increases in agonist-induced feeding relative to corresponding vehicle control values (Dunnett comparisons, $P<0.05$) The crosses (+) in this figure indicate significant decreases in agonist-induced feeding following either antagonist or AS ODN pretreatment relative to corresponding agonist-induced feeding values alone (Dunnett comparisons, $p<0.05$).



KOR-1 AS ODN Probes and βEND-Induced Feeding

βEND-induced feeding failed to be significantly altered by any of the AS ODN probes directed against either coding exons 1, 2, or 3 of the KOR-1 gene (**Figure 8**). These data indicate the relative lack of an effect for AS ODN probes targeting different coding exons of the KOR-1 gene in the mediation of this response.

KOR-3/ORL-1 AS ODN Probes and βEND-Induced Feeding

βEND-induced feeding failed to be significantly altered by any of the AS ODN probes directed against either coding exons 1, 2, or 3 of the KOR-3/ORL-1 gene (**Figure 9**). These data indicate the relative lack of an effect for AS ODN probes targeting different coding exons of the KOR-3/ORL-1 gene in the mediation of this response.

Discussion

The present study demonstrated that increased food intake following βEND was significantly and dose-dependently attenuated by pretreatment with either general (NTX), μ (βFNA), δ (naltrindole) or κ_1 (NorBNI) antagonists. In addition, βEND-induced feeding was significantly reduced following pretreatment with AS ODN probes directed against either coding exons 1, 3, or 4 of the MOR-1 gene, and coding exon 1 of the DOR-1 gene. In contrast, AS ODN probes directed against any coding exons of either the KOR-1 or KOR-3/ORL-1 clones were ineffective. Further, a control MS probe, that differed from an effective MOR-1 coding exon 1 AS ODN probe by the sequence reversal of only two pairs of bases, was also ineffective.

The potency of general and selective opioid receptor antagonists in reducing βEND-induced feeding was not uniform. The use of equimolar antagonist doses revealed

Figure 8. Alterations (mean, ±SEM) in food intake (g) following i.c.v. administration of either vehicle alone, β-END alone or ventricular pretreatment over three days (Days 1, 3 and 5) with AS ODN probes directed against specific coding exons of the KOR-1 opioid receptor clone with β-END administration occurring 24 h later (Day 6) (n=7/condition). Significant differences in food intake were observed following βEND and KOR-1 AS ODN treatment conditions relative to vehicle after 1 ($F(4,60)= 13.85$, $p<.0001$), 2 ($F= 16.54$, $p<.0001$) and 4 ($F= 23.99$, $p<.0001$) h. Difference-score analyses also displayed significant effects after 1 ($F(3,60) =5.97$, $p<.0012$), 2 ($F=7.84$, $p<.0002$), and 4 ($F=9.74$, $p<.0001$) h. The asterisks (*) in this figure indicate significant increases in agonist-induced feeding relative to corresponding vehicle control values (Dunnett comparisons, $P<0.05$) The crosses (+) in this figure indicate significant decreases in agonist-induced feeding following either antagonist or AS ODN pretreatment relative to corresponding agonist-induced feeding values alone (Dunnett comparisons, $p<0.05$).

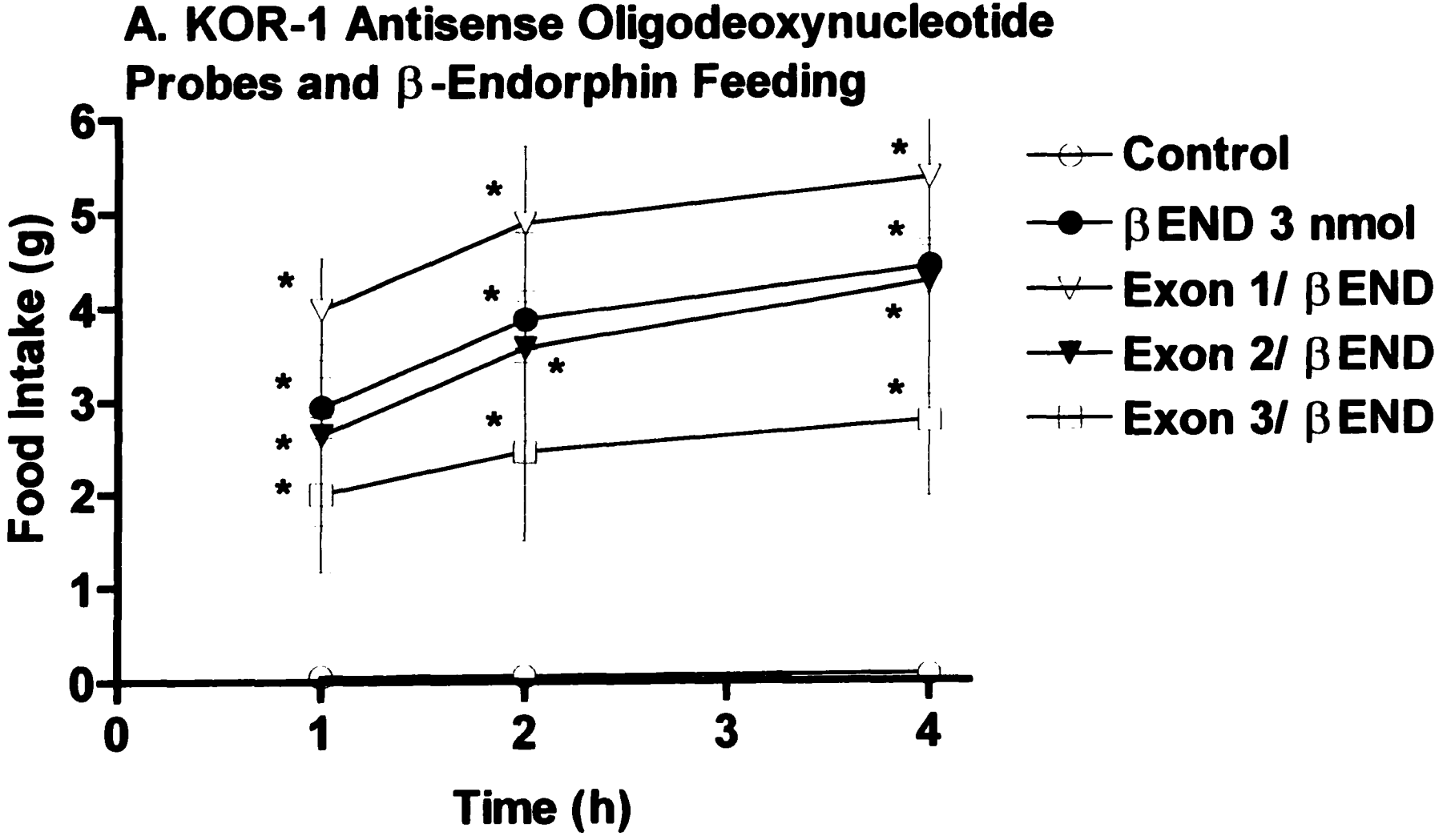
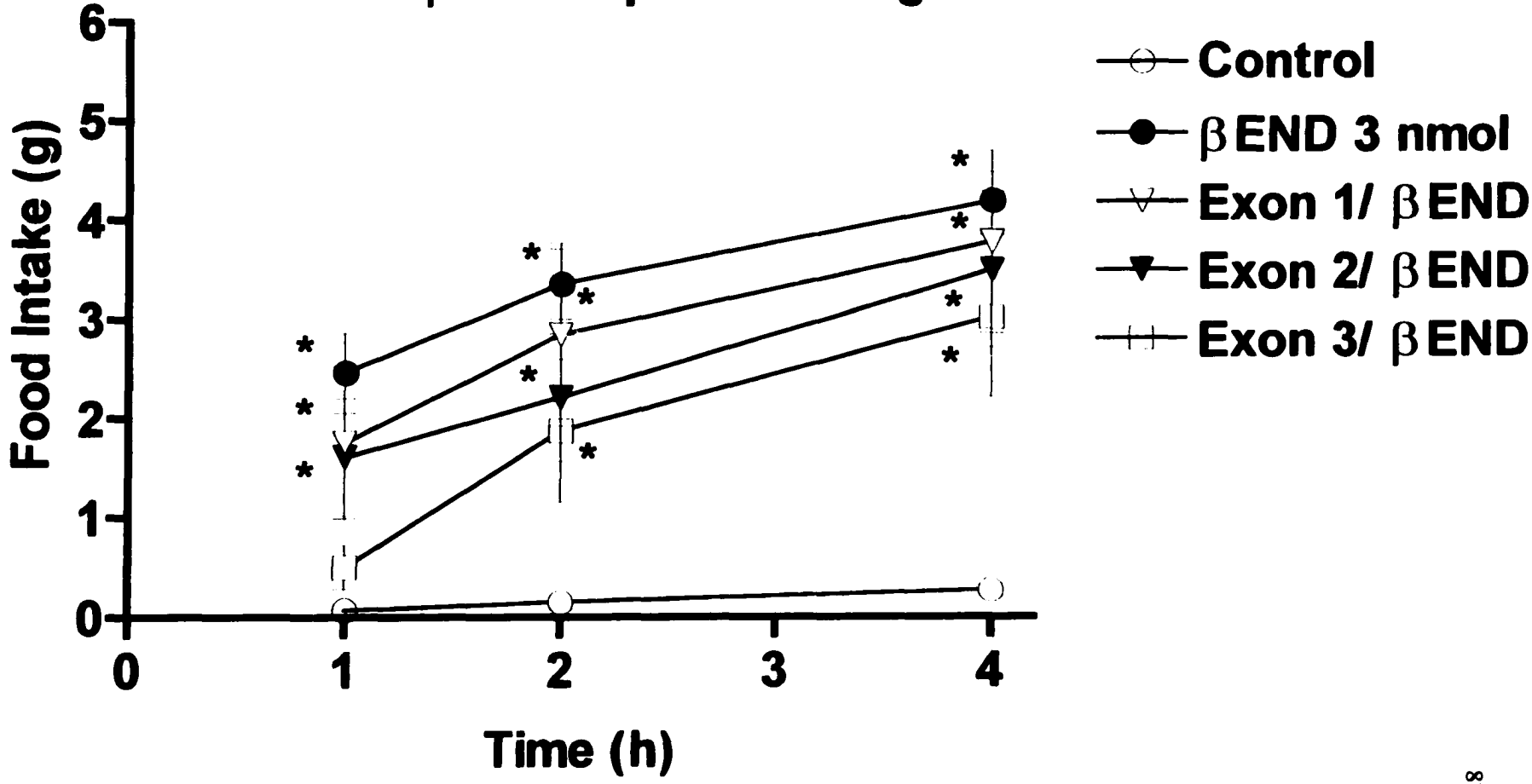


Figure 9. Alterations (mean, ±SEM) in food intake (g) following i.c.v. administration of either vehicle alone, β-END alone or ventricular pretreatment over three days (Days 1, 3 and 5) with AS ODN probes directed against specific coding exons of the KOR-3/ORL-1 opioid receptor clone with β-END administration occurring 24 h later (Day 6) (n=7/condition). Significant differences in food intake were observed following βEND and KOR-3/ORL-1 AS ODN treatment conditions relative to vehicle after 1 ($F(4,56)= 8.22$, $p<.0001$), 2 ($F= 10.89$, $p<.0001$) and 4 ($F= 16.36$, $p<.0001$) h. Difference-score analyses also displayed significant effects after 1 ($F(3,60) =7.28$, $p<.0003$), 2 ($F=8.06$, $p<.0001$), and 4 ($F=9.62$, $p<.0001$) h. The asterisks (*) in this figure indicate significant increases in agonist-induced feeding relative to corresponding vehicle control values (Dunnett comparisons, $P<0.05$) The crosses (+) in this figure indicate significant decreases in agonist-induced feeding following either antagonist or AS ODN pretreatment relative to corresponding agonist-induced feeding values alone (Dunnett comparisons, $p<0.05$).

B. KOR-3/ORL-1 Antisense Oligodeoxynucleotide Probes and β-Endorphin Feeding



that μ (βFNA) opioid antagonism significantly reduced βEND-induced feeding at far lower doses than general (NTX), κ_1 (NorBNI) or δ (naltrindole) opioid antagonists. It should be noted that the antagonist dose range employed has previously demonstrated non-selectivity in blocking feeding elicited by selective opioid agonists. Thus, comparable doses of βFNA and NorBNI respectively reduced feeding elicited by both μ (DAMGO) and κ (U50488H) opioid agonists to the same degree for each antagonist (Levine et al., 1990, 1991). However, in the present study, whereas a dose of 5 nmol of βFNA significantly reduced βEND-induced feeding across the time course, an equimolar dose of the other antagonists were ineffective. The present study confirmed the previously-observed attenuation of βEND-induced feeding by general opioid antagonists (Grandison and Guidotti, 1977; Leibowitz and Hor, 1982; Majeed et al., 1986; De Pedro et al., 1995). However, βEND-induced feeding was transiently (1 h), but significantly increased following pretreatment with the lowest (5 μg) dose of the general opioid receptor antagonist naltrexone. This finding is somewhat surprising and could reflect a disinhibitory effect of naltrexone upon other opioid receptors involved in the central control of feeding, which may be independent of the βEND-induced feeding response. Selective opioid receptor subtype antagonist analyses of βEND-induced feeding were previously limited to the goldfish, and indicated activity by μ-selective antagonists (βFNA, naloxonazine: De Pedro et al., 1996), which is consistent with the present results. Taken together, these antagonist data suggest that μ opioid receptors appear to play a sizable role in the mediation of βEND-induced feeding, yet other (δ and κ_1) opioid receptors might participate to a lesser degree.

These data underscore inherent limitations in the exclusive use and subsequent interpretation of selective antagonist data. In addition to the use of equimolar doses, it is essential that these antagonists exert functionally specific and selective effects at their respective receptors. Since these antagonists only worked at high equimolar doses relative to βFNA, it is conceivable that they could be exerting their effects through multiple rather than specific opioid receptors. Since none of the antagonist doses employed in the present study completely eliminated βEND-induced feeding, this suggests that blockade of multiple opioid receptors might be necessary to produce this effect. This is in contrast to the ability of comparable doses of βFNA to eliminate feeding elicited by the μ-selective agonists, morphine, M6G and DAMGO (Levine et al., 1991; Leventhal et al., 1997, 1998b), and comparable doses of NorBNI to eliminate feeding elicited by the κ_1-selective agonist, U50488H (Levine et al., 1990).

Thus, further converging evidence is required to determine the distinct pharmacological properties of βEND-induced feeding, and the use of AS ODN probes provided support for primary μ-receptor mediation of this response. AS ODN probes directed against either coding exons 1, 3, or 4 of the MOR-1 opioid receptor clone significantly reduced βEND-induced feeding. The unique sensitivity and specificity of βEND-induced feeding to disruption by an AS ODN probe directed against coding exon 1 of the MOR-1 clone was demonstrated by the failure of a control MS ODN probe to alter βEND-induced feeding. Other μ opioid-selective agonists induced feeding, which was reversed by βFNA pretreatment and which was further distinguished using AS ODN probes directed against specific coding exons of the MOR-1 clone (Leventhal et al., 1997,

1998b). Thus, feeding elicited by either morphine or DAMGO was blocked by AS ODN probes directed against either coding exons 1 or 4, but not coding exons 2 or 3 of the MOR-1 clone (Leventhal et al., 1997, 1998b). The activity of AS ODN probes directed against either coding exons 1 or 4 of the MOR-1 clone suggests that βEND shares highly similar molecular binding profiles to that of morphine and DAMGO in eliciting feeding. However, since an AS ODN probe directed against coding exon 3 of the MOR-1 clone significantly reduced βEND-induced feeding, this shows that this response shares similarities with feeding elicited by the active morphine metabolite, M6G, which is significantly reduced by pretreatment with AS ODN probes directed against either coding exons 2 or 3 of the MOR-1 clone (Leventhal et al., 1998b). The differential MOR-1 AS ODN sensitivity profiles displayed by morphine, DAMGO and M6G suggested that they were potentially acting upon different splice variants or different isoforms of the MOR-1 clone (see reviews: Pasternak and Standifer, 1995; Rossi and Pasternak, 1997), and the present data indicate that βEND-induced feeding appears to be mediated by multiple coding exon regions and perhaps different isoforms of the MOR-1 gene.

AS ODN probes directed against specific coding exons of the DOR-1, KOR-1, or KOR-3/ORL-1 opioid receptor clones provided converging data concerning specificity and selectivity of δ and κ_1 opioid antagonist data. The ability of an AS ODN probe directed against coding exon 1, but not coding exons 2 or 3 of the DOR-1 clone to transiently reduce βEND-induced feeding differs markedly from the selective ability of an AS ODN probe targeting coding exon 3 of the DOR-1 clone to eliminate feeding induced by the δ_2-opioid agonist Deltorphin II (Leventhal et al., 1998b). The ability of NorBNI at

moderate and high doses to reduce βEND-induced feeding suggests a κ_1 mechanism of action. NorBNI completely blocks feeding induced by the κ_1 opioid agonist, U50488H (Levine et al., 1990) which in turn is eliminated by an AS ODN probe directed against coding exon 3 of the KOR-1 opioid receptor clone (Leventhal et al., 1998a). Since none of the AS ODN probes directed against the KOR-1 clone significantly reduced βEND-induced feeding, this suggests that NorBNI's effect upon this feeding response was not mediated through the κ_1 opioid receptor. AS ODN probes directed against either coding exons 1, 2 or 3 of the KOR-3/ORL-1 clone each significantly reduce feeding elicited by orphanin FQ/nociceptin (Leventhal et al., 1998a), yet none of the probes were effective in altering feeding elicited by βEND, providing further evidence for the independent actions of these opioid receptor peptides in mediating their respective functional effects.

Thus, it appears that the primary opioid receptor mediating βEND-induced feeding is the pharmacologically-described μ opioid receptor. The gene(s) responsible for this action appears to be encoded by the MOR-1 clone since βEND-induced feeding share similar, though not identical profiles in AS ODN studies using morphine, DAMGO and M6G.

In addition to βEND, the endogenous opioid peptide, dynorphin A_{1-17} has also been reported to induce feeding following direct microinjection (Morley et al., 1982; Morley and Levine, 1983). The following study attempted to establish which opioid receptors participate in the mediation of dynorphin-induced feeding.

CHAPTER 4: SPECIFIC AIM TWO.

Pharmacological Characterization of the Dynorphin $A_{(1-17)}$-induced Feeding Response in Rats

Introduction

Dynorphin A_{1-17} is an endogenous opioid peptide derived by the enzymatic cleavage of prodynorphin. Since the isolation of the complete peptide from porcine pituitary tissue (Goldstein et al., 1981), this peptide has displayed significant binding activity at multiple opioid receptors. The ability of dynorphin to induce naloxone-reversible food intake is well established (Gosnell and Lipton, 1986; Gosnell et al., 1986; Cooper et al., 1985; Morley and Levine, 1983). Moreover, several studies have implicated that dynorphin, through its putative action at the κ-opioid receptor, plays a key role in the modulation of food intake (Chavkin and Goldstein, 1981; Levine et al., 1985; Morley and Levine, 1981, 1983) and appetite (Sanger, 1981). Support for the role of the κ-opioid receptor in feeding behavior has been identified in studies using other κ receptor agonists such as the selective κ_1 agonists U-50488H (Cooper et al., 1985), ketocyclazocine, butorphanol, tifluadom (Morley et al., 1982), as well as the dynorphin fragments (1-10), (1-11), (1-13) and (3-13) (Morley and Levine, 1983).

The significant efficacy of dynorphin A_{1-17} at multiple opioid receptor subtypes obscures the pharmacological profile of dynorphin-induced feeding. Given the paucity of data supporting an exclusive role for κ-opioid receptors in dynorphin induced feeding, the aim of the present study is to determine the precise pharmacological mechanism of dynorphin-induced feeding using selective opioid receptor antagonists and antisense

oligonucleotide probes directed against different opioid receptor genes. We will attempt to provide converging evidence to suggest a considerable role for the κ-opioid receptor in dynorphin induced feeding. The findings of the present study have been recently accepted for publication (Silva et al., 2001b).

Methods

Drugs: Dynorphin A_{1-17} (5-50 μg, Peninsula Laboratories, Belmont, CA), NTX (5-80 nmol, Sigma Chemical Co., St. Louis, MO), βFNA (5-80 nmol, Research Biochemicals Inc., Natick, MA), naltrindole (40-80 nmol, Research Biochemicals Inc.) and NorBNI (5-80 nmol, Research Biochemicals Inc.) were each dissolved in 0.9% normal saline.

Antisense probes: All phosphodiester oligodeoxynucleotides (Midland Certified Reagent Company, Midland, TX) were purified in our laboratory (G.C.R, G.W.P.). Each AS ODN probe was directed against the individual coding exons of either the MOR-1, DOR-1, KOR-1 or KOR-3/ORL-1 opioid receptor genes. All AS ODN probes were administered at 10 μg doses dissolved in 5 μl volumes of 0.9% normal saline based upon their previously-determined effectiveness in feeding studies (Leventhal et al., 1997, 1998a, 1998b).

Procedures: Following baseline determinations, the first group of thirteen animals were assessed for food intake after 1, 2 and 4 h following microinjection of dynorphin A_{1-17} at doses of 0, 5, 10, 20 and 50 μg in counterbalanced order at weekly intervals. Following the determination that a 50 μg dose of dynorphin A_{1-17} produced the most consistent feeding response (see Results), the test phase of the antagonist studies had subgroups of animals were pretreated with equimolar doses (5-80 nmol) of either the general (NTX, n=8), δ-selective (naltrindole, n=7), μ-selective (βFNA, n=8) or κ-selective (NorBNI, n=9) opioid receptor

antagonists. Dynorphin-induced food intake was subsequently assessed after 1, 2 and 4 h following microinjection of dynorphin (50 μg) as previously described. In the antisense studies, separate subgroups of animals received microinjections of AS ODN probes (10 μg) directed against either coding exons 1, 2, 3 or 4 of the MOR-1 gene (n=8/condition), coding exons 1, 2 or 3 of the DOR-1 gene (n=7/condition), coding exons 1, 2 or 3 of the KOR-1 gene (n=7/condition), coding exons of the KOR-3/ORL-1 gene (n=7/condition) or a MS ODN probe directed against coding exon 1 of the MOR-1 gene (n=6) which differed from its corresponding AS ODN probe by the sequence reversal of two pairs of bases (**Table 1**). During each 6-day testing phase, rats received microinjections of their particular AS ODN probes on days 1, 3 and 5 as previously described (Leventhal et al., 1997). Twenty-four h following the last AS or MS ODN treatment (day 6), all animals received dynorphin (50 μg), and food intake was assessed after 1, 2 and 4 h.

Results

Dynorphin-Induced Feeding Dose-Response Curve

Dynorphin produced significant dose-dependent increases in food intake after 1 [$F(4,56) = 10.94$, $p<0.0001$], 2 ($F = 11.73$, $p<0.0001$), and 4 ($F = 12.01$, $p<0.0001$) h. Pretreatment with the three highest (10-50 μg), but not the lowest (5 μg) dynorphin doses produced increases in spontaneous food intake (**Table 3**). Since the highest (50 μg) dynorphin dose produced the greatest magnitude of effects upon feeding responses across all test intervals, that were comparable to those observed for morphine, M6G and βEND, this i.c.v. dose was used in all subsequent studies.

NTX and Dynorphin-Induced Feeding

Table 3. Summary of dose-dependent actions (Mean, ±SEM) of dynorphin-induced feeding.

Dynorphin Dose (μg) (n)	Time (h) 1	2	4
0 (15)	0.05 (0.03)	0.12 (0.06)	0.27 (0.10)
5 (6)	1.07 (0.29)	1.30 (0.38)	1.43 (0.38)
10 (8)	1.88 (0.63)*	1.94 (0.62)*	2.38 (0.63)*
20 (13)	1.51 (0.21)*	1.58 (0.22)*	1.63 (0.21)*
50 (13)	2.29 (0.29)*	2.68 (0.31)*	2.97 (0.35)*

Note: Significant differences in food intake were observed among dynorphin doses after 1 ($F(4,56)= 10.94$, $P<0.0001$), 2 ($F= 11.73$, $P<0.0001$) and 4 ($F= 12.01$, $P<0.0001$) h. The asterisks (*) denote significant increases in intake relative to vehicle treatment (Tukey comparison, $P<0.05$).

Dynorphin induced feeding was significantly reduced only following pretreatment with the highest (80 nmol) NTX dose after 2 [$F(5,35) = 5.74$, $p<0.0006$] and 4 ($F = 9.00$, $p<.0001$) h (**Figure 10**), indicating small general opioid receptor mediation of dynorphin-induced feeding.

βFNA and Dynorphin-Induced Feeding

Dynorphin-induced feeding was significantly reduced by pretreatment with βFNA after 1 [$F(5,35) = 7.03$, $p<0.0001$], 2 ($F = 7.92$, $p<0.0001$), and 4 ($F = 15.61$, $p<0.0001$) h. Pretreatment with βFNA doses of 20 (2-4 h), 40 (1-4 h) and 80 (2-4 h) nmol significantly reduced dynorphin-induced feeding (**Figure 11**). The lowest (5 nmol) βFNA dose failed to significantly alter dynorphin-induced feeding. The potency and magnitude of βFNA effects suggest μ-opioid receptor mediation of dynorphin-induced feeding.

Naltrindole and Dynorphin-Induced Feeding

Dynorphin-induced feeding was significantly reduced only by the highest (80 nmol) naltrindole dose after 1 [$F(3,21) = 15.50$, $p<0.0001$], 2 ($F = 7.71$, $p<0.0016$), and 4 ($F = 9.49$, $p<0.0006$) h (**Figure 12**). However, pretreatment with a lower (40 nmol) naltrindole dose was completely ineffective in significantly altering dynorphin-induced feeding, suggesting minimal δ-opioid receptor mediation of dynorphin-induced feeding.

NorBNI and Dynorphin-Induced Feeding

Dynorphin induced feeding was significantly reduced following pretreatment with the three highest (20, 40, 80 nmol) NorBNI doses after 1 [$F(5,45) = 7.57$, $p<0.0001$], 2 ($F = 9.63$, $p<0.0001$), and 4 ($F = 7.92$, $p<0.0001$) h (**Figure 13**). Further, the lowest dose (5 nmol) of NorBNI significantly reduced dynorphin-induced feeding after 1 and 2,

Figure 10. Alterations (mean, ±SEM) in food intake (g) following i.c.v. administration of either vehicle alone, dynorphin (50 μg) alone or dynorphin following ventricular pretreatment (1 h) with the general opioid receptor antagonist, NTX at doses of either 5, 20, 40 or 80 nmol (n=8/condition). Significant differences in food intake were observed following all dynorphin treatment conditions relative to vehicle after 1 ($F(5,35)= 4.17$, $p<.0045$), 2 ($F= 5.74$, $p<.0006$) and 4 ($F= 9.00$, $p<.0001$) h. The asterisks (*) in this figure indicate significant increases in agonist-induced feeding relative to corresponding vehicle control values (Dunnett comparisons, $P<0.05$) The crosses (+) in this figure indicate significant decreases in agonist-induced feeding following either antagonist or AS ODN pretreatment relative to corresponding agonist-induced feeding values alone (Dunnett comparisons, $p<0.05$), but not 4 h. The potency and magnitude of NorBNI effects over this large dose range strongly suggests κ_1-opioid receptor mediation of dynorphin-induced feeding.

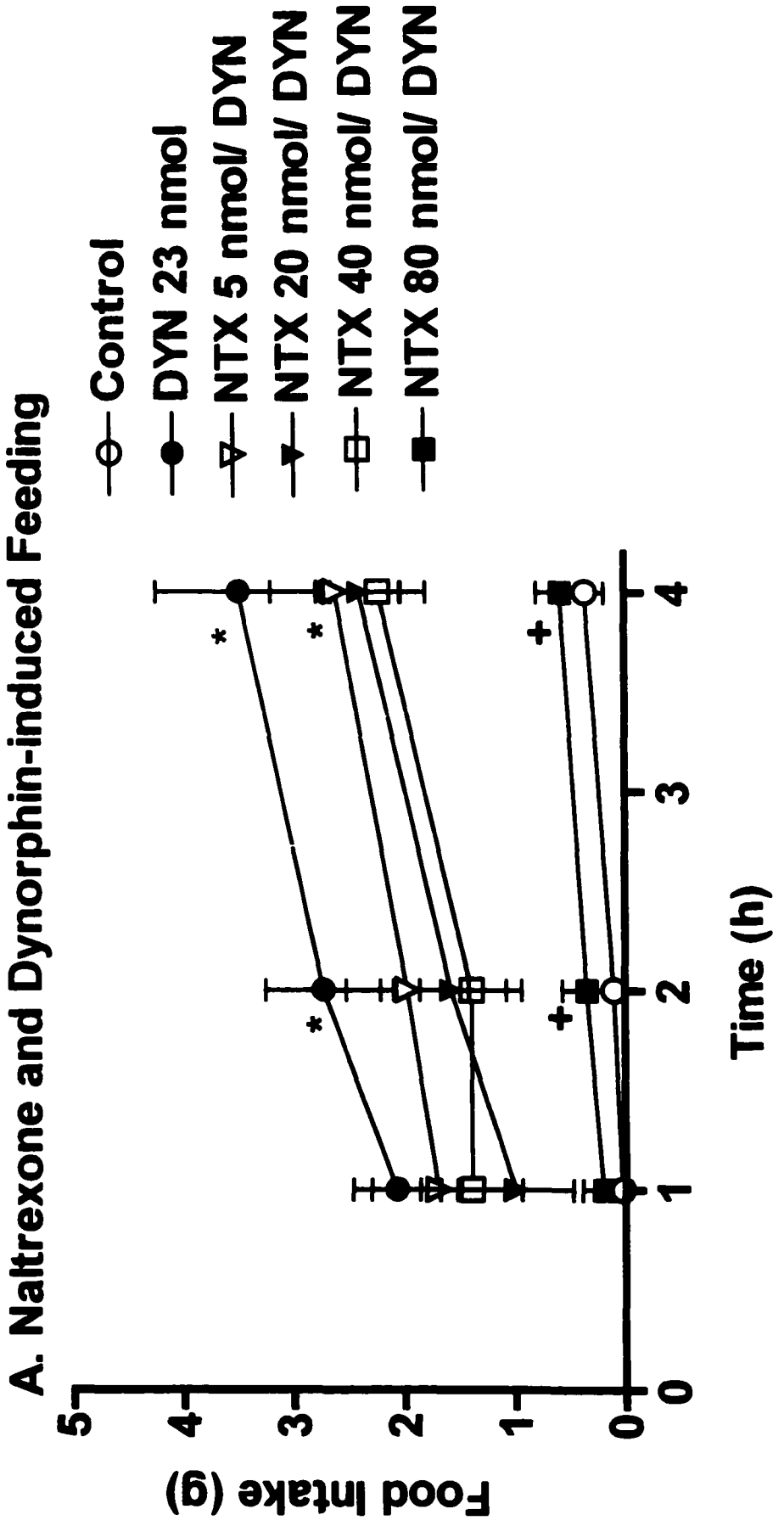


Figure 11. Alterations (mean, ±SEM) in food intake (g) following i.c.v. administration of either vehicle alone, dynorphin (50 μg) alone or dynorphin following ventricular pretreatment (24 h) with the μ-opioid receptor antagonist, βFNA (n=8/condition). Significant differences in food intake were observed following dynorphin and βFNA treatment conditions relative to vehicle after 1 ($F(5,35)= 7.03$, $p<.0001$), 2 ($F= 7.92$, $p<.0001$) and 4 ($F= 15.61$, $p<.0001$) h. The asterisks (*) in this figure indicate significant increases in agonist-induced feeding relative to corresponding vehicle control values (Dunnett comparisons, $P<0.05$) The crosses (+) in this figure indicate significant decreases in agonist-induced feeding following either antagonist or AS ODN pretreatment relative to corresponding agonist-induced feeding values alone (Dunnett comparisons, $p<0.05$).

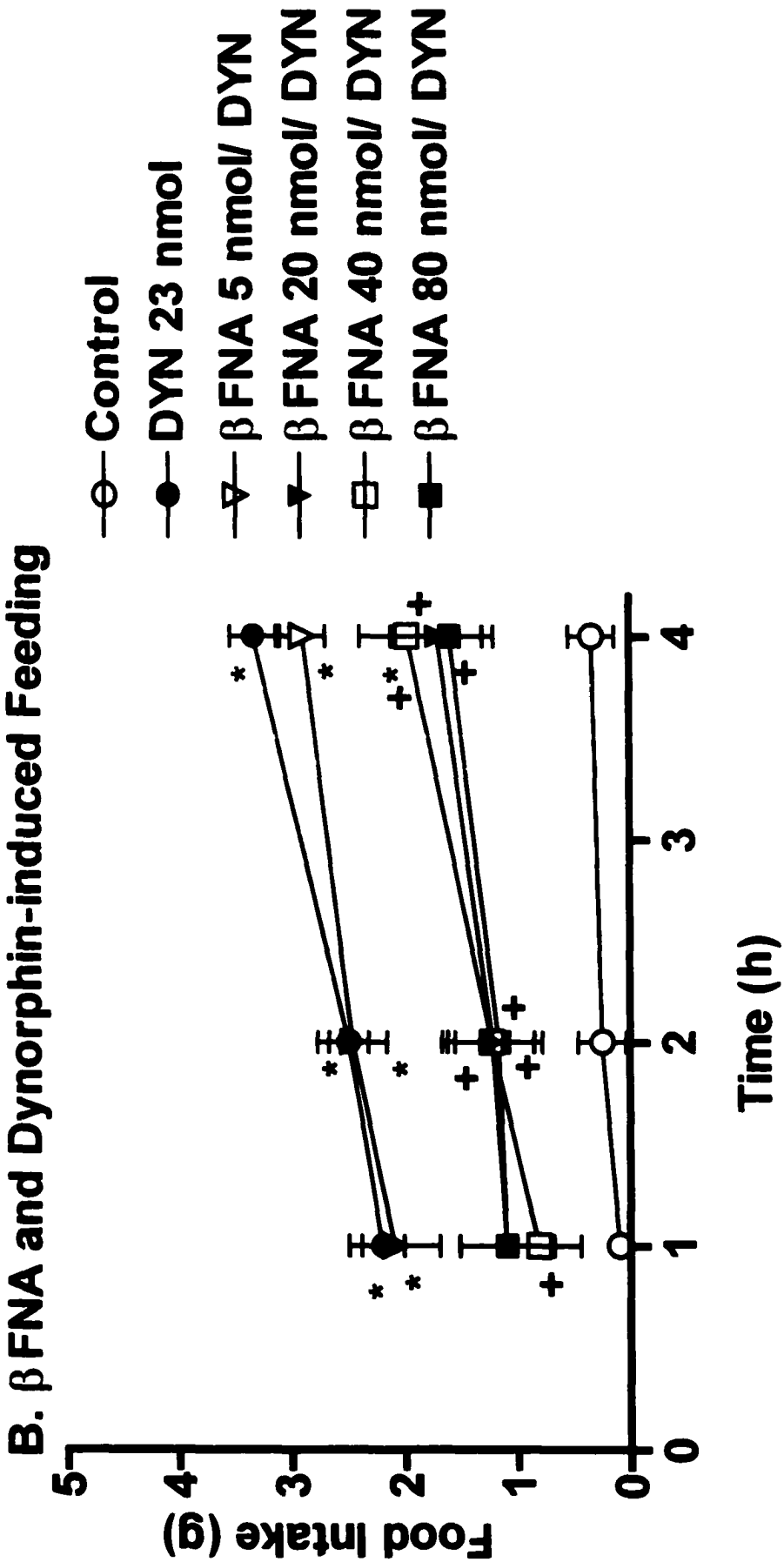


Figure 12. Alterations (mean, ±SEM) in food intake (g) following i.c.v. administration of either vehicle alone, dynorphin (50 μg) alone or dynorphin following ventricular pretreatment (1 h) with the δ-opioid receptor antagonist, naltrindole (n=7/condition). Significant differences in food intake were observed following dynorphin and naltrindole treatment conditions relative to vehicle after 1 ($F(3,21)= 15.50$, $p<.0001$), 2 ($F= 7.71$, $p<.0016$) and 4 ($F= 9.49$, $p<.0006$) h. The asterisks (*) in this figure indicate significant increases in agonist-induced feeding relative to corresponding vehicle control values (Dunnett comparisons, $P<0.05$) The crosses (+) in this figure indicate significant decreases in agonist-induced feeding following either antagonist or AS ODN pretreatment relative to corresponding agonist-induced feeding values alone (Dunnett comparisons, $p<0.05$).

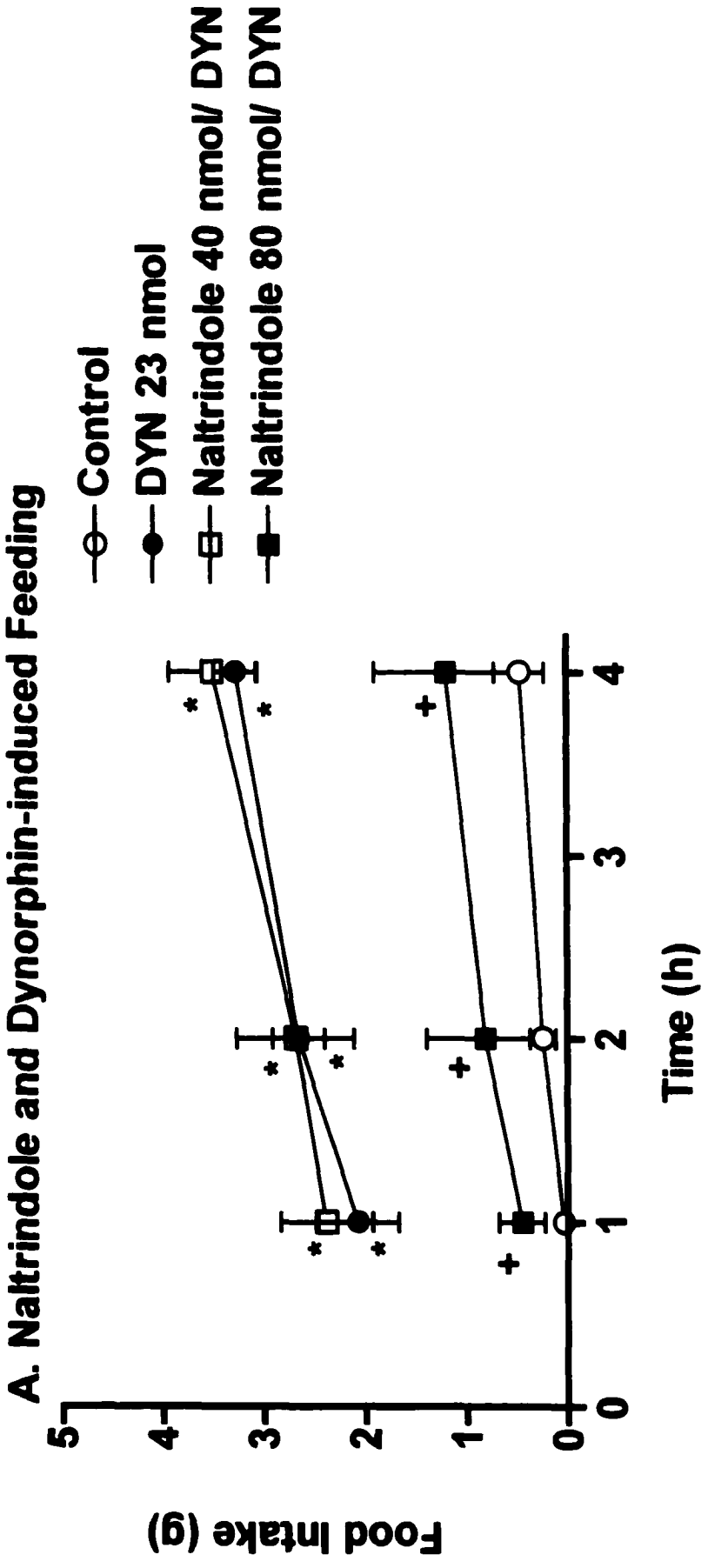
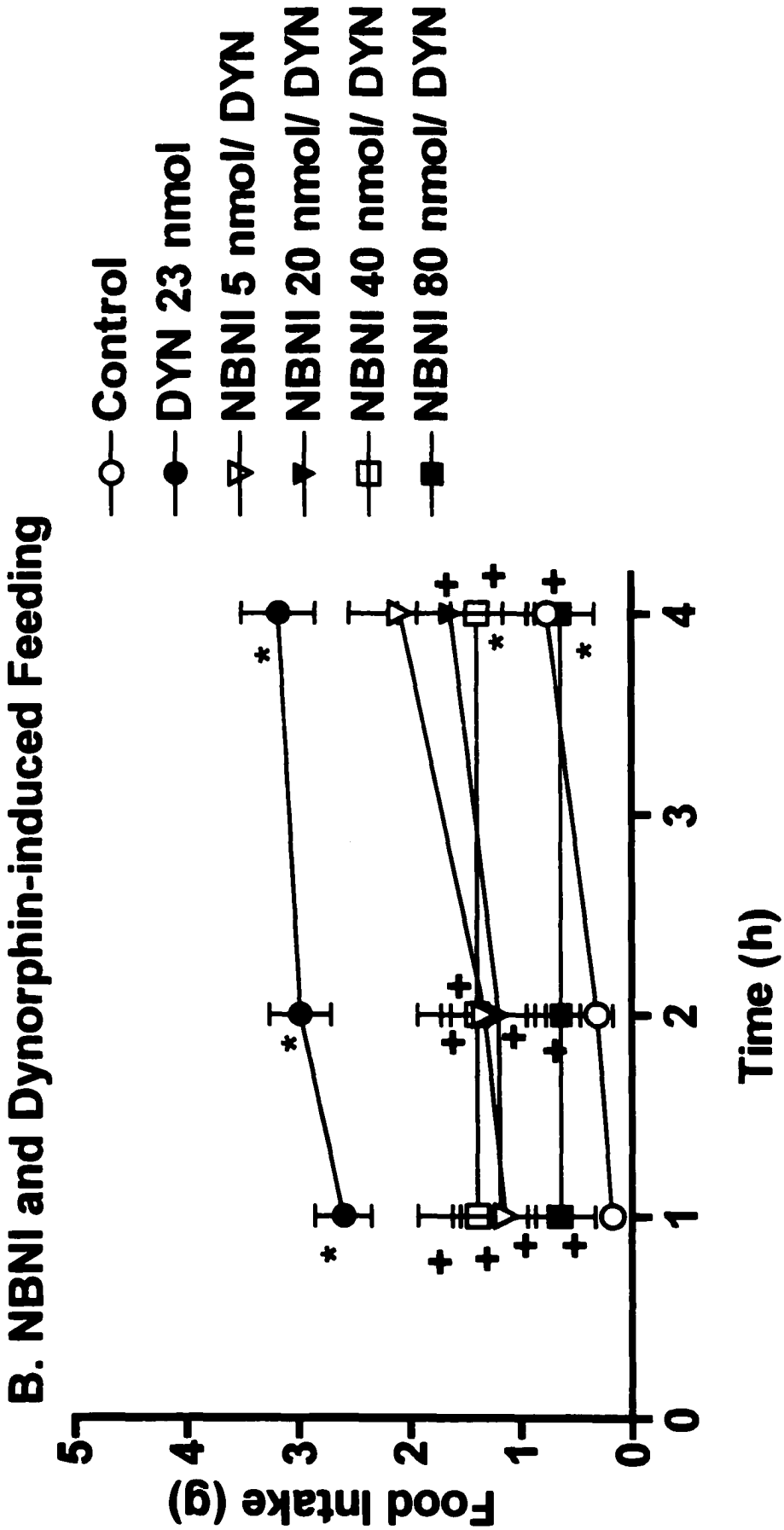


Figure 13. Alterations (g, ±SEM) in food intake following i.c.v. administration of either vehicle alone, dynorphin (50 μg) alone or dynorphin following ventricular pretreatment (24 h) with the κ_1-opioid receptor antagonist, NorBNI at doses of either 5, 20, 40 or 80 nmol (n=9/condition). Significant differences in food intake were observed following dynorphin treatment conditions relative to vehicle after 1 ($F(5,45)= 7.57$, $p<.0001$), 2 ($F= 9.63$, $p<.0001$) and 4 ($F= 7.92$, $p<.0001$) h. The asterisks (*) in this figure indicate significant increases in agonist-induced feeding relative to corresponding vehicle control values (Dunnett comparisons, $P<0.05$) The crosses (+) in this figure indicate significant decreases in agonist-induced feeding following either antagonist or AS ODN pretreatment relative to corresponding agonist-induced feeding values alone (Dunnett comparisons, $p<0.05$).



MOR-1 AS ODN Probes and Dynorphin-Induced Feeding

Dynorphin-induced feeding was significantly reduced by pretreatment with an AS ODN probe directed against coding exon 1 of the MOR-1 gene only after 1 [$F(5,120)=13.22$, $p<0.0001$] and 2 ($F=42.33$, $p<0.0001$) h (**Figure 14**). AS ODN probes directed against either coding exons 2, 3, or 4 of the MOR-1 gene failed to significantly reduce dynorphin-induced feeding. These data suggest that the integrity of the MOR-1 gene plays a relatively minor role in th mediation of dynorphin-induced feeding.

DOR-1 AS ODN Probes and Dynorphin-Induced Feeding

Dynorphin-induced feeding was significantly and transiently reduced by pretreatment with an AS ODN probe directed against coding exon 1 of the DOR-1 gene after 4 [$F(4,80) = 29.91$, $p<0.0001$] h (**Figure 15**). AS ODN probes directed against either coding exons 2 or 3 of the DOR-1 clone were ineffective. These data indicate the relatively limited actions upon dynorphin-induced feeding of AS ODN probes targeting different coding exons of the DOR-1 gene.

KOR-1 AS ODN Probes and Dynorphin-Induced Feeding

Dynorphin-induced feeding was significantly reduced by pretreatment with AS ODN probes directed against either coding exons 1 or 2 of the KOR-1 gene after 1 [$F(5,130) = 53.76$, $p<0.0001$), 2 ($F = 62.91$, $p<0.0001$), and 4 ($F = 78.48$, $p<0.0001$) h (**Figure 16**). In contrast, an AS ODN probe directed against coding exon 3 of the KOR-1 gene, as well as a control MS ODN probe both failed to significantly alter dynorphin-induced feeding. The efficacy of AS ODN probes directed against coding exons 1 or 2 of the KOR-1 gene in significantly reducing dynorphin-induced feeding suggests that the

Figure 14. Alterations (mean, ±SEM) in food intake (g) following i.c.v. administration of either vehicle alone, dynorphin (50 μg) alone or ventricular pretreatment over three days (Days 1, 3 and 5) with AS ODN probes directed against specific coding exons of the MOR-1 opioid receptor clone with dynorphin administration occurring 24 h later (Day 6) (n=8/condition). Significant differences in food intake were observed following dynorphin and MOR-1 AS ODN treatment conditions relative to vehicle after 1 ($F(5,120)= 13.22$, $p<.0001$), 2 ($F= 42.33$, $p<.0001$) and 4 ($F= 57.14$, $p<.0001$) h. The asterisks (*) in this figure indicate significant increases in agonist-induced feeding relative to corresponding vehicle control values (Dunnett comparisons, $P<0.05$) The crosses (+) in this figure indicate significant decreases in agonist-induced feeding following either antagonist or AS ODN pretreatment relative to corresponding agonist-induced feeding values alone (Dunnett comparisons, $p<0.05$).

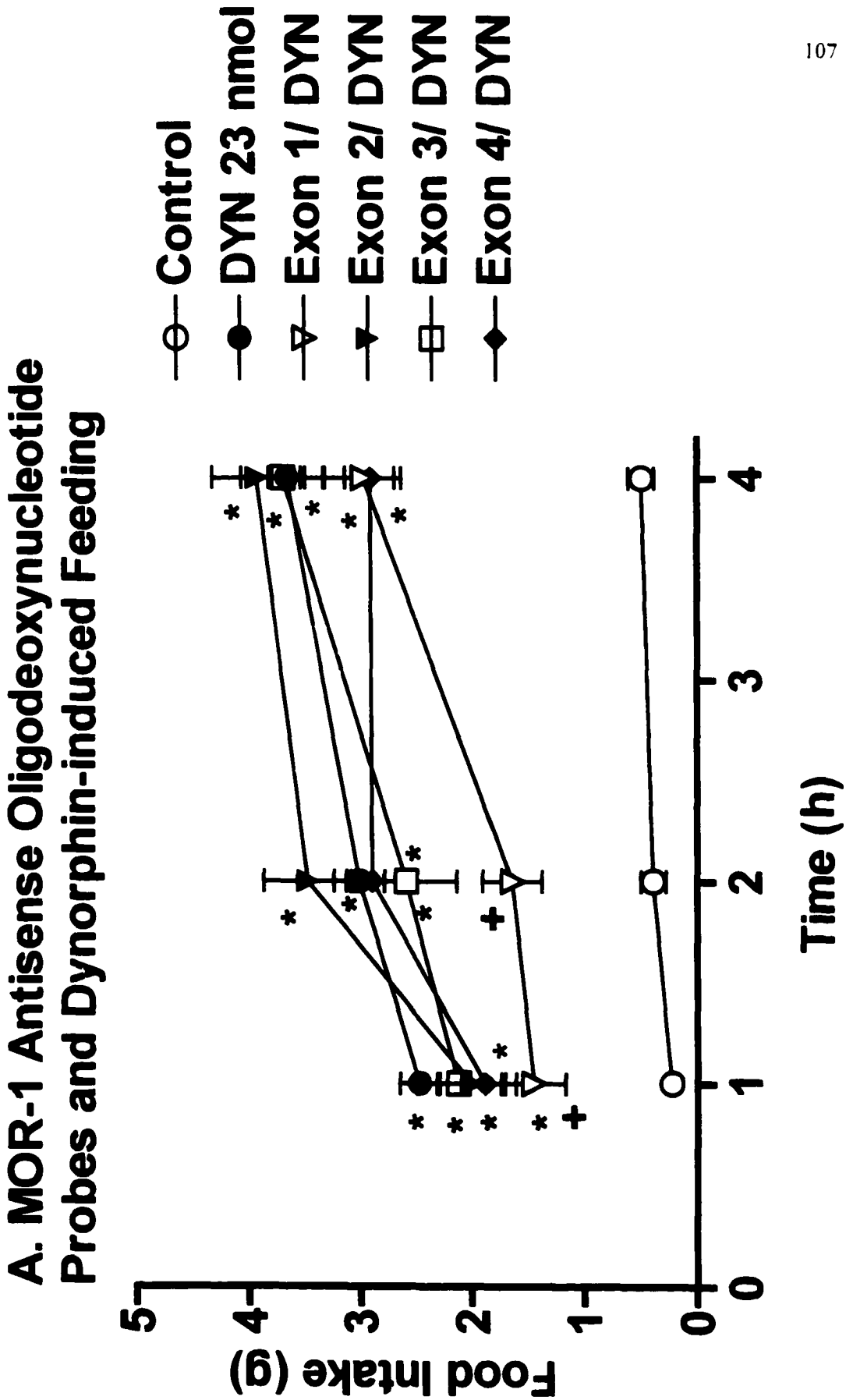


Figure 15. Alterations (mean, ±SEM) in food intake (g) following i.c.v. administration of either vehicle alone, dynorphin (50 μg) alone or ventricular pretreatment over three days (Days 1, 3 and 5) with AS ODN probes directed against specific coding exons of the DOR-1 opioid receptor clone with dynorphin administration occurring 24 h later (Day 6) (n=7/condition). Significant differences in food intake were observed following dynorphin and DOR-1 AS ODN treatment conditions relative to vehicle after 1 ($F(4,80)= 21.27$, $p<.0001$), 2 ($F= 25.81$, $p<.0001$) and 4 ($F= 29.91$, $p<.0001$) h. The asterisks (*) in this figure indicate significant increases in agonist-induced feeding relative to corresponding vehicle control values (Dunnett comparisons, $P<0.05$) The crosses (+) in this figure indicate significant decreases in agonist-induced feeding following either antagonist or AS ODN pretreatment relative to corresponding agonist-induced feeding values alone (Dunnett comparisons, $p<0.05$).

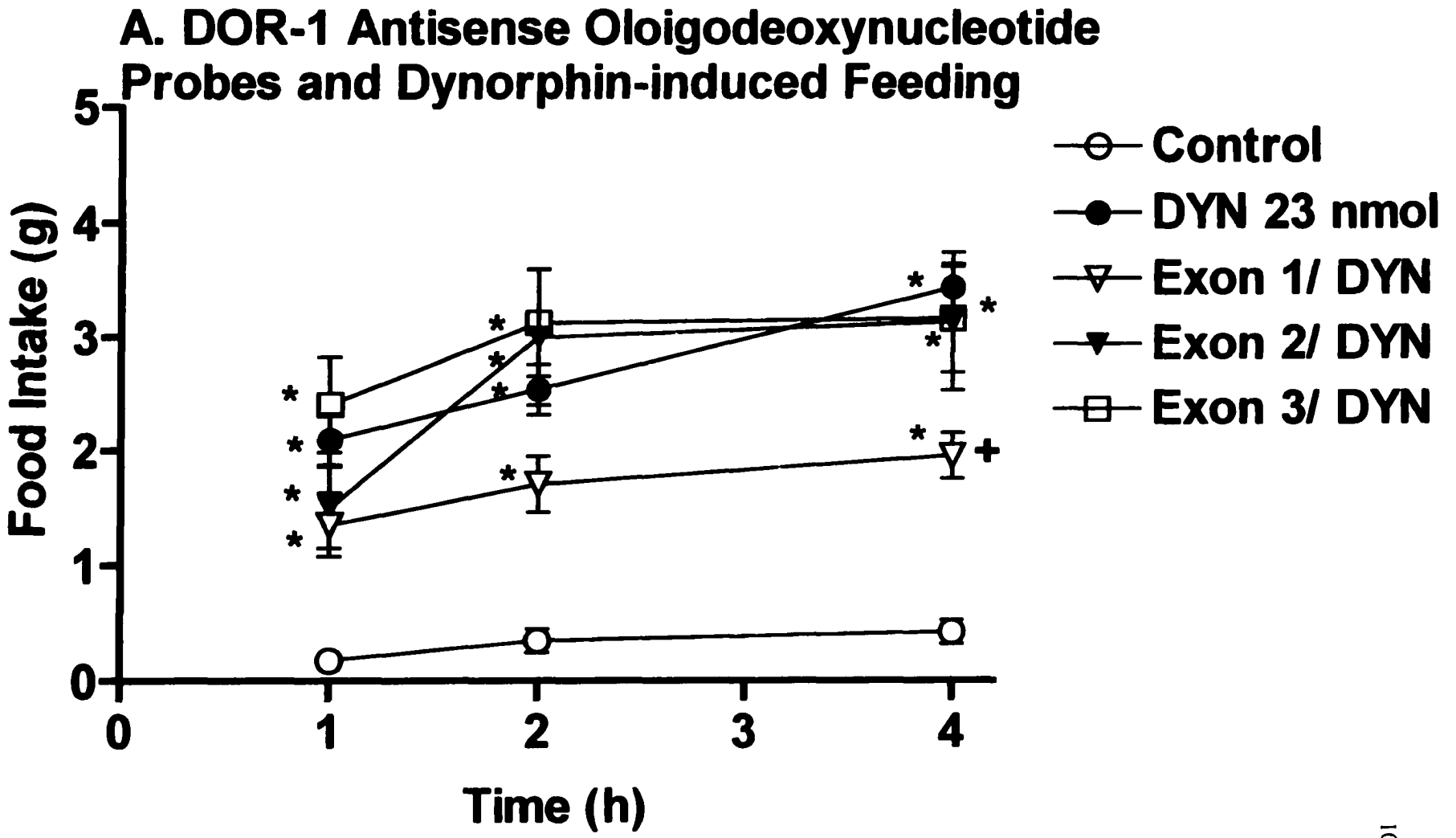
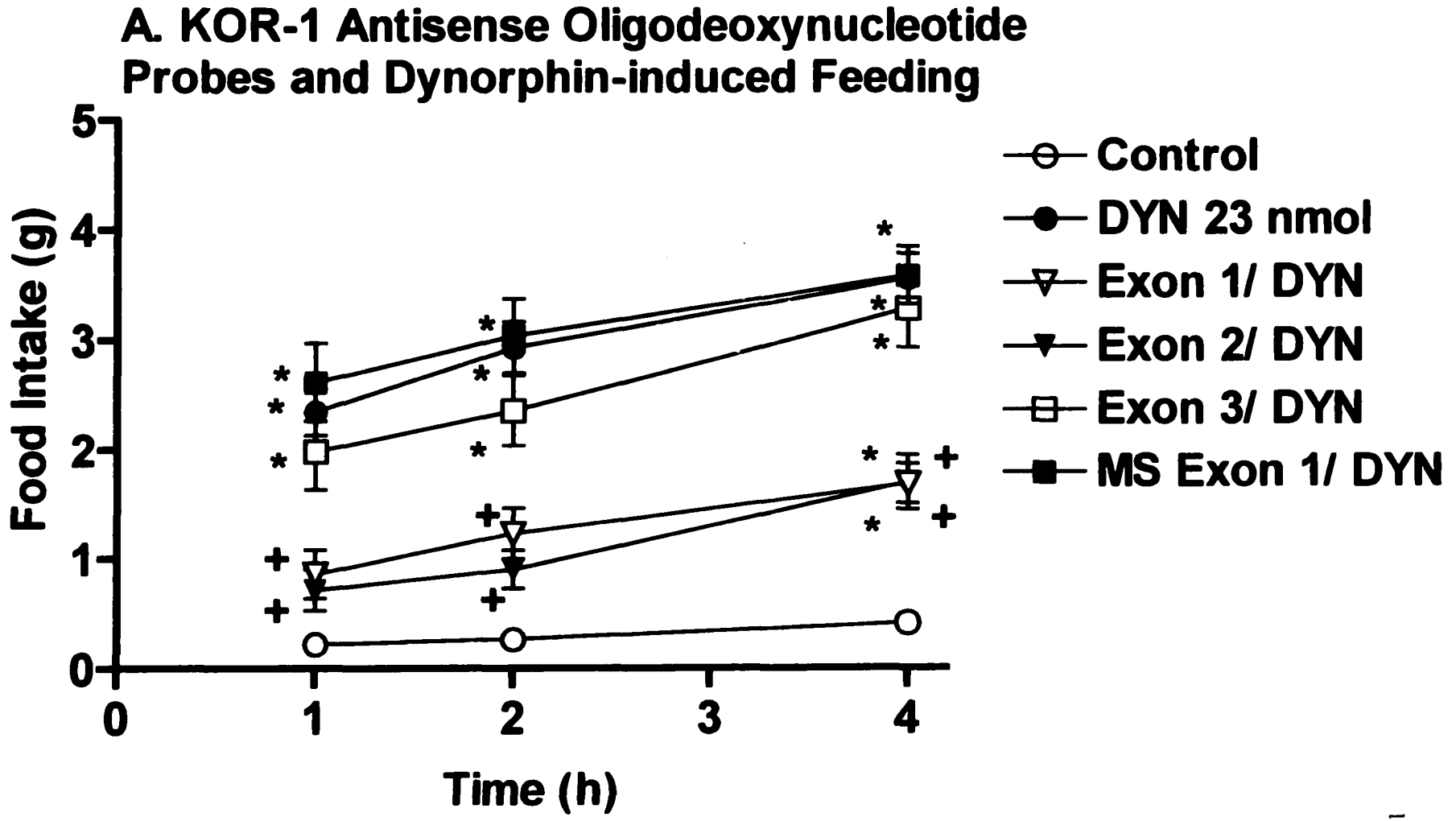


Figure 16. Alterations (mean, ±SEM) in food intake (g) following i.c.v. administration of either vehicle alone, dynorphin (50 μg) alone or ventricular pretreatment over three days (Days 1, 3 and 5) with AS or MS ODN probes directed against specific coding exons of the KOR-1 opioid receptor clone with dynorphin administration occurring 24 h later (Day 6) (n=7/condition). Significant differences in food intake were observed following dynorphin and KOR-1 AS ODN treatment conditions relative to vehicle after 1 ($F(5,130)= 53.76$, $p<.0001$), 2 ($F= 62.91$, $p<.0001$) and 4 ($F= 78.48$, $p<.0001$) h. The asterisks (*) in this figure indicate significant increases in agonist-induced feeding relative to corresponding vehicle control values (Dunnett comparisons, $P<0.05$) The crosses (+) in this figure indicate significant decreases in agonist-induced feeding following either antagonist or AS ODN pretreatment relative to corresponding agonist-induced feeding values alone (Dunnett comparisons, $p<0.05$).



full expression of dynorphin-induced feeding is dependent upon the functional expression of coding exons 1 and 2 of the KOR-1 gene.

KOR-3/ORL-1 AS ODN Probes and Dynorphin-Induced Feeding

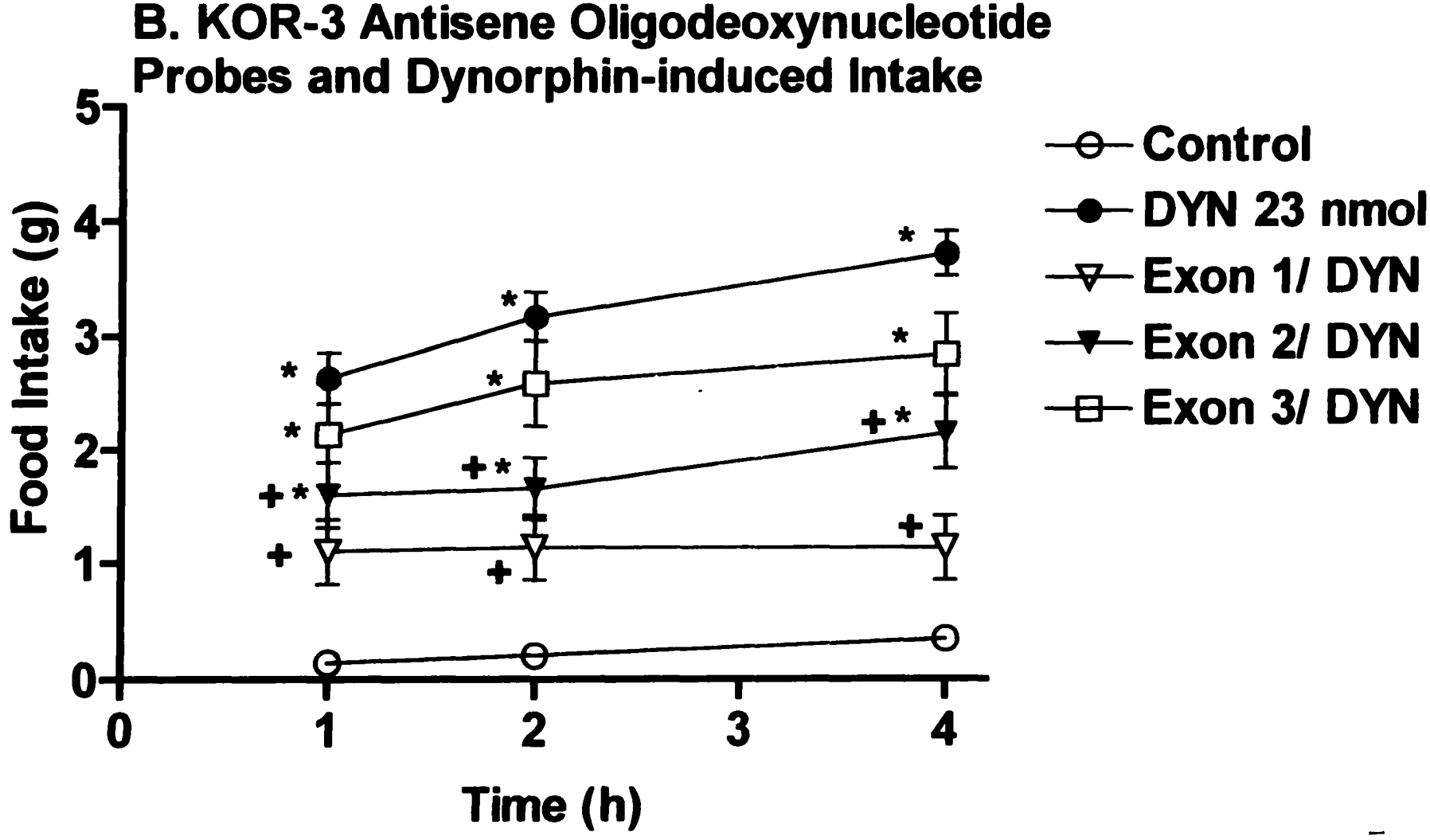
Dynorphin-induced feeding was significantly reduced by pretreatment with AS ODN probes directed against either coding exons 1 or 2 of the KOR-3/ORL-1 gene after 1 [$F(4,76) = 21.64$, $p<0.0001$), 2 ($F = 38.71$, $p<0.0001$), and 4 ($F = 49.77$, $p<0.0001$) h (**Figure 17**). In contrast, an AS ODN probe directed against coding exon 3 of the KOR-3/ORL-1 gene, as well as a control MS ODN probe both failed to significantly alter dynorphin-induced feeding. These data suggest that the expression of the dynorphin-induced ingestive response is dependent upon the functional expression of coding exons 1 and 2 of the KOR-3/ORL-1 gene.

Discussion

The present study demonstrated that increased food intake following dynorphin administration was significantly, dose-dependently and differentially reduced by pretreatment with either general (NTX), κ_1 (NorBNI), δ (naltrindole)- and μ (βFNA)-opioid antagonists. The relative potencies of the four antagonists upon dynorphin-induced feeding at a peak (2 h) intake interval demonstrated that lower doses of NorBNI were more effective in reducing dynorphin-induced feeding than were equivalent doses of βFNA, NTX and naltrindole respectively.

In addition, dynorphin-induced feeding was significantly reduced by AS ODN probes directed against either coding exons 1 and 2, but not 3 of the KOR-1 gene, coding exons 1 and 2, but not 3 of the KOR-3/ORL-1 gene, exon 1, but not 2 or 3 of the DOR-1

Figure 17. Alterations (mean, ±SEM) in food intake (g) following i.c.v. administration of either vehicle alone, dynorphin (50 μg) alone or ventricular pretreatment over three days (Days 1, 3 and 5) with AS ODN probes directed against specific coding exons of the KOR-3/ORL-1 opioid receptor clone with dynorphin administration occurring 24 h later (Day 6) (n=7/condition). Significant differences in food intake were observed following dynorphin and KOR-3/ORL-1 AS ODN treatment conditions relative to vehicle after 1 ($F(4,76)= 21.64$, $p<.0001$), 2 ($F= 38.71$, $p<.0001$) and 4 ($F= 49.77$, $p<.0001$) h. The asterisks (*) in this figure indicate significant increases in agonist-induced feeding relative to corresponding vehicle control values (Dunnett comparisons, $P<0.05$) The crosses (+) in this figure indicate significant decreases in agonist-induced feeding following either antagonist or AS ODN pretreatment relative to corresponding agonist-induced feeding values alone (Dunnett comparisons, $p<0.05$).



gene, and coding exon 1, but not 2, 3 or 4 of the MOR-1 gene. Furthermore, a control MS ODN probe, that differed from the highly effective KOR-1 coding exon 1 AS ODN probe by the sequence reversal of only three pairs of bases, was completely ineffective in altering dynorphin-induced feeding.

It has been strongly suggested that dynorphin-induced feeding acts through direct activation of the κ_1 opioid receptor. Dynorphin displays 5-30 fold greater affinity for κ_1 opioid receptors relative to μ and δ receptors (Mansour et al., 1995; Zhang et al., 1998), and its binding characteristics are very similar to the prototypical κ agonist, ethylketocyclazocine (Chavkin et al., 1982). Further, the ability of dynorphin and other κ_1 receptor ligands to increase feeding behavior is well established (Morley and Levine, 1981, 1983; Morley et al., 1982; Walker et al., 1980). Yet although dynorphin-induced feeding has been postulated to result from its direct interaction with the κ_1 opioid receptor (see reviews: Levine et al., 1985; Gosnell and Levine, 1996), this has never been formally evaluated. The results of the equimolar opioid antagonist paradigm revealed that κ_1 (NorBNI)-mediated opioid antagonism significantly reduced dynorphin-induced feeding at far lower doses (5-80 nmol) than either NTX (80 nmol) or naltrindole (80 nmol), and also, to a more potent degree than βFNA (20-80 nmol). The relatively high dose (80 nmol) of NTX required to block dynorphin-induced feeding strongly parallels the low sensitivity to naloxone antagonism displayed by dynorphin as well as other κ-selective ligands relative to μ- and δ-selective agonists (Chavkin et al., 1982; Goldstein et al., 1979). The ability of κ_1 and μ opioid antagonists to significantly reduce dynorphin-induced feeding is not unique to this agonist since both of these antagonists also significantly reduced feeding

elicited by the κ_1-selective agonist U50488H, the μ-selective agonist DAMGO or β-endorphin, albeit with different degrees of potency (Leventhal et al., 1997; Levine et al., 1990, 1991; Silva et al., 2001a). For example, the ability of NorBNI at doses as low as 5 nmol to reduce dynorphin-induced feeding compares favorably with this antagonist's ability to reduce feeding elicited by U50488H (1 nmol: Levine et al., 1990). Yet, the ability of βFNA at doses as low as 20 nmol to reduce dynorphin-induced feeding is in contrast to its greater ability to effectively reduce feeding elicited by either DAMGO (0.1-0.4 nmol: Leventhal et al., 1997; Levine et al., 1991) or β-endorphin (0.5 nmol: Silva et al., 2001a). The relative lack of efficacy observed for the δ opioid antagonist, naltrindole to reduce dynorphin-induced feeding was similar to its minimal effects upon β-endorphin-induced feeding (Silva et al., 2001a), and may reflect only a slight cross-activation of δ receptors by dynorphin.

The AS ODN studies provided compelling and converging evidence supporting the hypothesis that κ opioid receptors are primarily involved in the mediation of dynorphin-induced feeding. Dynorphin-induced feeding was eliminated by AS ODN probes directed against coding exons 1 and 2 of the KOR-1 gene. Such data are consistent with the ability of AS ODN probes directed against coding exon 1 of the KOR-1 gene to reduce feeding elicited by the κ_1-selective opioid agonist, U50488H, but not the μ-sensitive opioid agonists, M6G (Leventhal et al., 1998b) or βEND (Silva et al., 2001a). This effect is also largely consistent with the specific and selective actions of AS ODN probes directed against all three coding exons of the KOR-1 gene to reduce analgesic responses elicited by κ_1 opioid agonists (e.g., Chen et al., 1994; Pasternak et al., 1999). However, the inability

of AS ODN probes targeting coding exon 3 of the KOR-1 opioid receptor gene to significantly reduce dynorphin-induced feeding may be a result of this probe's reduced knockdown efficacy. That is, the ability of individual coding exon antisense probes to reduce protein concentrations might be different. In this case, antisense targeting coding exon 3 of the KOR-1 gene in this study may display a more limited knockdown efficacy than those used in previous analgesia studies, and thus may explain the discrepancy in the results between feeding and analgesia elicited by dynorphin and U50488H respectively. Alternatively, this discrepancy could be the result of differential inherent thresholds of κ opioid receptors for activation during analgesia and feeding. Nevertheless, the non-significant reductions in dynorphin-induced feeding observed in this study following pretreatment with KOR-1 coding exon 3 antisense appear to support these possibilities.

Interestingly, dynorphin-induced feeding was similarly reduced by AS ODN probes directed against coding exons 1 and 2, but not 3 of the KOR-3/ORL-1 gene. Whereas AS ODN probes directed against all three coding exons of the KOR-3/ORL-1 gene effectively eliminate feeding elicited by OFQ/N_{1-17} (Leventhal et al., 1998a), an AS ODN probe directed against coding exon 1 of the KOR-3/ORL-1 gene failed to affect M6G-induced feeding (Leventhal et al., 1998a). Importantly, an equi-effective feeding response elicited by β-endorphin was unaffected by AS ODN probes directed against coding exons 1 or 2 of the KOR-3/ORL-1 gene (Silva et al., 2001a).

The sensitivity of dynorphin-induced feeding to disruption by pretreatment with AS ODN probes targeting either coding exons 1 or 2 of both the KOR-1 and KOR-3/ORL-1 opioid receptor genes may occur as a result of subtleties in either receptor or neuronal

organization. The potential mechanism(s) by which dynorphin acts upon both the κ_1 and κ_3 opioid receptors to induce feeding remains unclear.

First, dynorphin and OFQ/N share significant primary structural similarities (Reinscheid et al., 1998). Specifically, the N-terminal domain known as the "message" domain of dynorphin is recognized by the KOR-3/ORL-1 receptor (Reinscheid et al., 1998). Moreover, as few as four point mutations in the amino acid sequence of the KOR-3/ORL-1 receptor results in the ability of this receptor to bind dynorphin A_{1-17} with high affinity (Meng et al., 1996). Therefore, the sensitivity of dynorphin-induced feeding to reduction by AS ODN probes targeting coding exons 1 and 2 of the KOR-3/ORL-1 gene suggests that this receptor may play a significant role in the mediation of this ingestive response. These data thereby suggest that this response may be dependent upon direct binding interactions between dynorphin and both κ_1 and κ_3 receptors.

Alternatively, the sensitivity of dynorphin-induced feeding to both KOR-1 and KOR-3 AS ODN pretreatment may reflect the involvement of both of these genes in a serial circuit mediating this ingestive response. For example, the direct activation of κ_1 receptors by dynorphin may activate a trans-neuronal circuit, which is dependent upon the downstream activation of κ_3 receptors for the full expression of dynorphin-induced feeding. According to this model, disruption of the KOR-3/ORL-1 gene by pretreatment with antisense results in observable reductions in dynorphin-induced feeding that are independent of the direct activation of κ_1 receptors by dynorphin.

Finally, the involvement of both the KOR-1 and KOR-3 opioid receptor genes in the mediation of dynorphin-induced feeding may be explained by the existence of opioid

receptor dimers. Recent discoveries have provided both biochemical and pharmacological evidence suggesting that opioid receptors may form functional, cross-modulating receptor complexes known as dimers (see review: Jordan et al., 2000). The common sensitivity displayed by the KOR-1 and KOR-3/ORL-1 genes may reflect the existence of a κ_1/κ_3 dimer complex. According to this model, dynorphin-induced feeding is sensitive to disruption by pretreatment with AS ODN probes targeting either the KOR-1 or KOR-3/ORL-1 opioid receptor genes since the receptors encoded by these genes form a dimer complex.

The sensitivity of dynorphin-induced feeding to disruption by pretreatment with AS ODN probes targeting either the KOR-1 or KOR-3/ORL-1 opioid receptor genes may occur via one or more of the aforementioned mechanisms, as well as by an unidentified mechanism(s). Nevertheless, the data reported here suggests that the full expression of dynorphin-induced feeding is dependent upon the integrity of both the KOR-1 and KOR-3/ORL1 genes.

The roles of other opioid receptor subtypes in the mediation of dynorphin-induced feeding are less compelling in light of their AS ODN probe effects. The limited effectiveness of the high (80 nmol) naltrindole dose in transiently (4 h) reducing dynorphin-induced feeding is consistent with reductions induced by an AS ODN probe (coding exon 1) directed against the DOR-1 gene. This is in contrast to the elimination of feeding over 4 h elicited by the δ_2 opioid agonist, deltorphin by pretreatment with the identical DOR-1 AS ODN probe (Leventhal et al., 1998b). Although the highest doses (20-80 nmol) of the selective μ-opioid receptor antagonist βFNA significantly reduced

dynorphin-induced feeding, pretreatment with an AS ODN probe directed against coding exon 1, but not coding exons 2, 3 or 4 of the MOR-1 gene only transiently (1-2 h) reduced this effect. In contrast, AS ODN probes directed against either coding exons 1, 3 or 4 of the MOR-1 gene eliminated β-endorphin-induced feeding (Silva et al., 2001a).

Thus, the endogenous opioid peptide, dynorphin A_{1-17}, stimulates food intake following microinjection into intracerebral sites historically implicated in ingestive behavior (Gosnell et al., 1986b; Hamilton and Bozarth, 1988; Klitenick and Wirtshafter, 1995; Majeed et al., 1986). The wide distribution of dynorphin's ingestive effects in the central nervous system together with its activation under a wide array of ingestion-related situations strongly suggests that this opioid peptide is an important modulator of food intake. The present data now strongly suggest that the primary receptor site(s) of action by which dynorphin A_{1-17} stimulates food intake is the κ_1, and also κ_3 opioid receptor. As with the elucidation of receptor mediation of β-endorphin-induced feeding in a companion study (Silva et al., 2001a), the combined use of selective antagonists and additional modern molecular tools such as the AS ODN technique allows for the discovery of precise receptor mechanisms mediating feeding and other behavioral actions of the endogenous opioid peptide system.

Opioid receptors mediate their effects by the direct activation of G-proteins, to which they are coupled. The catalytic subunit of heterotrimeric G-proteins is the α-subunit, which initiates an intracellular effector signaling cascade upon activation. The family of G-protein receptor complexes can be subdivided according to the identity of the α-subunit. The following two studies utilized AS ODN probes directed against individual

types of G-protein α-subunits to characterize the G-protein α-subunits involved in feeding elicited by the prototypical μ receptor agonists, morphine and M6G (Specific Aim 3), and also by the opioid peptides, βEND and dynorphin (Specific Aim 4).

CHAPTER 5: SPECIFIC AIM THREE.

Morphine and Morphine-6β-glucuronide-induced Feeding: Distinguishing Signal Transduction Profiles Using G-protein α-subunit Antisense Probes.

Introduction

Morphine and other opioid agonists such as heroin, butorphanol and levorphanol potently stimulate food intake when administered centrally (Gosnell and Levine, 1996). In humans, morphine is rapidly metabolized at the three and six positions (Jaffe and Martin, 1985), yielding a 3- and 6-glucuronide, respectively. The morphine metabolite, morphine-6β-glucuronide (M6G) significantly and dose dependently increases spontaneous food intake (Leventhal et al., 1998) and pain thresholds (Abbott and Palmour, 1988; Pasternak et al., 1987) when administered centrally.

Functional characterization of both morphine and M6G-induced feeding has implicated the μ-opioid receptor in the mediation of these responses since each is blocked by the selective μ-antagonists, βFNA and naloxonazine (Leventhal et al., 1998b; Mann et al., 1988). In contrast, M6G-induced feeding is unaffected by either selective κ_1, δ_1 or δ_2 opioid receptor subtype antagonists (Leventhal et al., 1998b). These data strongly suggest that morphine and M6G-induced feeding are each mediated by the selective activation of the pharmacologically-characterized μ-opioid receptor. A detailed mapping study of the four coding exons of the MOR-1 gene indicated that morphine- and M6G-induced feeding could be distinguished from each other on the basis of their differential sensitivities to MOR-1 AS ODN probes in that AS ODN probes directed against coding exons 1 and 4 selectively reduce morphine-induced feeding, whereas AS ODN probes directed against

coding exons 2 and 3 selectively reduce M6G-induced feeding (Leventhal et al., 1998b). These findings are strongly supported by similar findings using analgesic assays (Rossi et al., 1995a; Rossi and Pasternak, 1997; Rossi et al., 1995b). In addition, both knockout and transgenic paradigms involving the MOR-1 gene provide further evidence supporting the existence of a novel M6G receptor (Brown et al., 1997; Rossi et al., 1996).

Many different G-proteins have been implicated in μ-opioid mediated functions. For example, administration of antisera raised against $G_i\alpha_2$ significantly reduced the analgesic activity of DAMGO, morphine, βEND, DPDPE and deltorphin II, whereas pretreatment with antisera against $G_{x2}\alpha$ attenuated DAMGO, βEND and morphine analgesia in mice (Garzon et al., 1994; Sanchez-Blazquez et al., 1993). Identical results were reported using AS ODN probes targeted against $G_i\alpha_2$ and $G_{x2}\alpha$ (Raffa et al., 1996; Sanchez-Blazquez et al., 1995). Thus, the identity of G-protein α-subunits establishes a specificity profile among G-protein receptor complexes that allows for their classification.

To determine whether the distinctions between feeding responses induced by morphine and M6G involve different G-protein α-subunits, the present study evaluated the effects of ventricularly-administered AS ODN probes directed against either the $G_i\alpha_1$, $G_i\alpha_2$, $G_i\alpha_3$, $G_o\alpha$, $G_s\alpha$, $G_{xz}\alpha$ or $G_q\alpha$ subunits as well as a control nonsense probe upon spontaneous food intake induced by morphine and M6G in rats. Additionally, antisense probe-induced alterations in body weight and ad libitum food intake were examined. The findings of this study have been recently published (Silva et al., 2000).

Methods

Drugs: The opioid agonists, M6G (250, 500 ng, Research Technology Branch, NIDA, Rockville, MD), and morphine (5 μg, Pennick Laboratories), were each dissolved in 0.9% normal saline. All phosphodiester oligodeoxynucleotides (Midland Certified Reagent Company, Midland, TX) were dissolved in 0.9% normal saline at a concentration of 5 μg/μl at a dose of 25 μg, and were specific for the G-protein α-subunits (**Table 2**) for which they were targeted.

Protocol 1:. Because these opioid agonists produce initial sedative and hypoactive effects (see review by: Gosnell and Levine, 1996), each animal received two separate microinjections of morphine (5 μg, i.c.v.) or M6G (250 or 500 ng, i.c.v.) on different days without measuring intake. Following this pretreatment, a morphine dose of 5 μg or M6G doses of either 250 or 500 ng were administered to separate groups of animals, and food intake was assessed at 1, 2 and 4 h later.

Protocol 2: Following stabilization of baseline food intake and establishment of morphine- or M6G-induced feeding, separate subgroups of rats were exposed to the following AS ODN treatments over a 4-day paradigm. Following a vehicle injection (day 1), rats received an AS ODN (25 μg) or nonsense probe 24 h thereafter (day 2). The 25 μg AS ODN dose was used because it has been shown to be most effective in reducing morphine- and M6G-induced analgesia as well as producing minimal debilitation (Rossi et al., 1995b). Animals then received either morphine or M6G treatment at 24 (day 3) and 48 (day 4) h. This time course was chosen since significant downregulation of G-protein α-subunits occurs maximally between 24 and 48 h following a single AS ODN injection (Rossi and Pasternak,

1997; Rossi et al., 1995b; Standifer et al., 1996). To assess any body weight changes induced by G-protein AS ODN treatment, body weight was assessed immediately prior to each treatment. To assess any ad libitum 24-h intake changes induced by G-protein AS ODN treatment, food intake was measured between days 1 and 2 (pre-measure), and days 2 and 3 (24-h AS ODN effect) prior to any opioid agonist treatment.

In assessing G-protein α-subunit AS ODN effects upon morphine-induced feeding, subgroups of at least eight rats received microinjections (i.c.v.) of either (1) vehicle (5 μl, 0.9 % normal saline), (2) morphine (5 μg, 5μl), G-protein α-subunit AS ODN probes (25 μg, 5μl) directed against either (3) $G_i\alpha_1$, (4) $G_i\alpha_2$, (5) $G_i\alpha_3$, (6) $G_s\alpha$, (7) $G_o\alpha$, (8) $G_{xz}\alpha$, (9) $G_q\alpha$ or (10) a nonsense (NS) ODN 24 h prior to receiving morphine (5 μg). In assessing G-protein α-subunit AS ODN effects upon M6G-induced feeding, subgroups of at least six rats received microinjections (i.c.v.) of either (1) vehicle (5 μl, 0.9% normal saline), (2) M6G (500 ng), G-protein α-subunit AS ODN probes (25 μg, 5μl) directed against either (3) $G_i\alpha_1$, (4) $G_i\alpha_2$, (5) $G_i\alpha_3$, (6) $G_s\alpha$, (7) $G_o\alpha$, (8) $G_{xz}\alpha$, (9) $G_q\alpha$ or (10) a nonsense (NS) ODN 24 h prior to receiving M6G (500 ng). After assessing that G-protein AS ODN effects upon M6G-induced feeding at a 500 ng dose were largely ineffective, a lower (250 ng) M6G dose was employed that elicited a similar magnitude of feeding. Other AS ODN probes have previously been shown to alter intake following lower, but not higher doses of either 2-deoxy-D-glucose (Burdick et al., 1998; Leventhal et al., 1996), DAMGO (Leventhal et al., 1997) or mercaptoacetate (Stein et al., 2000). Thus, subgroups of rats (n=8-10/condition) received either: 1) vehicle, 2) M6G (250 ng), G protein α-subunit AS ODN probes directed against either 3) $G_i\alpha_1$, 4) $G_i\alpha_2$, 5) $G_i\alpha_3$, 6) $G_s\alpha$, 7) $G_o\alpha$, 8) $G_{xz}\alpha$, 9) $G_q\alpha$, or 10) a NS ODN. During

testing, food intake was measured at 1,2 and 4 h following morphine or M6G injection based on previous studies (Gosnell and Levine, 1996; Leventhal et al., 1998b). Two weeks following AS ODN treatments, animals with patent cannulae were retested for either morphine or M6G-induced feeding, and one week thereafter, were retested with a second different AS ODN or nonsense probe over the 4-day paradigm.

Results

Morphine- and M6G-Induced Feeding

Microinjections of morphine and the two doses of M6G significantly increased food intake at each of the time points relative to control values after 1 [$F(3,154) = 29.70$, $P<0.0001$), 2 ($F=73.00$, $P<0.0001$), and 4 ($F=246.55$, $P<0.0001$) h. Over the 4-h time course, intake induced by morphine at a dose of 5 μg (4.47± 0.17 g) failed to differ from intake induced by M6G at a dose of 250 ng (4.96±0.17 g) **(Table 4)**. In turn, both of feeding induced by both of these conditions was significantly lower than intake induced by M6G at a dose of 500 ng (5.58±0.22 g). These data suggest that the morphine (5 μg) and M6G (250 ng) doses constitute equi-effective feeding conditions, whereas the M6G (500 ng) dose produces a more robust ingestive action.

G-protein AS ODN Treatment and Morphine-Induced Feeding

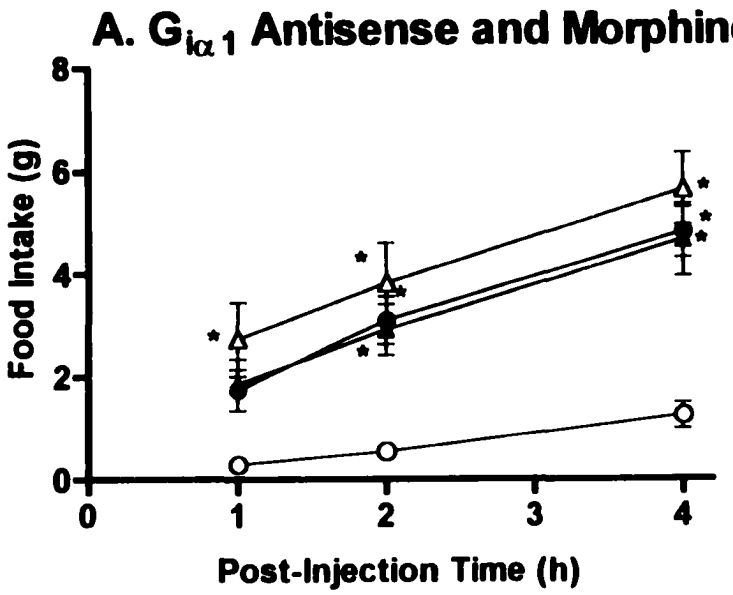
Pretreatment with the $G_i\alpha_2$ AS ODN probe significantly reduced morphine-induced feeding across the time course at 24 and 48 h following AS ODN treatment **(Figure 18B)**. In contrast, pretreatment with either the $G_i\alpha_1$ or $G_i\alpha_3$ AS ODN probes failed to exert effects **(Figure 18A and C)**. Importantly, a nonsense control probe also failed to alter morphine-induced feeding **(Figure 18D)**. Morphine-induced feeding was also unaffected by AS ODN

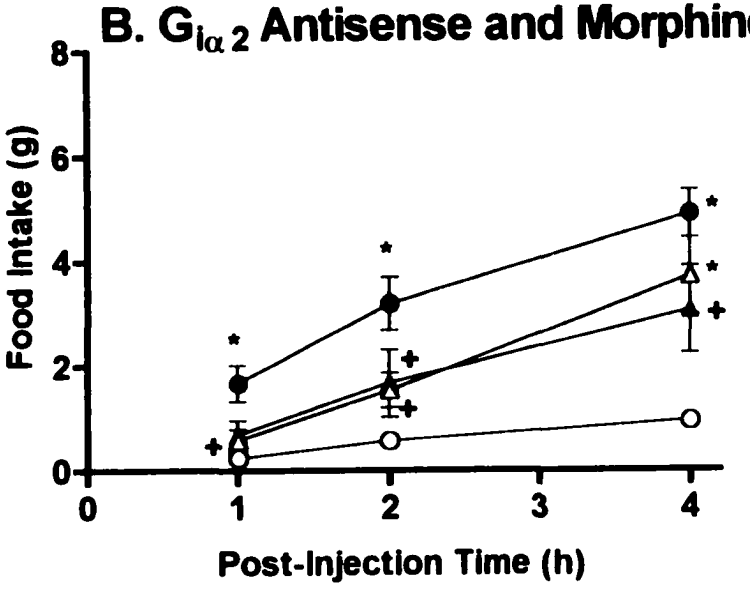
Table 4. Maximal changes (%) in feeding induced by morphine (5 μg) and M6G (250 ng) following G-protein antisense oligodeoxynucleotide probes.

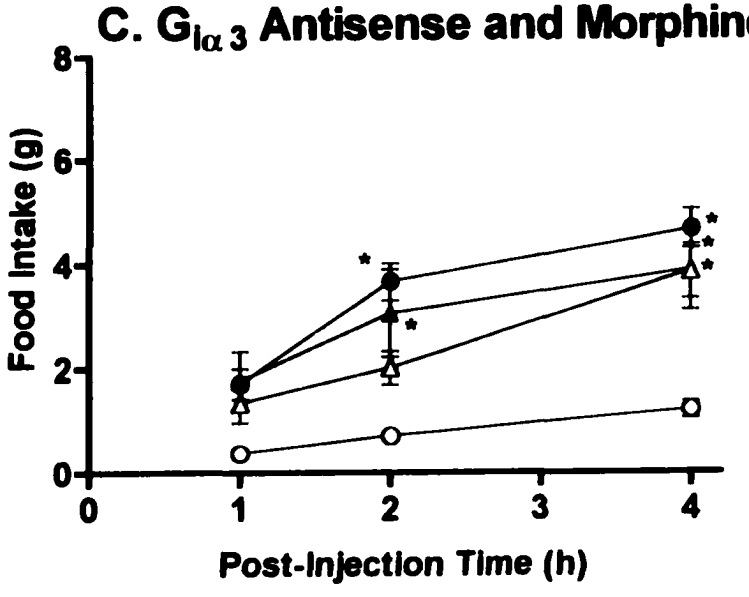
AS Probe	Morphine 24 h	Morphine 48 h	M6G 24 h	M6G 48 h
$G_{i\alpha1}$	+23	-4	-50*	-56*
$G_{i\alpha2}$	-63*	-60*	-34	-27
$G_{i\alpha3}$	-23	-22	-58*	-40*
Nonsense	-12	0	+6	-20
G_s	+52*	+30	-28	-37
G_o	+3	+12	-2	-11
G_q	+8	-15	-11	+19
$G_{z/z}$	+9	-17	-69*	-44*

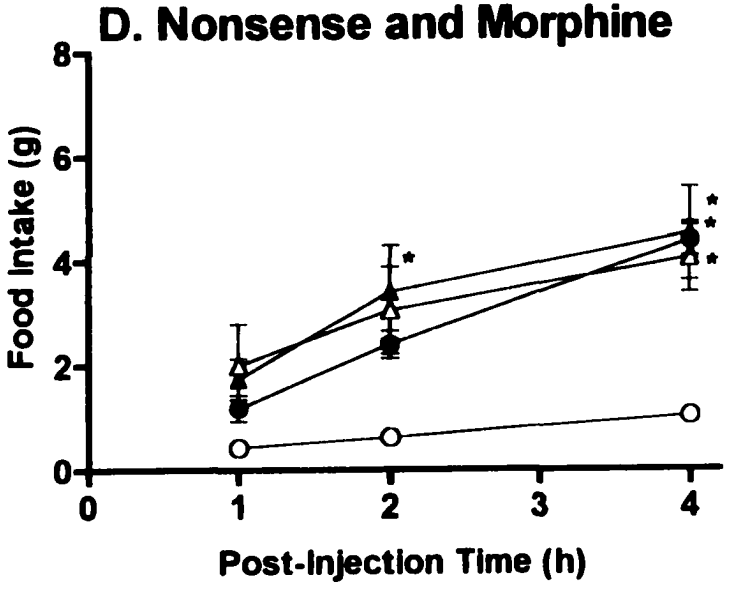
* denotes significant increase or decrease relative to the agonist alone (Dunnett comparisons. $p<.05$).

Figure 18. Alterations (Mean. ± SEM) in spontaneous food intake following vehicle (open circles) and morphine (closed circles) as well as morphine-induced feeding observed 24 (open triangles) and 48 (closed triangles) h following AS ODN probes directed against either the $G_i\alpha_1$ (Panel A), $G_i\alpha_2$ (Panel B), $G_i\alpha_3$ (Panel C) subunits or a nonsense probe (Panel D). Significant differences were observed among treatment conditions (n=8-10/condition) for the $G_i\alpha_1$ (1 h: $F(3,39) = 5.03$, $p < .007$; 2 h: $F = 8.20$, $p < .001$; 4 h: $F = 11.62$, $p < .0001$), the $G_i\alpha_2$ (1 h: $F(3,47) = 5.53$, $p < .003$; 2 h: $F = 6.72$, $p < .001$; 4 h: $F = 10.62$, $p < .0001$), the $G_i\alpha_3$ (1 h: $F(3,39) = 3.54$, $p < .028$; 2 h: $F = 8.16$, $p < .001$; 4 h: $F = 10.54$, $p < .0001$) and nonsense (1 h: $F(3,31) = 2.08$, $p < .133$; 2 h: $F = 4.80$, $p < .011$; 4 h: $F = 11.86$, $p < .0001$) treatments. The asterisks (*) in this figure indicate significant increases in agonist-induced feeding relative to corresponding vehicle control values (Dunnett comparisons, $P<0.05$) The crosses (+) in this figure indicate significant decreases in agonist-induced feeding following either antagonist or AS ODN pretreatment relative to corresponding agonist-induced feeding values alone (Dunnett comparisons, $p<0.05$).









probes directed against the $G_o\alpha$ **(Figure 19B)**, $G_q\alpha$ **(Figure 19C)**, and $G_{z}\alpha$ **(Figure 19D)** subunits, but was transiently enhanced at the 4-h intake point 24 h following pretreatment with the $G_s\alpha$ AS ODN probe **(Figure 19A)**.

G-protein AS ODN Treatment and M6G-Induced Feeding

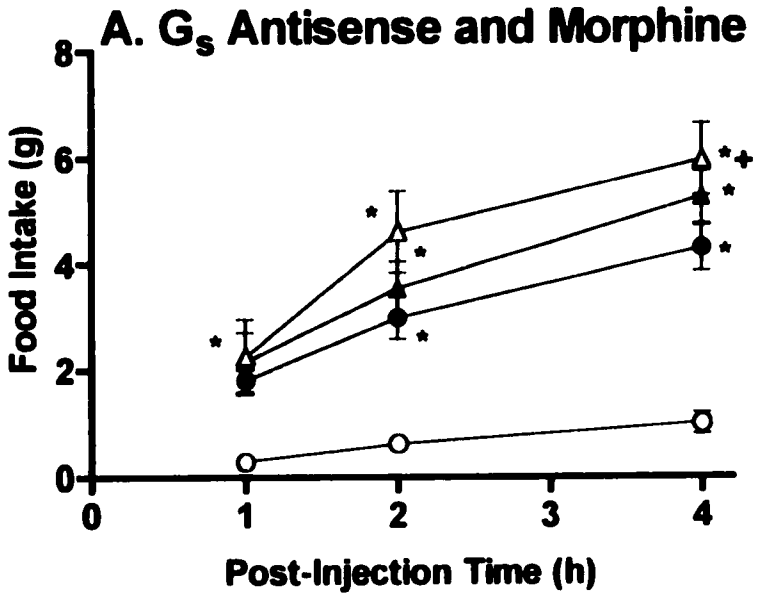
Feeding was significantly increased by the active morphine metabolite, M6G at doses of 250 **(Figures 20 and 21)** and 500 **(Table 5)** ng relative to control values. However, none of the G-protein α-subunit AS ODN probes significantly attenuated M6G-induced feeding when the 500-ng dose was used **(Table 5)**.

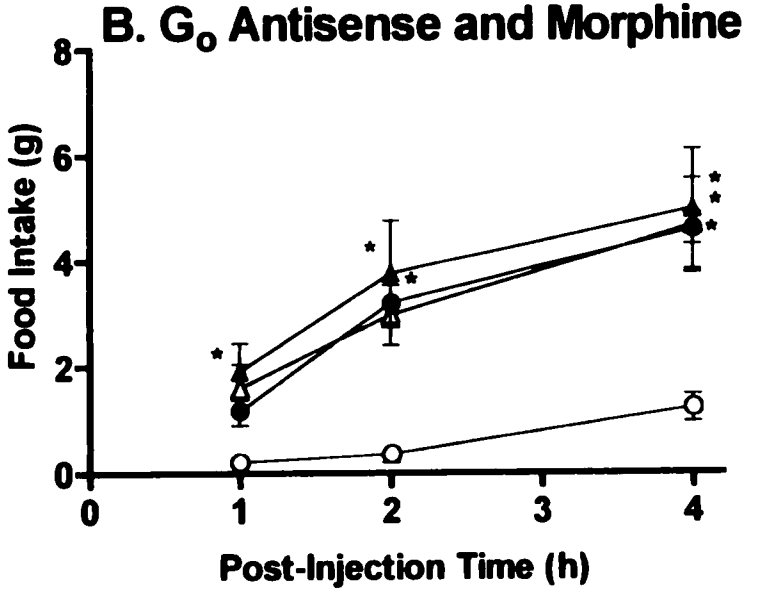
In contrast to morphine-induced feeding, pretreatment with either the $G_i\alpha_1$ **(Figure 20A)** or $G_i\alpha_3$ **(Figure 20C)** AS ODN probes significantly reduced M6G (250 ng)-induced feeding at 24 and 48 h following treatment. Correspondingly, pretreatment with the $G_i\alpha_2$ AS ODN probe failed to exert effects **(Figure 20B)**. Again importantly, a nonsense control probe also failed to alter M6G-induced feeding **(Figure 20D)**. M6G-induced feeding was also unaffected by AS ODN probes targeting the $G_s\alpha$ **(Figure 21A)**, $G_o\alpha$ **(Figure 21B)** and $G_q\alpha$ **(Figure 21C)** subunits. M6G-induced feeding was also significantly reduced by $G_{z}\alpha$ AS ODN probe at 24 and 48 h following treatment **(Figure 21D)**.

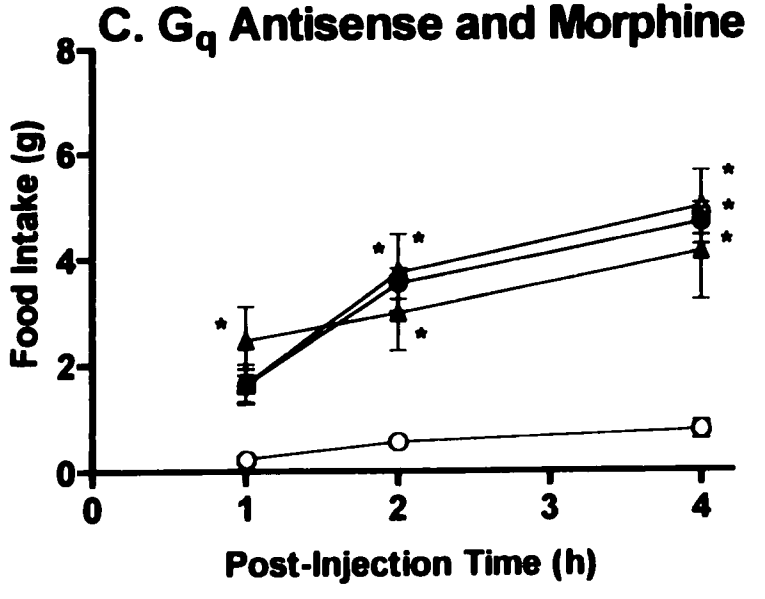
G-protein AS ODN Treatment: Cumulative Body Weight and Intake

The selective alterations in feeding induced by morphine and M6G following the AS ODN treatment could not be accounted for by nonspecific alterations in either body weight **(Table 6)** or spontaneous food intake **(Figure 22)**. Small, but significant decreases in body weight were observed following pretreatment with an AS ODN probe targeting the $G_s\alpha$

Figure 19. Alterations (Mean. ± SEM) in spontaneous food intake following vehicle and morphine as well as morphine-induced feeding observed 24 and 48 h following AS ODN probes directed against either the $G_s\alpha$ (Panel A), $G_o\alpha$ (Panel B), $G_q\alpha$ (Panel C) or $G_{z}\alpha$ (Panel D) subunits. Significant differences were observed among treatment conditions (n=8-10/condition) for the $G_s\alpha$ (1 h: $F(3,39) = 4.64$, $p < .001$; 2 h: $F = 13.50$, $p < .0001$; 4 h: $F = 25.15$, $p < .0001$), the $G_o\alpha$ (1 h: $F(3,35) = 3.89$, $p < .022$; 2 h: $F = 5.83$, $p < .004$; 4 h: $F = 6.17$, $p < .003$), the $G_q\alpha$ (1 h: $F(3,39) = 5.62$, $p < .004$; 2 h: $F = 8.97$, $p < .0003$; 4 h: $F = 13.48$, $p < .0001$) and the $G_{z}\alpha$ (1 h: $F(3,39) = 2.43$, $p < .087$; 2 h: $F = 7.70$, $p < .001$; 4 h: $F = 13.61$, $p < .0001$) treatments. The asterisks (*) in this figure indicate significant increases in agonist-induced feeding relative to corresponding vehicle control values (Dunnett comparisons, $P<0.05$) The crosses (+) in this figure indicate significant decreases in agonist-induced feeding following either antagonist or AS ODN pretreatment relative to corresponding agonist-induced feeding values alone (Dunnett comparisons, $p<0.05$).







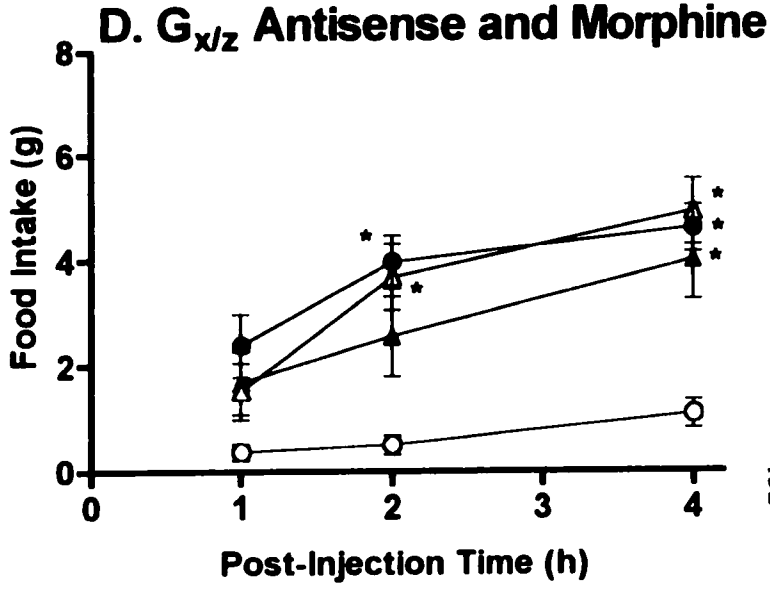
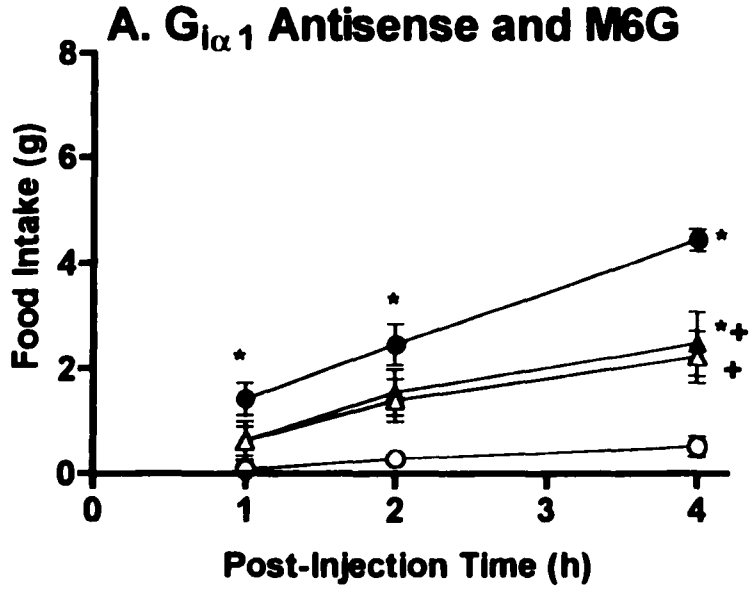
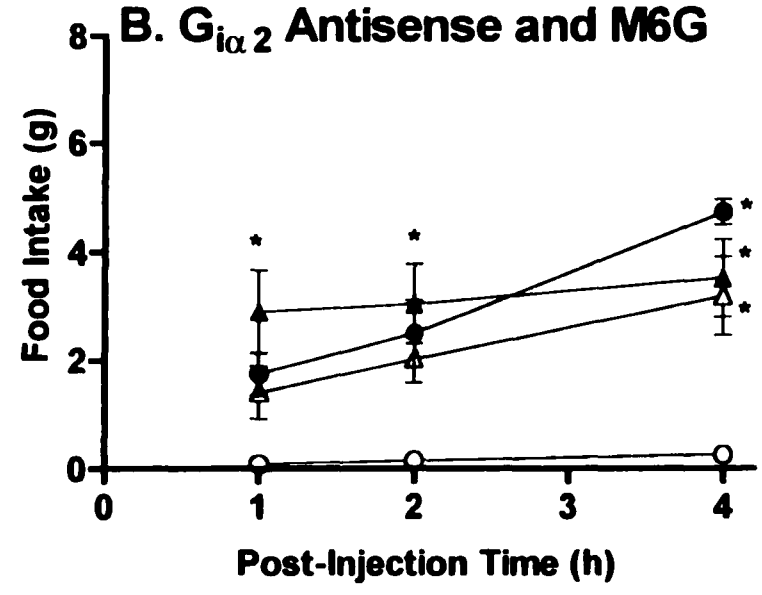


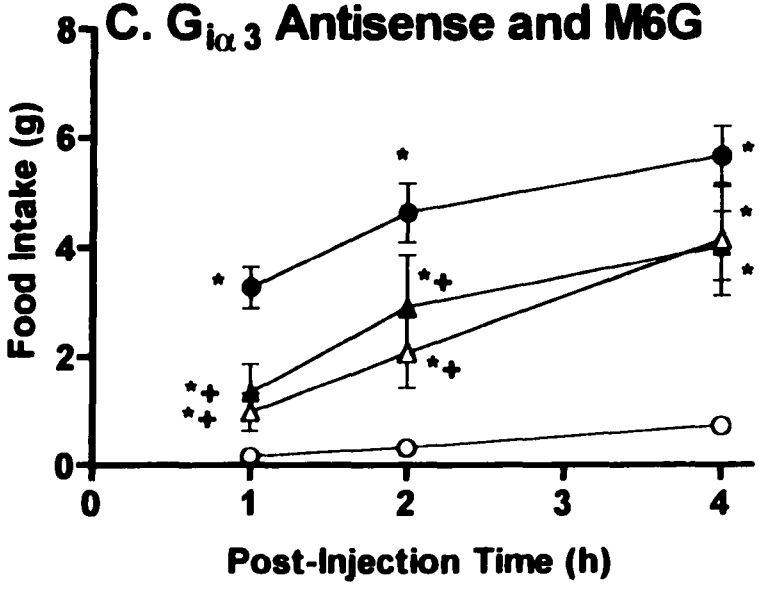
Table 5. Antisense oligdeoxynucleotide (AS ODN) probes did not significantly alter M6G-induced at the 500 ng dose.

AS Probe	BL 1	BL 2	BL 4	M6G 1	M6G 2	M6G 4	AS 24h M6G 1	AS 24h M6G 2	AS 24h M6G 4	AS 48h M6G 1	AS 48h M6G 2	AS 48h M6G 4
$G_{i\alpha1}$	0.0 (0.0)	0.1 (0.1)	0.2 (0.1)	0.9 (0.5)	2.7 * (0.5)	5.0 * + (0.4)	4.6* + (1.4)	7.4* + (1.9)	9.8 + (2.1)	1.4 (0.7)	2.3 (0.9)	5.5 * (1.0)
$G_{i\alpha2}$	0.2 (0.1)	0.2 (0.2)	0.3 (0.1)	1.3 (0.5)	3.0 (0.9)	6.2 * + (0.6)	3.4 * (1.2)	4.8 * (1.2)	6.4 (1.8)	3.4 (1.2)	4.7 * (1.6)	7.7 * (2.8)
$G_{i\alpha3}$	0.5 (0.1)	0.5 (0.1)	0.8 (0.2)	1.9 (0.5)	2.6 * (0.4)	5.2 * (0.5)	3.0 (1.0)	3.8 (1.0)	5.2 (1.3)	2.4 (0.5)	3.6 (0.6)	5.2 (0.9)
$G_{o\alpha}$	0.0 (0.0)	0.1 (0.1)	0.2 (0.1)	1.4 * (0.4)	2.9 * (0.9)	5.7 * (0.7)	3.9 (1.5)	6.2 (2.4)	8.2 (2.9)	3.0 (1.8)	4.9 (2.7)	8.4 (3.3)
$G_{s\alpha}$	0.3 (0.3)	0.6 (0.3)	0.6 (0.3)	1.1 (0.5)	3.5 * (0.6)	6.1 * (0.4)	2.2 (1.3)	4.4 (0.9)	6.6 (0.9)	2.8 (1.4)	4.8 (1.3)	8.4 (1.0)
$G_{q\alpha}$	0.2 (0.2)	0.2 (0.1)	0.2 (0.1)	1.9 * (0.3)	3.3 * (0.4)	5.8 * (0.6)	3.0 (1.2)	4.3 (1.1)	6.0 (1.3)	3.6 (1.4)	5.0 (1.4)	6.6 (1.7)
$G_{11/12\alpha}$	0.2 (0.2)	0.3 (0.1)	0.3 (0.1)	2.0 (0.7)	2.9 * (0.8)	5.7 * (0.6)	2.5 (1.0)	3.0 (1.1)	4.1 (1.5)	2.6 (1.2)	4.3 (1.5)	7.2 (1.3)

Figure 20. Alterations (Mean, ± SEM) in spontaneous food intake following vehicle (open circles) and M6G (250 ng: closed circles) as well as M6G-induced feeding observed 24 (open triangles) and 48 (closed circles) h following AS ODN probes directed against either the $G_i\alpha_1$ (Panel A), $G_i\alpha_2$ (Panel B), $G_i\alpha_3$ (Panel C) subunits or a nonsense probe (Panel D). Significant differences were observed among treatment conditions (n=8-10/condition) for the $G_i\alpha_1$ (1 h: $F(3,23) = 5.00$, $p < .01$; 2 h: $F = 7.36$, $p < .003$; 4 h: $F = 14.50$, $p < .0001$), the $G_i\alpha 2$ (1 h: $F(3,27) = 5.29$, $p < .01$; 2 h: $F = 5.65$, $p < .01$; 4 h: $F = 15.47$, $p < .0001$), the $G_i\alpha_3$ (1 h: $F(3,23) = 14.58$, $p < .0001$; 2 h: $F = 16.04$, $p < .0001$; 4 h: $F = 14.00$, $p < .0001$) and nonsense (1h: $F(3,51) = 6.30$, $p < .002$; 2 h: $F = 13.93$, $p < .0001$; 4 h: $F = 28.52$, $p < .0001$) treatments. The asterisks (*) in this figure indicate significant increases in agonist-induced feeding relative to corresponding vehicle control values (Dunnett comparisons, $P<0.05$) The crosses (+) in this figure indicate significant decreases in agonist-induced feeding following either antagonist or AS ODN pretreatment relative to corresponding agonist-induced feeding values alone (Dunnett comparisons, $p<0.05$).







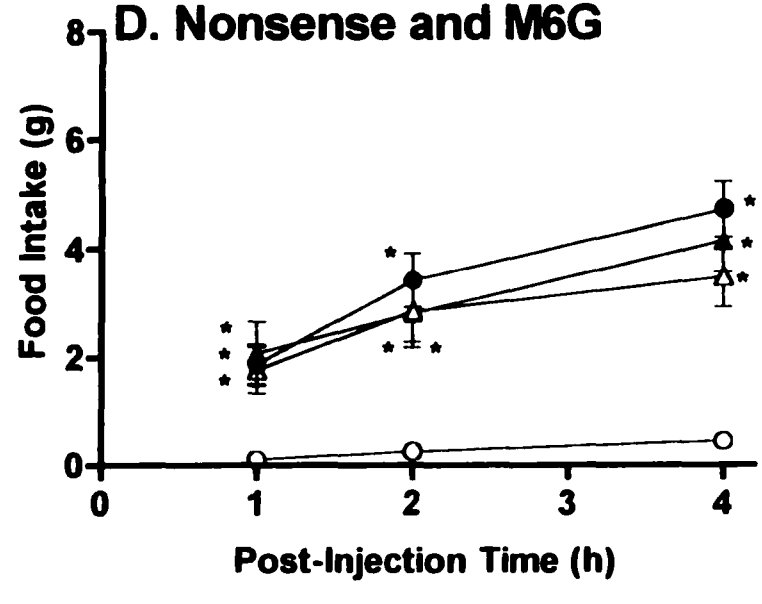
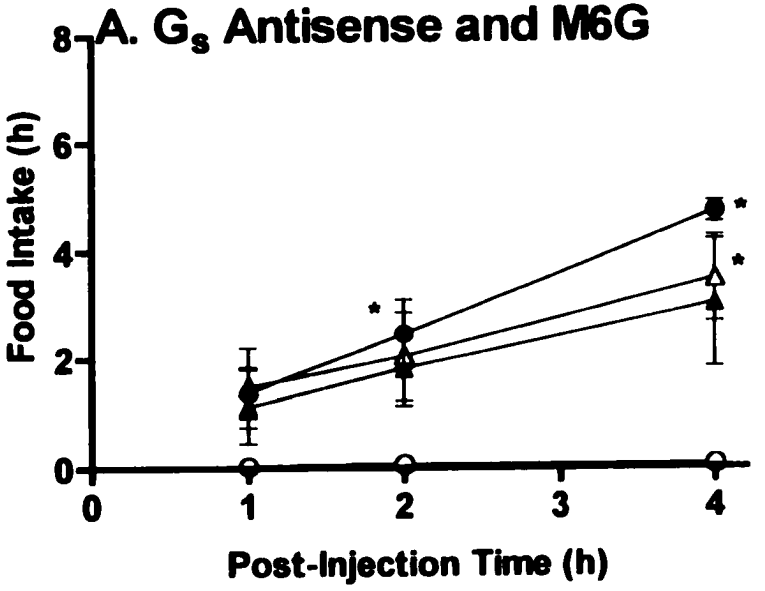
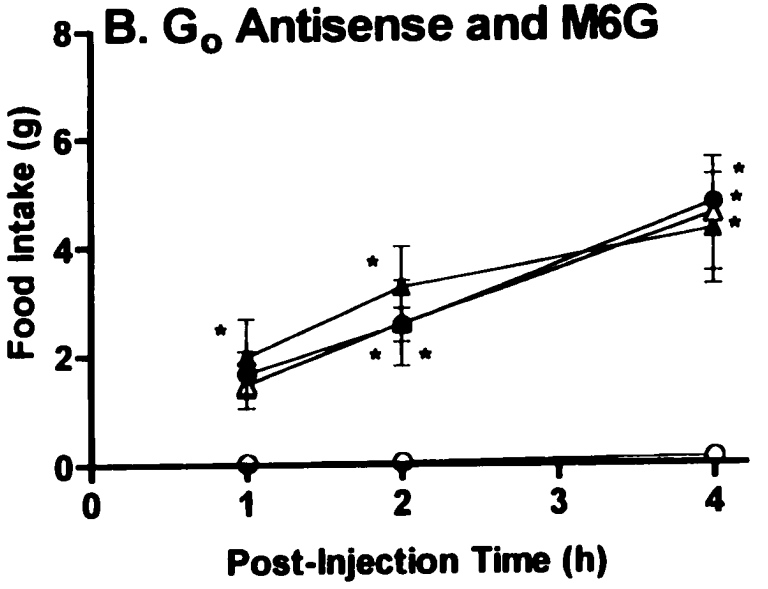
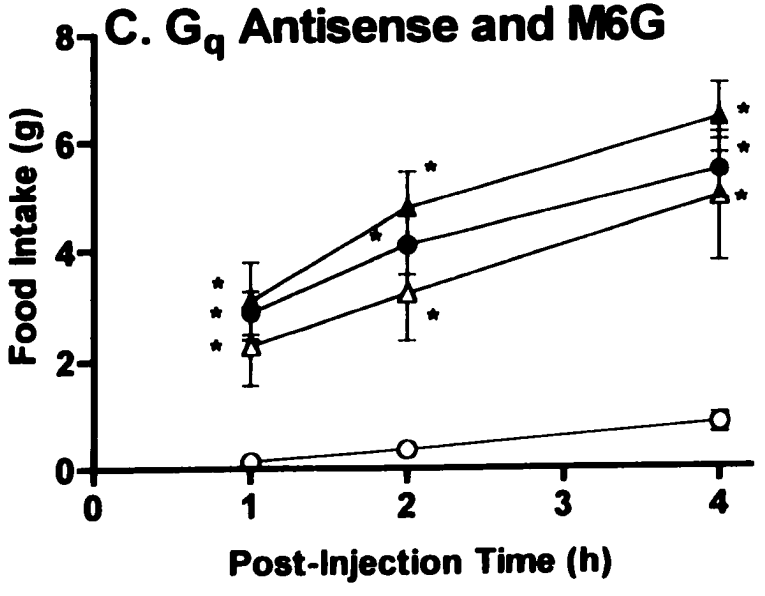


Figure 21. Alterations (Mean, ± SEM) in spontaneous food intake following vehicle and M6G (250 ng) as well as M6G-induced feeding observed 24 and 48 h following AS ODN probes directed against either the $G_s\alpha$ (Panel A), $G_o\alpha$ (Panel B), $G_q\alpha$ (Panel C) or $G_{x_z}\alpha$ (Panel D) subunits. Significant differences were observed among treatment conditions (n=8-10/condition) for the $G_s\alpha$ (1 h: $F(3,27) = 2.80$, $p < .07$; 2 h: $F = 5.41$, $p < .01$; 4 h: $F = 9.35$, $p < .001$), the $G_o\alpha$ (1 h: $F(3,27) = 5.49$, $p < .01$; 2 h: $F = 9.27$, $p < .001$; 4 h: $F = 11.15$, $p < .0002$), the $G_q\alpha$ (1 h: $F(3,23) = 9.32$, $p < 001$; 2 h: $F = 15.07$, $p < .0001$; 4 h: $F = 13.15$, $p < .0002$) and the $G_{x_z}\alpha$ (1 h: $F(3,23) = 19.54$, $p < .0001$; 2 h: $F = 18.83$, $p < .0001$; 4 h: $F = 17.73$, $p < .0001$) treatments. The asterisks (*) in this figure indicate significant increases in agonist-induced feeding relative to corresponding vehicle control values (Dunnett comparisons, $P<0.05$) The crosses (+) in this figure indicate significant decreases in agonist-induced feeding following either antagonist or AS ODN pretreatment relative to corresponding agonist-induced feeding values alone (Dunnett comparisons, $p<0.05$).







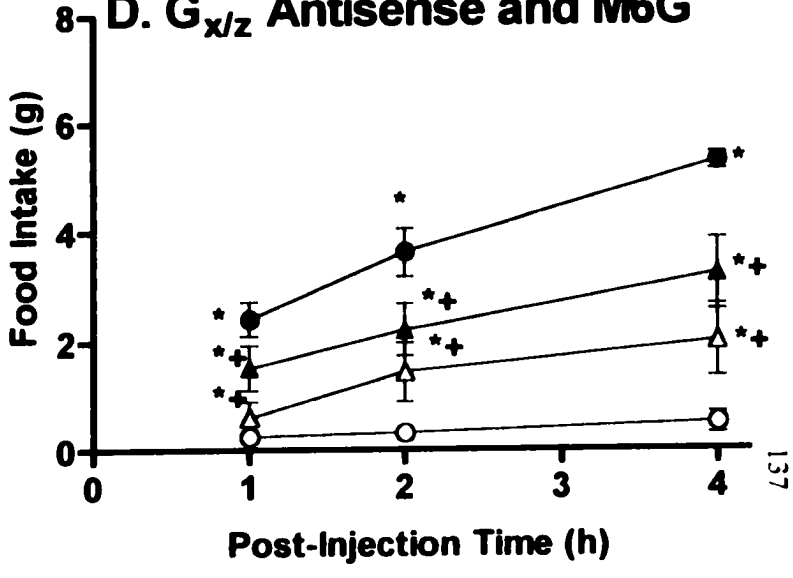
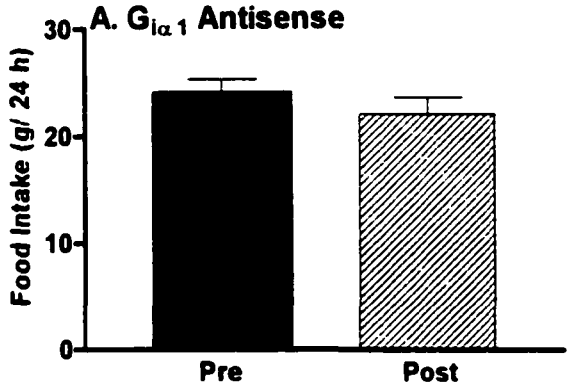
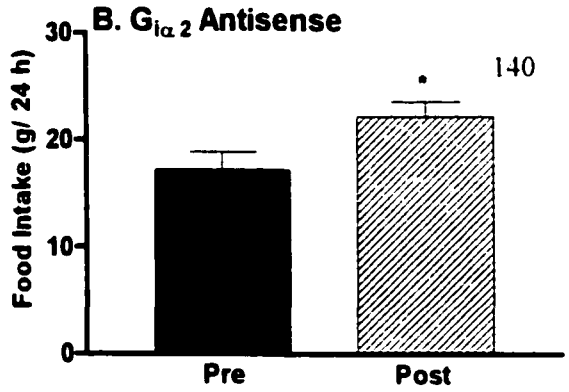


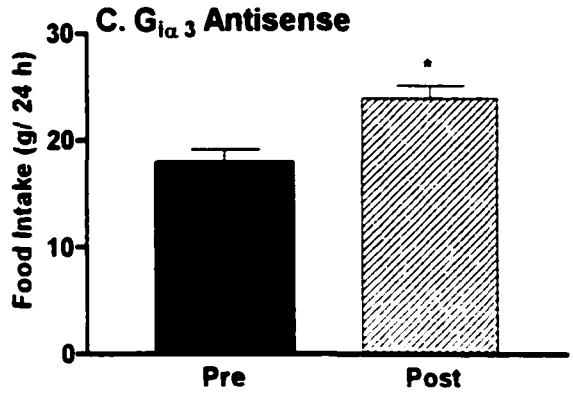
Table 6. Alterations (Mean, ± SEM) in body weight (n=8-10/condition) following pretreatment with antisense probes targeting individual G-protein α-subunits.

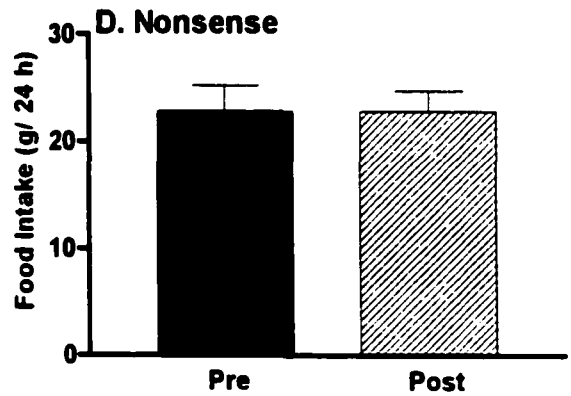
	Body Weight (g)		
AS ODN probe	**Pre**	**24 h**	**48 h**
$G_{i}\alpha_{1}$	463.1 (11.5)	458.3 (9.8)	458.1 (8.8)
$G_{i}\alpha_{2}$	457.7 (10.3)	458.9 (10.6)	452.4 (10.7)
$G_{i}\alpha_{3}$	467.7 (15.8)	470.9 (15.8)	466.1 (15.1)
$G_{o}\alpha$	490.7 (17.4)	481.6 (17.5)*	477.1 (16.9)*
$G_{s}\alpha$	517.7 (18.3)	517.3 (17.5)	511.2 (18.1)*
$G_{q}\alpha$	482.6 (18.7)	482.1 (18.5)	474.2 (18.5)*
$G_{z}\alpha$	475.9 (17.8)	475.1 (17.0)	468.0 (17.0)
NS ODN	523.1 (19.0)	522.5 (19.5)	519.7 (19.9)

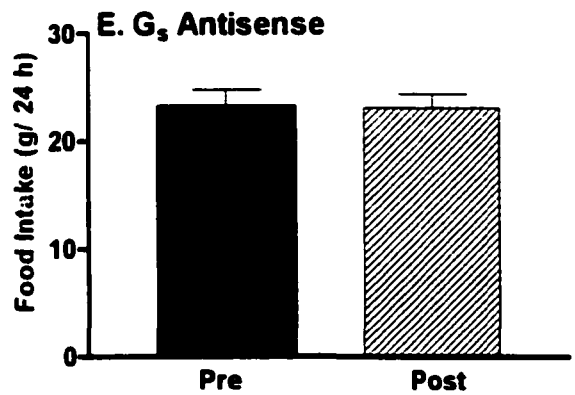
Figure 22. Alterations (Mean, ± SEM) in ad libitum (24 h) food intake (n=8-10/condition) following either $G_i\alpha_1$ (Panel A: $t(19) = 1.28$, $p < .22$), $G_i\alpha_2$ (Panel B: $t(22) = 3.32$, $p < .003$), $G_i\alpha_3$ (Panel C: $t(18) = 4.82$, $p < .0001$), nonsense (Panel D: $t(12) = .001$, $p < .996$), $G_s\alpha$ (Panel E: $t(16) = .121$, $p < .91$), $G_o\alpha$ (Panel F: $t(20) = 3.15$, $p < .01$), $G_q\alpha$ (Panel G: $t(20) = 1.04$, $p < .31$) probes. The asterisks (*) in this figure indicate significant differences in ad libitum (24 h) food intake feeding relative to corresponding vehicle control values (Dunnett comparisons, $P<0.05$).

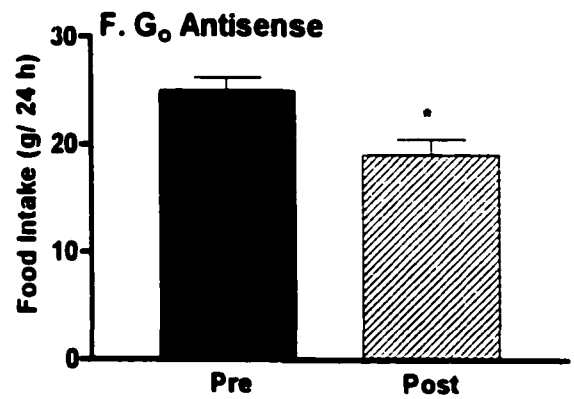


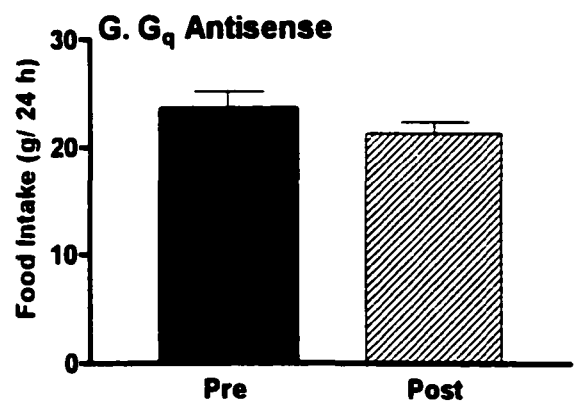


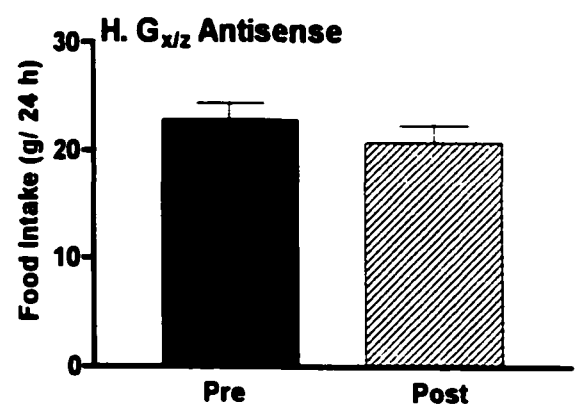












[F(2,56) = 5.95, p<0.001], $G_o\alpha$ [F(2,62) = 8.83, p<0.001] and $G_q\alpha$ [F(2,56) = 6.21, p<0.005] subunits. Importantly, the control nonsense probe failed to alter body weight. In contrast, small but significant increases in cumulative intake were observed following administration of AS ODN probes directed against the $G_i\alpha_2$ [t(22) = 3.32, p<0.003] and the $G_i\alpha_3$ [t(18) = 4.82, p<0.0001] subunits. A small but significant decrease in cumulative intake was observed following pretreatment with the AS ODN probe targeting the $G_o\alpha$ subunit [t(20) = 3.15, p<0.01].

Discussion

This study demonstrated that feeding responses induced by morphine and its active metabolite, M6G are differentially sensitive to AS ODN probes targeted against different G-protein α-subunits. Specifically, morphine- but not M6G-induced feeding, was significantly reduced by an AS ODN probe targeted against the $G_i\alpha_2$ subunit, and was significantly increased by an AS ODN probe targeted against the $G_s\alpha$ subunit. Correspondingly, M6G-induced feeding, but not morphine-induced feeding was significantly reduced by AS ODN probes targeted against either the $G_i\alpha_1$, $G_i\alpha_3$ and $G_{z2}\alpha$ subunits. A nonsense control probe failed to alter either morphine-induced feeding or M6G-induced feeding. Moreover, other AS ODN probes directed against either the $G_o\alpha$ or $G_q\alpha$ subunits also failed to alter feeding induced by morphine or M6G. Also, the effective AS ODN probes directed against either the $G_i\alpha_1$, $G_i\alpha_2$, $G_i\alpha_3$ or $G_{z2}\alpha$ subunits failed to alter the body weights of the tested rats, and either failed to alter or actually slightly increased spontaneous food intake, suggesting that these probes did not produce debilatory, nonspecific effects.

The active morphine metabolite, M6G significantly increased spontaneous food intake at both 250 and 500ng doses with the latter dose producing significantly greater feeding responses over the 4-h time course relative to the former dose. AS ODN probes directed against several G-protein α-subunits significantly attenuated M6G-induced feeding elicited by the 250 ng, but not the 500 ng dose. The inability of AS ODN probes to reduce intake at higher effective doses of an orexogenic agent, yet reduce intake at lower effective doses of the same orexogenic agent is not novel. Indeed, specific AS ODN probes directed against specific coding exons of different opioid receptor clones have been shown to reduce feeding induced by lower, but not higher effective doses of either the μ-selective opioid agonist, DAMGO (Leventhal et al., 1997), the glucoprivic agent, 2-deoxy-D-glucose (2DG) (Leventhal et al., 1996; Burdick et al. 1998) or the lipoprivic agent, mercaptoacetate (Stein et al., 2000). Since AS ODN effects are limited by modest (40%) reductions in receptor protein levels following administration (Pasternak and Standifer, 1995), this may explain why AS ODN probes are efficacious in blocking ingestive responses induced by these μ opioid agonists at lower, but not higher effective doses. A similar rationale of an asymptotic and rate limiting loss in G-protein subunits by their effective AS ODN probes may explain these dose-dependent effects upon M6G-induced feeding.

The decreases in morphine-induced feeding induced by an AS ODN probe directed against the $G_i\alpha_2$ subunit provide similar profiles to that observed in supraspinal morphine analgesia studies using antibodies targeting individual G-proteins (Sanchez-Blazquez et al., 1993; Sanchez-Blazquez and Garzon, 1992; Garzon et al., 1994) or an AS ODN probe

targeted against the $G_i\alpha_2$ subunit (Raffa et al., 1994, 1996; Sanchez-Blazquez et al., 1995; Standifer et al., 1996). Since only the $G_i\alpha_2$ AS ODN probe was effective in attenuating morphine-induced feeding as well as morphine-induced analgesia, it appears that the μ-receptor, a primary receptor target of morphine, mediates morphine-induced feeding either through its direct coupled activation with the $G_i\alpha_2$ subunit, and/or with another unidentified transmitter system downstream that also requires activation of the $G_i\alpha_2$ subunit (Ross, 1989). Evidence supporting the former contention includes significant reductions in [^{3}H]DAMGO specific binding to the μ opioid receptor in the periaqueductal gray following either antisera (Sanchez-Blazquez et al., 1993) or AS ODN probes (Rossi et al., 1995b) directed against the $G_i\alpha_2$ subunit. In contrast to reductions in morphine-induced feeding observed following pretreatment with the $G_i\alpha_2$ AS ODN probe, morphine-induced feeding was significantly enhanced by the AS ODN probe directed against the $G_s\alpha$ subunit. Activation of the $G_s\alpha$ subunit potently stimulates adenylyl cyclase activity, thereby activating the effector signaling pathway (Roerig, 1998; Standifer and Pasternak, 1997; Taussig and Gilman, 1995). This potentiation of morphine-induced feeding is similar to the enhancement of morphine-induced supraspinal analgesia following pretreatment with antisera which functionally inactivated the $G_s\alpha$ subunit (Sanchez-Blazquez and Garzon, 1992). In this model, the behavioral effects produced by morphine-induced $G_i\alpha$ activation and the subsequent inhibition of adenylyl cyclase activity may be antagonized by basal activation of adenylyl cyclase activity of $G_s\alpha$. AS ODN probes directed against the $G_s\alpha$ subunit may act by reducing adenylyl cyclase

activity, and thereby potentiating the behavioral response induced by morphine-induced activation of opioid receptors mediated by $G_i\alpha$ (Cruciani et al., 1999).

Antisense ODN probes directed against either the $G_i\alpha_1$, $G_i\alpha_3$, or $G_{z}\alpha$ subunits significantly reduced M6G-induced feeding. The G-protein sensitivity profile noted for M6G-induced feeding differs dramatically from that observed for morphine. Standifer and co-workers (1996) showed that supraspinal analgesia induced by M6G, but not morphine was significantly reduced in mice after administration of AS ODN probes targeted against $G_i\alpha_1$, $G_i\alpha$, and $G_{z}\alpha$ G-protein α-subunits. The divergent sensitivity profiles of morphine and M6G suggest that the pharmacological mechanisms subserving each of these μ-opioid agonists may be distinctly different. Although all of the G-proteins involved in both morphine and M6G signaling share the common action of inhibiting adenylyl cyclase activity by the activation of the $G_i\alpha$ subunit (Standifer et al., 1996), these results demonstrate that the individual signaling pathways through which they produce their effects are clearly divergent.

Morphine- and M6G-induced feeding may be distinguished from one another by proposing a novel M6G receptor with a different functional α-subunit profile and a pharmacologically distinct effector system. Using this model, alterations in agonist-induced feeding observed in this study may be due to specific inactivation of the individual opioid receptors by G-protein down-regulation via AS ODN treatment. Binding studies showing significant reductions in [^{3}H]DAMGO specific binding to the μ opioid receptor in response to $G_i\alpha_2$ AS ODN treatment (Rossi et al., 1995b; Sanchez-Blazquez et al., 1993) suggest that G-protein AS ODN probes may produce specific loss of function by

functionally inactivating their associated receptors. That is, reductions in agonist-induced feeding observed in this study may reflect specific inactivation of different subtypes of the μ opioid receptor, each displaying different G-protein α-subunit sensitivity profiles. However, an alternative explanation for the loss of function associated with α-subunit AS ODN treatment may involve a trans-neuronal network in which behavioral effects are mediated through the interaction of multiple signals produced by the agonist. Ross (1989) has proposed a scheme for signal routing in G-protein-mediated networks. This work proposes that signals can diverge or converge at different levels of the neuronal network. For example, agonist-induced feeding may be attenuated by the down-regulation of receptor α-subunits and/or down-regulation occurring further downstream in the signaling pathway. Downstream synapses may contain receptors which are sensitive to down-regulation by α-subunit AS ODN probes, but have no direct involvement in agonist binding. This scheme proposes that loss of function can occur as a result of disruption at any level of the trans-neuronal network. Thus, the ability of a given G-protein α-subunit AS ODN to alter agonist-induced feeding does not imply that the opioid receptor actually couples to the specific G-protein being targeted. This suggests that the G-protein sensitivity profiles of morphine and M6G may not represent interactions at relevant opioid receptors. Instead, each response may comprise an intricate functional network composed of multiple types of G-protein receptor complexes including receptors for non-opioid neurotransmitter systems located downstream of μ-receptors. It is clear from our observations, as well as from analgesia studies, that morphine and M6G have pharmacologically distinct effector pathways involving different G-protein receptor

complexes. The level at which the divergence of the signaling pathways occurs remains unclear.

In conclusion, G-protein AS ODN probes differentially modulate feeding responses induced by morphine and its active metabolite, M6G such that AS ODN probes targeted against the $G_i\alpha_2$ subunit significantly reduces feeding induced by morphine, but not M6G. In contrast, AS ODN probes targeted against either the $G_i\alpha_1$, $G_i\alpha_3$ or $G_{x/z}\alpha$ subunits reduce feeding induced by M6G, but not morphine. These effects occurred independently of any G-protein AS ODN-mediated effect upon either body weight or spontaneous food intake per se. The divergent sensitivity profiles of morphine and M6G suggest that the pharmacological mechanisms subserving each of these μ-opioid agonists may be distinctly different.

The distinct G-protein AS ODN sensitivity profiles displayed by morphine and M6G in this study suggested that feeding induced by other opioid agonists could also be distinguished in this way. The following study used an identical G-protein α-subunit AS ODN paradigm to identify the G-protein α-subunit effector systems mediating feeding elicited by βEND and dynorphin.

CHAPTER 6: SPECIFIC AIM FOUR.

G-protein Sensitivity Profile of Endogenous Opioid-induced Feeding: Functional Characterization of β-endorphin and Dynorphin A_{1-17} Using G-protein α-subunit Antisense Probes in Rats.

Introduction

The administration of the endogenous opioid peptides β-endorphin and dynorphin have been shown to produce a robust feeding response in rats (Grandison and Guidotti, 1977; Gosnell et al., 1986a). The μ-subtype of opioid receptors has been primarily implicated in β-endorphin-induced-feeding, since this response is blocked by pretreatment with the selective μ antagonist such as β-funaltrexamine (βFNA) in goldfish (DePedro et al., 1995). These findings are supported by the results from Specific Aim One, in that βEND-induced feeding was potently blocked by βFNA as well as by pretreatment with AS ODN probes targeting coding exons 1, 3, and 4 of the MOR-1 gene. In contrast, dynorphin-induced feeding is postulated to be primarily mediated through the activation of the κ_1-opioid receptor (Chavkin and Goldstein, 1982). κ-opioid receptor-mediation of dynorphin-induced feeding was confirmed in Specific Aim Two, in which the selective κ opioid receptor antagonist NorBNI, as well an AS ODN probes targeting coding exons 1 and 2 of both the KOR-1 and KOR-3 genes effectively blocked dynorphin-induced feeding. Although antagonist studies suggest that the orexogenic properties of these peptides result from the putative activation of individual opioid receptors, it appears that the functional selectivity of antagonists may not be sufficient to examine subtleties in the opioid receptor effector signaling pathways mediating these ingestive effects.

Opioid receptors mediate their effects through the putative activation of guanine nucleotide binding proteins (G-proteins) to which they are coupled (Childers. 1988; Pasternak and Standifer. 1995; Reisine and Bell. 1993; Standifer et al.,1996; Uhl et al., 1994). G-proteins have been implicated in opioid-mediated functions using AS ODN probes directed against the α-subunit (Pasternak and Standifer. 1995). Specifically, the antinociceptive activities of selective μ and δ opioid receptor agonists are reduced by AS ODN probes targeted against the $G_i\alpha_2$ and $G_{x/z}\alpha$ subunits (Sanchez-Blazquez et al., 1995; Raffa et al., 1996). Analgesia elicited by morphine and its active metabolite, morphine-6β-glucuronide (M6G) can be differentiated by this technique such that AS ODN probes directed against either $G_i\alpha_2$ or $G_o\alpha$ reduced morphine, but not M6G analgesia, whereas AS ODN probes directed against $G_i\alpha_1$ or $G_{x/z}\alpha$ reduced M6G, but not morphine analgesia (Rossi et al., 1995b; Standifer et al., 1996). Similar distinctions have been made for feeding responses elicited by morphine and M6G using AS ODN probes directed against G-protein α-subunits. Thus, morphine-induced feeding was significantly reduced by an AS ODN probe directed against $G_i\alpha_2$, and was significantly potentiated by an AS ODN probe directed against $G_s\alpha$ (Silva et al., 2000). In contrast, M6G-induced feeding was significantly reduced by AS ODN probes directed against either $G_i\alpha_1$, $G_i\alpha_3$ or $G_{x/z}\alpha$ (Silva et al., 2000).

Thus, βEND and dynorphin-induced feeding can be further characterized by the elucidation of the individual types of G-proteins that modulate these ingestive effects. The mediation of both βEND and dynorphin-induced feeding can be determined on the basis of their individual G-protein activation profile. A complete understanding of the signal

transduction mechanisms which modulate both βEND and dynorphin-induced feeding would allow for the elucidation of the distinct effector pathways responsible for these effects. In addition, the G-protein sensitivity profiles displayed by each of these endogenous peptides could be directly compared to those previously observed for morphine ($G_i\alpha_2$, $G_s\alpha$)- and M6G ($G_i\alpha_1$, $G_i\alpha_3$, $G_{z/z}\alpha$)-induced feeding (Silva et al., 2000). To determine whether the distinctions between feeding responses induced by βEND and dynorphin involve different G-protein α-subunits, the present study will evaluate the effects of ventricularly-administered AS ODN probes directed against either the $G_i\alpha_1$, $G_i\alpha_2$, $G_i\alpha_3$, $G_o\alpha$, $G_s\alpha$, $G_{z/z}\alpha$, or $G_q\alpha$ α-subunits as well as a control nonsense probe upon spontaneous food intake induced by βEND and dynorphin in rats. The findings of the present study have been recently submitted for publication (Silva et al., 2001c).

Methods

Opioid agonists and AS ODN probes. The opioid agonists, βEND (Peninsula Laboratories, Belmont, CA), and dynorphin A_{1-17} (Peninsula Laboratories), were each dissolved in distilled water at a concentration of 2 μg/μl. All phosphodiester oligodeoxynucleotides (Midland Certified Reagent Company, Midland, TX) were dissolved in 0.9% normal saline at a concentration of 5μg/μl, and were specific for the G protein α subunits for which they were targeted. These particular AS ODN sequences were chosen because of their previously-demonstrated effectiveness in specifically and selectively reducing both feeding and analgesia induced by either morphine or M6G (Rossi et al., 1995b; Silva et al., 2000; Standifer et al., 1996). All sequences were subject

to a GenBank search, were found to be unique to their respective G protein α-subunit, and were based on rat sequences with the exception of $G_{z}\alpha$ (human).

Following baseline measurements, separate groups of 35 and 40 rats were assessed for food intake after 1, 2 and 4 h following microinjection of either βEND (Peninsula Laboratories) at a dose of 10 μg, or dynorphin A_{1-17} (Peninsula) at a dose of 50 μg respectively. Both agonists were administered in 5 μl volumes in distilled water to guarantee solubility of the compounds. The doses of βEND and dynorphin were chosen based on their previously reported peak efficacies and their equi-effective magnitudes of response (Silva et al., 2001a, 2001b).

Protocol 1: Following stabilization of baseline control intake and establishment of βEND-induced feeding, separate subgroups of rats underwent the following 3-day paradigm. First, animals received an AS ODN (25 μg) or nonsense probe microinjection at 3 h into the light cycle (Day 1). Animals then received βEND (10 μg) 24 (Day 2) and 48 (Day 3) h thereafter with food intake measurements taken 1, 2 and 4 h following each agonist treatment. This time course was chosen since significant down-regulation of G protein α subunits occurs maximally between 24 and 48 h following a single AS ODN injection (Rossi and Pasternak, 1997; Rossi et al., 1995b; Standifer et al., 1996). The 25 μg dose was chosen because it has been shown to be most effective in reducing morphine- and M6G-induced feeding as well as producing minimal effects upon ad libitum food intake and body weight (Silva et al., 2000). The subgroups (n=8-10/condition) of rats received: (1) control treatment, (2) βEND (10 μg), AS ODN probes (25 μg) directed against either (3) $G_{i}\alpha_{1}$, (4) $G_{i}\alpha_{2}$, (5) $G_{i}\alpha_{3}$, (6) $G_{s}\alpha$, (7) $G_{o}\alpha$, (8) $G_{z}\alpha$, (9) $G_{q}\alpha$, or (10) a

nonsense (NS) ODN 24 h prior to βEND administration. Ten to fifteen days following the first AS ODN treatment, rats with a patent cannula were retested with a second AS ODN or nonsense probe over a second 3-day paradigm.

Protocol 2: Following stabilization of baseline control intake and establishment of dynorphin-induced feeding, separate subgroups of rats underwent the identical 3-day paradigm described in protocol 2 except that dynorphin (50 μg) was used as the agonist. The subgroups of rats received: (1) control treatment, (2) dynorphin A_{1-17} (50 μg), AS ODN probes (25 μg) directed against either (3) $G_i\alpha_1$, (4) $G_i\alpha_2$, (5) $G_i\alpha_3$, (6) $G_z\alpha$, (7) $G_o\alpha$, (8) $G_{z/z}\alpha$, (9) $G_q\alpha$, or (10) a NS ODN 24 h prior to dynorphin administration. Again, ten to fifteen days following the first AS ODN treatment, rats with a patent cannula were retested with a second AS ODN or nonsense probe over a second 3-day paradigm.

Results

βEND-Induced and Dynorphin-Induced Feeding

In assessing whether βEND and dynorphin produced equi-effective increases in food intake across the time course, significant differences in intake were observed between injection groups (1 h: $F(1,38)=4.77$, $p<0.035$; 2 h: $F = 2.18$, n.s.; 4 h: $F = 0.37$, n.s.), between control and agonist treatments (1 h: $F(1,38)=204.16$; $p<0.0001$; 2 h: $F=375.22$, $p<0.0001$; 4 h: $F=846.50$; $p<0.0001$), and for the interaction between groups and treatments (1 h: $F(1,38)=0.43$, n.s.; 2 h: $F=9.93$, $p<0.003$; 4 h: $F=9.58$, $p<0.004$). **Table 7** summarizes the effectiveness of βEND (10 μg) and dynorphin (50 μg) to elicitfeeding across the time course. Both βEND and dynorphin significantly increased food intake after 1, 2, and 4 h relative to their respective control conditions, and failed to

Table 7. β-endorphin and dynorphin A_{1-17} significantly increased food intake (g, ± S.E.M.) across the 4 h time course relative to their respective control values.

	1 h	2 h	4 h
A. βEND Group (n= 35)			
Control	0.2 (0.1)	0.3 (0.1)	0.5 (0.1)
βEND (10 μg)	2.0* (0.2)	3.5* (0.2)	4.1* (0.2)
B. Dynorphin Group (n= 40)			
Control	0.4 (0.1)	0.5 (0.1)	0.5 (0.1)
Dynorphin A_{1-17} (50 μg)	2.4* (0.2)	3.0*+ (0.2)	3.7* (0.1)

differ from each other in the magnitudes of their responses after 1 and 4 h. βEND produced a marginally (0.5 g), but significantly greater amount of intake than dynorphin at the 2 h time point. Thus, these doses of the two agonists significantly increased food intake to a similar degree as observed previously (Silva et al., 2001a, 2001b), and therefore allowed for reliable comparisons of G-protein AS ODN effects upon βEND-induced and dynorphin A_{1-17}-induced feeding.

G-Protein AS ODN Treatment and βEND-Induced Feeding

Significant differences were observed in intake among treatment conditions after 1 ($F(17,197)=5.07$, $p<0.0001$), 2 ($F=6.84$, $p<0.0001$) and 4 ($F=7.69$, $p<0.0001$) h. βEND significantly increased food intake relative to control values after 1, 2 and 4 h. βEND-induced feeding was significantly reduced at the 4 h intake point 24 h following the $G_i\alpha_1$ AS ODN probe; this effect was not observed 48 h after treatment (**Figure 23A**). In contrast, βEND-induced feeding was significantly increased across the time course 24 h following the $G_i\alpha_2$ AS ODN probe; this effect was also not observed 48 h after treatment (**Figure 23B**). βEND-induced feeding was also significantly increased at the 1 h intake point 24 and 48 h following the $G_i\alpha_3$ AS ODN probe (**Figure 23C**). AS ODN probes directed against either $G_s\alpha$, $G_o\alpha$, $G_q\alpha$ or $G_{z2}\alpha$ failed to alter βEND-induced feeding at any time point24or 48 h following probe administration (**Figures 23D, 24A-24C**). Importantly, a control nonsense probe also failed to alter βEND-induced feeding at any time point 24 or 48 h following probe administration (**Figures 24D**).

G-Protein AS ODN Treatment and Dynorphin-Induced Feeding

Significant differences were observed in intake among treatment conditions after 1

Figure 23. Alterations (Mean, ±SEM) in food intake (g) following i.c.v. administration of either vehicle alone, βEND (10 μg) alone, or βEND following ventricular pretreatment (24 and 48 h) with AS ODN probes (n=8-10/condition) directed against either $G_i\alpha_1$ (Panel A), $G_i\alpha_2$ (Panel B), $G_i\alpha_3$ (Panel C) or $G_s\alpha$ (Panel D). Significant differences in βEND-induced food intake were observed following pretreatment with AS ODN probes targeting either $G_i\alpha_1$ (4 h: 24 h), $G_i\alpha_2$ (2, 4 h: 24 h) and $G_i\alpha_3$ (1 h: 24 and 48 h). No significant differences in βEND-induced food intake were observed following pretreatment with AS ODN probes targeting $G_s\alpha$. The asterisks (*) in this figure indicate significant increases in agonist-induced feeding relative to corresponding vehicle control values (Dunnett comparisons, $P<0.05$) The crosses (+) in this figure indicate significant decreases in agonist-induced feeding following either antagonist or AS ODN pretreatment relative to corresponding agonist-induced feeding values alone (Dunnett comparisons, $p<0.05$).

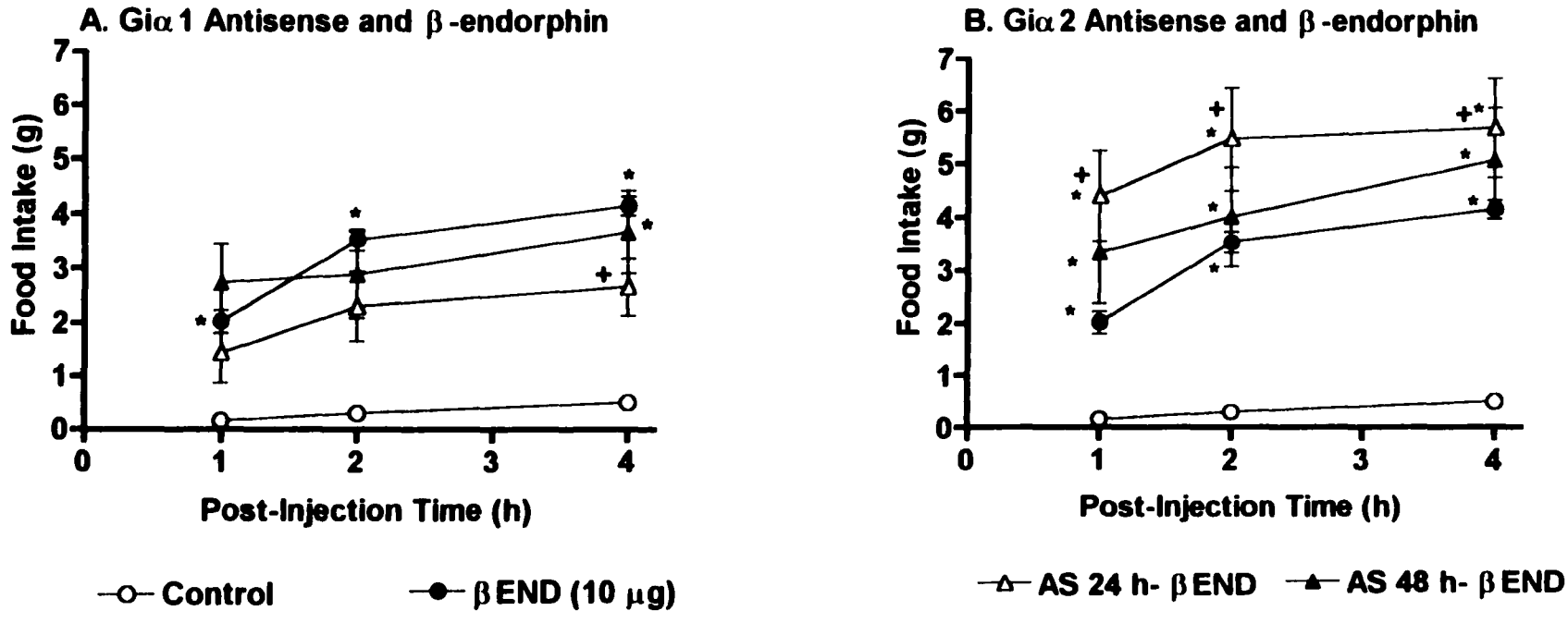
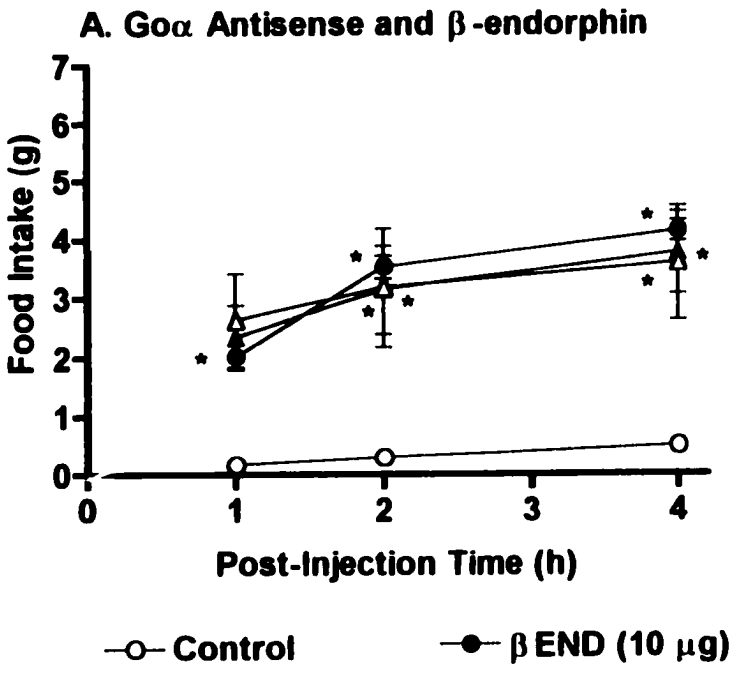
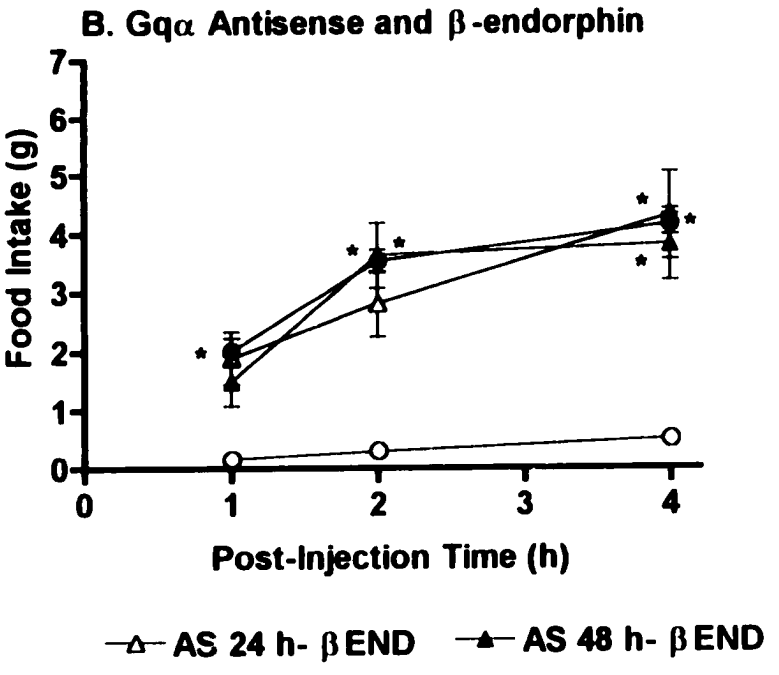
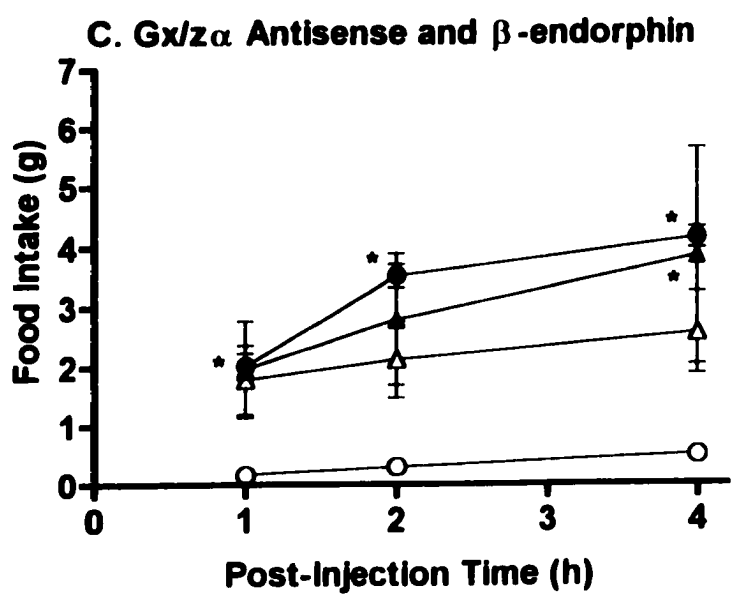
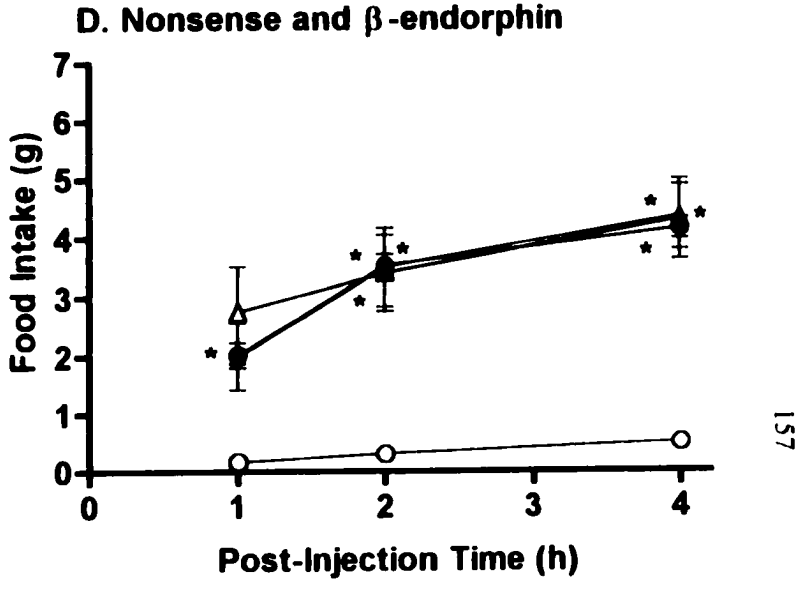


Figure 24. Alterations (Mean, ±SEM) in food intake (g) following i.c.v. administration of either vehicle alone, βEND (10 μg) alone, or βEND following ventricular pretreatment (24 and 48 h) with AS ODN probes (n=8-10/condition) directed against either $G_o\alpha$ (Panel A), $G_q\alpha$ (Panel B), $G_{z2}\alpha$ (Panel C) or the NS ODN probe (Panel D). No significant differences in βEND-induced food intake were observed following pretreatment with AS ODN probes targeting either the $G_o\alpha$, $G_q\alpha$, $G_{z2}\alpha$ subunits or the control NS ODN probe. The asterisks (*) in this figure indicate significant increases in agonist-induced feeding relative to corresponding vehicle control values (Dunnett comparisons, $P<0.05$) The crosses (+) in this figure indicate significant decreases in agonist-induced feeding following either antagonist or AS ODN pretreatment relative to corresponding agonist-induced feeding values alone (Dunnett comparisons, $p<0.05$).







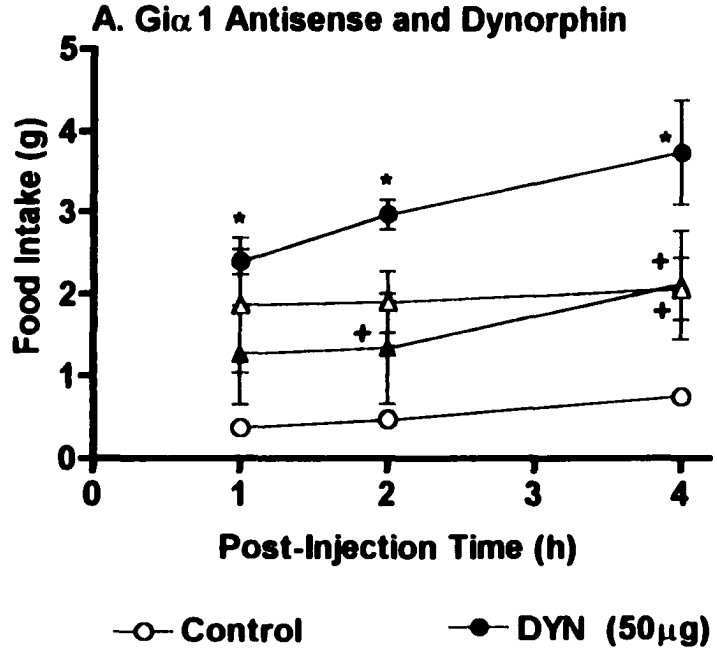


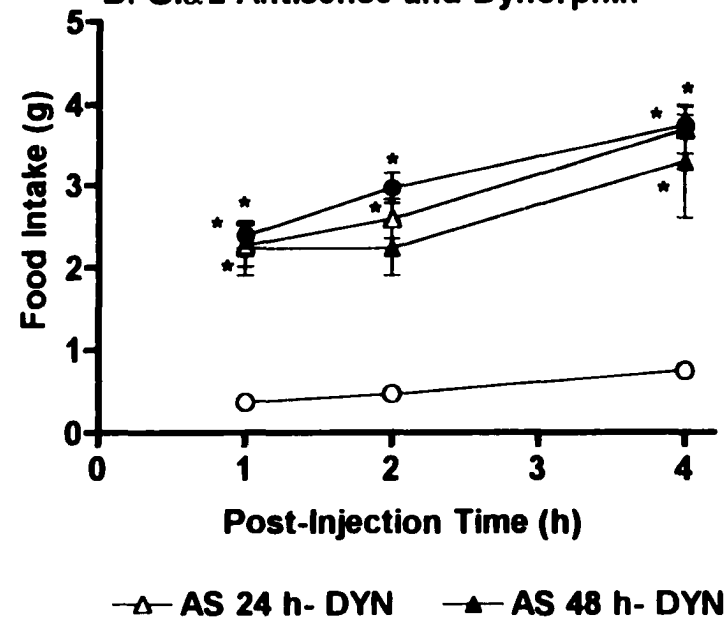
($F(17,187)=3.89$, $p<0.0001$), 2 ($F= 4.26$, $p<0.0001$) and 4 ($F=6.43$, $p<0.0001$) h. Dynorphin significantly increased food intake relative to control values after 1, 2 and 4 h. Dynorphin-induced feeding was significantly reduced at the 2 h intake point 48 h following the $G_i\alpha_1$ AS ODN probe, and at the 4 h intake point 24 and 48 h following the $G_i\alpha_1$ AS ODN probe **(Figure 25A)**. Further, dynorphin-induced feeding was reduced at the 4 h intake point 48 h following the $G_o\alpha$ AS ODN probe **(Figure 26A)**. In contrast, AS ODN probes directed against either $G_i\alpha_2$, $G_i\alpha_3$, $G_s\alpha$, $G_q\alpha$ or $G_{z/z}\alpha$ failed to alter dynorphin-induced feeding at any time point 24 or 48 h following probe administration **(Figures 25B-25D, 26B-26C)**. Again, a control nonsense probe also failed to alter dynorphin-induced feeding at any time point 24 or 48 h following AS ODN treatment **(Figure 26D)**.

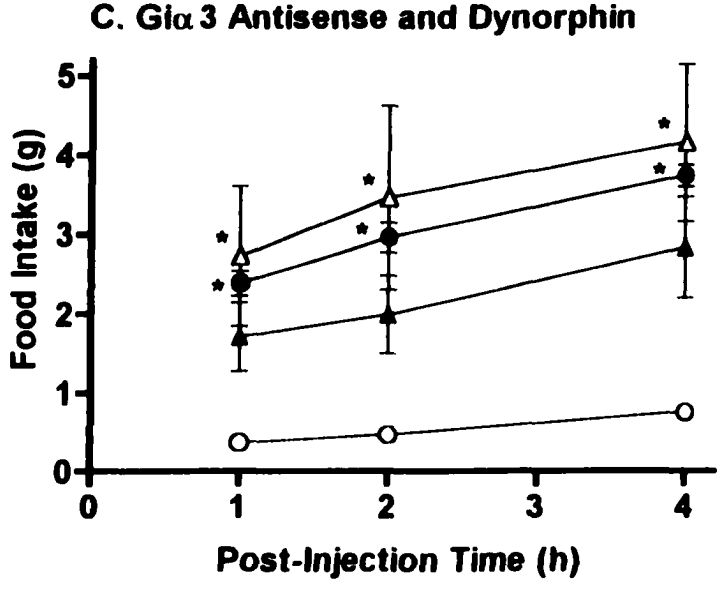
Discussion

Feeding elicited by βEND and dynorphin were each differentially sensitive to pretreatment with AS ODN probes directed against different G-protein α-subunits. Both βEND-induced feeding and dynorphin-induced feeding were significantly reduced by pretreatment with an AS ODN probe directed against $G_i\alpha_1$, albeit with slightly different time courses and durations of effects. This common sensitivity of both agonists to this particular probe suggests a common effector pathway for the ingestive effects mediated by both of these endogenous peptides. Additionally, βEND-induced feeding was significantly increased by AS ODN probes directed against either the $G_i\alpha_2$ or $G_i\alpha_3$ subunits. Moreover, dynorphin-induced feeding was significantly reduced by an AS ODN probe directed against the $G_o\alpha$ subunit.

Figure 25. Alterations (Mean, ±SEM) in food intake (g) following i.c.v. administration of either vehicle alone, dynorphin (50 μg) alone, or dynorphin following ventricular pretreatment (24 and 48 h) with AS ODN probes (n=8-10/condition) directed against either $G_i\alpha_1$ (Panel A), $G_i\alpha_2$ (Panel B), $G_i\alpha_3$ (Panel C) or $G_s\alpha$ (Panel D). Significant differences in dynorphin-induced food intake were observed following pretreatment with AS ODN probes targeting $G_i\alpha_1$ (2 h: 24 h; 4 h: 24 and 48 h). No significant differences in dynorphin-induced food intake were observed following pretreatment with AS ODN probes targeting either $G_i\alpha_2$, $G_i\alpha_3$ or $G_s\alpha$. The asterisks (*) in this figure indicate significant increases in agonist-induced feeding relative to corresponding vehicle control values (Dunnett comparisons, $P<0.05$) The crosses (+) in this figure indicate significant decreases in agonist-induced feeding following either antagonist or AS ODN pretreatment relative to corresponding agonist-induced feeding values alone (Dunnett comparisons, $p<0.05$).







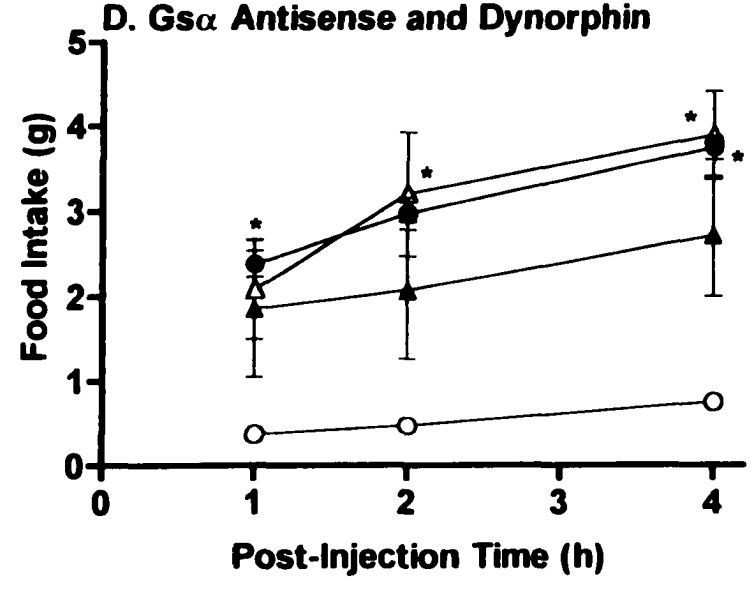
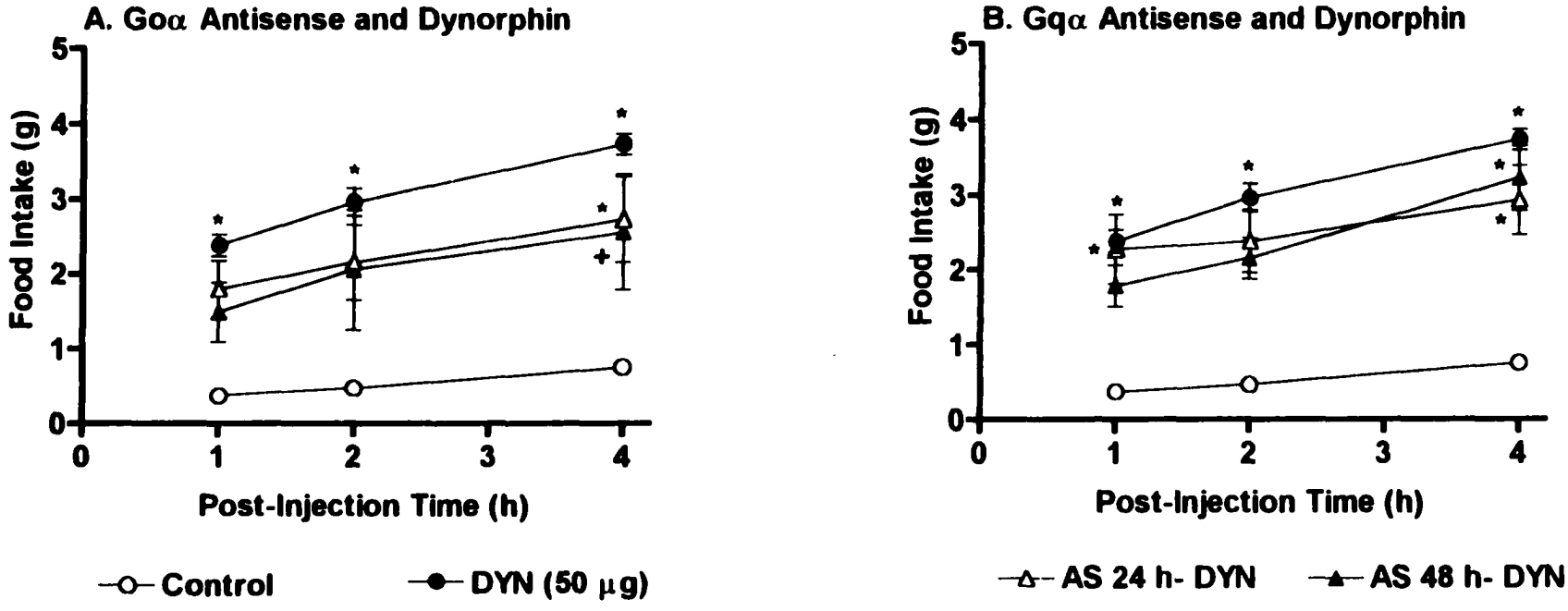
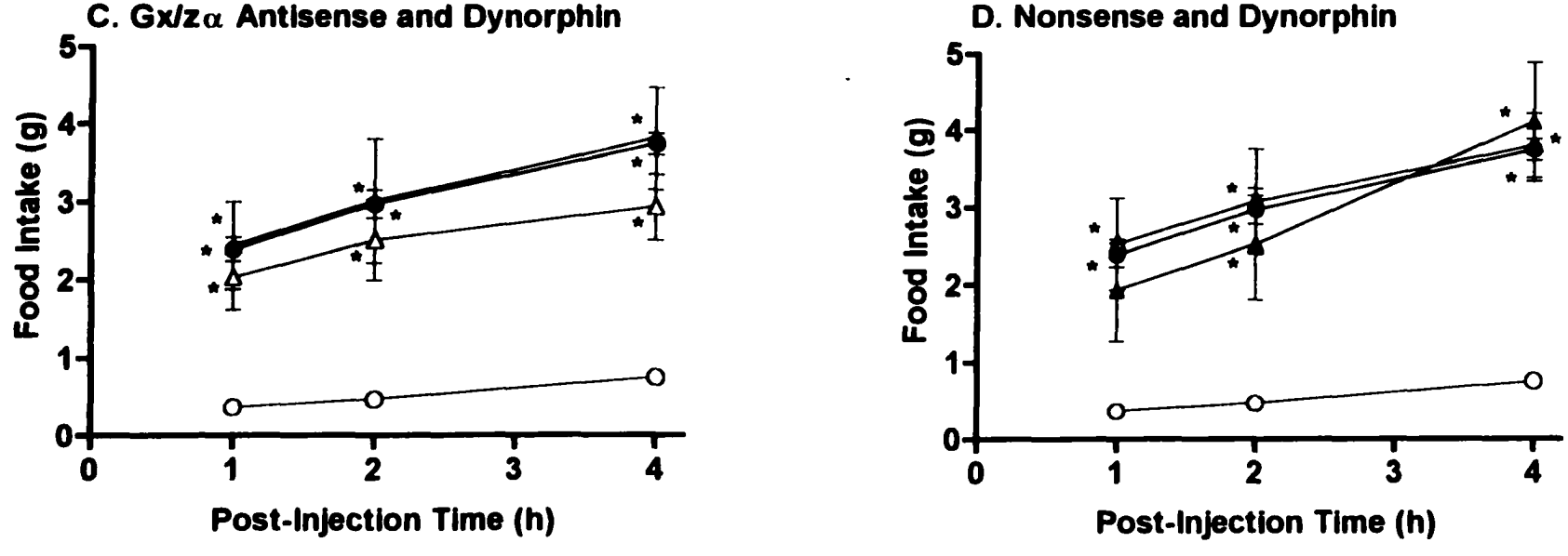


Figure 26. Alterations (Mean, ±SEM) in food intake (g) following i.c.v. administration of either vehicle alone, dynorphin (50 μg) alone, or dynorphin following ventricular pretreatment (24 and 48 h) with AS ODN probes (n=8-10/condition) directed against either $G_o\alpha$ (Panel A), $G_q\alpha$ (Panel B), $G_{z2}\alpha$ (Panel C) or the NS ODN probe (Panel D). No significant differences were observed following pretreatment with AS ODN probes targeting either the $G_o\alpha$, $G_q\alpha$, $G_{z2}\alpha$ subunits or the control NS ODN probe. The asterisks (*) in this figure indicate significant increases in agonist-induced feeding relative to corresponding vehicle control values (Dunnett comparisons, $P<0.05$) The crosses (+) in this figure indicate significant decreases in agonist-induced feeding following either antagonist or AS ODN pretreatment relative to corresponding agonist-induced feeding values alone (Dunnett comparisons, $p<0.05$). significantly increased by AS ODN probes directed against either $G_i\alpha_2$ or $G_i\alpha_3$ Moreover, dynorphin-induced feeding was significantly reduced by an AS ODN probe directed against $G_o\alpha$





The $G_i\alpha_1$ subunit is implicated in the feeding responses elicited by either βEND or dynorphin based upon reductions elicited by pretreatment with an AS ODN probe directed against it. In this regard, these βEND and dynorphin effects share similarities with the ability of this AS ODN probe directed against $G_i\alpha_1$ to reduce analgesia and feeding elicited by the morphine metabolite, M6G (Rossi et al., 1995b; Silva et al., 2000; Standifer et al., 1996) as well as analgesia elicited by heroin and methadone (Sanchez-Blazquez et al., 2001). In contrast, since the analgesic and ingestive actions of morphine are insensitive to pretreatment with this AS ODN probe (Rossi et al., 1995b; Standifer et al., 1996; Silva et al., 2000), these results would suggest that βEND and dynorphin share a common effector pathway with M6G and heroin that differs from that of morphine. The common sensitivity of feeding elicited by these opioid agonists to reduction by pretreatment with the μ-selective antagonist βFNA (Leventhal et al., 1998b; Silva et al., 2001a, 2001b) supports these findings.

It should be noted however that the G-protein AS ODN profiles observed for βEND and dynorphin are not identical to those observed for M6G (Silva et al., 2000). Thus, βEND-induced feeding is significantly increased by pretreatment with an AS ODN probe directed against $G_i\alpha_3$, yet this probe significantly reduces feeding elicited by M6G (Silva et al., 2000). Further, dynorphin induced feeding is significantly decreased by pretreatment with an AS ODN probe targeting the $G_o\alpha$ subunit, yet this probe is ineffective against M6G-induced feeding (Silva et al., 2000). Moreover, AS ODN mapping studies have demonstrated that feeding elicited by βEND, dynorphin, and M6G display differential sensitivities to pretreatment with AS ODN probes targeting the opioid

receptor genes themselves. Thus, βEND-induced feeding is reduced by AS ODN probes targeting either coding exons 1, 3, or 4 of the MOR-1 gene; dynorphin-induced feeding is reduced by AS ODN probes targeting either coding exons 1 or 2 of both the KOR-1 and KOR-3/ORL-1 genes; M6G-induced feeding is reduced by AS ODN probes directed against either coding exons 2 or 3 of the MOR-1 clone (Leventhal et al., 1998b; Silva et al., 2001a, 2001b). Therefore, the dissimilar G-protein- and opioid receptor-AS ODN profiles displayed by βEND-, dynorphin- and M6G-induced feeding suggests that the effects of the $G_i\alpha_1$ AS ODN probe do not occur via the direct disruption of receptor-effector interactions within the same neuron, but rather reflect disruption of common G-proteins at different downstream effector sites (Ross, 1989). That is, downstream synapses may contain receptors which are sensitive to down-regulation by $G_i\alpha_1$ AS ODN treatment, but have no direct involvement in agonist binding. This model proposes that the reductions in food intake elicited by βEND, dynorphin and M6G may occur as a result of disruption at any level of the trans-neuronal network.

Therefore, the G-protein sensitivity profiles displayed by βEND- and dynorphin-induced feeding, may not only reflect a common receptor-level effector pathway mediated by the $G_i\alpha_1$ subunit, but may also comprise an intricate functional network composed of multiple types of G-protein receptor complexes including receptors for other transmitter systems located downstream of that particular receptor. The common sensitivity βEND- and dynorphin-induced feeding as well as feeding elicited by other opioid agonists including M6G to $G_i\alpha_1$ antisense treatment, suggests that this neuronal network plays a

central role in opioid-mediated behavior, and may represent a final common pathway for a distinct class of opioid agonists.

CHAPTER 7. GENERAL DISCUSSION

Opioid Pharmacology of βEND-Induced Feeding

The present series of studies have examined the role of the endogenous opioid peptides, β-endorphin (βEND) and dynorphin A_{1-17} in the mediation of ingestive behavior. Results from Chapter 3 showed that feeding induced by βEND was primarily mediated by the μ opioid receptor. βEND-induced feeding was significantly reduced by moderate (20-40nmol) doses of general (naltrexone), δ (naltrindole), and κ (NorBNI) opioid receptor antagonists as well as by lower (0.5-40 nmol) doses of the μ-selective opioid antagonist, βFNA (**Table 8**). βEND-induced feeding was also significantly reduced by pretreatment with AS ODN probes targeting either coding exons 1, 3, or 4 of the MOR-1 gene and was transiently (1 d) reduced following pretreatment with an AS ODN probe targeting coding exon 1 of the DOR-1 gene (**Table 9**). The effective use of opioid antagonists as well as antisense probes targeting opioid receptor clones provided converging evidence implicating a μ opioid receptor-mediated mechanism for βEND-induced feeding. The unique sensitivity and specificity of βEND-induced feeding to disruption by pretreatment with AS ODN probes targeting either coding exons 1, 3, or 4 of the MOR-1 opioid receptor clone, was further demonstrated by the failure of a control MS ODN probe, which differed from the MOR-1 coding exon 1 AS ODN by the sequence reversal of only two pairs of bases (**Table 1**), to alter βEND-induced feeding. In addition, the use of this control missense condition ensured that AS ODN effects did not occur as a result of nonspecific factors such as malaise, or other compensatory mechanisms resulting from antisense-induced opioid receptor down-regulation. The high sequence homology between

Table 8. Comparison of μ-, δ-, and κ-selective Opioid Receptor Antagonist-Induced Reductions of Feeding Elicited by Morphine, M6G, βEND and Dynorphin A_{1-17}.

	Selective Opioid Antagonist		
	μ (βFNA)	**δ (naltrindole)**	**κ (NBNI)**
Opioid Agonist			
Morphine	+++	++	N/A
M6G	+++	++	+
β-END	+++	+	++
Dynorphin	++	+	+++

+++ Denotes large reductions relative to parallel agonist condition.
++ Denotes moderate reductions relative to parallel agonist condition.
+ Denotes small reductions relative to parallel agonist condition.
- Denotes no significant effect.

Table 9. Comparison of MOR-1, DOR-1, KOR-1 and KOR-3/ORL-1 Exon-specific AS ODN-Induced Reductions of Feeding Elicited by Morphine, M6G, βEND and Dynorphin A_{1-17}.

	Opioid Agonist			
	Morphine	**M6G**	**β-END**	**Dynorphin**
AS ODN Probe				
MOR-1				
Coding exon 1	+++	-	+	++
Coding exon 2	-	+++	-	-
Coding exon 3	-	+++	+++	-
Coding exon 4	+++	-	+++	-
DOR-1				
Coding exon 1	N/A	N/A	+	+
Coding exon 2	N/A	N/A	-	-
Coding exon 3	N/A	N/A	-	-
KOR-1				
Coding exon 1	N/A	N/A	-	+++
Coding exon 2	N/A	N/A	-	+++
Coding exon 3	N/A	N/A	-	-
KOR-3/ORL-1				
Coding exon 1	N/A	N/A	-	+++
Coding exon 2	N/A	N/A	-	+++
Coding exon 3	N/A	N/A	-	-

+++ Denotes large reductions relative to parallel agonist condition.
++ Denotes moderate reductions relative to parallel agonist condition.
+ Denotes small reductions relative to parallel agonist condition.
- Denotes no significant effect.

ineffective control MS ODN probes and their corresponding AS ODN probes demonstrates the unique specificity of this technique relative to the exclusive use of opioid receptor antagonists.

Consistent with the present results, other studies have confirmed that βEND-induced feeding is sensitive to pretreatment with μ antagonists such as βFNA and naloxonazine (De Pedro et al., 1996). In addition, detailed AS ODN mapping studies of other μ agonists such as DAMGO, morphine and morphine-6β-gulcuronide (M6G) have demonstrated that μ-mediated ingestive effects are uniquely and differentially sensitive to pretreatment with AS ODN probes targeting individual coding exons of the MOR-1 clone. Specifically, feeding elicited by either morphine or DAMGO was potently blocked by pretreatment with AS ODN probes targeting either coding exons 1 or 4, but not coding exons 2 or 3 of the MOR-1 clone (Leventhal et al., 1997, 1998b). In contrast, M6G-induced feeding was blocked by pretreatment with AS ODN probes directed against either coding exons 2 or 3, but not coding exons 1 or 4 of the MOR-1 clone (Leventhal et al., 1998b). The differential MOR-1 AS ODN sensitivity profiles displayed by morphine, DAMGO and M6G suggested that these agonists were potentially activating different splice variants or isoforms of the MOR-1 clone (see review: Pasternak, 2001; Pasternak and Standifer, 1995; Rossi and Pasternak, 1997). The present findings demonstrating the unique sensitivity of βEND-induced feeding to pretreatment with AS ODN probes directed against either coding exons 1, 3, or 4 of the MOR-1 clone, suggest that βEND-induced feeding may result from activation of multiple μ-opioid receptor isoforms. However, the lack of cloning data to support the existence of pharmacologically defined

μ-opioid receptor isoforms seriously weakens this conclusion. Thus, it appears that the opioid receptor mediating βEND-induced feeding is the pharmacologically described μ opioid receptor. The genes responsible for this effect are encoded by the MOR-1 clone since βEND-induced feeding shares a similar, although not identical AS ODN profile to those observed for morphine, DAMGO and M6G.

Opioid Pharmacology of Dynorphin A_{1-17}-Induced Feeding

The results from Chapter 4 extended the specificity of the AS ODN technique to the examination of feeding induced by dynorphin A_{1-17}. This study confirmed that dynorphin-induced feeding is mediated primarily by the activation of κ opioid receptors. Dynorphin-induced feeding was potently reduced by pretreatment with the κ (NorBNI)- and μ (βFNA)-selective opioid antagonists, whereas the general (naltrexone) and δ (naltrindole)-selective opioid antagonists were moderately effective (**Table 8**). Whereas pretreatment with AS ODN probes directed against either coding exons 1 or 2 of both the KOR-1 and KOR-3/ORL-1 genes potently reduced dynorphin-induced feeding, AS ODN probes directed against coding exon 1 of the DOR-1 gene and coding exon 1 of the MOR-1 gene only transiently reduced this effect (**Table 9**). Again, the inability of a MS ODN probe to alter dynorphin-induced feeding illustrates the specificity of the AS ODN technique. The ability of AS ODN probes directed against either coding exons 1 or 2, but not 3 of both the KOR-1 and KOR-3/ORL-1 clones to potently block dynorphin-induced feeding suggests that both the $κ_1$ and the $κ_3$ opioid receptors are involved in the mediation of this response.

Since AS ODN probes directed against each of the three coding exons of the KOR-1 clone were capable of reducing U50488H-induced analgesia (Pasternak et al., 1999), and AS ODN probes targeting coding exon 1 of the KOR-1 clone reduced U50488H-induced feeding (Leventhal et al., 1998b), the inactivity of the coding exon 3 AS ODN probe in this study argues against the possibility that the receptor responsible for dynorphin-induced feeding is completely encoded by the KOR-1 gene itself. However, the failure of the KOR-1 coding exon 1 MS ODN, to alter dynorphin-induced feeding served as an important control measure to indicate the specificity of the AS ODN directed against coding exon 1 of the KOR-1 gene in altering dynorphin-induced feeding. Also, the efficacy of AS ODN probes directed against either coding exons 1 or 2 of *both* the KOR-1 and KOR-3/ORL-1 clones is somewhat surprising given the dissociation between κ_1 and κ_3 opioid agonists in feeding studies. Thus, whereas κ_1 (U50488H)-induced feeding is equi-effectively reduced by both naltrexone and NorBNI, κ_3 (naloxone benzoylhydrazone)-induced feeding is reduced by naltrexone, but not NorBNI (Koch et al., 1992). However, the pharmacologically-identified κ_3 receptor subtype and the molecularly-defined KOR-3/ORL-1 gene have not been definitively linked (Pan et al., 1994, 1995).

The ability of both μ (βFNA) and δ (naltrindole) opioid receptor antagonists to weakly block dynorphin-induced feeding suggests the potential involvement of these receptors in the mediation of this ingestive response. Although the efficacy of these selective opioid receptor antagonists was limited, the involvement of these receptor subtypes cannot be refuted based on these antagonist results. The equimolar dose-ranges

used in this study were intended to control for molar differences between antagonists. However, since the affinities of these antagonists for their respective receptors vary, pharmacokinetic differences between these drugs may contribute to their differential effects. Therefore, future investigations would benefit from the use of selective opioid receptor subtype antagonists with comparable affinity values although such antagonists are not currently available. Further, the transient efficacy of AS ODN probes targeting either coding exon 1 of the MOR-1 clone or coding exon 1 of the DOR-1 clone again implicates only the partial involvement of both the μ and δ opioid receptor subtypes in the mediation of the dynorphin-induced feeding response. Thus, the relatively weak effects observed for both the naltrindole and the DOR-1 AS ODN conditions upon dynorphin-induced feeding in this study suggest that the δ receptor plays a minor role in the mediation of this ingestive response. However, the reductions in dynorphin-induced feeding observed following βFNA and MOR-i coding exon 1 AS ODN treatment may corroborate binding studies showing that although dynorphin binds primarily to κ opioid receptors, it also displays binding at μ opioid receptors (Mansour et al., 1998) and may require the latter receptor subtype for the full expression of its functional effects.

G-protein Sensitivity Profiles of Morphine- and M6G-Induced Feeding

The results from Chapter 5 demonstrated that feeding induced μ-selective opioid agonists, morphine and M6G were differentially sensitive to AS ODN probes targeting different G-protein α-subunits. The use of AS ODN probes directed against G-protein α-subunits allowed for a detailed analysis of the effector mechanisms involved in feeding induced by these μ receptor agonists. The different AS ODN sensitivity profiles displayed

by morphine- ($G_i\alpha_2$) and M6G ($G_i\alpha_1$, $G_i\alpha_3$, $G_{z2}\alpha$)-induced feeding constitute important double dissociation controls indicating the specificity of the different AS ODN probes **(Table 10)**. That is, the G-protein AS ODN profiles observed for morphine and M6G-induced feeding do not overlap. Two further and important controls also indicate specificity fo the different AS ODN probes. First, a nonsense control probe as well as other AS ODN probes targeting $G_o\alpha$ or $G_q\alpha$ subunits, failed to alter either morphine- or M6G-induced feeding. Secondly, none of the effective AS ODN probes significantly altered either body weight of spontaneous food intake, thereby suggesting that the AS ODN treatments did not induce nonspecific deficits. The latter control suggested that the reductions in agonist-induced intake observed for each of the agonists did not occur as a result of systemic illness induced by AS ODN treatment.

The divergent sensitivity profiles of morphine and M6G suggest that the pharmacological mechanisms subserving each of these μ-opioid agonists may be distinctly different. Although all of the G-proteins involved in both morphine and M6G signaling share the common action of inhibiting adenylyl cyclase activity by the activation of the $G_i\alpha$ subunit (Standifer et al., 1996), these results demonstrate that the individual signaling pathways through which they produce their effects are clearly divergent.

Morphine- and M6G-induced feeding are distinguished from one another by proposing the existence of a novel M6G with a different functional α-subunit profile and a pharmacologically distinct effector system. The similarity in the G-protein ASODN profile of feeding and analgesic responses to M6G, relative to the G-protein AS ODN

Table 10. Summary of G-protein α-subunit AS ODN-Induced Reductions on Feeding Elicited by Morphine, M6G, βEND and Dynorphin A_{1-17}.

	G-protein α-subunit AS ODN Probe						
	$G_i\alpha_1$	$G_i\alpha_2$	$G_i\alpha_3$	$G_o\alpha$	$G_s\alpha$	$G_q\alpha$	$G_{12}\alpha$
Opioid Agonist							
Morphine	-	+++	-	-	-	-	-
M6G	++	-	+++	-	-	-	+++
βEND	+	xx	xx	-	-	-	-
Dynorphin	++	-	-	+	-	-	-

+++ Denotes large reductions relative to parallel agonist condition.
++ Denotes moderate reductions relative to parallel agonist condition.
+ Denotes small reductions relative to parallel agonist condition.
- Denotes no significant effect.
xx Denotes a significant increase relative to parallel agonist condition.

profile of feeding and analgesic responses to morphine, suggests that a novel M6G receptor may exist. Such a receptor may result from alternative splice variants of the MOR-1 clone or from a distinct gene encoding a novel μ opioid receptor subtype (see reviews: Pasternak and Standifer, 1995; Rossi and Pasternak, 1997). Indeed, a recent molecular cloning study has demonstrated the existence of at least 8 different isoforms of the μ opioid receptor (Pan et al., 1999, 2000; Pasternak and Pan, 2000). Using this model, alterations in agonist-induced feeding would be a result of specific inactivation of the individual opioid receptors by G-protein downregulation via AS ODN treatment. Binding studies showing significant reductions in [^{3}H]DAMGO specific binding to the μ opioid receptor in response to $G_i\alpha_2$ AS ODN treatment (Rossi et al., 1995b; Sanchez-Blazquez et al., 1993), suggest that G-protein AS ODN probes may produce specific loss of function by functionally inactivating their associated opioid receptors. In this context, reductions in agonist-induced feeding observed in this study may reflect specific inactivation of different subtypes of the μ opioid receptor, each displaying different G-protein α-subunit sensitivity profiles.

However, an alternative explanation for the loss of function associated with α-subunit AS ODN treatment may involve a trans-neuronal network in which behavioral effects are mediated through the interaction of multiple signals produced by the agonist (Ross, 1989). In this model, signals can diverge or converge at different levels of the neuronal network. This model therefore proposes that loss of function can occur as a result of disruption at any level of the trans-neuronal network. This suggests that the G-protein sensitivity profiles of morphine and M6G may not only represent interactions at

relevant opioid receptors, but may also comprise an intricate functional network composed of multiple types of G-protein receptor complexes located downstream of μ opioid receptors. This study confirmed earlier analgesia studies (see review: Rossi and Pasternak, 1997) suggesting that morphine and M6G have pharmacologically distinct effector pathways involving different G-protein receptor complexes. However, further investigations are necessary to determine the level at which the divergence of the signaling pathways occurs.

G-protein Sensitivity Profiles of βEND- and Dynorphin A_{1-17}-Induced Feeding

The results from Chapter 6 extended the G-protein α-subunit AS ODN technique to feeding induced by β-endorphin and dynorphin A_{1-17}. βEND-induced feeding was significantly reduced by pretreatment with the $G_i\alpha_1$ AS ODN probe and was significantly potentiated following pretreatment with the $G_i\alpha_2$ AS ODN probe **(Table 10)**. Dynorphin-induced feeding was significantly reduced following pretreatment with AS ODN probes targeting either the $G_i\alpha_1$ or $G_o\alpha$ subunits **(Table 10)**. The sensitivity of feeding induced by both βEND and dynorphin to disruption by an AS ODN probe directed against the $G_i\alpha_1$ subunit suggests a common effector pathway for the ingestive effects mediated by both of these endogenous opioid peptides. The sensitivity of βEND-induced feeding to reduction by pretreatment with $G_i\alpha_1$ AS ODN is similar to the ability of this AS ODN probe to reduce both analgesia and feeding elicited by other μ agonists including M6G (Rossi et al., 1995a; Standifer et al., 1996; Silva et al., 2000), as well as analgesia elicited by heroin and methadone (Sanchez-Blazquez et al., 2001). In contrast, the insensitivity of βEND-induced feeding to pretreatment with an AS ODN probe targeting the $G_i\alpha_2$ subunit,

distinguishes this response from that observed for morphine-induced feeding. Therefore, these results suggest that βEND-induced feeding shares a common effector pathway with M6G and heroin that differs from that of morphine. Given the possibility of the existence of a novel μ opioid receptors (see review: Pasternak, 2001), the results of this study suggest that βEND-induced feeding may result from the direct activation of novel μ opioid receptors. However, this conclusion is substantially weakened by the following results. First, the inability of an AS ODN probe targeting the $G_{z/z}\alpha$ subunit to significantly alter βEND-induced feeding directly contrasts the efficacy of this AS ODN in reducing both feeding and analgesia induced by M6G. Secondly, the potentiation of βEND-induced feeding by pretreatment with AS ODN probes targeting the$G_i\alpha_3$ subunit, is in direct contrast with the ability of the $G_i\alpha_3$ AS ODN probe to reduce M6G-induced feeding.

The sensitivity of dynorphin-induced feeding to reduction by pretreatment with AS ODN probes targeting either $G_i\alpha_1$ or $G_o\alpha$ confirms previous in vitro evidence suggesting that dynorphin exerts its effects by direct activation of the κ opioid receptor and its coupled $G_i\alpha_1$ or $G_o\alpha$ subunit (Kohno et al., 2000; Misawa et al., 1995; Ueda et al., 1996). Thus, these findings suggest that dynorphin-induced feeding is mediated by the activation of the κ opioid receptor and the initiation of a signaling cascade involving either the $G_i\alpha_1$ or $G_o\alpha$ subunits. However, the possibility that AS ODN probes targeting $G_i\alpha_1$ and $G_o\alpha$ disrupt the dynorphin-induced signaling cascade *downstream* of the κ receptor sites, cannot be ignored and thus represents an alternative mechanism. The common sensitivity to disruption by $G_i\alpha_1$ AS ODN treatment displayed by both βEND- and dynorphin-

induced feeding, suggests that these individual effector pathways may converge at a given level of the neuronal network mediating their effects.

Limitations of the AS ODN Technique

A potential limitation of the conclusions of this dissertation involves the use of the AS ODN technique. The exact mechanism of AS ODN-induced disruption of cellular protein levels remains unclear, but is believed to involve disruption of cellular transcription and/or translation processes (Myers and Dean, 2000). However, previous work has established that the antisense technique results in a 30-40% loss of protein concentrations (see review: Pasternak and Standifer, 1995). Therefore, the sensitivity of a system to modest reductions such as these, will determine whether the AS ODN treatment will yield observable effects. Importantly, the opioid system is particularly sensitive to this level of protein downregulation. However, the present studies do not include biochemical verification of either opioid receptor or G-protein α-subunit protein downregulation. Thus, the efficacy of individual AS ODN treatment conditions could not be directly attributed to alterations in protein concentration. Despite this shortcoming, these studies utilized converging lines of evidence when possible including the detailed antagonist paradigm used in Experiments 1 and 2 in which an equimolar dose range was used to assess the involvement of individual opioid receptor subtypes in feeding elicited by either βEND or dynorphin. Further, parallel findings using opioid agonist-induced analgesia supported the validity of the present data and provided converging evidence. Specifically, the pattern of G-protein α-subunit AS ODN effects observed for morphine- and M6G-induced feeding in Experiment 3 were nearly identical to those observed in identical

analgesic assays (Rossi et al., 1995b; Standifer et al., 1996). Moreover, each of the paradigms used in this dissertation included a control MS ODN or NS ODN control condition, thereby ensuring the specificity of the AS ODN technique.

A related issue involves the distribution of AS ODN probes within the central nervous system. Since all paradigms used a standard i.c.v. microinjection protocol in which the AS ODN probes were injected into the lateral ventricle, it is likely that these AS ODN probes interacted with periventricular sites. Thus, neural structures and areas known to modulate opioid-induced feeding such as the PVN, VMH, nucleus accumbens, NTS and parabrachial nucleus are viable potential targets. However, AS ODN probes may interact with more distant sites including the amygdala. VTA and lateral hypothalamus cannot be ruled out as potential AS ODN targets. Thus, individual neuroanatomical sites involved in the opioid-induced feeding response should be assessed for their sensitivity to disruption by AS ODN treatments.

A significant limitation of the current dissertation involves the interaction of neurotransmitter systems mediating opioid-induced feeding responses. Opioids are one of a number of neurotransmitter systems which mediate ingestive behavior. Disruptions of opioid receptor protein levels induced by AS ODN treatments may interfere with the effector signaling cascades of other neurotransmitter systems involving multiple neuronal networks. Nowhere is this more evident than in Experiments 3 and 4 in which G-protein α-subunit AS ODN probes were used. Opioid receptors belong to a superfamily of receptor proteins characterized by their direct coupling to G-proteins. These G-protein receptor complexes as well as their α-subunits are highly conserved (Ross, 1989) and thus

may exist as identical subunits on different types of receptors. The sequence-specificity of G-protein α-subunit AS ODN probes cannot discriminate between α-subunits coupled to opioid receptors and those coupled to other types of receptors. Therefore, other neurotransmitter systems may be affected by the actions of α-subunit AS ODN probes. Disruption of other neurotransmitter function may occur, thereby obscuring opioid-mediated effects. For example, orexogenic neurotransmitters and neuropeptides such as neuropeptide Y (NPY), galanin, and dopamine are each directly coupled to G-proteins. The α_2-adrenergic receptor, which has been implicated in the mediation of the ingestive actions of norepinephrine (see review: Leibowitz, 1987) is coupled to the $G_i\alpha$ and $G_o\alpha$ subunits. The orexogenic peptides NPY and galanin, also mediate their ingestive effects through their respective receptors that are directly coupled to $G_i\alpha 1$ and $G_o\alpha$ subunits (Brown et al., 1995; Freitag et al., 1995; Michel et al., 1995; Wang et al., 1999). These difficulties are compounded by the fact that both opioid and non-opioid receptors can exist within the same cell, as well as by the fact that opioid mediation of ingestive behavior involves interactions with other neurotransmitter systems. Other components of the intracellular signaling cascade may serve as more selective targets for disruption. However, the role of other neurotransmitter systems could be determined by the identification of systems that include G-protein α-subunit profiles which differ from the typical $G_i\alpha$ profiles displayed by opioid receptors. An excellent candidate neurotransmitter system is the dopamine system which contains a subclass of receptors that mediate their effects by the putative activation of $G_s\alpha$ opioid receptors. The distinct $G_s\alpha$ subunit sensitivity profile displayed by this receptor system could be distinguished

from the $G_i\alpha$ sensitivity profile displayed by the opioid system thus identifying the role of each of these systems in opioid-induced feeding. Therefore, these two systems could be distinguished from one another using G-protein AS ODN probes because they display completely non-overlapping sensitivities.

The results of the current dissertation have widespread implications for rational drug design. The endogenous opioid peptide system plays a critical role in the mediation of food intake and appetite, thus serving as a natural starting point for examining the etiology and treatment of eating disorders. Specifically, basal levels of pituitary and plasma βEND are elevated in genetically obese mice and rats (Margules et al., 1978). In contrast, hypothalamic βEND is decreased in chronically food-restricted rats (Kim et al., 1996). Further, several studies have shown substantially reduced CSF and plasma βEND levels in individuals with clinically diagnosed anorexia and bulimia nervosa (Brewerton et al., 1992; Demitrack et al., 1993). Similarly, dynorphin levels are decreased in patients with clinically diagnosed anorexia nervosa (see review: Yim and Lowy, 1984). Thus, it is clear that these endogenous opioid peptides play a sizeable role in the development of these and other eating disorders. Elucidation of the receptor pharmacology of these peptides provides a detailed mechanism for the functional effects of these peptides. The detailed pharmacology of this system would allow for the identification of therapeutic targets for treatment of these disorders.

The findings presented in this dissertation have provided a more detailed understanding of the receptor pharmacology and effector signaling mechanisms involved in βEND- and dynorphin-induced feeding. Pharmacological characterization of a given

behavior typically involves the manipulation of relevant ligand-receptor interactions and a subsequent analysis of behavioral changes associated with such manipulations. Within the framework of this type of paradigm, stimulation or inhibition of specific ligands, receptors or their gene expression is directly responsible for alterations in behavior. However, the sophistication and availability of powerful molecular techniques now offer the opportunity to examine how changes in behavior can alter ligand and receptor function as well as gene expression. Several studies have now demonstrated that changes in behavioral state alters βEND and dynorphin gene expression. For example, behavioral challenge conditions such as chronic food restriction and streptozotocin-induced diabetes markedly increased dynorphin peptide concentrations (Berman et al., 1994, 1995) and prodynorphin mRNA levels (Berman et al., 1997; Welch et al., 1996) in brain regions known to be involved in the mediation of ingestive behavior such as the paraventricular nucleus, the dorsal medial, ventral medial and medial preoptic hypothalamus as well as the central nucleus of the amygdala. Similarly, streptozotocin-induced diabetes also induced significant increases in POMC mRNA expression in the arcuate nucleus (Kim et al., 1999). Thus, by establishing the receptor pharmacology of these endogenous opioid peptides, the findings of this dissertation provide an important link between their biological function and their impact on feeding behavior.

Over the last three decades, the role of the endogenous opioid system in the modulation of many aspects of ingestive behavior has been firmly established. Indeed, opioids have been implicated in the modulation of a number of ingestive conditions including weight maintenance, homeostatic controls under deprivation, glucoprivic,

lipoprivic, and stressful states, as well as palatability (see review: Bodnar, 1998). However, the endogenous opioid system also modulates a number of other physiological functions including reproduction, immune function, gastrointestinal motility, analgesia, and memory function (see review: Levine et al., 1985). Thus, the role of opioids in the modulation of ingestive behaviors can be considered part of a larger physiological system that has evolved to maintain homeostatic function, and it will be interesting to note whether specific receptor subtypes of opioid receptors exist in order to mediate highly specific and relevant behavioral sequelae of which ingestion, or any of the other individual behaviors, can be construed as a component..

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