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ANTISENSE MAPPING OF OPIOID RECEPTOR CLONES:

ROLE IN INGESTIVE BEHAVIOR IN RATS

by

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Abstract
Antisense Mapping of Opioid Receptor Clones: Role In Ingestive Behavior in Rats
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The endogenous opioid system is one of a number of neurotransmitter and peptide systems that modulate ingestive behavior. Previous studies investigating opioid modulation of ingestive behavior employed selective agonists and antagonists which are cross reactive and lack relative specificity. The cloning of the opioid receptors made it possible to employ molecular knockdown techniques, including antisense oligodeoxynucleotides (AS ODNs) to investigate opioid receptor clones in behaving animals. AS ODN probes directed against each of the four exons of the MOR-1 clone were found to significantly reduce spontaneous food intake and body weight, whereas a missense control failed to exert effects. Thus, the MOR-1 clone encodes the receptor modulating spontaneous weight and intake. Subsequently, AS ODN probes directed against either exons 1 or 4, but not exons 2 or 3, of the MOR-1 clone were found to block hyperphagia, operationally defined as an increase in spontaneous food intake, induced by the μ agonists, DAMGO and morphine. In contrast, AS ODN probes directed against either exons 2 or 3, but not 1 or 4 of the MOR-1 clone blocked hyperphagia induced by the potent morphine metabolite, M6G. Pharmacological studies with selective opioid antagonists confirmed the μ receptor mediation of hyperphagia induced by DAMGO and M6G. M6G-induced hyperphagia was also unaffected by AS ODN probes directed against the DOR-1, KOR-1 and KOR-3/ORL-1 clones which were each capable of blocking intake induced by their respective agonists. Thus, the AS ODN profile for hyperphagia induced by DAMGO, morphine and M6G was identical to that observed in analgesic assays, and argues for the existence of multiple splice variants of the MOR-1 gene. Finally, AS

ODNs directed against each of the three exons of the KOR-3/ORL-1 clone blocked hyperphagia intake induced by the recently-identified opioid peptide, OFQ/N, indicating that the KOR-3/ORL-1 clone encodes the receptor mediating OFQ/N-induced food intake. These data provide critical molecular evidence for the opioid mediation of ingestion, an important homeostatic behavior, indicating that the AS ODN technique should be employed as a useful tool to investigate the molecular substrates mediating other motivated behaviors, including those involved with addiction.

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This dissertation is dedicated to my family, my parents, Drs. Alice and Marvin Leventhal and my sister, Tama Leventhal, whose love and support I could not have done without.

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List of Abbreviations

AS ODN: antisense oligodeoxynucleotide

ßFNA: ß-funaltrexamine, general μ receptor antagonist

CTOP: Cys^2-Tyr^3-Orn^5-Pen^7, general μ receptor antagonist

DADL: D-Ala^2,D-Leu^5-enkephalin, general δ receptor agonist

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

DSLET: D-Ser^2,Leu^5-enkephalin-Thr^6, general δ receptor agonist

DTLET: [D-Thr^2]-leucine enkephalin-Thr, general δ receptor agonist

GI: gastrointestinal

i.c.v.: intracerebroventricular

KOR-1: κ opioid receptor clone

KOR-3: κ_3 opioid receptor clone

MS ODN: missense oligodeoxynucleotide

M6G: morphine-6ß-glucuronide, morphine metabolite

MOR-1: μ opioid receptor clone

NalBzOH: naloxone benzolhydrazone, selective κ_3 receptor agonist

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

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

VMH: ventral medial nucleus of the hypothalamus

VTA: ventral tegmental area

CHAPTER 1. INTRODUCTION

The endogenous opioid system is one of a number of transmitter systems that plays a role in modulating ingestive behavior. General opioid receptor antagonists reduce food intake, whereas general opioid receptor agonists stimulate food intake. The differential activity of various opioid compounds led to the proposition that there were multiple opioid receptor subtypes. The development of selective opioid receptor subtype agonists and antagonists subsequently led to the pharmacological characterization of the multiple opioid receptor subtypes. Many studies employing selective opioid agonists for μ (morphine & DAMGO), δ_1 (DPDPE), δ_2 (Deltorphin II), κ_1 (U50,488H), and κ_3 (NalBzOH) receptors and selective opioid antagonists for μ (ßFNA), μ_1 (naloxonazine), δ_1 (DALCE), δ_2 (NTII), and κ_1 (NorBNI) receptors investigated opioid mediation of ingestive behavior under various conditions. Selective agonists for all opioid receptor subtypes stimulate food intake, whereas selective antagonists for each of the opioid receptor subtypes reduce food intake and body weight. However, opioid receptor subtype antagonists often do not selectively block the actions of their respective receptor subtype agonist, and often a receptor subtype antagonist blocks the action of other opioid receptor subtype agonists. This lack of correspondence observed between opioid antagonists and agonists in ingestive behavior challenges the selectivity and specificity of available pharmacological agents.

Advances in the field of molecular biology led to the cloning of the δ (DOR-1), μ (MOR-1), κ (KOR-1) opioid receptors and a novel opioid receptor clone (κ_3-like/orphanin: KOR-3/ORL-1). The DOR-1, KOR-1 and KOR-3/ORL-1 genes contain three coding exons while the MOR-1 gene encodes four exons. The AS ODN, technique used to investigate opioid receptor clones, utilizes short (18-25 nucleotide bases) probes complementary to specific, unique regions of mRNA from a targeted gene. The selectivity and specificity of AS

ODN probes far exceeds that of available antagonists. Initial studies in analgesic assays validated the efficacy of this technique in that AS ODNs probes targeted against regions of the DOR-1 clone selectively reduced analgesia elicited by selective δ_1 (DPDPE) and δ_2 (deltorphin) opioid agonists. AS ODN probes directed against either MOR-1 or KOR-1 clones respectively reduced analgesia elicited by μ (morphine/DAMGO) or κ_1 (U50,488H) opioid agonists. These studies not only confirmed the importance of the cloned receptors to the traditional opioid receptor classifications based upon pharmacological and biochemical evidence, but also demonstrated the effectiveness of utilizing AS ODNs in investigating the relationship of the cloned receptors to opioid actions *in vivo*.

Subsequently, experiments demonstrated that AS ODNs can effectively downregulate opioid receptor mRNA at any region of the receptor, therefore allowing for the targeting of selected exons of each opioid receptor clone. Thus, studies examined the role of individual exons in mediating selective opioid agonist actions through functional mapping using AS ODN probes. Specifically, AS ODN probes targeted against either exons 1 or 4 of the MOR-1 clone blocked morphine-induced analgesia, whereas probes targeting either exons 2 or 3 were ineffective. In contrast, the active morphine metabolite, M6G, displayed an exact opposite profile such that probes targeting either exons 2 or 3 of the MOR-1 clone blocked M6G-induced analgesia, whereas probes targeting either exons 1 or 4 were ineffective. Thus, these data suggested the possibility of novel opioid receptors resulting from alternative splice variants of the MOR-1 gene.

Due to the success of the AS ODN approach in evaluating the functional effects of opioid receptor clones in agonist-induced analgesia, and given the fact that the selectivity of some opioid agonists and antagonists have been challenged in certain ingestive paradigms, the AS ODN technique will be employed to investigate opioid modulation of ingestive behavior.

Specifically, the role of the cloned opioid receptors in mediating both spontaneous food intake and body weight as well as opioid agonist-induced hyperphagia was elucidated. There are two ways to define hyperphagia in the ingestive behavior field. The first way is an increase in food intake that leads to obesity and the second way is a situationally induced increase in food intake. In the current dissertation this latter definition is used to operationalize hyperphagia. Mapping studies, previously characterized in analgesic assays, were employed to investigate another opioid-mediated behavior to determine whether various opioid-mediated behaviors are subserved by common exons of the receptor clones, or if different behaviors are subserved by distinct exons of the receptor clones. Such data provide information that has implications for rational drug design. The Specific Aims of the dissertation were as follows:

1. **To evaluate alterations in spontaneous food intake and body weight following AS ODN probes targeted against each of the four exons of the MOR-1 clone and a mismatch probe.**
2. **To evaluate alterations in hyperphagia elicited by the μ-selective opioid agonist, DAMGO following AS ODN probes targeted against each of the four exons of the MOR-1 clone and a mismatch probe, and compare any activity with the selective μ opioid antagonist ßFNA.**
3. **To evaluate whether the active morphine metabolite, M6G, produces hyperphagia and determine which AS ODN probes targeted the MOR-1, DOR-1, KOR-1 and KOR-3/ORL-1 clones altered this response and compare it with selective opioid antagonists.**

4. **To evaluate alterations in hyperphagia elicited by OFQ/N following AS ODN probes targeted against each of the three exons of the KOR-3/ORL-1 clone and a mismatch probe.**

The following sections provide background information regarding: I) opioid peptides, II) opioid receptor subtypes, III) opioid receptor clones, IV) the AS ODN technique, and V) opioids and ingestive behavior.

I. Opioid Peptides.

Opioid peptides are derived from one of five gene precursor molecules: proopiomelanocortin (POMC), proenkephalin, prodynorphin, proorphanin/pronociceptin and endomorphins. The first three opioid peptide precursors all share a common opiate-active pentapeptide core (Try-Gly-Gly-Phe), while the latter two peptide precursors vary from this classical opioid motif (see review: Sherman, Akil and Watson, 1989; Mansour, Fox, Akil and Watson, 1995; Meunier, Mollereau, Toll, Suadeau, Moisand, Alvinerie, Butour, Guillemot, Ferrara, Monsarrat, Mazargull, Vassart, Parmentier and Constentin, 1995; Reinscheid, Nothacker, Bourson, Ardati, Henningsen, Bunzow, Grandy, Langen, Monsma and Civelli, 1995; Zadina, Hackier, Ge and Kastin, 1997).

A. POMC. The C-terminus of POMC contains the 31-amino acid peptide ß-endorphin, and its 91 amino acid precursor ß-lipotropin which also gives rise to α- and τ-endorphin (Eipper and Mains, 1978; Mains, Eipper and Ling, 1977; Roberts, Seeburg, Shine, Herbert, Baxter and Goodman, 1979). POMC can also be cleaved into ACTH (18-39), α-melanotropin and corticotropin-like intermediate lobe protein. However, of all the POMC-derived peptides, ß-endorphin is the only opioid peptide (Mains et al., 1977).

Whereas the pituitary is the major site of POMC synthesis, the brain contains two distinct POMC-derived cell groups (Khachaturian, Lewis, Schaffer and Watson, 1985). The first cell group is in the arcuate nucleus and surrounding periarcuate nuclei of the hypothalamus (Watson, Akil, Richard and Barchas, 1978). These cells project extensively throughout the brain (Khachaturian et al., 1985). Specifically, rostrally-projecting fibers course through periventricular diencephalic and telencephalic areas, innervating many hypothalamic and limbic structures, including the preoptic area, septum, and bed nucleus of the stria terminalis. Lateral projections extend through the medial-basal hypothalamic region

to the temporal cortex and amygdala. Caudally-projecting fibers innervate the periventricular thalamus, the periaqueductal gray (PAG), the nucleus raphe magnus (NRM), the nucleus reticularis gigantocellularis (NRGC), the nucleus tractus solitarius (NTS) and the nuclei reticularis lateralis, parabrachialis, ambiguus, as well as the dorsal motor nucleus of vagus. The second cell group containing ß-endorphin is located in the caudal region of the NTS which projects laterally to the lateral reticular nucleus (Khachaturian et al., 1985).

B. Proenkephalin. Proenkephalin contains several opioid peptides including leu-enkephalin, met-enkephalin, met-enkephalin-Arg-Phe and met-enkephalin-Arg-Gly-Leu (Kimura, Lewis, Stern, Rossier, Stein and Undenfriend, 1980; Comb, Herbert and Crea, 1982). Hughes was the first to isolate leu- and met-enkephalin from brain and to demonstrate their opioidergic activity (Hughes, Smith, Kosterlitz, Fothergill, Morgan and Morris, 1975). Enkephalins are found as interneurons in many neuronal systems from the telencephalon to the spinal cord. Specifically, immunoreactive enkephalin perikarya are found in such telencephalic structures as the cerebral cortex, olfactory tubercle, amygdala, hippocampus, bed nucleus of the stria terminalis and preoptic area, such diencephalic structures as the hypothalamus and periventricular and lateral geniculate nuclei of the thalamus, such mesencephalic structures as the superior and inferior colliculi, PAG and interpeduncular nucleus, and such metencephalic and myelencephalic structures as the parabrachial, dorsal tegmental, vestibular and raphe nuclei, NRM, NRGC, NTS, lateral reticular nucleus, spinal trigeminal nucleus and spinal cord dorsal gray (Hokfelt, Elde, Johansson, Terenius and Stein, 1977; Khachaturian, Lewis, Hollt and Watson, 1983; Khachaturian et al., 1985; Sar, Stumpf, Miller and Chang and Cuatrecasas, 1978). Extrinsic enkephalinergic pathways project from the central and medial nuclei of the amygdala to the PAG and adjacent dorsal raphe nucleus (Rizvi, Ennis, Behbehani and Shipley, 1991), and also project from the PAG to the NRM

(Beitz, 1982).

C. Prodynorphin. Prodynorphin is cleaved into three leu-enkephalin-containing peptides: alpha and beta-neoendorphin, dynorphin A and dynorphin B (Goldstein, Fischli, Lowney, Hunkapiller and Hood, 1981; Kangawa, Minamino, Chino, Sakakibara and Matsuo, 1981). There are several peptides synthesized from dynorphin A that are biologically active including dynorphin A_{1-8}, dynorphin A_{1-17} and several other intermediate-length peptides (Goldstein et al., 1981; Seizinger, Hollt and Herz, 1981; Suda, Tozawa, Tachibana, Demura and Shizume, 1982). Immunoreactive dynorphin perikarya are located in telencephalic (cerebral cortex, striatum, amygdala and hippocampus), diencephalic (supraoptic, paraventricular (PVN), and arcuate nucleus of hypothalamus), mesencephalic (PAG) and metencephalic/myelencephalic (parabrachial and spinal trigeminal nucleus, NTS, lateral reticular nucleus) structures as well as the dorsal and ventral horns of the spinal cord. Further, most dynorphin perikarya in the PVN of the hypothalamus co-exist with vasopressin in the magnocellular nuclei (Watson, Akil, Fischili, Goldstein, Zimmerman and Nilaver, 1982).

D. Proorphanin/Pronociceptin. Orphanin/nociceptin (OFQ/N) is a recently discovered (Meunier et al., 1995; Reinscheid et al., 1995) heptadecapeptide which is structurally similar to dynorphin A. Unlike classical opioid peptides, OFQ/N does not have a Tyr-Gly-Gly-Phe core at the N-terminus, 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. Like POMC, the pre-pro-orphanin/nociceptin gene contains additional pairs of basic amino acid residues that delineate two putative biologically-active peptides that are respectively 17 and 35 amino acids long immediately downstream of OFQ/N (Meunier et al., 1995; Reinscheid et al., 1995). The pre-pro-orphanin/nociceptin gene also contains a

precursor peptide that is biologically active called nocistatin (Okuda-Ashitake, Minamino, Tachibana, Yaoshihara, Nishiuchi, Kimura and Ito, 1998). Further, OFQ/N contains two pairs of basic amino acids, raising the possibility that it may be subject to post-translational processing into either OFQ/N_{1-11} or OFQ/N_{1-7} which may have biological activity (see review: Henderson and McKnight, 1997). Immunohistochemical and autoradiographic studies have identified OFQ/N in the bed nucleus of the stria terminalis, medial preoptic area, lateral septum, amygdala and median eminence. A dense plexus of OFQ/N terminal fibers are also present in the superficial layer of the dorsal horn, in the sensory trigeminal complex, raphe nuclei and PAG (Henderson and McKnight, 1997).

E. Endomorphins. Endomorphins are the most recent opioid peptides to be isolated from the brain (Zadina et al., 1997). Their N-terminus sequence differs from the classical opioid peptides: endomorphin-1 (Try-Pro-Trp-Phe-NH_2) and endomorphin-2 (Try-Pro-Phe-Phe-NH_2). Preliminary radioimmunoassay studies of endomorphin-1 suggest that it is found in the thalamus, hypothalamus, cortex and striatum (Zadina et al., 1997). Further, endomorphin-2-like immunoreactivity has been localized in the medulla and dorsal root and dorsal root ganglia of the spinal cord (Martin-Schild, Zadina, Gerall, Vigh and Kastin, 1997).

II. Opioid Receptor Subtypes.

Following the discovery of the opiate receptor by three independent laboratories (Pert and Snyder, 1973; Simon, Hiller and Edelman, 1973; Terenius, 1973), Martin provided the first evidence in chronic spinal dogs of multiple opioid receptor subtypes (Martin, Eades, Thompson, Huppler and Gilbert, 1976). Three distinct receptors were proposed based upon the lack of cross-tolerance among three different opioid agonists: μ (morphine), κ (ketocyclazocine), and σ (SKF 10047). Subsequent studies characterized the σ receptor as a nonopioid receptor because its actions are not blocked by general opioid antagonism (Vaupel,

1983; Zukin, Brady, Slifer and Balster, 1984). Bioassay and biochemical characterization of enkephalins led to the identification of an enkephalin-preferring receptor called the δ receptor, since enkephalins were more potent than morphine in the mouse vas deferens assay. The μ receptor was characterized in the guinea pig ileum assay where morphine was more potent than the enkephalins (Lord, Waterfield, Hughes and Kosterlitz, 1977) (See Table 1).

Although both endogenous opioid peptides and receptors have been localized, there is relatively poor anatomical correspondence between them (see review: Akil, Watson, Young, Lewis, Khachaturian and Walker, 1984). Further, there appears to be cross-reactivity between opioid peptides and receptors in binding assays. ß-endorphin selectively binds both μ and δ opioid receptors, but not κ opioid receptors. Enkephalins and dynorphins bind preferentially to δ and κ opioid receptors respectively *in vitro*. Moreover, all proenkephalin and prodynorphin peptides can bind to μ, κ and δ opioid receptors depending on the peptide product and species (Corbett, Paterson, McKnight, Magnan and Kosterlitz, 1982; Quirion nd Weiss, 1983). Unlike classical opioid peptides, the newer peptides, OFQ/N and endomorphin, demonstrate selective biochemical correspondence with their endogenous opioid receptors. Whereas OFQ/N shows little or no affinity for μ, κ and δ opioid receptors, it displays high affinity for the orphanin receptor (Meunier et al., 1995; Reinscheid et al., 1995). Endomorphin-1 and endomorphin-2 have a high affinity and selectivity for the μ receptor, and it has been suggested that they are the actual endogenous ligands for the μ receptor (Zadina et al., 1997).

A. μ opioid receptors. μ receptors are widely distributed throughout the forebrain, midbrain and hindbrain. Binding of the μ receptor is most dense in the neocortex, caudate-putamen, nucleus accumbens, thalamus, hippocampus, amygdala, inferior and superior colliculi, NTS, spinal trigeminal nucleus and dorsal horn. Moderate binding is observed in

Table 1 Pharmacological and Molecular Classification of Opioid Receptors

Receptor	Clone	Agonist	Antagonist
μ	MOR-1	Morphine DAMGO	βFNA Naloxonazine
δ	DOR-1	DPDPE Deltorphin	DALCE NTII
κ	KOR-1	U50,488H	NorBNI
none	KOR-3, ORL-1	Orphanin FQ Nociceptin	none

the PAG and raphe nuclei, and little binding is observed in the hypothalamus, preoptic area and globus pallidus (Mansour, Khachaturian, Lewis, Akil and Watson, 1988). The μ receptor has been characterized pharmacologically using the μ-selective agonist (i.e., D-Ala^2, met-Phe^4,Gly(ol)^5-enkephalin, DAMGO: Handa, Lane, Lord, Morgan, Rance and Smith, 1981) and antagonists (i.e, ß-funaltrexamine, ßFNA: Portoghese, Larson, Sayre, Fries and Takemori, 1980; Takemori, Larson and Portoghese, 1981) and (Cys^2-Tyr^3-Orn^5-Pen^7, CTOP: Gulya, Pelton, Hruby and Yamamura, 1986).

μ_1 and μ_2 opioid receptors. The μ receptor has been further classified into μ_1 and μ_2 receptor subtypes based on pharmacological assays in which naloxazone and naloxonazine selectively antagonize μ_1 receptor actions *in vitro* and *in vivo* (Hahn, Carroll-Buatti and Pasternak, 1982; Ling, Simantov, Clark and Pasternak, 1986; Pasternak and Hahn, 1980; Pick, Paul and Pasternak, 1991). The μ_1 receptor binds opiates and most enkephalins with similar high affinity while the μ_2 receptor binds morphine more potently than enkephalins (see review: Pasternak and Wood, 1986). Autoradiographic studies revealed similar, but not identical, distributions of μ_1 and μ_2 receptors (Goodman and Pasternak, 1985; Moskowitz and Pasternak, 1985). μ_1 binding is denser in the frontal cortex, striatum, ventral palladium, nucleus accumbens, medial thalamus, interpeduncular nucleus, median raphe and PAG. μ_2 binding is denser in the parietal, occipital and temporal cortices, hippocampus, amygdala, dorsal motor nucleus of vagus and NTS. Behavioral studies have also distinguished the actions of μ_1 and μ_2 receptors in spinal and supraspinal analgesia (Bodnar, Williams, Lee and Pasternak, 1988; Paul, Bodnar, Gistrak and Pasternak, 1989; Pick, Paul and Pasternak, 1991).

B. δ opioid receptors. Autoradiographic studies indicate that δ receptor binding is densest in the olfactory-related neural areas, neocortex, caudate-putamen, nucleus accumbens

and amygdala. In contrast, little or no binding is observed in the thalamus, hypothalamus and brainstem (Mansour et al., 1988). Initial studies characterizing the δ receptor utilized enkephalin analogues as general δ agonists, D-Ser^2,Leu^5-enkephalin-Thr^6 (DSLET) and D-Ala^2,D-Leu^5-enkephalin (DADL) (Lord et al., 1977; Mosberg, Hurst, Hruby, Gee, Yamamura, Galligan and Burks, 1983a), and general δ antagonists, ICI 174864 (Cotton, Giles, Miller, Shaw and Timms, 1984) and naltrindole (Portoghese, Sultana, Nagase and Takemori, 1988). Subsequently, more selective δ ligands were developed.

δ_1 and δ_2 opioid receptors. The development of selective δ receptor agonists and antagonists led to the classification of δ_1 and δ_2 receptor subtypes. The δ_1 receptor has been pharmacologically characterized by the agonist D-Pen^2,D-Pen^5-enkephalin (DPDPE: Mosberg, Hurst, Hruby, Galligan, Burks, Gee and Yamamura, 1983b) and long-term actions of the antagonist D-Ala^2,Leu^5,Cys^6-enkephalin (DALCE) (Bowen, Hellewell, Kelemen, Huey and Steward, 1987; Jiang, Bowen, Mosberg, Rothman, Porreca, 1990a). The δ_2 receptor has been pharmacologically characterized by the agonist D-Ala^2,Glu^4-deltorphin (Jiang, Takemori, Sultana, Portoghese, Bowen, Mosberg and Porreca, 1991) and the antagonist naltrindole-5'-isothiocyanate (NTII) (Portoghese, Sultana and Takemori, 1990). Behavioral studies have also distinguished the actions of δ_1 and δ_2 receptors in spinal and supraspinal analgesia (Jiang et al., 1991; Mattia, Farmer, Takemori, Sultana, Portoghese, Mosberg, Bowen and Porreca, 1992).

C. κ opioid receptors. κ receptor binding is densest in the caudate-putamen, nucleus accumbens, amygdala, hypothalamus, neural lobe of the pituitary, median eminence, and NTS and moderate in the PAG, raphe nuclei, spinal trigeminal nucleus and dorsal horn (Mansour et al., 1988).

κ_1 and κ_2 opioid receptors. Selective agonists and antagonists have also distinguished

multiple κ receptor subtypes. The κ_1 receptor subtype has been characterized using the agonist U50,488H (VanVoigtlander, Lahti and Ludens, 1983) and the antagonist nor-binaltorphamine (NorBNI: Portoghese, Lipkowski, Takemori, 1987). The κ_2 receptor has been demonstrated in biochemical assays as being U50,488H-insensitive, but has not been demonstrated *in vivo* (Zukin, Eghbali, Olive, Unterwald and Temple, 1988).

κ_3 receptors. A κ_3 receptor has also been identified as a U50,488H-insensitive site which selectively binds the agonist, naloxone benzolhydrazone (NalBzOH: Clark, Liu, Price, Hersh, Edelson and Pasternak, 1989; Gistrak, Paul, Hahn and Pasternak, 1989; Paul, Levison, Howard, Pack, Hahn and Pasternak, 1990). Hyperphagia induced by centrally-administered NalBzOH was also insensitive to NorBNI pretreatment (Koch, Pasternak, Arjune and Bodnar, 1992).

D. Pitfalls of Selective Agonists and Antagonists. Many of the selective agonists and antagonists described above exhibit a high degree of selectivity and specificity. However, under certain conditions, the selectivity of some of the agonists and antagonists has been challenged. For example, repeated administration of the selective κ antagonist NorBNI equally blocked the analgesic actions of μ, δ and κ agonists (Spanagel, Almeida and Shippenberg, 1994). The characterization of the opioid receptor subtypes using selective agonists and antagonists has been aided by the recent cloning of the traditional opioid receptor subtypes. The isolation of the cDNA's encoding the opioid receptors has allowed for biochemical, molecular, and functional analysis confirming distinctions made employing selective agonists and antagonists.

III. Opioid Receptor Clones.

In 1992, expression cloning yielded the amino acid sequence of the δ opioid receptor clone (DOR-1) which provided the first molecular evidence for the existence of opioid

receptors (Evans, Keith, Morrison, Magendzo and Edwards, 1992; Kieffer, Befort, Gaveriaux-Ruff and Hirth, 1992). Subsequently, based on the amino acid sequence of the DOR-1 clone, several groups successfully cloned the cDNAs encoding the μ- (MOR-1), κ- (KOR-1), and κ_3 (KOR-3) (see review: Uhl, Childers and Pasternak, 1994). Initially the KOR-3 clone was thought to be the κ_3 receptor, but was later shown to exhibit a high degree of sequence homology to a newly-identified orphanin-opioid receptor clone (ORL-1) which is subsequently referred to as the KOR-3/ORL-1 clone. Each of the opioid receptor genes encodes a G-protein coupled receptor that has seven transmembrane-spanning domains. The clones share homology of approximately 65-70% of their amino acid sequence, primarily in their transmembrane-spanning regions and intracellular loops (Reisine and Bell, 1993). The DOR-1, KOR-1 and KOR-3/ORL-1 genes contain three coding exons, whereas the MOR-1 gene encodes four exons, with the last exon encoding a very short sequence. These cloned opioid receptors are G_i/G_o-coupled, and therefore inhibit adenylate cyclase and the formation of cAMP (see review: Reisine and Bell, 1993). Activation of these opioid receptors also leads to an increase in potassium conductance and closure of high-voltage calcium channels (DiChiara and North, 1992).

A. DOR-1 clone. In 1992, the δ receptor was isolated and cloned from mouse independently in two separate laboratories, and has since transformed the field of molecular opioid pharmacology (Evans et al., 1992; Kieffer et al., 1992). Both groups used a similar approach to isolate the receptor which entailed preparing a cDNA library from a NG108-15 cell line, which expresses high densities of δ receptors. The cDNA library was transfected into Chinese Hamster Ovary (COS) cells, which doesn't express endogenous opioid receptors, and than screened for cells that bound a radioactively-labelled selective δ-agonist. The isolated cDNA encoded a 372 amino acid protein, the sequence of which resembled the

somatostatin receptor, had all the characteristics of a G-protein coupled receptor, and demonstrated all of the properties previously described in pharmacological studies of the endogenous δ receptor.

The DOR-1 clone displayed a much higher affinity for enkephalin peptides and delta receptor agonists and antagonists than for dynorphin, μ and κ agonists and κ antagonists (Evans et al., 1993; Kieffer et al., 1992; Yasuda, Raynor, Kong, Breder, Takeda, Reisine and Bell, 1993). The DOR-1 clone also mediated agonist-induced inhibition of cAMP formation, suggesting that it was functionally coupled to adenylate cyclase (Evans et al., 1992; Kieffer et al., 1992). Both *in situ* hybridization techniques and immunohistochemical techniques made it possible to localize neuroanatomical distribution of opioid receptor clones. *In situ* hybridization and immunohistochemical localization have identified DOR-1 receptor clone in the anterior olfactory nucleus, neocortex, caudate-putamen, nucleus accumbens, olfactory tuberacle, diagonal band of Broca, globus pallidus, ventral palladium, septal nuclei, amygdala, ventromedial nucleus of the hypothalamus (VMH) and pontine nuclei, brainstem and spinal cord (Arvidsson, Dado, Reidl, Lee, Law, Loh, Elde and Wessendorf, 1995a; Mansour et al., 1995). These distributions correlate very well with observations made in prior autoradiographic studies using δ ligands.

B. MOR-1 clone. Shortly after the cloning of the δ-receptor, a clone encoding the μ-opioid receptor (i.e., MOR-1) was isolated from rat and human (Chen, Mestek, Liu, Hurley and Yu, 1993; Fukada, Kato, Mori, Nishi and Takeshima, 1993; Thompson, Mansour, Akil and Watson, 1993; Wang, Imai, Eppler, Gregor, Spivak and Uhl, 1993). The MOR-1 clone was identified using homology cloning strategies based on the δ-receptor cDNA and protein microsequence data from purified opioid receptor preparations (Uhl et al., 1994). *In vitro* studies determined that the MOR-1 clone displays high affinity for μ-selective opioid agonists

(DAMGO) and antagonists (ßFNA and naloxonazine), and low affinity for δ- (DPDPE) and κ- (U50,488H) selective agonists (Chen et al., 1993; Wang et al., 1993). Like DOR-1, the MOR-1 clone also mediates agonist-induced inhibition of cAMP formation, indicating that is coupled to adenylate cyclase (Chen et al., 1993). *In situ* hybridization and immunohistochemical localization have identified the MOR-1 receptor clone 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 coeruleus, nucleus ambiguus, NTS and spinal cord which correlates well with previous autoradiographic studies using μ ligands (Arvidsson, Reidl, Chakrabarti, Lee, Nakano, Dado, Loh, Law, Wessendorf and Elde, 1995b; Mansour et al., 1995).

C. KOR-1 clone. The κ-opioid receptor (KOR-1) was also cloned from mouse and rat using homology cloning strategies (Chen, Mestek, Liu and Yu, 1993; Meng, Xie, Thompson, Mansour, Goldstein, Watson and Akil, 1993; Minami, Toya, Katao, Maekawa, Nakamura, Onogi, Kaneko and Satoh, 1993; Nishi, Takeshima Fukada, Kato and Mori, 1993; Yasuda et al., 1993). *In vitro* studies demonstrated that the KOR-1 clone has a high affinity for dynorphin, and selective κ-agonists (U50,488H) and κ-antagonists (NorBNI), yet a low affinity for enkephalins (Yasuda et al., 1993). Like DOR-1 and MOR-1, the KOR-1 clone mediates inhibition of cAMP formation and agonist-induced inhibition of calcium channel activity (Yasuda et a., 1993). *In situ* hybridization has identified KOR-1 receptor mRNA in the nucleus accumbens, ventral tegmental area (VTA), caudate-putamen, olfactory tuberacle, bed nucleus of the stria terminalis, medial preoptic area, PVN, supraoptic, dorsomedial and VMH, amygdala, midline thalamic nucleus, PAG, raphe nuclei, locus coeruleus, spinal trigeminal nucleus and NTS. These data, correlate moderately well with previously

autoradiographic studies using κ ligands such that previously-unrecognized sites were detected in the mRNA studies (Mansour et al., 1995).

D. KOR-3/ORL-1 clone. A previously unrecognized receptor κ_3-opioid and orphanin-opioid receptor (KOR-3/ORL-1) clone was the fourth member of the opioid receptor family to be cloned in mouse, rat and human by screening cDNA libraries with cDNA probes based on the classical opioid receptor subtypes (Bunzow, Saez, Mortrud, Bouvier, Williams, Low and Grandy, 1994; Fukuda, Kato, Mori, Nishi, Takeshima, Iwabe, Miyata, Houtani and Siguimoti, 1994; Keith, Maung, Anton and Evans, 1994; Mollereau, Paramentier, Mailleux, Butour, Moisand, Chalon, Caput, Vassart and Meunier, 1994; Pan, Cheng, Xu and Pasternak, 1994; Pan, Cheng, Xu, Rossi, Jacobson, Ryan-Moro, Brooks, Dean, Standifer and Pasternak, 1995; Wang, Johnson, Imai, Persico, Ozenberger, Eppler and Uhl, 1994; Wick, Minnerath, Lin, Elde, Law and Loh, 1994). OFQ/N, the endogenous ligand for the receptor binds with high affinity to the KOR-3/ORL-1 clone and mediates agonist-induced inhibition of adenylate cyclase (Meunier et al., 1995; Reinscheid et al., 1995). When transfected into cell lines the KOR-3/ORL-1 clone does not demonstrate reliable opioid binding. Although potent agonist drugs like etorphine, cyclazocine and nalorphine each inhibited adenylate cyclase activity, morphine, DAMGO, U50,488H and DPDPE were all inactive (Pan et al., 1995). Thus, unlike the classical opioid receptors, the KOR-3/ORL-1 clone has a low affinity for traditional opioids and opiates. *In situ* hybridization and immunohistochemical localization have identified high levels of KOR-3/ORL-1 receptor mRNA in the PVN, VMH, amygdala, pyriform cortex, dorsal raphe nucleus, locus coeruleus and pons. Moderate expression was observed in cerebellum, cortex, thalamus, hippocampus, PAG and spinal cord (Anton, Fein, To, Li, Silberstein and Evans, 1996; Fukada et al., 1994; Chen et al, 1994; Wick et al., 1994).

E. Summary of Opioid Receptor Clones. The cloning of the opioid receptors confirmed prior biochemical data obtained utilizing selective opioid agonists and antagonists regarding affinity, specificity and localization. Importantly, the cloning of the opioid receptors led to the discovery of a novel opioid receptor (KOR-3/ORL-1) followed by the isolation of an endogenous ligand (OFQ/N) for this receptor. However, the cloned receptors have yet to account for the existence of multiple receptor subtypes of the classical opioid receptors. There are two ways to investigate the opioid receptor clones *in vivo*: 1) the knockout and 2) the knockdown approach. However, the knockout approach entails elimination of a protein or gene. Further, when knocking out a gene, it is possible that other processes may compensate or take over lost function thus making it difficult to assess functional relevance of the deleted gene (Wahlestedt, 1994). Also the knockout technique is very labor intensive and expensive. Further, it is an irreversible process and the prototypical animal in which the technique is performed is the mouse which is very difficult to assess in ingestive assays. The opioid field has lagged in the development of knockout and transgenic mice. The knockdown approach entails temporarily eliminating a protein or gene by utilizing antisense oligodeoxnucleotides (AS ODN), this technique avoids the possibility of compensatory changes encountered in the knockout animals and has proven quite fruitful in evaluating cloned receptor actions *in vivo* (Wahlestedt, 1994). Although there is not a complete elimination of the targeted protein, the technique is easy to perform, less expensive, and importantly any region or exon of the receptor clone can be targeted. Further, the AS ODN technique is reversible allowing for evaluation of the targeted protein both in the presence and absence of the protein in question. Also the technique can be performed in the rat which is the prototypical animal model used to investigate ingestive behavior. Thus, the following section will focus upon the knockdown technique in evaluating the functional

activity of cloned opioid receptors (See Table 1).

IV. Antisense Oligodeoxynucleotides.

Recent advances in molecular biology, specifically in the area of cloning, have piqued interest in utilizing the AS ODN technique. This technique utilizes short nucleotide probes that are 18-25 bases long complimentary to nonhomologous regions of mRNA from a specific gene, and are therefore highly specific to that gene. The theory behind this strategy is that the AS ODN interacts with targeted nucleic acid at some time during the sequence occurring during protein synthesis (Wahlestedt, 1994). The idea of utilizing AS ODNs as sequence-specific inhibitors of gene expression was first proposed in 1967 (Belikova, Zarytova and Grineva, 1967); however the technology was not available to successfully utilize the technique. In 1978, Zamecnick and Stephenson (1978) were able to successful apply this strategy and demonstrate sequence-specific inhibition of Rous sarcoma virus expression *in vitro*. The 18-25 bases length of the AS ODN probe allows for uptake into the cell, and diminishes the chances of a longer probe breaking down into smaller fragments which may differ in selectivity to the parent probe (Wahlestedt, 1994). Whereas many AS ODNs are designed to bind to the initiation codon, the 5'-capping region or 3'-untranslated mRNA regions can be targeted as well (Wahlestedt, 1994). There is evidence that the mechanism of AS ODN uptake is predominantly through pinocytosis in which substances from the cell membrane can be incorporated into the interior of the cell (Stein, 1993). *In vitro* studies have shown that AS ODNs are rapidly 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). The most plausible theory as to how this technique works is that AS ODNs exert their effects by inducing translational arrest, possibly by providing substrates for the enzyme that degrades the RNA strand of an RNA-DNA duplex (Stein, 1992). Thus, by annealing to the

targeted mRNA, the AS ODN can selectively downregulate the mRNA encoding the protein of interest. One of the major advantages of this approach is that by altering the sequence of three to four base pairs (i.e., mismatch) or scrambling the sequence (i.e., sense) of the AS ODN probe, the specificity of the resultant probe is eliminated, thereby serving as an effective control condition for *in vivo* studies (Wahlestedt, 1993).

Experiments have successfully used this approach to downregulate NMDA, dopamine, muscarinic cholinergic, $GABA_B$, and neuropeptide Y receptors *in vivo* (Holopainen and Wojcik, 1993; Wahlestedt Golanov, Yamamoto, Yee, Ericson, Yoo, Inturrisi and Reis, 1993a; Wahlestedt, Pich, Koob, Yee and Helig, 1993b; Zhang and Creese, 1993). However, the success of the AS ODN technique in mediating *in vivo* actions depends upon a clearly-delineated function for the peptide or receptor in question. Consequently, the existence of a clearly-delineated pain-inhibitory opioid-receptor mediated pathway, as well as a reliable behavioral assay in which to examine this *in vivo* action, have made the opioid transmitter-receptor system ideal for successful employment of the AS ODN technique in analgesic assays.

Initial studies employing AS ODNs in the study of opioid pharmacology validated the efficacy of the technique through a series of converging molecular, biochemical and behavioral experiments (Standifer, Chien, Wahlestedt, Brown and Pasternak, 1994; Jenab, Su, Chien, Pan, Inturrisi and Pasternak, 1995). AS ODNs probes targeted against different regions of the entire length of the DOR-1 clone lowered binding of the selective δ-agonist DPDPE *in vitro* by 40-50% and *in vivo* by 25-30%. In contrast, neither mismatch nor sense probes were effective, confirming the specificity of the effect. Further, AS ODNs targeted against different regions of the DOR-1 clone lowered both mRNA and protein levels, and intrathecally-administered AS ODNs targeted against the DOR-1 clone, but not a mismatch

ODN in mice treated over five days significantly lowered spinal analgesia elicited by the selective δ_1-agonist DPDPE and δ_2-agonist deltorphin (Standifer et al., 1994). In contrast, analgesia elicited by selective μ- or κ_1-agonists were insensitive to DOR-1 AS ODNs demonstrating the selectivity of the effect. DPDPE-induced analgesia gradually recovered over the subsequent five days demonstrating that the loss of analgesia was not due to toxicity but rather to receptor inactivation. Subsequently, others confirmed the effectiveness of DOR-1 AS ODNs treatment in reducing selective δ-mediated analgesia (Lai, Bilsky, Rothman and Porreca, 1994; Tseng, Collins and Kampine, 1994).

These studies not only confirmed the importance of the DOR-1 clone to the traditional δ receptor, but also demonstrated the effectiveness of utilizing AS ODNs in investigating the relationship of the cloned receptors to opioid actions *in vivo*. Subsequent tissue culture studies demonstrated that AS ODNs can effectively downregulate opioid receptor mRNA at any region of the receptor, thereby allowing for any exon of the opioid receptor clone to be targeted (Rossi, Pan, Brown and Pasternak, 1995; Standifer et al., 1994). This led to a series of studies in which opioid receptor clones were functionally mapped using AS ODN to investigate the role of individual exons of the receptor clones (see review: Pasternak and Standifer, 1995).

A. DOR-1 clone and AS ODNs. Previously-described studies confirmed the functional role of the DOR-1 clone in opioid-mediated analgesia using AS ODN probes (Lai et al., 1994; Standifer et al., 1994; Tseng et al., 1994). By employing a mapping strategy which utilizes probes specific to that exon of the receptor clone it is possible to determine which individual exons of the receptor clone are mediating particular agonist actions. At the spinal level, AS ODN probes targeted against each of the three exons of DOR-1 clone reduced analgesia elicited by either the selective δ_1-agonist DPDPE or δ_2-agonist deltorphin

(Rossi, Su, Leventhal, Su and Pasternak, 1997). At the supraspinal level, centrally-administered AS ODN probes targeted against each of the three exons of the DOR-1 clone blocked deltorphin-mediated analgesia. However, supraspinal DPDPE-induced analgesia was reduced by probes targeting exon 3, but not exons 1 or 2 of the DOR-1 clone (Rossi et al., 1997). A mismatch probe was ineffective in altering spinal and supraspinal analgesia. Thus, based on opioid receptor subtype classifications of agonists, as well as the AS ODN profile of the DOR-1 clone using mapping strategies, it appears that the DOR-1 gene encodes a δ_2 receptor subtype.

B. MOR-1 clone and AS ODNs. Initial studies confirmed the relevance of the MOR-1 clone to the previously-described actions of the μ opioid receptor subtype (Chen, Adams, Geller, DeRiel, Adler and Liu-Chen, 1995; Rossi, Pan, Cheng and Pasternak, 1994). Specifically, AS ODN probes targeted against the MOR-1 clone blocked selective μ-agonist induced analgesia elicited by morphine in the rat (Rossi, et al., 1994). AS ODN mapping of the MOR-1 clone was done in mouse and rat (Rossi, Pan, Brown and Pasternak, 1995a; Rossi, Brown, Leventhal, Yang and Pasternak, 1996; Rossi, Leventhal Pan, Cole, Su, Bodnar and Pasternak, 1997). AS ODNs targeted against exon 4 effectively blocked intrathecally-administered morphine-induced analgesia. In contrast, AS ODNs targeted against either exons 1, 2, or 3 were ineffective in reducing spinal morphine analgesia. At the supraspinal level, AS ODNs targeted against either exons 1 or 4 of the MOR-1 clone blocked ventricular morphine-induced analgesia, whereas probes targeted against either exons 2 or 3 were ineffective. Interestingly, the morphine metabolite, morphine-6ß-glucuronide (M6G), which labels μ receptors with an affinity slightly less than morphine in binding assays (Paul, Standifer, Inturrisi and Pasternak, 1989), but is a 100-fold more potent in central analgesic assays (Abbott and Palmour, 1988; Frances, Gout, Monsarrat, Cros and Zajac, 1992; Gong,

Hedner, Hedner, Bjorkman and Nordberg, 1991; Pasternak, Bodnar, Clark and Inturrisi, 1987; Paul et al., 1989) displayed the opposite antisense profile than its parent compound, morphine. Thus, AS ODN probes directed against either exons 2 or 3 of the MOR-1 clone blocked M6G-induced analgesia, whereas AS ODNs directed against either exon 1 or 4 were ineffective. Further, analgesia induced by the putative μ-agonists heroin, fentanyl and etonitazine were also reduced by AS ODNs directed against either exon 1 or 2 of the MOR-1 clone (Rossi et al., 1996). These studies raise the possibility that analgesia induced by μ receptor subtype agonists may result from either alternative splice variants of the MOR-1 gene and/or the possibility of novel μ receptor subtype families.

AS ODN mapping has also been employed to investigate the role of morphine's inhibition of gastrointestinal (GI) transit (Rossi et al., 1995). AS ODNs targeted against exon 4 effectively reduced morphine's inhibition of GI transit. In contrast, AS ODNs targeted against either exons 1, 2, or 3 were ineffective in this response. Both morphine-induced inhibition of GI transit and spinal analgesia have been pharmacologically characterized as μ_2-mediated actions since both are blocked by general μ (ßFNA), but not by μ_1 (naloxonazine) antagonism (Paul and Pasternak, 1988; Paul et al., 1989). Since both actions are selectively blocked by AS ODNs targeted against exon 4 of the MOR-1 clone, this suggests that the μ_2-opioid receptor subtype may be encoded by exon 4 of the MOR-1 clone.

C. KOR-1 clone and AS ODNs. Initial studies confirmed the relevance of the KOR-1 clone to the previously-described actions of the κ_1 opioid receptor subtype (Adams, Chen, DeRiel, Adler and Liu-Chen, 1994; Chien, Brown, Pan and Pasternak, 1994). Specifically, these studies demonstrated that centrally administered AS ODN probes targeted against the KOR-1 clone reduced selective κ-mediated analgesia elicited by U50,488H. AS ODN mapping of the KOR-1 clone revealed that probes targeted against either exons 1, 2, or 3 of

the clone selectively blocked both spinal and supraspinal U50,488H-induced analgesia (Rossi and Pasternak, personal communication), suggesting that the KOR-1 gene encodes the previously described κ_1-opioid receptor subtype.

D. KOR-3/ORL-1 clone and AS ODNs. Initial studies suggested that the KOR-3 clone was the previously-described κ_3 opioid receptor subtype (Pan et al., 1994; Pan, Cheng et al., 1995). Specifically, centrally-administered AS ODN probes targeted against the KOR-3/ORL-1 clone selectively blocked analgesia elicited by the selective κ_3 agonist NalBzOH, and was ineffective against selective μ, δ, or κ_1 agonists (Pan et al., 1995). AS ODN mapping of the KOR-3/ORL-1 clone revealed that probes targeted against either exons 2 or 3 effectively blocked supraspinal NalBzOH-mediated analgesia, while a probe targeted against exon 1 was ineffective. However, the ineffectiveness of all AS ODN probes targeted against each of the three exons of the KOR-3/ORL-1 clone to reduce NalBzOH-induced analgesia suggests that KOR-3/ORL-1 clone does not correspond to the previously describe κ_3-opioid receptor subtype. Further, it was also determined that the KOR-3 clone shared a high degree of sequence homology with the ORL-1 clone suggesting that both clones encoded a novel opioid receptor subtype.

OFQ/N, the putative endogenous ligand for the KOR-3/ORL-1 clone (Meunier et al., 1995; Reinscheid et al., 1995) has subsequently been employed in AS ODN mapping of the KOR-3/ORL-1 in both mouse and rat (Rossi, Leventhal, Bolan and Pasternak, 1997; Rossi, Perlmutter, Leventhal, Talatti and Pasternak, in press). The *in vivo* pharmacology of OFQ/N is complex with early studies demonstrating either pronociceptive effects of OFQ/N (Meunier et al., 1995), or antiopioid effects of OFQ/N (Mogil, Grisel, Reinscheid, Civelli, Belknap and Grandy, 1996). Subsequent studies indicated that OFQ/N initially produces a brief hyperalgesia followed by a longer-acting naloxone-reversible analgesia in mice (Rossi et al.,

1997). AS ODN mapping of the KOR-3/ORL-1 clone in mice revealed that OFQ/N-induced hyperalgesia was blocked by a AS ODN probe targeting exon 1, whereas AS ODN probes targeting either exons 1, 2, or 3 were ineffective in reducing OFQ/N-mediated analgesia. Rats display a different profile following OFQ/N administration in that analgesia, but not hyperalgesia is observed (Rossi et al., in press). AS ODN mapping of the KOR-3/ORL-1 clone in the rat revealed that probes targeting either exon 2 or 3 , but not exon 1 effectively blocked OFQ/N-mediated analgesia.

The ability of AS ODN probes directed against specific exons of different opioid receptor clones to selectively alter analgesic responses induced by opioid receptor agonists demonstrate the promise of this knockdown technique, and yielded a conceptual framework by which to study opioid-mediated responses. The following section describes the role of the endogenous opioid system in another important homeostatic function, control of food intake and body weight.

V. Opioids and Ingestive Behavior.

The role of opioids in ingestive behavior was first suggested by the observation (Flowers, Dunham and Barbour, 1929) that chronic morphine treatment increased water intake, and that opiate withdrawal increased food intake in rats. Further, morphine increased basal metabolic rates in dogs, suggesting opioid involvement in both energy expenditure and intake (Barbour, Gregg and Hunter, 1930). Morphine-tolerant rats also ate large amounts of food following daily morphine treatment (Martin, Wikler, Eades and Pescor, 1963). The role of the central endogenous opioid system in opioid modulation of ingestive behavior was initially confirmed by the observation that ß-endorphin microinjected into the VMH increased food intake (Grandison and Guidotti, 1977), whereas general opioid antagonism decreased food and water intake in deprived rats (Holtzman, 1974). These initial studies on the role of

the opioids and opiates in ingestive processes were subsequently followed by evaluation of selective opioid agonists and antagonists upon ingestive behavior across a range of situations and conditions.

A. Endogenous opioid peptides and ingestive behavior. The role of endogenous opioid peptides in specific brain sites in mediating ingestive behavior has been investigated primarily by studying spontaneous intake during the light cycle when rats typically have low intake. Thus, ß-endorphin stimulated food intake following injections into the lateral ventricle and PVN (Leibowitz and Hor, 1982) as well as the VMH (Grandison and Guidotti, 1977). Food intake induced by either ß-endorphin or α-Neo-endorphin also occurs following microinjections into the nucleus accumbens (Majeed, Przewlocka, Wedzony and Przewlocki, 1986). Met- and leu-enkephalin injections in the VMH elicit feeding as well (Tepperman and Hirst, 1983). Dynorphin increases feeding in mice (Walker, Katz and Akil, 1980), and in nondeprived rats (Morley and Levine, 1983). Whereas dynorphin$_{1-17}$ increases feeding in the nucleus accumbens (Majeed et al., 1986), VMH and PVN (Gosnell, Morley and Levine, 1986), dynorphin$_{1-13}$ increases intake in the VTA (Hamilton and Bozarth, 1988). Finally, OFQ/N increases feeding following injections into the lateral ventricles (Pomonis, Billington and Levine, 1996), VMH and nucleus accumbens (Stratford, Holahan and Kelley, 1997).

B. General opioid antagonism and ingestive behavior. The role of the endogenous opioid system has been investigated using both general and selective opioid receptor antagonists under spontaneous and challenge situations. One advantage of employing antagonists as compared to agonists is that by selectively blocking the endogenous system, it is possible to observe changes in behavior in the absence of receptor activity. In contrast, exogenous agonist treatment is additive to endogenous peptide activity, resulting in effects induced by peptide levels greater than that elicited by physiological activity per se. Systemic

naloxone decreased feeding in rodents under spontaneous (Jalowiec, Panksepp, Zolovick, Najam an Herman, 1981) and nocturnal (Brands, Thornhill, Hirst and Gowdy, 1979; Jalowiec et al., 1981; Lowy, Maickel, and Yim, 1980) conditions as well as following food deprivation (Brands et al., 1979), 2-deoxy-D-glucose glucoprivation (2DG) (Lowy et al., 1980), insulin glucoprivation (Lowy et al., 1980), stress induced by either mild tail-pinch or social defeat (Lowy et al., 1980; Morley and Levine, 1980), and hypothalamic stimulation-induced feeding (Carr and Simon, 1983; Jenck, Gratton and Wise, 1986). General opioid antagonism reduces palatable intake induced by exposure to high fat (Cooper, Jackson, Morgan, Carter, 1985; Islam and Bodnar, 1990), thereby supporting a hypothesis that opioids selectively modulate fat intake (Marks-Kaufman and Kanarek, 1981, 1990). Naloxone reduces other forms of palatable intake elicited by sucrose (Siviy and Reid, 1983), saccharin (Cooper, 1983) and maltose dextrin (Beczkowska, Koch, Bostock, Leibowitz and Bodnar, 1993) exposure. Chronic administration of naloxone can also decrease food intake and body weight in rats (Mann, Pasternak, Hahn, Curreri, Lubin and Bodnar, 1988; Marks-Kaufman, Balmagiya and Gross, 1984; Olson, DeLatte, Kastin, McLean, Phillpott and Olson, 1985).

C. Selective opioid agents and ingestive behavior. Whereas selective opioid receptor agonists for μ, δ, and κ receptors each produce hyperphagia, selective antagonism of either receptor subtype typically reduce spontaneous food intake and body weight (see review: Bodnar et al., 1996; Gosnell and Levine, 1996). Table 2 summarizes the roles of selective opioid receptor subtypes in modulating different forms of ingestive behavior.

1. μ receptor compounds and food intake. Chronic systemic administration of morphine, heroin, codeine and levorphanol each increased spontaneous food intake (Martin et al., 1963; Sanger and McCarthy, 1980; Thornhill, Hirst and Gowdey, 1976). Central injections of the μ agonist DAMGO, also increase spontaneous food intake in rats (Gosnell,

Table 2. Summary of Opioid Receptor Subtype Antagonist Reductions as a Function of Ingestive Situations

	βFNA (μ)	Naloxonazine (μ_1)	NorBNI (κ_1)	Naltrindole (δ)	DALCE (δ_1)
Spontaneous feeding					
Intake	41%*	32%*	54%*	Ns	Ns
Weight	9%*	11%*	Ns	?	7%*
Deprivation feeding					
Overall	50%*	75%*	28%*	Ns	Ns
Carbohydrate	53%*	92%*	Ns	Ns	Ns
Fat	38%*	62%*	Ns	Ns	Ns
Glucoprivic feeding					
Insulin overall	54%*	Ns	Ns	?	Ns
2DG overall	90%*	Ns	68%*	Ns	Ns
2DG carbohydrate	51%*	Ns	Ns	Ns	Ns
2DG fat	89%*	Ns	88%*	Ns	Ns
Palatabale/stress feeding					
High fat	37%*	Ns	79%*	Ns	Ns
Sucrose	34%*	Ns	55%*	Ns	Ns
Saccharin	Ns	Ns	Ns	94%*	Ns
Maltose dextrin	44%*	Ns	Ns	Ns	Ns
Tail Pinch	28%*	32%*	Ns	Ns	Ns

*: Significant difference ($p<0.05$); Ns: not significant, 2DG: 2-Deoxy-D-glucose
Data represents degree of inhibition relative to appropriate control condition

Levine and Morley, 1986). Intracerebral sites of action of μ-agonist-induced feeding include the VMH (Tepperman and Hirst, 1982), PVN (McLean and Hoebel, 1983; Stanley, Lanthier and Leibowitz, 1989; Woods and Leibowitz, 1985), amygdala (Gosnell, 1988; Stanley et al., 1989), VTA (Mucha and Iverson, 1986; Noel and Wise; 1995; Stanley et al., 1989) and nucleus accumbens (Mucha and Iverson, 1986; Bakshi and Kelley, 1993a, 1993b). μ-agonists also alter intake of palatable ingesta, including sucrose, glucose, saccharin and sodium chloride in both intake (Bertino, Abelson, Marglin, Neuman, Burkhardt and Reid, 1988; Cooper, 1983; Czirr and Reid, 1986; Gosnell and Majchrzak, 1989, 1990; Ruegg, Yu and Bodnar, 1997); and operant (Gosnell and Patel, 1993) conditions. Although some studies suggest that morphine selectively increases fat intake (Marks-Kaufman, 1982; Marks-Kaufman and Kanarek, 1990), others suggest selective increases in fat intake only in food-restricted rats (Shor-Posner, Azar, Filart, Tempel and Leibowitz, 1986). Indeed morphine increases intake of fat, protein and carbohydrate in nondeprived rats (Bhakthavatsalam and Leibowitz, 1986), which led to the contention that opioid and particularly μ agonists stimulate intake of the preferred macronutrient (Gosnell, Krahn and Majchrzak, 1990).

The μ receptor is involved in a wide variety of ingestive situations based on antagonist studies (Table 2). Antagonism of μ receptors with ßFNA decreased spontaneous food intake and body weight in rats under acute and chronic conditions (Arjune, Standifer, Pasternak and Bodnar, 1990; Cole, Leventhal, Pasternak, Bowen and Bodnar, 1995), and reduces intake following food deprivation (Arjune et al., 1990) and 2DG and insulin glucoprivation (Arjune et al., 1990; Beczkowska et al., 1992). ßFNA also reduced intake of fat, sucrose and maltose dextrin (Beczkowska, Koch, Bostock, Leibowitz and Bodnar, 1992; Beczkowska et al., 1993; Islam and Bodnar, 1990) as well as feeding elicited by tail-pinch or electrical stimulation of the lateral hypothalamus (Koch and Bodnar, 1993; Papadouka and

Carr, 1994). Sites of action at which ßFNA reduces intake under deprivation, glucoprivic and palatable conditions include the PVN and nucleus accumbens, but not the VTA (Koch et al., 1995; Bodnar, Glass, Ragnauth and Cooper, 1995; Ragnauth et al., 1997). Antagonism of the μ_1 receptors with naloxonazine significantly reduced spontaneous intake and body weight under acute and chronic conditions (Cole et al., 1995; Mann et al., 1988), deprivation-induced feeding (Koch and Bodnar, 1994; Simone, Bodnar, Goldman and Pasternak, 1985) and tail-pinch feeding (Koch and Bodnar, 1993). In contrast, naloxonazine failed to alter food intake under glucoprivic or palatable conditions (see review: Bodnar, 1996). The ability of μ and μ_1 antagonists to alter intake under spontaneous, deprivation or stress-related conditions implies a role for the pharmacologically-defined μ_1 receptor in these ingestive responses. The ability of μ, but not μ_1 antagonists to alter palatable and glucoprivic intake implies a role for the pharmacologically-defined μ_2 receptor in these ingestive responses. These data provide further evidence for multiple μ receptors that may be defined by molecular AS ODN techniques.

Finally, hyperphagia induced by selective μ agonists appears to be mediated by more than one receptor subtype in antagonist studies. DAMGO-induced hyperphagia is reduced by pretreatment with either the selective μ-antagonist ßFNA or the selective κ_1-antagonist NorBNI (Levine, Grace, Billington and Portoghese, 1990; Levine, Grace and Billington, 1991). These data either challenge the selectivity of the agents employed, or imply that different opioid receptors are modulating agonist effects in sites that are connected in a series. Again, the AS ODN technique can distinguish among these possibilities.

2. δ receptor compounds and food intake. Central injections of δ-agonists increase spontaneous food intake (Gosnell et al., 1986) in such sites as the VMH (Tepperman and Hirst, 1983), nucleus accumbens (Majeed et al., 1986), amygdala (Stanley et al., 1989) and

PVN (McLean and Hoebel, 1983; Gosnell et al., 1986). Selective δ_1 and δ_2 receptor subtype agonists each increase spontaneous food intake in rats (Yu, Ruegg and Bodnar, 1997) with intracerebral sites of action of δ_1 (DPDPE)-induced feeding in VTA (Noel and Wise, 1995) and nucleus accumbens (Bakshi and Kelley, 1993; Majeed et al., 1986). δ receptor agonists also stimulate intake under palatability and challenge situations. The general δ-agonist, [D-Thr^2]-leucine enkephalin-Thr (DTLET), significantly increased saccharin and sodium chloride intake in rats (Gosnell and Majchrzak, 1989, 1990). Whereas both DPDPE (δ_1) and deltorphin (δ_2) increased sucrose intake in rats (Ruegg et al., 1997), only deltorphin significantly enhanced 2DG-induced hyperphagia (Yu et al., 1997). The ability of selective δ antagonists to block selective δ agonists effects in feeding studies produced conflicting results. Thus, DSLET-induced feeding was blocked by central pretreatment with either μ or κ_1 (Levine et al., 1990) antagonists. Further, DPDPE-induced hyperphagia was reduced by δ_2 (NTII), but not δ_1 (DALCE) antagonism (Yu et al., 1997), yet deltorphin-induced hyperphagia was blocked by DALCE and NTII (Yu et al., 1997). Whereas δ receptor subtype agonists reliably stimulate intake, a role for the δ receptor in modulating ingestion under a variety of situations appears quite limited in antagonist studies (Table 2). Both chronic δ_1 (DALCE) and δ_2 (NTII) antagonism reduces spontaneous intake under deprivation, glucoprivic and palatable conditions (see review: Bodnar, 1996) except for a reduction in saccharin intake (Beczkowska et al., 1993). δ_2 receptor antagonism in the VTA produced small reductions of intake following deprivation and glucoprivation (Ragnauth et al., 1997). The ability of δ agonists, but not δ antagonists, to alter intake is consistent with a modulatory rather direct role for this receptor in ingestion in which agonists enhance the efficacy of a "final common pathway" for feeding which does not include δ receptors in its circuitry.

3. κ receptor compounds and food intake. Systemic administration of κ-selective

drugs including, cyclazocine, ketocyclazocine bremazocine, butorphenol and U50,488H each increase food intake (Gosnell et al., 1986; Levine and Morley, 1983a; Morley, Levine, Grace and Kneip, 1982; Morley and Levine, 1983b; Sanger and McCarthy, 1981). Whereas central injections of both selective κ_1 (U50,488H) and κ_3 (NalBzOH) agonists increase spontaneous food intake (Gosnell et al., 1986; Koch et al., 1992), neither the VTA nor nucleus accumbens produce U50,488H-induced feeding (Bakshi and Kelley, 1993; Noel and Wise, 1993). Both U50,488H and NalBzOH stimulate sucrose intake (Lynch and Burns, 1990; Ruegg et al., 1997). The κ receptor has been implicated in ingestive behavior in antagonists studies with NorBNI producing potent reductions in nocturnal, sucrose and high-fat intake as well as intake induced by 2DG (Arjune and Bodnar, 1990; Beczkowska et al., 1992). In contrast NorBNI produces only marginal reductions in deprivation-induced intake (Levine et al., 1990; Koch and Bodnar, 1994) and chronic spontaneous intake (Cole et al., 1995). NorBNI fails to alter intake induced by either insulin, tail-pinch, saccharin or maltose dextrin (see review: Bodnar, 1996). These data limit κ opioid receptor mediation of intake to rather circumscribed situations. The marked involvement of μ, relative to κ and δ, receptor subtype antagonists in ingestion prompted the emphasis placed on studying effects of AS ODNs directed against the MOR-1 clone.

VI. Rationale.

The four specific aims of this dissertation are:

1. **To evaluate alterations in spontaneous food intake and body weight following AS ODN probes targeted against each of the four exons of the MOR-1 clone and a mismatch probe.**
2. **To evaluate alterations in hyperphagia elicited by the μ-selective opioid agonist, DAMGO following AS ODN probes targeted against each of the four exons of the MOR-1 clone and a mismatch probe, and compare any activity with the selective μ opioid antagonist ßFNA.**
3. **To evaluate whether the active morphine metabolite, M6G, produces hyperphagia and determine which AS ODN probes targeted the MOR-1, DOR-1, KOR-1 and KOR-3/ORL-1 clones altered this response and compare it with selective opioid antagonists.**
4. **To evaluate alterations in hyperphagia elicited by OFQ/N following AS ODN probes targeted against each of the three exons of the KOR-3/ORL-1 clone and a mismatch probe.**

Specific Aim One: **MOR-1 AS ODN probes: Effects upon spontaneous food intake and body weight measures.**

Both μ and μ_1-selective antagonists decrease spontaneous food intake and body weight and decrease food intake following either food deprivation or stress (see review: Bodnar,

1996). Therefore, control of spontaneous intake and body weight appears to be an excellent ingestive situation under which AS ODNs directed against the MOR-1 clone should act. Previous studies investigating morphine-induced analgesia (Rossi et al., 1997a) employed a treatment protocol in which the AS ODNs (10 μg) were injected every other day over a 5 day period and then tested 24 hours after the last injection (day 6). This treatment protocol and dosing regime were employed because they correlate with the turnover and synthesis of the opioid receptor (about 72 hours) and therefore maximizes the effects of the AS ODNs. Further, the 10 μg dose was selected because a higher 25 μg dose produced non-specific reductions in morphine-induced analgesia in that these AS ODN effects persisted well after the termination (7 days) of AS ODN treatment (Rossi et al., 1997a). In contrast, the chosen 10 (μg) dose produced potent reductions in morphine-induced analgesia yet this analgesic response systematically recovered over a time course that was consistent with the receptor turnover and synthesis. Detailed mapping studies based upon the MOR-1 clone have revealed interesting results in analgesic assays such that AS ODN probes directed against either exons 1 or 4 blocked morphine-induced analgesia, while probes directed against either exons 2 or 3 were ineffective. Conversely, antisense probes directed against exons 2 or 3 potently blocked the analgesic actions of M6G, while probes directed against either exons 1 or 4 were inactive.

The first specific aim examined the effects of centrally-administered AS ODNs directed against each of the four exons of the MOR-1 clone upon spontaneous food intake and body weight and compared these effects with those obtained for morphine-induced analgesia. This study, completed and published in Brain Research (1996) 719: 78-84, had the following competing hypotheses:

1a. If the MOR-1 clone encodes the receptor responsible for mediating food intake and body weight, then AS ODNs probes targeting each of the four exons of the MOR-1 clone

should significantly reduce food intake and body weight.

1b. If the receptor responsible for mediating food intake and body weight is identical to that receptor mediating traditional μ agonist-induced analgesia, then AS ODNs probes targeting either exon 1 or 4 of the MOR-1 clone should significantly reduce food intake and body weight, whereas probes targeting either exon 2 or 3 should be ineffective.

1c. If the receptor responsible for mediating food intake and body weight is identical to that receptor mediating M6G-induced analgesia, then AS ODNs probes targeting either exon 2 or 3 of the MOR-1 clone should significantly reduce food intake and body weight, whereas probes targeting either exon 1 or 4 should be ineffective.

2. A MS ODN (exon 1) probe, identical to the AS ODN probe except that two base pairs have been switched, should fail to reduce food intake and body weight.

3. Morphine-induced analgesia should be blocked by AS ODN probes targeting either exon 1 or 4, but not exons 2 or 3 of the MOR-1 clone.

Specific Aim Two: MOR-1 AS ODN probes: Effects upon DAMGO-induced hyperphagia.

Although MOR-1 encodes a μ opioid receptor, its relationship to the pharmacologically-defined μ receptor subtypes has been unclear. Using the AS ODN technique to map individual exons within the MOR-1 clone, it proved possible to determine which individual exons modulate μ-mediated analgesia (Rossi et al., 1995a, 1995b, 1996, 1997). AS ODN probes directed against exons 1 and 4 of the MOR-1 clone blocked morphine and μ agonist-mediated analgesia, while probes targeted against exons 2 and 3 of the MOR-1 clone were ineffective. In contrast, AS ODN probes directed against exons 2 and 3 of the MOR-1 clone blocked analgesia induced by the morphine metabolite, M6G, while AS ODNs directed against exons 1 and 4 of the MOR-1 clone were ineffective. Analgesic

responses induced by heroin, fentanyl, and etonitazine are reduced by AS ODNs directed against either exons 1 or 2 of the MOR-1 clone (Rossi et al., 1996). Thus, these studies raised the possibility that various μ receptor subtypes could result from alternative splice variants of the MOR-1 clone (Pasternak and Standifer, 1995).

μ-selective opioid agonists such as morphine and DAMGO stimulate food intake following systemic and central administration (Bakshi and Kelley, 1993; Gosnell et al., 1986a, 1986b; Sanger and McCarthy, 1980) which is blocked by μ-selective opioid antagonists (Levine et al., 1991). The present study examined the profile of DAMGO-induced hyperphagia following AS ODNs directed against the MOR-1 clone to determine whether it was similar to that observed for spontaneous intake and body weight (Specific Aim One) or similar to that observed for DAMGO-induced analgesia. This study, completed and published in The Journal of Pharmacology and Experimental Therapeutics (1997) 282: 1402-1407, had the following competing hypotheses.

1a. If the receptor mediating DAMGO-induced hyperphagia is identical to that mediating spontaneous food intake and body weight, then AS ODNs probes targeting each of the four exons of the MOR-1 clone should significantly reduce DAMGO-induced hyperphagia.

1b. If the receptor responsible for DAMGO-induced hyperphagia is identical to that mediating traditional μ agonist-induced analgesia, then AS ODNs probes targeting either exon 1 or 4 of the MOR-1 clone should significantly reduce DAMGO-induced hyperphagia, whereas probes targeting either 2 or 3 should be ineffective.

2. A MS ODN probe similar to one of the effective AS ODN probes except that two base pairs have been switched should fail to reduce DAMGO-induced hyperphagia.

3. Since AS ODNs produce a modest reduction in receptor protein levels (30-40%) then it would be expected that those AS ODN probes that effectively reduce DAMGO-induced

hyperphagia should do so at lower but not higher doses of DAMGO.

4. DAMGO-induced hyperphagia should be dose-dependently be blocked by the μ antagonist, ßFNA.

Specific Aim Three: MOR-1 AS ODN probes: Effects upon M6G-induced hyperphagia.

In addition to the ability of endogenous opioid peptides and peptide analogues to stimulate food intake, morphine and other opiates such as heroin, butorphanol, codeine and levorphanol also produce a robust feeding response (see review: Gosnell and Levine, 1996). Morphine is rapidly metabolized and glucuronidated at both the three and six positions (Jaffe & Martin, 1985). Although M6G labels μ receptors with an affinity slightly less than morphine in binding assays (Paul et al., 1989), it is 100-fold more potent (Paul et al., 1989) centrally on both thermal (Abbott and Palmour, 1988; Pasternak et al., 1987; Shimomura, Kamata, Ueki, Oguri, Yoshimura and Tsukamato, 1971; Sullivan, McQuay, Bailey and Dickenson, 1989) and visceral (Frances et al., 1992) nociceptive tests than morphine. However, rats do not metabolize morphine into M6G so any pharmacological or behaviorally-induced effects of morphine and subsequent alterations by antagonists or AS ODNs occur independent of M6G. The present study examined whether M6G, like its parent compound, morphine, produces long-acting (4 h) ingestive effects and examined the profile of M6G-induced hyperphagia following AS ODNs directed against the MOR-1 clone to determine whether the AS ODN profile was similar to that observed for spontaneous intake and body weight (Specific Aim One) or similar to that observed for M6G-induced analgesia (Rossi et al., 1995a; 1995b; 1997). This study, completed, and in press in The Journal of Pharmacology and Experimental Therapeutics, had the following competing hypotheses.

1. M6G will produce dose-dependent increases in spontaneous food intake.

2a. If the receptor mediating M6G-induced hyperphagia is identical to that mediating spontaneous food intake and body weight, then AS ODNs probes targeting each of the four exons of the MOR-1 clone should significantly reduce M6G-induced hyperphagia.

2b. If the receptor responsible for M6G-induced hyperphagia is identical to that mediating M6G-induced analgesia, then AS ODNs probes targeting either exons 2 or 3 of the MOR-1 clone should significantly reduce M6G-induced hyperphagia, whereas probes targeting either exons 1 or 4 should be ineffective.

2c. A MS ODN probe similar to one of the effective AS ODN probes except that the order of three nucleotide bases was altered should fail to reduce M6G-induced hyperphagia.

3. M6G-induced hyperphagia should be dose-dependently reduced by pretreatment with the μ antagonist ßFNA but not by equimolar doses of either δ_1 (DALCE), δ_2 (NTII) and κ_1 (NorBNI) opioid antagonists.

4. Morphine-induced hyperphagia should have an opposite MOR-1 AS ODN profile than its metabolite, M6G, such that morphine-induced hyperphagia should significantly be reduced by an AS ODN probe targeting exon 1, but not exon 2 of the MOR-1 clone.

5. M6G-induced hyperphagia should fail to be reduced by AS ODNs targeting regions of the DOR-1, KOR-1, or KOR-3/ORL-1 clones. However these probes should reduce hyperphagia elicited by their respective selective agonists. Specifically, an AS ODN probe targeting the DOR-1 clone should significantly reduce deltorphin II-induced hyperphagia, an AS ODN probe targeting the KOR-1 clone should significantly reduce U50,488H-induced hyperphagia, and an AS ODN probe targeting the KOR-3/ORL-1 clone should significantly reduce OFQ/N-induced hyperphagia.

Specific Aim Four: KOR-3/ORL-1 AS ODN probes: Effects upon OFQ/N-induced hyperphagia.

A novel receptor, termed the orphan opioid receptor-like (ORL-1/KOR-3) clone (e.g., Pan et al., 1995; Mollereau et al., 1994), displays little affinity for traditional opioid peptides. OFQ/N (Meunier et al., 1995; Reinscheid et al., 1995) binds with high affinity to the ORL-1/KOR-3 clone, and has been characterized for hyperalgesic (Meunier et al., 1995; Reinscheid et al., 1995) and analgesic (Rossi et al., 1997) actions. OFQ/N-induced analgesia is reduced by AS ODNs targeted against either exons 2 or 3 of the ORL-1/KOR-3 clone in rat (Rossi et al., 1998). OFQ/N, like other opioid peptides, stimulates food intake following ventricular, hypothalamic and accumbens administration that is blocked by naloxone (Pomonis et al., 1996; Stratford et al., 1997). The lack of a selective antagonist for the ORL-1/KOR-3 receptor makes it difficult to assert that this receptor mediates OFQ/N-induced hyperphagia. The present study examined the profile of OFQ/N-induced hyperphagia following AS ODNs directed against the KOR-3/ORL-1 clone to determine whether it was similar to that observed for OFQ/N-induced analgesia. This study, completed and published in European Journal of Pharmacology (1998) 349: R1-R3, had the following hypotheses.

1. If the receptor responsible for OFQ/N-induced hyperphagia is identical to that mediating OFQ/N-induced analgesia, then AS ODNs probes targeting either exon 2 or 3 of the KOR-3/ORL-1 clone should significantly reduce OFQ/N-induced hyperphagia, whereas a probe targeting exon 1 should be ineffective.

2. A MS ODN probe similar to one of the effective AS ODN probes except that the order of three base pairs has been switched should fail to reduce OFQ/N-induced hyperphagia.

3. OFQ/N-induced hyperphagia should be significantly and dose-dependently reduced by naltrexone.

CHAPTER 2. GENERAL METHODS

I. Subjects, surgery and cannula verification. Adult male albino Sprague-Dawley rats, 90-120 days of age, were purchased from Charles River Laboratories, Kingston, NY and were housed individually in wire mesh cages and maintained on a 12-h light: 12-h dark cycle with water and chow available *ad libitum*. Each rat was pretreated with chlorpromazine (3 mg/kg, i.p.) and anesthetized with Ketamine HCl (120 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 top of the skull. Each cannula was secured to the skull by three anchor screws with dental acrylic. All animals were allowed at least 2 weeks to recover from stereotaxic surgery before behavioral testing began. Rats weighed between 275 and 300 g before surgery, and weighed 400 to 550 g after completion of testing. After completion of behavioral testing, all animals were killed with an overdose of anesthetic, and cannula placements were verified visually.

II. Drugs. To characterize effects of specific opioid receptor clones upon opioid-mediated ingestion and hyperphagia, selective opioid receptor antagonists, agonists, and AS ODNs were employed.

A. Opioid antagonists and agonists. ßFNA (Research Biochemicals Intl., Natick, MA), an irreversible selective μ receptor antagonist, NorBNI (Research Biochemicals Intl., Natick, MA), a short acting κ antagonist, and NTII (Research Biochemicals Intl., Natick, MA) a long-acting δ_2 antagonist, were each dissolved in 0.9 % normal saline, and DALCE (synthesized by Dr. W.D Bowen) a long-acting δ_1 antagonist was dissolved in 0.2 M HCl in distilled water with the pH adjusted to 7.5-8.0 with 0.2 M NaOH.

DAMGO (Peninsula Laboratories, Belmont, CA), morphine (Pennick Laboratories, NJ), M6G (Research Technology Branch of the National Institute of Drug Abuse, Rockville, MD), deltorphin II (Peninsula, Belmont, CA), and U50,488H (Upjohn) were each dissolved in 0.9% normal saline. OFQ/N was synthesized by the Core Facility at Memorial Sloan-Kettering Cancer Center (NY, NY). Following purification by HPLC, structures of the peptide was verified by mass spectroscopy and peptide content was obtained. OFQ/N was dissolved in 0.9% normal saline.

All drugs were administered i.c.v in 5 μl volumes over 30 s through a stainless steel internal cannula (28-gauge, Plastic One, Roanoke, VA) which was attached to a Hamilton microsyringe by polyethylene tubing. ßFNA, NTII and DALCE were administered 24 h prior to agonist administration to allow for full development of irreversible antagonists effects, while NorBNI was administered 30 min prior to opioid agonist administration. All opioid agonists were administered just prior to behavioral testing.

B. AS ODNs. All phosphodiester oligodeoxynucleotides (Midland Certified Reagent Company, Midland, TX) were dissolved in 0.9% normal saline at a concentration of 0.5 μg/μl and purified. Table 3 summarizes the sequences the AS ODNs (19-22 bases long) that were directed against four regions of the MOR-1 clone, three regions of the KOR-3/ORL-1 clone, regions of the DOR-1 and KOR-1 clones, and mismatches for MOR-1 and KOR-3/ORL-1 probes. These sequences 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, 1997). Analysis of the 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 cDNA's.

Table 3 Sequence of Antisense Oligodeoxynucleotide and Missense Oligodeoxynucelotide

Probe	Sequence	Location	Nucleotide Position
MOR-1/AS1	CGC CCC AGC CTC TTC CTC T	Exon 1	195-213
MOR-1/MS1	CGC CCC GAC CTC TTC CCT T		
MOR-1/AS2	TTG GTG GCA GTC TTC ATT TTG G	Exon 2	572-593
MOR-1/MS2	TTG GCT GCA GTC GTC ATT TTG G		
MOR-1/AS3	TGA GCA GGT TCT CCC AGT ACC A	Exon 3	959-979
MOR-1/AS4	GGG CAA TGG AGC AGT TTC TG	Exon 4	1457-1476
KOR-3/AS1	GGG GCA GGA AAG AGG GAC TCC	Exon 1	301-321
KOR-3/AS2	GAC GAG GCA GTT CCC CAG GA	Exon 2	486-505
KOR-3/AS3	GGG CTG TGC AGA AGC CGA GA	Exon 3	1189-1208
KOR-3/MS3	GGG TCG GTC AGA GAC CGA GA		
KOR-1	GGT GCC TCC AAG GAC TAT CGC	Exon 3	761-782
DOR-1	AAC ACG CAG ATC TTG GTC AC	Exon 3	662-681

AS: Antisense, MS: Missense

All AS ODNs and MS ODNs were administered i.c.v. in 2 μl volumes over 15 s through a stainless steel internal cannula (28-gauge, Plastic One, Roanoke, VA) which was attached to a Hamilton microsyringe by polyethylene tubing. Three infusions were administered at 48-h intervals. This time course of treatment allows for both the downregulation of the synthesis of new receptors and the turnover of existing receptors (see review: Pasternak and Standifer, 1995).

III. General procedures. To assess daily food intake and body weight in Specific Aim One, rats were monitored over one week. Chow intake was adjusted for spillage that was collected on paper placed under the cage; weight and intake data from the last three days were used as the baseline measures. Measurements of food intake and body weight were monitored daily throughout the treatment paradigm.

To assess agonist-induced changes in spontaneous food intake (Specific Aim Two-Four), all rats were tested over 4 to 10 days at 3 to 9 h into the light cycle, an interval during which intake should be minimal, to ensure stability of baseline spontaneous food intake. Preweighed pellets were placed directly on the floor of the wire mesh cage to optimize accessibility. Cumulative intakes were assessed at 2 and 4 h (DAMGO, M6G, morphine, deltorphin II and U50,488H) and at 30 min, 1 h and 2 h (OFQ/N), adjusting for spillage that was collected on paper placed under the cage. In Specific Aim Four, the latency to commence feeding was recorded with a 15 min cutoff.

IV. Statistical analyses. Separate split-plot analyses of variance for each specific time point were performed to ascertain significant differences among vehicle treatments and different AS ODN and antagonist treatments. Tukey corrected comparisons ($p < .05$) were used to discern significant differences among vehicle treatments and individual AS ODN or antagonist treatments at specific time points. A between-subjects ANOVA was employed

because it is the most conservative approach since subject variance and error variance due to subjects is not parceled out. This inclusion of the sources of variability makes it more difficult to achieve significant results but is a more accurate representation of the design used in the study.

CHAPTER 3: SPECIFIC AIM ONE.

MOR-1 AS ODN Probes: Effects Upon Spontaneous Food Intake and Body Weight Measures.

Introduction

The endogenous opioid system has been implicated in a wide variety of ingestive behaviors based upon agonist and antagonist studies (see review: Bodnar, 1996; Gosnell and Levine, 1996). All of the major opioid receptor classes (Pasternak, 1993) have been implicated in these behaviors. μ, κ and δ agonists stimulate spontaneous food intake (see review: Gosnell and Levine, 1996). Both μ and μ_1-selective antagonists decrease spontaneous food intake and body weight and decrease food intake following either food deprivation or stress (see review: Bodnar, 1996). μ antagonists also reduce food intake under glucoprivic, palatable, and electrically-stimulated conditions (see review: Bodnar, 1996).

Following the successful cloning of DOR-1, which encodes a δ opiate receptor, clones were subsequently identified for a μ (MOR-1), κ_1 (KOR-1) and a κ_3-related/orphanin (KOR-3 or ORL-1) opioid receptor (see review: Uhl et al., 1994). AS ODN approaches have correlated the cloned receptors with the established pharmacology (see review: Standifer and Pasternak, 1995). AS ODN directed against a segment of the DOR-1 clone selectively reduced spinal δ, but not κ_1 or μ, analgesia (Standifer et al., 1994. These actions corresponded quite well to the downregulation of mRNA and δ receptor protein (Standifer et al., 1995. AS ODN directed against a segment of the KOR-1 receptor selectively blocked κ_1 (U50,488H) analgesia, and probes targeting the initial segment of MOR-1 eliminated morphine analgesia (see review: Pasternak and Pasternak, 1995). In contrast, MS ODN in which two pairs of bases from the antisense sequence had been switched failed to produce appreciable effects.

More detailed studies based upon the MOR-1 clone have revealed interesting results. The ability of antisense probes targeting different exons of DOR-1 to downregulate δ binding in NG108-15 cells equally well (Standifer et al., 1994) suggested that AS approaches could be used to map the presence of individual exons in the receptors mediating a response. Using this approach, the MOR-1 clone was mapped in the mouse with a series of AS ODN against all four exons (Rossi et al., 1995a; 1995b; 1996). As expected, the three antisense probes against exon 1 all blocked morphine analgesia, as did the probe targeting the coding region of exon 4. However, almost all the AS ODN directed against exons 2 and 3 were ineffective. Conversely, these same antisense probes against exons 2 and 3 potently blocked the analgesic actions of M6G, while the AS ODN against exon 1 and 4 were inactive. Similar results were observed in rats (Rossi et al., 1994). Thus, these studies raised the possibility that various μ receptor subtypes could result from alternative splicing of MOR-1 gene (Pasternak and Standifer, 1995). μ receptors are involved in different forms of ingestive behavior (see reviews: Bodnar, 1996; Gosnell and Levine, 1996).

The present study, completed and published in Brain Research (1996) 719: 78-84, examined the effects of centrally-administered AS ODNs directed against each of the four exons of the MOR-1 clone on ingestive behavior, and compared these results with those obtained with morphine analgesia (Rossi et al., 1995a; 1995b; 1996).

Methods

Procedure: Rats were monitored over one week for body weight, pellet chow intake and water intake; data from the last three days were used as the baseline measures. Groups of rats, matched on the basis of body weight and food intake, received i.c.v. microinjections of AS1 (n=28), AS2 (n=17), AS3 (n=12), AS4 (n=14) or MS1 (n=12) ODNs of the MOR-1 clone, or vehicle (n=24, 0.9% saline) on Days 1, 3 and 5 as previously described.

Measurements of food and water intake, as well as body weight were monitored daily throughout the treatment paradigm. Twenty-four h after the third injection (Day 6) rats received morphine (7.5 μg, i.c.v., Pennick Laboratories, NJ), and analgesia was measured in the radiant heat tail-flick assay 30 min later. Baseline latencies ranged from 3-3.5 s, and a 15 s cutoff was used to minimize tissue damage.

Results

MOR-1 AS ODNs and body weight:

Significant differences in body weight were observed among conditions ($F(5,162) = 2.26$, $p < .05$), across days ($F(5,810) = 18.02$, $p < .0001$) and for the interaction between conditions and days ($F(25,810) = 9.56$, $p < .0001$). Body weights of vehicle-treated rats significantly increased over the 5 day treatment period (Figure 1, Table 4). In contrast, each of the four AS ODNs directed against the MOR-1 clone significantly decreased weight 24 h after each injection: AS1 (7-9 g), AS2 (8-12 g), AS3 (7-14 g), AS4 (10-17 g) (Table 4, Figure 1). A compensatory rebound in weight occurred for each AS-treated group 48 h after each AS ODN injection (Table 4). The MS1 ODN failed to significantly alter weight after the first two injections, and significantly increased weight (9 g) after the third injection (Table 4, Figure 1). Throughout the study, all animals displayed normal ambulatory, grooming and ingestive behaviors, suggesting that the losses in body weight were not due to any generalized debilitation or illness.

MOR-1 AS ODNs and food intake

Significant differences in food intake were observed among conditions ($F(5,162) = 10.30$, $p < .0001$), across days ($F(5,810) = 113.00$, $p < .0001$) and for the interaction between conditions and days ($F(25,810) = 7.73$, $p < .0001$). Food intake of vehicle-treated rats was relatively constant over the test period with small significant reductions (2.5-4 g) after the first

Figure 1. Alterations in body weight relative to their respective baseline values (PRE) in rats receiving three central microinjections at 48 h intervals of either a 0.9% saline (SAL) vehicle, AS ODN probes directed against either exons 1 (AS1), 2 (AS2), 3 (AS3) or 4 (AS4) of the MOR-1 clone, or a missense probe (MS1). PRE values refer to the average of three days prior to AS ODN treatment. Post values refer to 24 h after the last AS ODN injection (Day 6). Significant differences are denoted relative to SAL (*) (Tukey comparisons, $p < .05$). All PRE values failed to differ from each other.

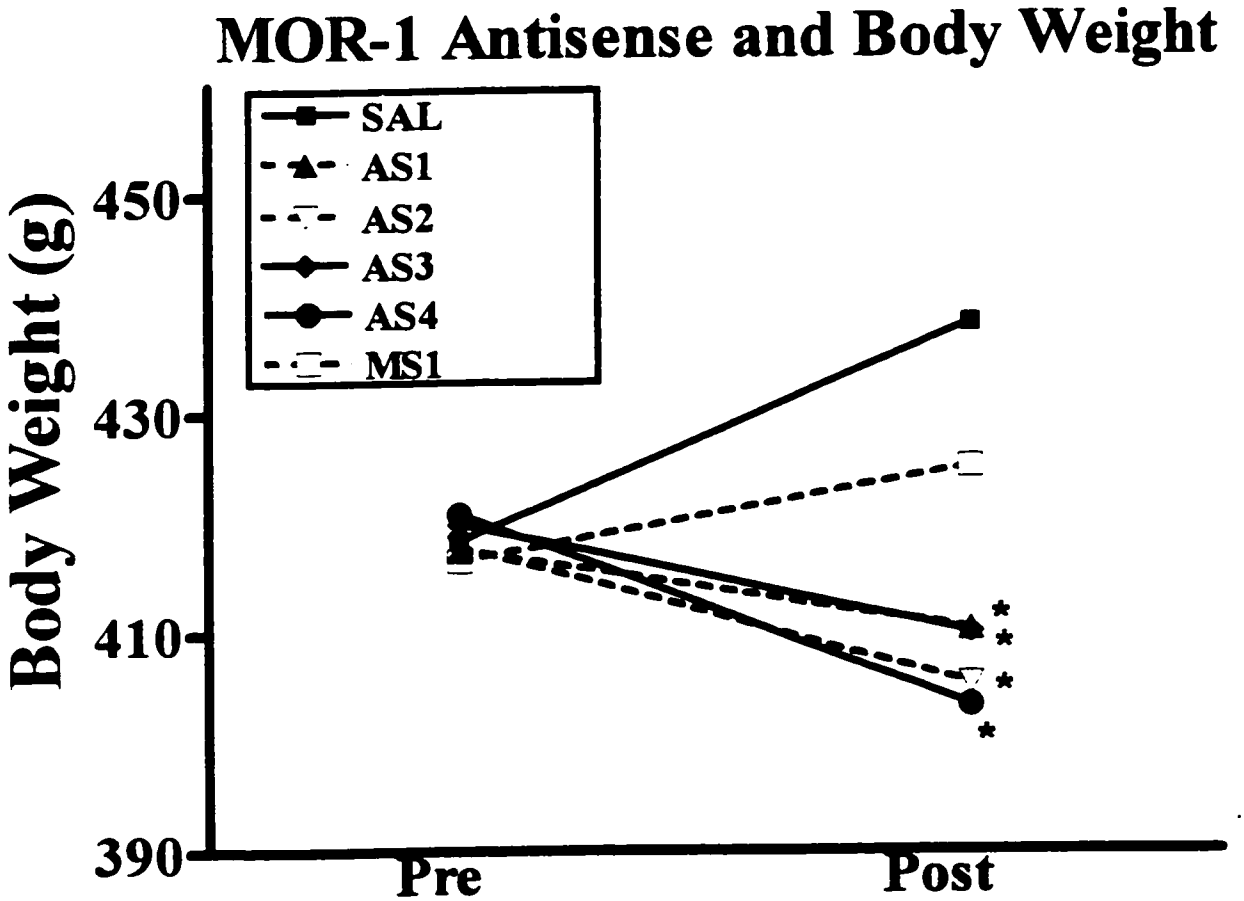


Table 4 Alterations (± S E M) in Body Weight and Food Intake Following Treatment with Vehicle (Veh), AS1, AS2, AS3 or MS1 of the MOR-1 Clone

A. Body weight (g):	*N*	PRE	Day 1	Day 2	Day 3	Day 4	Day 5
Veh	24	418 4 (4 9)	420 9 (5 1)	427 1 (5 0)	428 0 (5 4)*	432 8 (5 4)	438 2 (5 5)*
AS1	28	417 6 (4 6)	408 8 (5 6)*	415 5 (6 8)	409 2 (6 8)*	416 0 (7 1)	410 4 (7 1)*
AS2	17	417 9 (5 1)	409 4 (4 8)*	414 4 (4 8)	411 8 (6 3)	413 8 (6 8)	405 4 (7 7)*
AS3	12	419.8 (2.2)	412 4 (3 3)*	418 4 (3 2)	405 2 (3 4)*	415 7 (3 0)	410 1 (4 6)*
AS4	14	420 9 (4 7)	405 4 (4 9)*	414 4 (5 2)	410 7 (5 9)*	406 3 (5 6)*	403 4 (6 2)*
MS1	12	416 6 (4 1)	409 2 (5 4)	417 8 (4 8)	416 1 (5 8)	420 8 (6 9)	425 1 (7 1)*
B. Food intake (g):							
Veh	24	26.9 (0.6)	22 9 (1 0)*	25 0 (0 5)	25 1 (0 9)	26 5 (0 6)	24 3 (0 7)*
AS1	28	26 3 (0 6)	17 7 (1 4)*	21 8 (1 5)*	19 5 (1 4)*	24 0 (1 1)*	17 4 (1 1)*
AS2	17	26.7 (0 7)	18 6 (1 2)*	22 4 (0 9)*	19 3 (1 3)*	24 1 (1 2)*	15 8 (1 2)*
AS3	12	25.2 (0 5)	17 9 (0.9)*	19 7 (1 2)*	19 7 (1 5)*	22 1 (0 9)*	19 1 (1 3)*
AS4	14	29.4 (0.6)	19.6 (1.6)*	27 5 (1.3)	23 3 (1.6)*	22 6 (0 8)*	16 1 (1.1)*
MS1	12	25.8 (0.6)	16.4 (0.9)*	21 6 (0 6)*	18 8 (1 2)*	23 0 (1 3)	21 1 (0 8)

* :denotes significant difference relative to Veh ($p<0.05$)

and third injections (Figure 2, Table 4). Each of the four AS ODN directed against the MOR-1 clone significantly decreased food intake to a greater degree: AS1 (7.3-9 g), AS2 (7-11 g), AS3 (5.4-7.2 g), AS4 (6.2-13.3 g) (Figure 2, Table 4). Although the MS ODN significantly reduced food intake (7-9.4 g) after the first two injections, the reduction (4.7 g) after the third injection was considerably smaller (Figure 2, Table 4). A compensatory rebound in food intake occurred for all antisense and mismatch conditions 48 h after each injection (Table 4).

MOR-1 AS ODNs and morphine analgesia

Significant differences in latencies were observed among conditions ($F(5,96) = 7.06$, $p < .0001$), between pre- and post-morphine testing ($F(1,96) = 226.00$, $p < .0001$) and for the interaction between treatments and time ($F(5,96) = 5.44$, $p < .0002$). Whereas morphine significantly increased latencies in all groups, the magnitude of analgesia was significantly reduced in rats receiving AS ODNs directed against exon 1 (63%) and to a lesser degree by AS ODNs directed against exon 4 (33%) (Figure 3). In contrast, the magnitude of central morphine analgesia was not affected in rats receiving the MS ODN or AS ODNs directed against exons 2 or 3 of the MOR-1 clone.

Discussion

The present study demonstrated the importance of the receptor encoded by MOR-1 in spontaneous ingestive behavior in the rat. AS ODNs directed against each of the four exons of the MOR-1 clone significantly reduced body weight and food intake. The reductions (8-17 g) in body weight observed in the AS ODN groups were specific since groups given corresponding microinjections of either vehicle or a MS ODN increased their body weight by 19 and 9 g, respectively. Furthermore, all animals displayed normal ambulatory, grooming and ingestive behaviors without any observable generalized debilitation or illness. One

Figure 2. Alterations in food intake relative to their respective baseline values (PRE) in the same rats. Significant difference are denoted relative to SAL (*) (Tukey comparisons, $p < .05$). All PRE values failed to differ from each other.

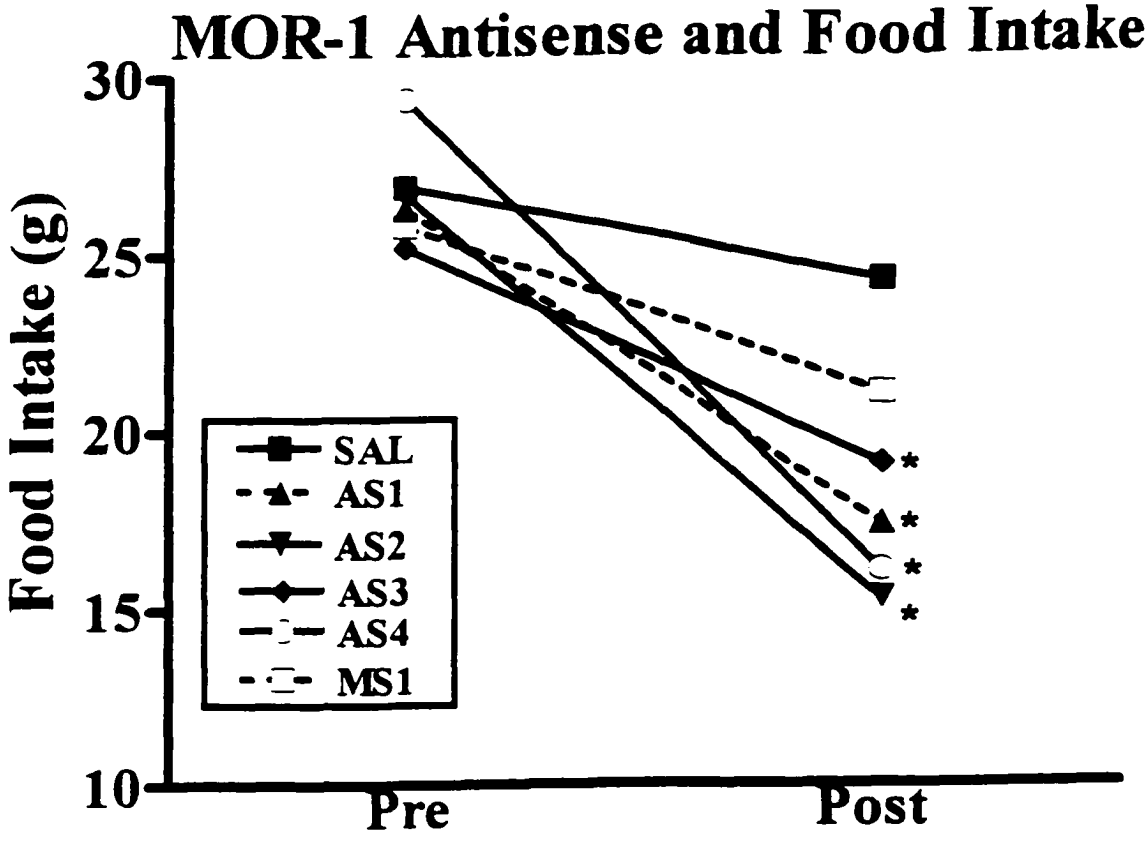
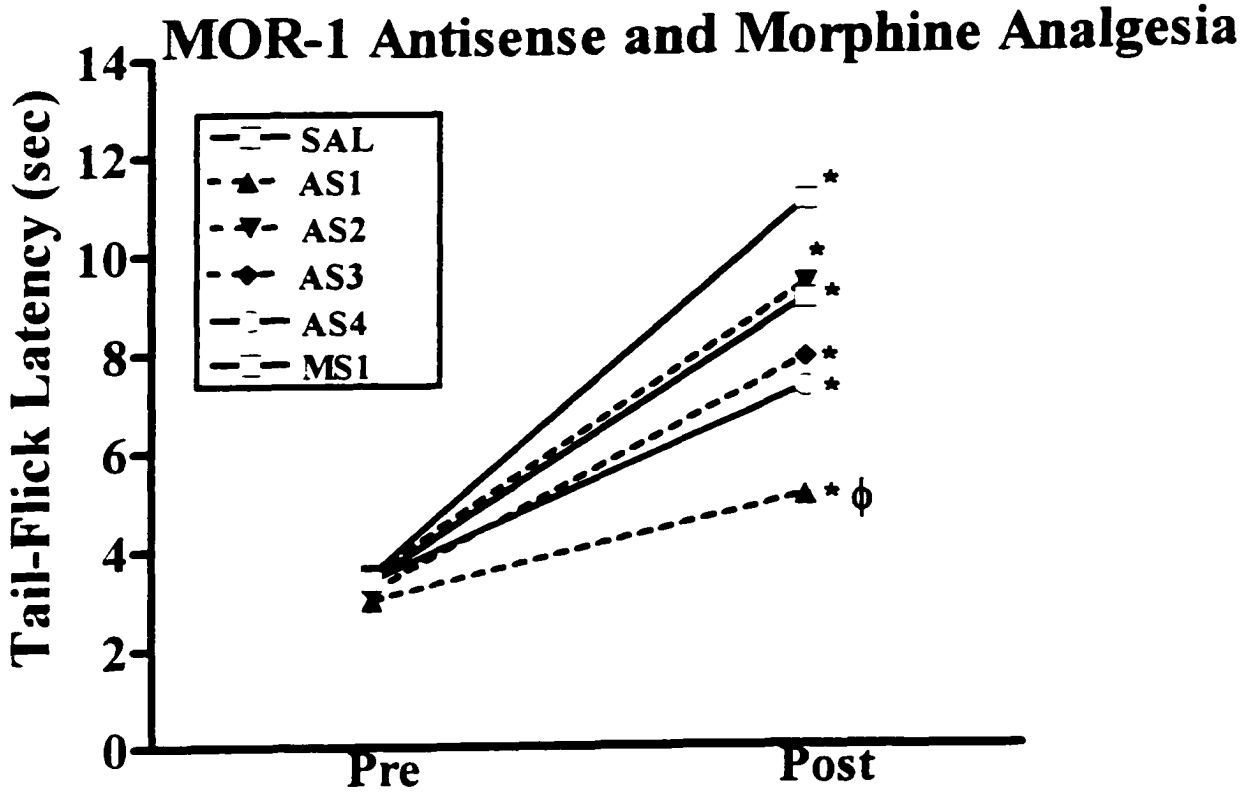


Figure 3. Alterations in morphine (7.5 μg, i.c.v.) analgesia in rats receiving central treatment of either vehicle (SAL), AS1, AS2, AS3 or AS4 of the MOR-1 clone, or a MS1. Significant differences are denoted relative to corresponding PRE value (*) or relative to SAL (phi) (Tukey comparisons, $p < .05$). All PRE values failed to differ from each other.



potential limitation in interpreting these data is the use of daily intake measures. Thus, one cannot detect, short-term (e.g., min or h) alterations in intake followed by subsequent compensatory changes in intake, or possible circadian alterations. Further, reductions in food intake and bodyweight 24 h following AS ODN injections were followed by compensatory increases in weight and intake between 24 and 48 h after each treatment. The AS ODN-induced changes in body weight were modest and may reflect activation of either negative feedback loops and/or compensatory mechanisms that may stimulate nonopioid systems. The results with feeding and weight maintenance are unique in that this is the first function which is blocked by AS ODN probes against all four exons of MOR-1, implying that MOR-1 itself encodes the receptor mediating these actions and not a putative splice variant. This contrasts with the analgesic actions of morphine and M6G and the inhibition of gastrointestinal transit by morphine which are sensitive to only selected exons in MOR-1 (see review: Pasternak and Standifer, 1995). All four AS ODNs directed against MOR-1 decrease food intake 24 h after the third injection by 9.3 to 13.3 g. Although intake for the vehicle-treated (2.3 g) and MS1-treated (4.4 g) groups was lowered over the treatment paradigm, the magnitude of the decreases was smaller and the animals still gained weight.

The activity of the AS ODN directed against the MOR-1 clone in this ingestive paradigm is similar to other AS ODN studies evaluating the role of nonopioid peptides in ingestive behavior. AS ODNs directed against the mRNA encoding galanin microinjected into the PVN decrease fat intake and body weight, while probes targeting neuropeptide Y mRNA decrease food intake and insulin secretion when administered into the hypothalamic arcuate nucleus (Akabayashi, Koenig, Watanabe, Alexander and Leibowitz, 1994; Akabayashi, Wahlestedt, Alexander and Leibowitz, 1994).

Thus, this study confirmed prior reports regarding the role of MOR-1 in morphine

analgesia and identified the first opioid action which is effectively blocked by the antisense probes targeting each of the four exons of the MOR-1 clone.

CHAPTER 4: SPECIFIC AIM TWO.

MOR-1 AS ODN Probes: Effects Upon DAMGO-induced Hyperphagia.

Introduction

A role for the endogenous opioid system in the central regulation of many types of ingestive behaviors has been characterized using selective opioid agonists and antagonists (see reviews: Bodnar, 1996; Gosnell and Levine, 1996; Morley et al., 1983). Agonists for all three major classes of opioid receptors (i.e., μ, κ, δ) typically stimulate spontaneous food intake, while general opioid antagonists decrease spontaneous intake and body weight. Further, specific opioid receptor subtype antagonists against μ, κ and δ receptors differentially reduce food intake as a function of the ingestive situation.

The efficacy and specificity of AS ODNs directed against opioid receptor clones have been confirmed functionally and biochemically (see review: Pasternak and Standifer, 1995), particularly in analgesic assays. AS ODNs directed against the 5'-untranslated regions of either the DOR-1, MOR-1 or KOR-1 clones selectively and respectively reduced δ-, μ- and κ_1-mediated forms of analgesia (see review: Pasternak and Standifer, 1995). Although MOR-1 encodes a μ opioid receptor, its relationship to the pharmacologically-defined μ receptor subtypes has been unclear. Using the AS ODN technique to map individual exons within the MOR-1 clone, it proved possible to determine which individual exons modulate μ-mediated analgesia (Rossi et al., 1995a, 1995b, 1996, 1997). AS ODN probes directed against either exons 1 or 4 of the MOR-1 clone blocked morphine and μ agonist-mediated analgesia, while probes targeted against either exons 2 or 3 of the MOR-1 clone were ineffective. In contrast, AS ODN probes directed against either exons 2 or 3 of the MOR-1 clone blocked analgesia induced by the morphine metabolite, M6G, while AS ODNs directed against either exons 1 or 4 of the MOR-1 clone were ineffective. Thus, these studies raised the possibility that various

μ receptor subtypes could result from alternative splice variants of the MOR-1 clone (Pasternak and Standifer, 1995).

The AS ODN strategy has recently been applied to opioid modulation of ingestive behavior (Leventhal, Cole, Rossi, Pan, Pasternak and Bodnar, 1996). Body weight and food intake were significantly reduced by AS ODNs directed against each of the four exons of the MOR-1 clone. In contrast, a MS ODN control was ineffective. The sensitivity of spontaneous intake and body weight to all four exons suggest that the receptor responsible for this action is encoded by the MOR-1 clone. μ-selective opioid agonists such as morphine and DAMGO stimulate food intake following systemic and central administration (Bakshi and Kelley, 1993; Gosnell et al., 1986a, 1986b; Sanger and McCarthy, 1980) which is blocked by μ-selective opioid antagonists (Levine et al., 1991). The present study, completed and published in The Journal of Pharmacology and Experimental Therapeutics (1997) 282: 1402-1407, examined the profile of DAMGO-induced hyperphagia following AS ODNs directed against the MOR-1 clone to determine whether it was similar to that observed for spontaneous intake and body weight (Specific Aim One) or similar to that observed for DAMGO-induced analgesia.

Methods

Drugs: DAMGO (0.5-5 μg, Peninsula Laboratories, Belmont, CA) and ßFNA (0.2-20 μg, Research Biochemicals Intl., Natick, MA) were dissolved in 0.9% normal saline and administered i.c.v. in 5 μl volumes over 30 s through an internal cannula. ßFNA was administered 24 h prior to agonist administration to allow for full development of irreversible μ antagonist effects (Portoghese et al., 1980).

Procedures: All rats were tested over 4-10 days at 3-9 h into the light cycle to insure the stability of baseline spontaneous food intake. Preweighed pellets were placed directly onto

the floor of the wire mesh cages to optimize accessibility since this factor can interfere with DAMGO-induced feeding (Badiani, Leone, Noel and Stewart, 1995). Cumulative intakes were assessed after 2 and 4 h before and after each condition, and adjusted for spillage which was collected by paper under the cage. Following intake stabilization, all rats received a vehicle condition (5 μl 0.9% normal saline, i.c.v.). Since DAMGO is known to produce sedative and hypoactive effects, each animal was treated twice with DAMGO (1 μg, i.c.v.) without measuring intake. In assessing DAMGO-induced hyperphagia, rats received one of four 0 (n=87), 0.5 (n=24), 1.0 (n=39), 5.0 (n=24) μg doses, and intake was assessed after 2 and 4 h. Intake elicited by each DAMGO dose was matched across the subgroups of rats receiving AS ODNs and the MS control. During the test phase of the experiment, rats received one of four AS ODN sequences (Table 5) for the following DAMGO doses: AS1: 0.5 μg (n=7), 1.0 μg (n=8), 5.0 μg (n=9); AS2: 0.5 μg (n=6), 1.0 μg (n=8); AS3: 0.5 μg (n=5), 1.0 μg (n=8); AS4: 0.5 μg (n=4), 1.0 μg (n=8), 5.0 μg (n=7) (10 μg, 2 μl, i.c.v.) or a MS: 1.0 μg (n=6) on days 1, 3, and 5 as previously described. Twenty-four h after the last AS ODN or MS ODN treatment (day 6), rats were retested with their respective DAMGO dose (0.5-5μg), and food intake was assessed after 2 and 4 h.

In order to confirm the μ-selective actions of DAMGO-induced hyperphagia, separate, but identically-screened groups of rats screened for DAMGO-induced hyperphagia, received the μ- selective antagonist, ßFNA (0.2-20 μg, n=5/6 each: Arjune et al., 1990; Levine et al., 1991; Ukai and Holtzman, 1988) 24 h prior to DAMGO treatment. Cumulative intakes were again assessed 2 and 4 h after DAMGO.

Results

DAMGO-induced hyperphagia

Significant dose-dependent differences in DAMGO-induced hyperphagia were

observed after 2 ($F(3,86) = 217.89$, $p < .0001$) and 4 ($F = 291.43$, $p < .0001$) h. DAMGO significantly increased food intake relative to vehicle after 4 h (Figure 4). These effects were dose-dependent in that the 5 μg dose was significantly greater than the 0.5 and 1 μg doses, and the 1 μg dose was correspondingly greater than the 0.5 μg dose (Figure 4). The pattern of effects observed after 2 h in this and the other conditions were identical to that depicted at 4 h.

MOR-1 AS ODNs and DAMGO (0.5 μg)-induced hyperphagia

Significant differences in food intake were observed among conditions after 2 ($F(5,23) = 67.92$, $p < .0001$) and 4 ($F = 69.87$, $p < .0001$) h. The 0.5 μg dose of DAMGO significantly increased intake after 2 and 4 h which was significantly reduced by pretreatment with AS ODNs directed against either exons 1 (AS1: 76%) or 4 (AS4: 70%) of the MOR-1 clone (Figure 5A). In contrast, neither AS ODN probes directed against exons 2 (AS2: 17% increase) or 3 (AS3: 10% increase) of the MOR-1 clone failed to alter DAMGO hyperphagia (Figure 5A).

MOR-1 AS ODNs and DAMGO (1.0 μg)-induced hyperphagia

Significant differences in food intake were observed among conditions after 2 ($F(6,37) = 66.42$, $p < .0001$) and 4 ($F = 73.38$, $p < .0001$) h. The 1.0 μg dose of DAMGO significantly increased intake after 2 and 4 h which was significantly reduced by pretreatment with either AS ODNs against either exons 1 (AS1: 100%) or 4 (AS4: 53%) of the MOR-1 clone (Figure 5B). In contrast, neither AS2 (10% increase) nor AS3 (7% increase) significantly altered DAMGO hyperphagia (Figure 5B). Further, the MS ODN failed to significantly alter DAMGO-induced hyperphagia at this dose (Figure 5B).

MOR-1 AS ODNs and DAMGO (5.0 μg)-induced hyperphagia

Significant differences in food intake were observed among conditions after 2

Figure 4. Alterations (Mean, ±SEM) in food intake following i.c.v. administration of either 0, 0.5, 1.0 or 5.0 μg doses of DAMGO. Significant differences are denoted relative to either vehicle (phi), 0.5 μg (*) or 1.0 μg (#) DAMGO doses (Tukey comparisons, $p < 0.01$).

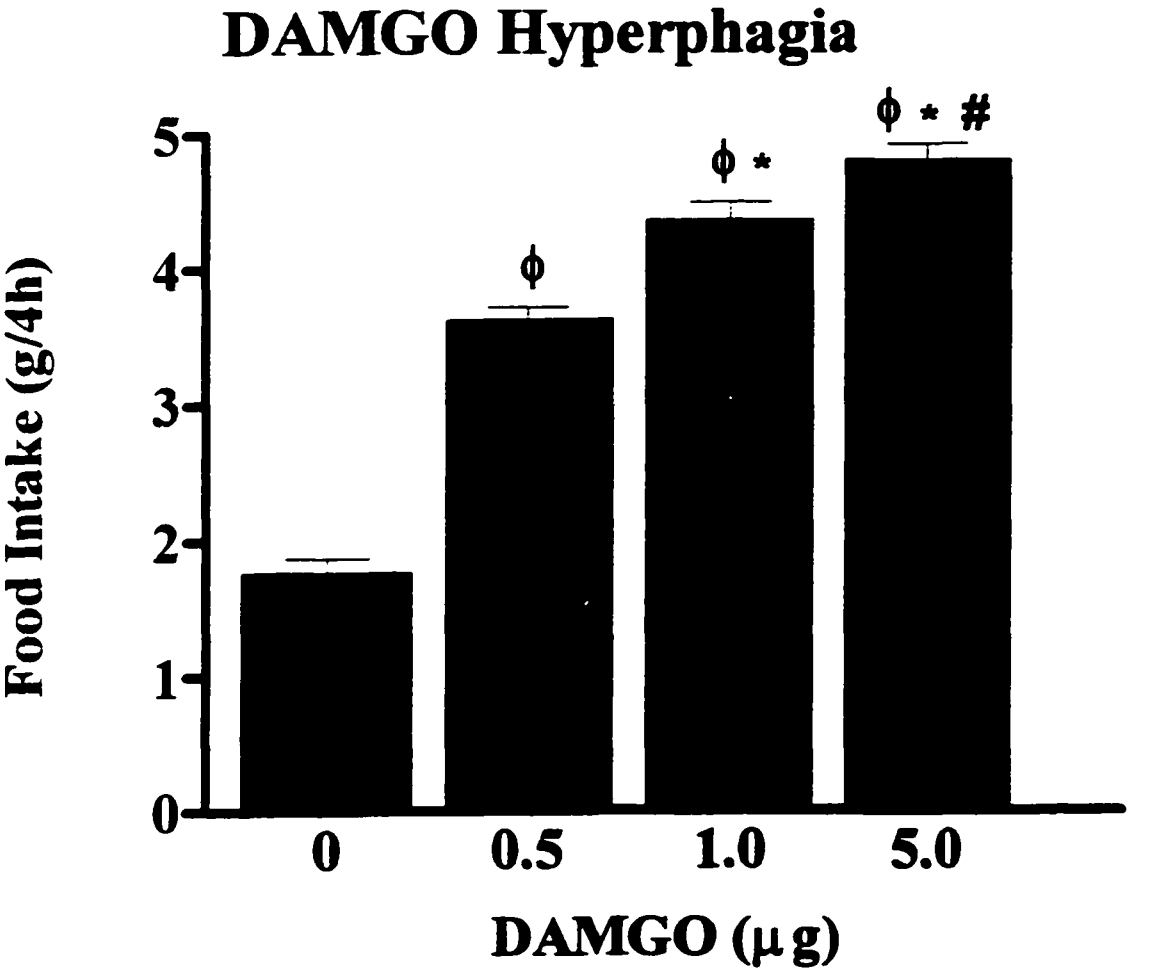
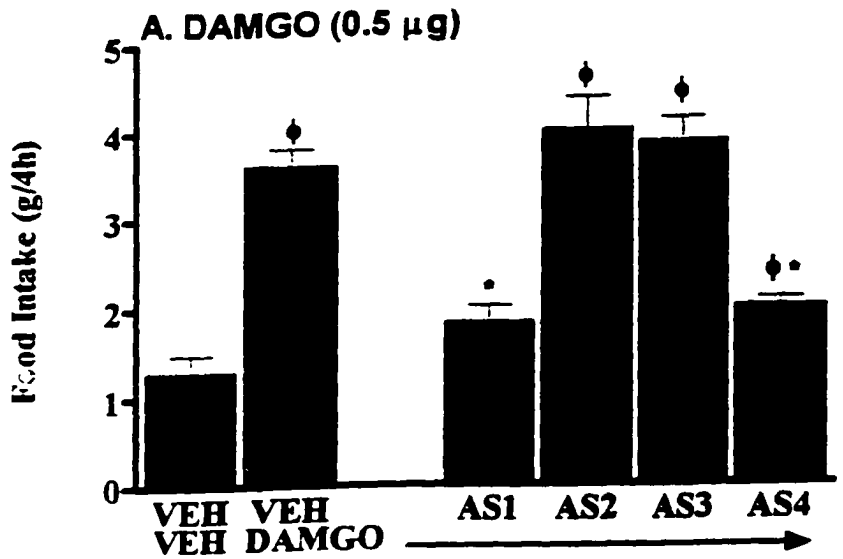
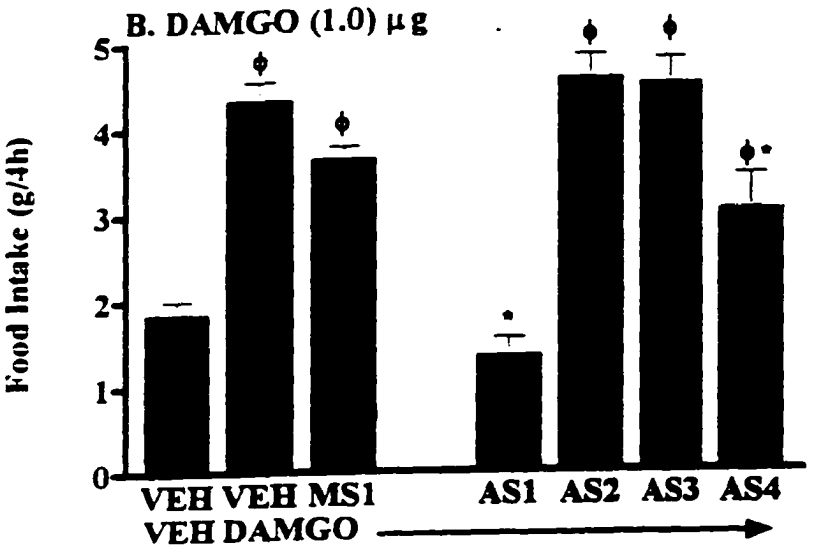
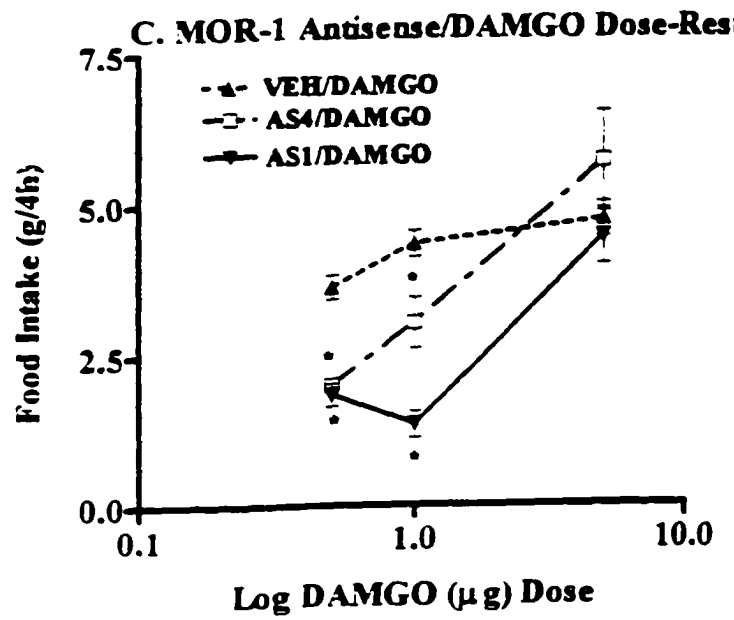


Figure 5. Alterations (Mean, ±SEM) in DAMGO-induced hyperphagia at doses of A) 0.5 or B) 1.0 μg following i.c.v. administration of either AS ODN probes directed against exons 1 (AS1), 2 (AS2), 3 (AS3), or 4 (AS4) of the MOR-1 clone or a missense probe (MS1). C) Alterations (Mean, ±SEM) in food intake as a function of the DAMGO dose (0.5-5.0 μg, i.c.v.) following administration of vehicle, or effective AS ODNs probes directed against exons 1 (AS1) or 4 (AS4) of the MOR-1 clone. Significant differences are denoted relative to either vehicle (VEH-VEH, phi) or VEH-DAMGO (*) values (Tukey comparisons, $p < 0.01$)







($F(4,17)= 15.93$, $p<.0001$) and 4 ($F= 21.74$, $p<.0001$) h. The 5.0 μg dose of DAMGO significantly increased intake after 2 and 4 h. In contrast to the differential effectiveness of AS ODNs upon DAMGO-induced hyperphagia at lower doses, none of the AS ODNs or MS ODN significantly altered hyperphagia induced by a 5 μg dose of DAMGO (Figure 5C).

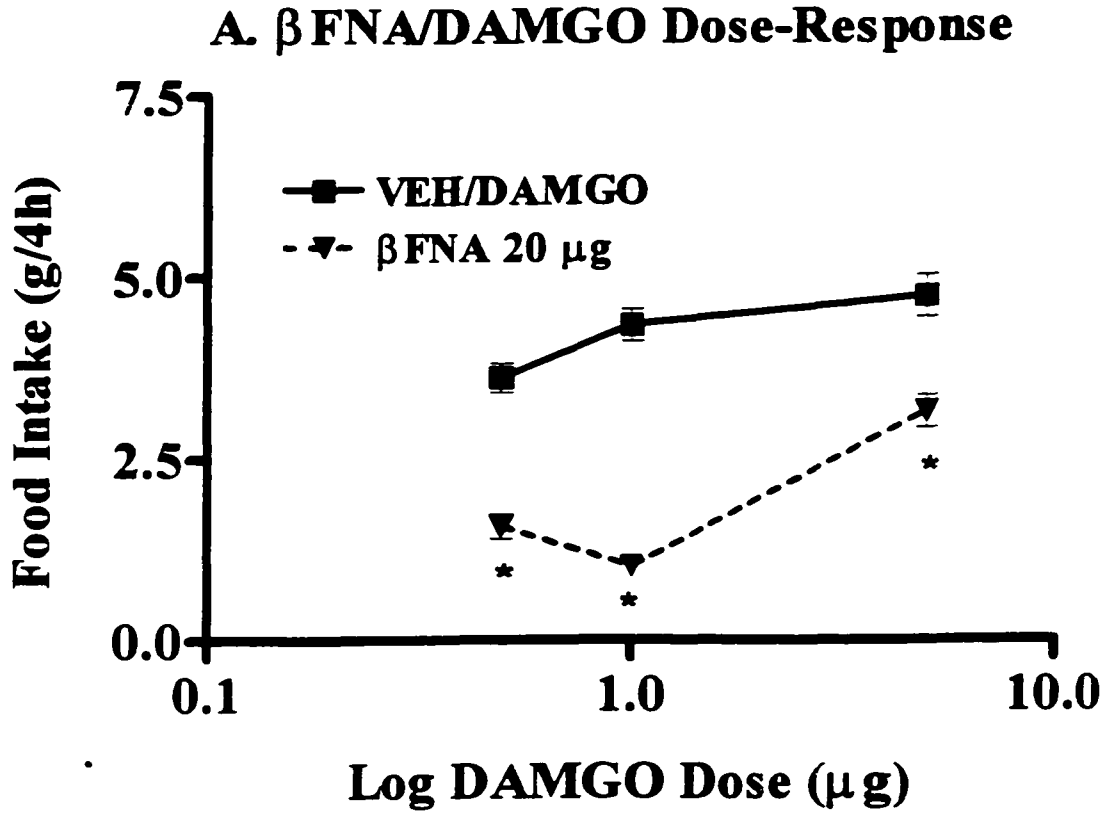
ßFNA and DAMGO-induced hyperphagia

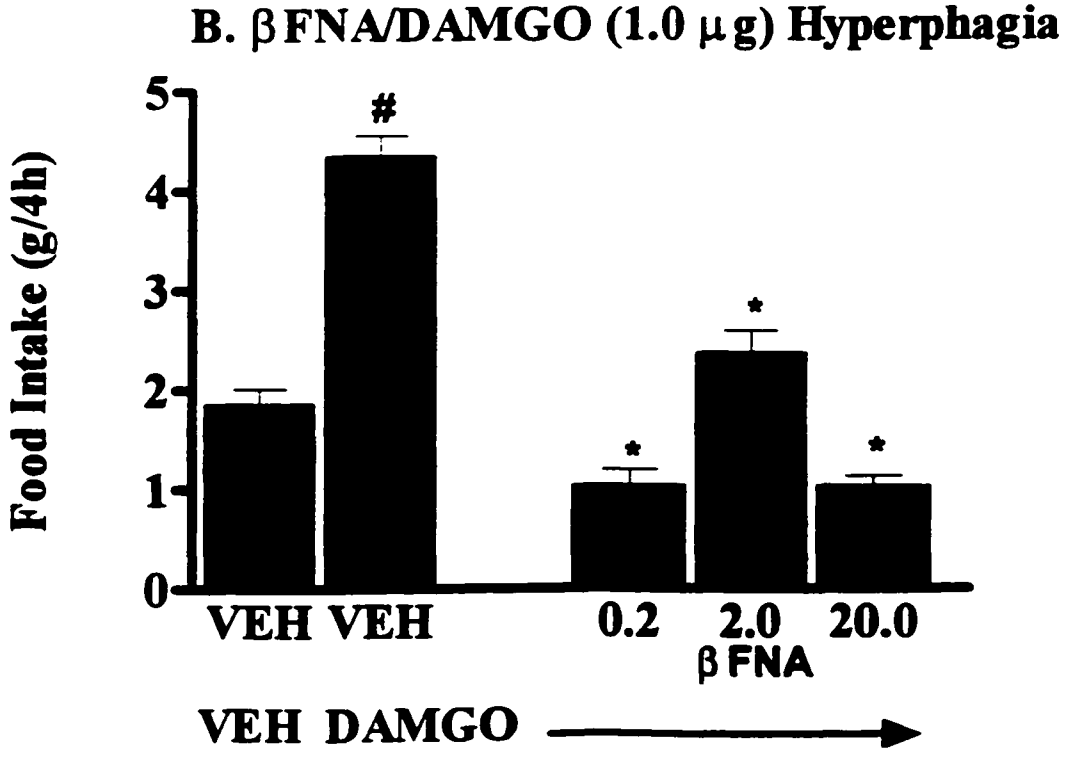
Significant differences were observed for DAMGO-induced hyperphagia among conditions for doses of 0.5 ($F(2,23)= 91.24$, $p<.0001$), 1.0 ($F(4,37)= 112.28$, $p<.0001$) and 5.0 ($F(2,17)= 31.53$, $p<.0001$) μg. A fixed 20 μg dose of ßFNA significantly and dose-dependently reduced DAMGO-induced hyperphagia at doses of 0.5 (88%), 1.0 (100%) and 5.0 (59%) μg (Figure 6A). Further, hyperphagia induced by a fixed 1.0 μg dose of DAMGO was significantly reduced by a dose range (0.2-20 μg) of ßFNA (80-100%: Figure 6B).

Discussion

The present findings confirmed the major hypotheses of this Specific Aim. First, specific AS ODNs directed against the MOR-1 clone blocked hyperphagia elicited by the μ agonist, DAMGO. In contrast, a MS ODN control failed to alter the magnitude of DAMGO-induced hyperphagia. Second, the observed pattern of AS ODNs directed against the MOR-1 clone were specific to the targeted regions of the clone in that AS ODNs directed against either exons 1 or 4 significantly reduced DAMGO-induced hyperphagia. In contrast, AS ODNs directed against either exons 2 or 3 of the MOR-1 clone failed to exert significant effects. This pattern of AS ODN effects for μ agonist-induced hyperphagia mirrored the pattern of AS ODN effectiveness observed for μ agonist-induced analgesia (Rossi et al., 1995a, 1995b, 1996, 1997). The present pattern of results for DAMGO-induced hyperphagia stands in marked contrast to reductions in spontaneous food intake and body weight following

Figure 6. A) Alterations (Mean, ±SEM) in the dose-response function of DAMGO-induced hyperphagia following pretreatment with a fixed (20 μg) dose of the μ opioid antagonist, ßFNA. B) Alterations (Mean, ±SEM) in DAMGO (1.0 μg)-induced hyperphagia following pretreatment with a dose (0.2-20 μg) range of ßFNA. Significant differences induced by ßFNA are denoted relative to either vehicle (VEH-VEH, #) or VEH-DAMGO (*) values (Tukey comparisons, $p < 0.01$).





AS ODNs directed against each of the four exons of the MOR-1 clone (Leventhal et al., 1996). Third, there are dose-dependent limitations to the AS ODN approach, such that effective AS ODNs reduced hyperphagia at DAMGO doses of 0.5 and 1 μg, but not at a higher 5 μg, dose. Hence, the presence or absence of AS ODN effects upon agonist-induced hyperphagia appears to depend upon the dose of the agonist employed as well as the efficacy of the AS ODN. This pattern can be explained by the modest (40%) reductions in receptor protein levels by AS ODN administration (see review: Pasternak and Standifer, 1995). Finally, the ability of the μ-selective antagonist, ßFNA to reduce DAMGO-induced hyperphagia confirmed previous findings (Levine et al., 1991). However, the magnitude of μ antagonist effects upon dose-response relationships for DAMGO-induced hyperphagia paralleled AS ODN effects such that the effectiveness of a fixed (20 μg) ßFNA dose to reduce DAMGO-induced hyperphagia declined as a function of increased DAMGO doses.

The reductions in DAMGO-induced hyperphagia by AS ODNs directed against only exons 1 or 4 parallel effects observed for μ analgesic actions, suggesting that a common splice variant of the MOR-1 clone may be mediating both analgesic and hyperphagic actions. It should be noted that inactivity of a particular AS ODN might result from a variety of technical factors, including unanticipated mRNA structures. This concern is alleviated by the fact that the inactive probes in the DAMGO-induced hyperphagia paradigm are active in reducing both M6G analgesia (Rossi et al., 1995a, 1995b, 1997) and spontaneous food intake and body weight (Leventhal et al., 1996). Further, although the same mRNA receptor substrate mediates both responses, the substrate is probably located in different supraspinal loci. Thus, μ-mediated analgesic responses are most potently elicited from the PAG, the rostral ventral medulla and the locus coeruleus (Bodnar, Paul and Pasternak, 1991; Fang and Fields, 1986; Smith, Perotti, Crisp, Cabral, Long and Scalziti, 1988). In contrast, μ-

mediated hyperphagic responses are most potently elicited from the PVN (Koch et al., 1995), the nucleus accumbens (Bakshi and Kelley, 1993; Bodnar et al., 1995; Cador, Kelley, LeMoal and Stinus, 1986; Majeed, Przewlocka, Wedzony and Przewlocka, 1986) and the ventral tegmental area (Mucha and Iversen, 1986; Noel and Wise, 1993, 1995). The differential actions of multiple MOR-1 splice variants may explain some of the different μ-mediated ingestive effects obtained using selective opioid antagonists. Thus, μ antagonism with ßFNA significantly reduces food intake under spontaneous, deprivation, glucoprivic and palatable conditions (see review: Bodnar, 1996). In contrast, μ_1 antagonism with naloxonazine significantly reduces food intake only under spontaneous and deprivation conditions.

Two possible profiles of AS ODN effects upon DAMGO-induced hyperphagia were hypothesized: the observed differential pattern described in the previous sections consistent with μ agonist-induced analgesia, and equal effectiveness of AS ODNs directed against each exon of the MOR-1 clone as observed in spontaneous intake and weight studies (see Chapter 3; Leventhal et al., 1996). That the former, but not latter hypothesis was confirmed strongly suggests that μ receptor mediation of μ agonist-induced hyperphagia and μ receptor mediation of spontaneous intake and weight are different. Spontaneous food intake is an outcome of multiple factors acting on organisms, and experimental alterations of any of them may increase feeding. For instance, μ opioid agonists increase feeding by altering the palatability of certain constituents of food, including either the macronutrient itself (e.g., fat: Marks-Kaufman, 1982; Marks-Kaufman and Kanarek, 1980), or the preference for a preferred macronutrient (Gosnell, Krahn and Majchrzak, 1990). It is conceivable that DAMGO elicits feeding by acting on such specific mechanisms, and that AS ODNs directed against either exons 1 and 4 block this effect. In contrast, body weight and spontaneous intake may be

influenced by additional factors mediated by all four exons of the MOR-1 clone. Such a distinction could not be made in traditional opioid antagonist studies. The μ antagonist, ßFNA significantly reduced both DAMGO-induced hyperphagia (Levine et al., 1991) and significantly reduced spontaneous intake and weight under both acute (Arjune et al., 1990; Ukai and Holtzman, 1988) and chronic (Cole et al., 1995) microinjection conditions. Therefore, the AS ODN approach appears to be a more precise tool in dissecting differences in the functional role of specific receptors. The selective actions of AS ODNs directed against different exons of the MOR-1 clone in reducing DAMGO-induced hyperphagia support the hypothesis that multiple splice variants of the MOR-1 clone exist raising the possibility of further opioid receptor subclassifications.

CHAPTER 5: SPECIFIC AIM THREE.

MOR-1 AS ODN Probes: Effects Upon M6G-induced Hyperphagia.

Introduction

In addition to the ability of endogenous opioid peptides and peptide analogues to stimulate food intake, morphine also produces a robust feeding response (see review: Gosnell and Levine, 1996). Morphine is rapidly metabolized and glucuronidated at both the three and six positions (Jaffe & Martin, 1985). Although M6G labels μ receptors with an affinity slightly less than morphine in binding assays (Paul et al., 1989), it is 100-fold more potent (Paul et al., 1989) centrally on both thermal (Abbott and Palmour, 1988; Pasternak et al., 1987; Shimomura, Kamata, Ueki, Oguri, Yoshimura and Tsukamato, 1971; Sullivan, McQuay, Bailey and Dickenson, 1989) and visceral (Frances et al., 1992) nociceptive tests than morphine. The present study, completed and is in press in The Journal of Pharmacology and Experimental Therapeutics, examined whether M6G, like its parent compound, morphine, produces long-acting (4 h) ingestive effects and examined the profile of M6G-induced hyperphagia following AS ODNs directed against the MOR-1 clone to determine whether the profile of AS ODNs was similar to that observed for spontaneous intake and body weight (Specific Aim One) or similar to that observed for M6G-induced analgesia.

Methods

Protocol 1. All rats in this and subsequent protocols were tested over a 4-10 day adaptation period at 3-9 h into the light cycle to insure stability of baseline spontaneous food intake during this phase of the light cycle. Preweighed pellets were placed on the floor of the wire mesh cages to optimize accessibility since this factor can interfere with opioid-induced feeding (see review: Gosnell and Levine, 1996). Cumulative intakes were assessed 1, 2 and 4 h after each condition, and were adjusted for spillage collected beneath each cage. After intake

stabilization, five rats received a vehicle condition (Veh, 5 μl, 0.9% normal saline, i.c.v). Since opioid agonists produce sedative and hypoactive effects (see review: Gosnell and Levine, 1996), each animal received two microinjections of M6G (500 ng, i.c.v.) without measuring intake. Then M6G doses of 10, 100, 500 and 1000 ng were administered to the five rats at weekly intervals, and food intake was assessed at 1, 2 and 4 h later. That dose of M6G which increased food intake to a similar degree as the μ agonist, DAMGO (1 μg, i.c.v) was chosen to allow comparison of antagonist and AS ODN effects relative to DAMGO-induced hyperphagia (Leventhal et al., 1997).

Protocol 2. Following stabilization of intake and adaptation to potential sedative and hypoactive effects of M6G, subgroups of rats were exposed to the following conditions at weekly intervals: control (5 μl, 0.9% normal saline, i.c.v., n=18), M6G (500 ng, n=18), ßFNA at doses of 0.4 (n=6), 4.0 (n=7) and 40 (n=7) nmol paired with M6G, NorBNI (40 nmol) paired with M6G (n=5), DALCE (40 nmol) paired with M6G (n=5), and NTII (40 nmol) paired with M6G (n=6). Cumulative food intake was assessed 4 h after vehicle or M6G administration since this interval significantly and reliably increased M6G-induced intake. Rats receiving specific antagonists were matched for intake elicited by M6G alone. An additional group of rats was evaluated to determine whether ßFNA (40 nmol) pretreatment altered intake (2 and 4 h) following vehicle treatment.

Protocol 3. All rats were stabilized for intake and adaptation to potential sedative and hypoactive effects of each of the following opioid agonists. In assessing MOR-1 AS ODN effects upon M6G-induced hyperphagia, subgroups of rats received: a) vehicle (5 μl, 0.9% normal saline, i.c.v., n=31), b) M6G (500 ng, i.c.v., n=31), AS ODNs (10 μg, 2 μl, i.c.v.) directed against exons c) 1 (AS1, n=6), d) 2 (AS2, n=7), e) 3 (AS3, n=6), or f) 4 (AS4, n=6) of the MOR-1 clone prior to M6G, and g) a MS ODN directed against exon 2 of the

MOR-1 clone (MS2, n=6) prior to M6G. In assessing MOR-1 AS ODN effects upon morphine-induced hyperphagia, subgroups of rats received: a) vehicle (n=16), b) morphine (5 μg, i.c.v., n=16), and AS ODNs directed against exons c) 1 (AS1, n=8) or d) 2 (AS2, n=8) of the MOR-1 clone prior to morphine. In assessing DOR-1 AS ODN effects upon hyperphagia induced by either M6G or deltorphin II, subgroups of rats received: a) vehicle (n=10), b) M6G (500 ng, n=5), c) deltorphin II (20 μg, n=4), and an AS ODN directed against exon 3 of the DOR-1 clone prior to either d) M6G (n=6) or e) deltorphin II (n=4). In assessing KOR-1 AS ODN effects upon hyperphagia induced by either M6G or U50,488H, subgroups of rats received: a) vehicle (n=10), b) M6G (500 ng, n=5), c) U50,488H (20 μg, n=5), and an AS ODN directed against exon 3 of the KOR-1 clone prior to either d) M6G (n=5) or e) U50,488H (n=5). In assessing KOR-3/ORL1 AS ODN effects upon hyperphagia induced by either M6G or OFQ/N, subgroups of rats received: a) vehicle (n=13), b) M6G (500 ng, n=6), c) OFQ/N (18 μg, n=7), and an AS ODN directed against exon 3 of the KOR-3/ORL1 clone prior to either d) M6G (n=6) or e) OFQ/N (n=7). AS ODNs were injection on days 1, 3, 5. Twenty-four h after the last AS ODN treatment (day 6), rats were microinjected with either M6G, morphine, deltorphin II or U50,488H, and food intake was assessed after 4 h. The chosen dose and intake interval for each of these agonists were based upon previous studies (e.g., Gosnell and Levine, 1996; Sanger and McCarthy, 1980; Yu et al., 1997). Intake was only measured after 2 h following OFQ/N given its shorter duration of action (Leventhal et al., 1998).

Results

M6G-induced hyperphagia

Significant differences in intake were observed across M6G doses after 2 ($F(4,20)=2.92$, $p<.047$) and 4 ($F=29.02$, $p<.0001$) h, but not after 1 h ($F=2.06$, n.s.). M6G

significantly and dose-dependently stimulated food intake following doses of either 100 ng after 4 h, 500 ng after 2 and 4 h, and 1000 ng after 2 and 4 h (Figure 7). In contrast, the 10 ng dose of M6G failed to alter intake at any time interval. Since the 500 ng dose of M6G produced the most comparable increase in intake relative to those doses of DAMGO used in a prior AS ODN study (Leventhal et al., 1997), this dose was employed in antagonist and AS ODN paradigms.

Opioid antagonists and M6G-induced hyperphagia

Significant differences in intake were observed among equimolar antagonist conditions relative to vehicle and M6G treatment alone ($F(5,53)= 15.58$, $p< .0001$). The significant increase in food intake following M6G after 4 h was eliminated by pretreatment with a 40 nmol dose of the μ antagonist, ßFNA (Figure 8A). In contrast, equimolar doses of either δ_1, δ_2 or κ_1 opioid antagonists failed to alter M6G-induced hyperphagia. In assessing the dose-dependent effects of ßFNA upon M6G-induced hyperphagia, significant differences were observed among conditions ($F(4,37)= 7.92$, $p< .0001$). M6G-induced hyperphagia was significantly reduced by the 40, but not by either the 0.4 or 4 nmol doses of ßFNA (Figure

MOR-1 AS ODN treatment and M6G-induced hyperphagia

Significant differences in intake were observed among AS ODN and MS ODN conditions relative to vehicle and M6G treatment alone ($F(6,84)= 15.18$, $p< .0001$). M6G-induced hyperphagia was differentially affected by MOR-1 AS ODN pretreatment such that it was significantly reduced by AS ODNs directed against either exons 2 (AS2: 66%) or 3 (AS3: 68%) of the MOR-1 clone (Figure 9A). In contrast, AS ODNs directed against either exons 1 (AS1) or 4 (AS4) of the MOR-1 clone failed to significantly affect M6G-induced hyperphagia. Further, the MS ODN control in which three bases had been changed in the effective exon 2 AS ODN sequence (Table 3), failed to significantly alter M6G-induced hyperphagia (Figure

Figure 7. Alterations (Mean, $\pm$ SEM) in cumulative food intake following i.c.v administration of the selective morphine metabolite, M6G. Significant differences are denoted relative to corresponding vehicle control values (*) (Tukey comparisons, $p < .05$).

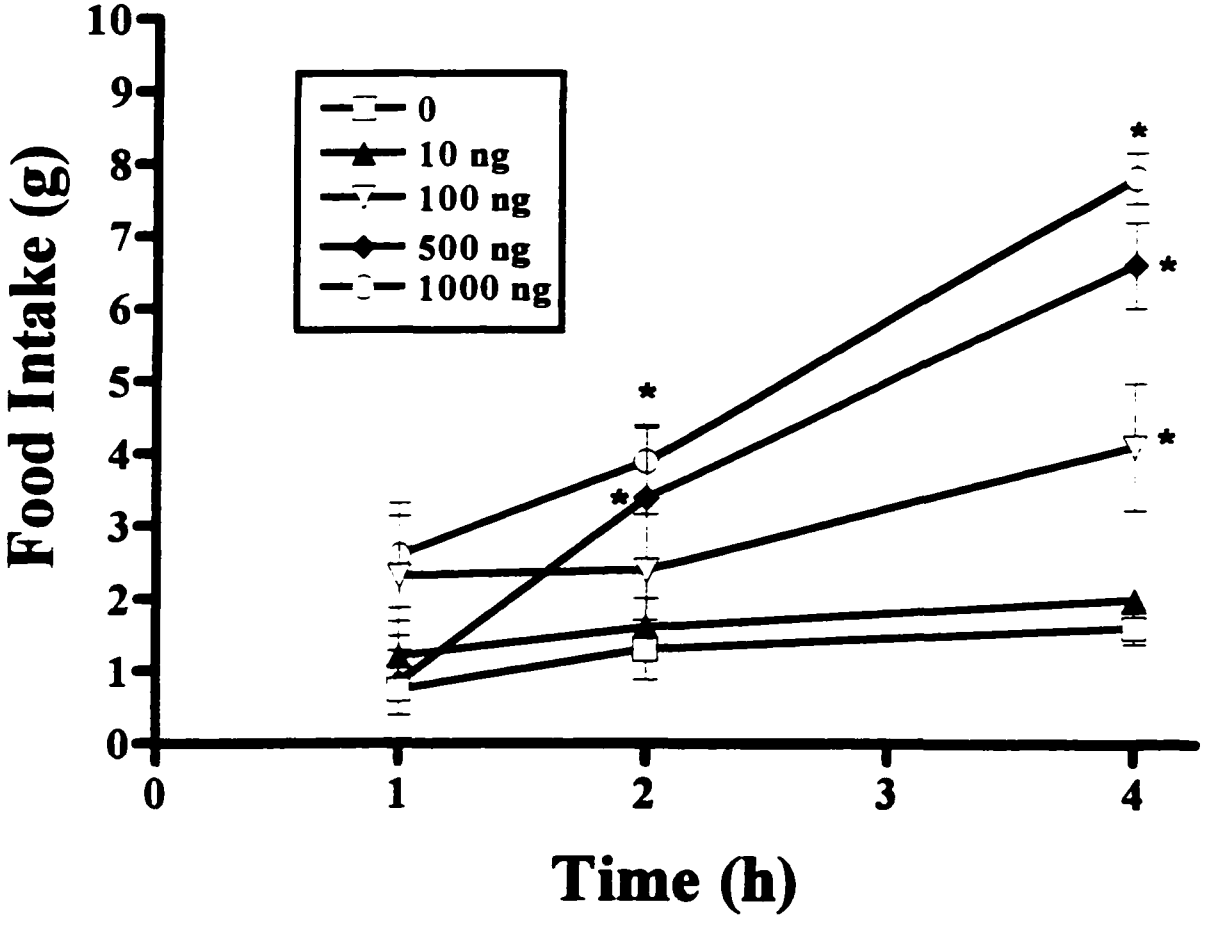
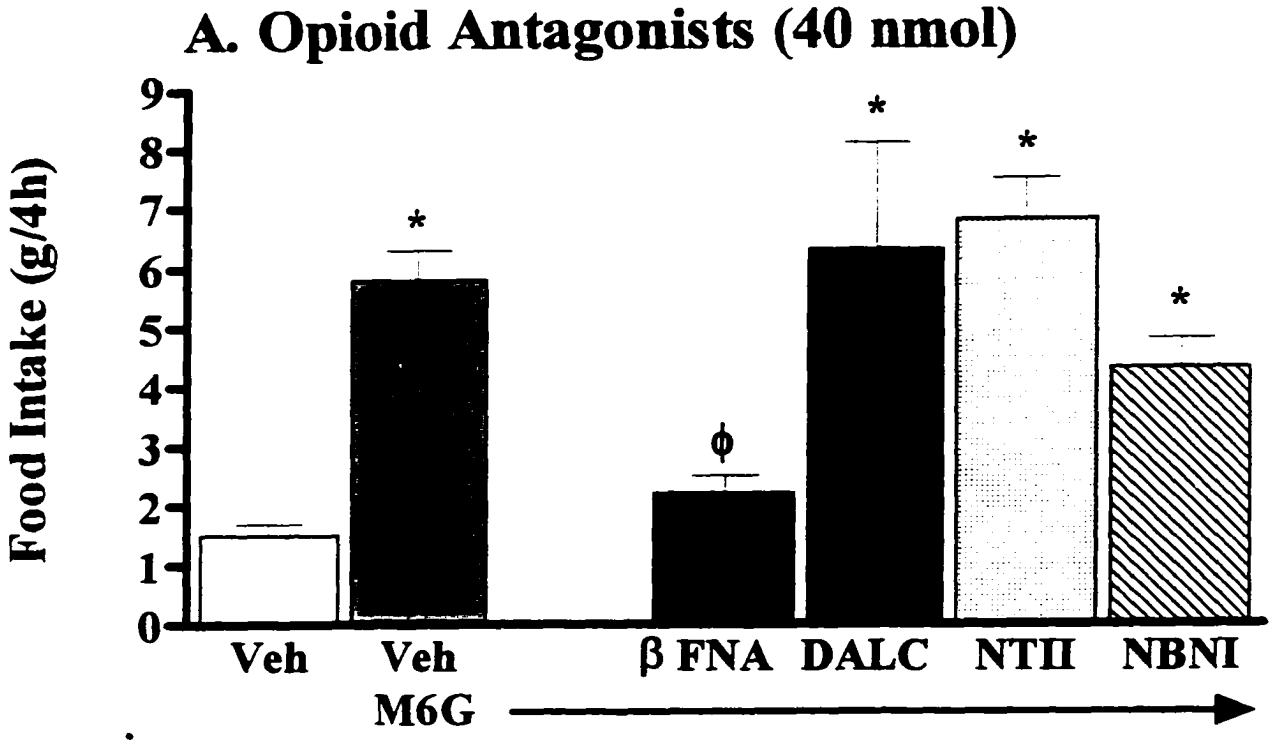


Figure 8. A) Alterations (Mean, ±SEM) in M6G (500 ng)-induced hyperphagia (4h) following central pretreatment with an equimolar dose (40 nmol, i.c.v) of either μ (ßFNA), δ_1 (DALC), δ_2 (NTII) or κ_1 (NBNI) opioid antagonists. B) Alterations (g, ±SEM) in M6G-induced hyperphagia (4h) in rats following central pretreatment with a dose-range (0.4-40 nmol, i.c.v) of ßFNA. Significant differences are denoted relative to either corresponding vehicle control values (*) or significant decreases relative to Veh/M6G values (phi) (Tukey comparisons, $p < .05$).

8B). The 40 nmol dose of ßFNA failed to significantly alter intake following vehicle treatment ($t(10) = 1.51$, n.s.; data not shown).



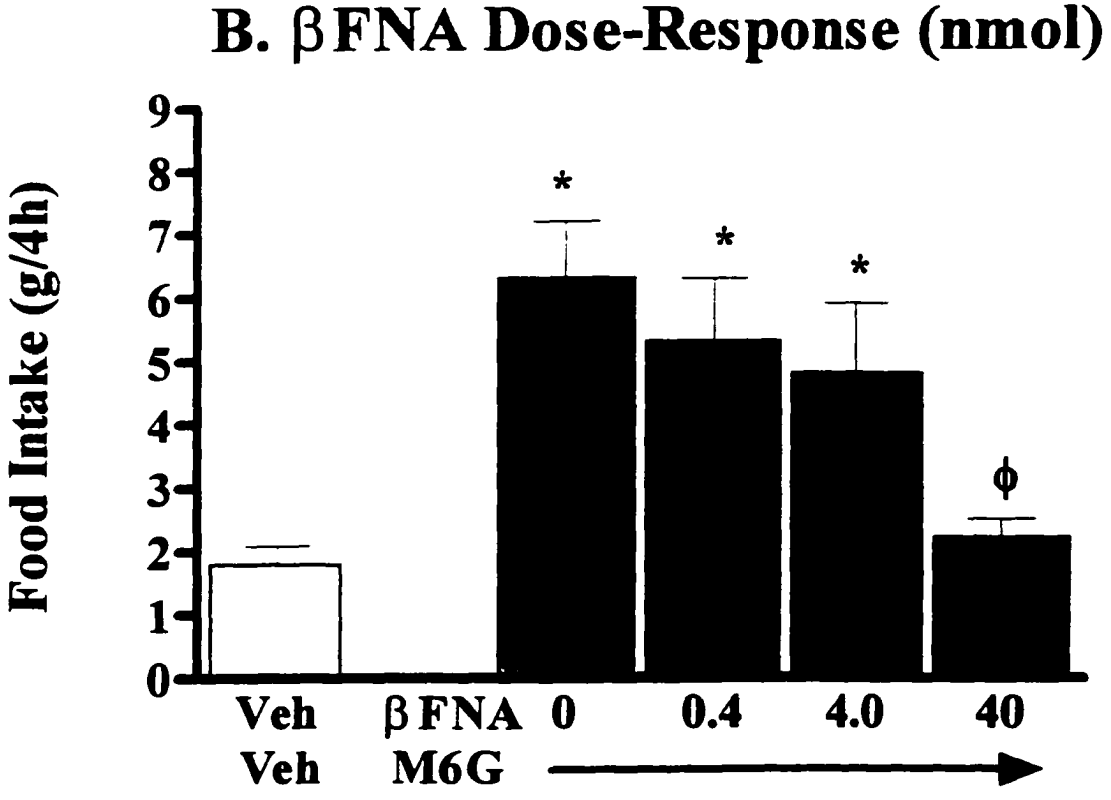
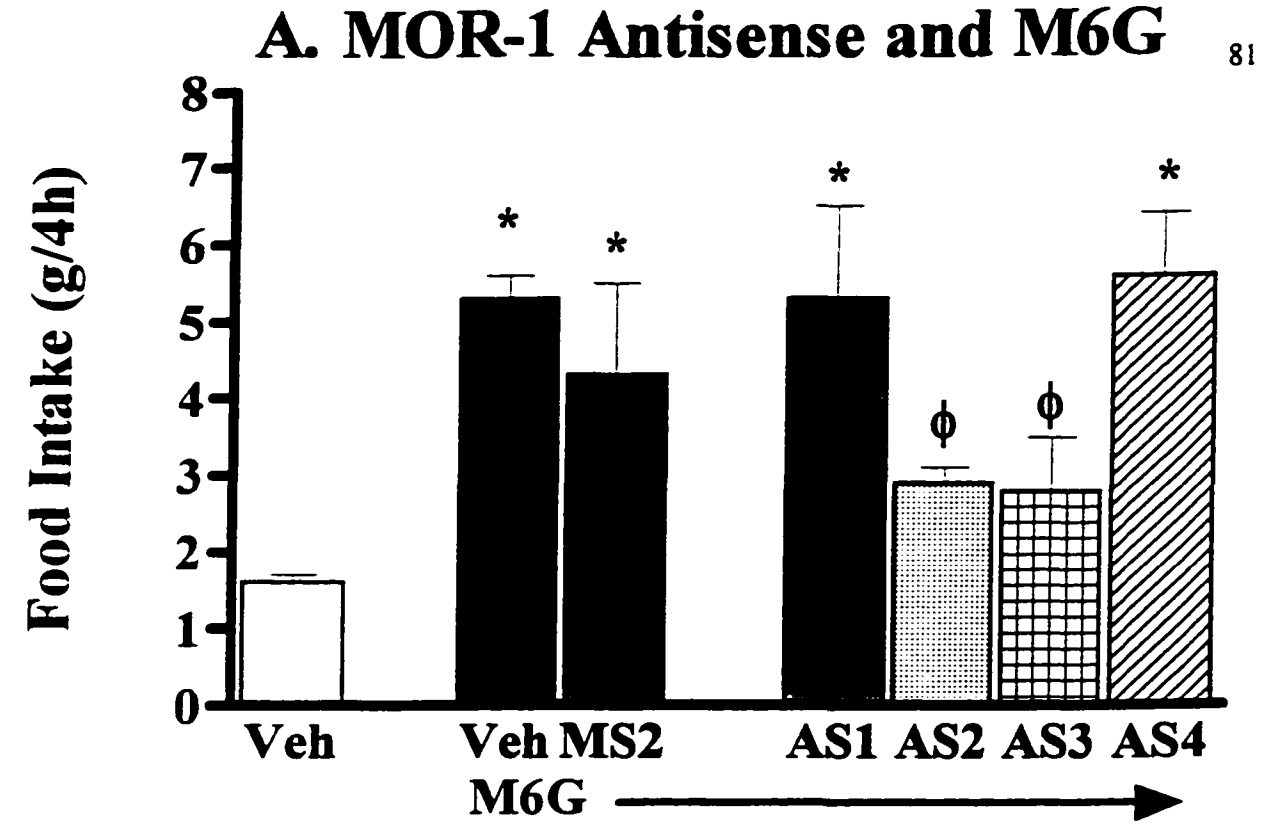
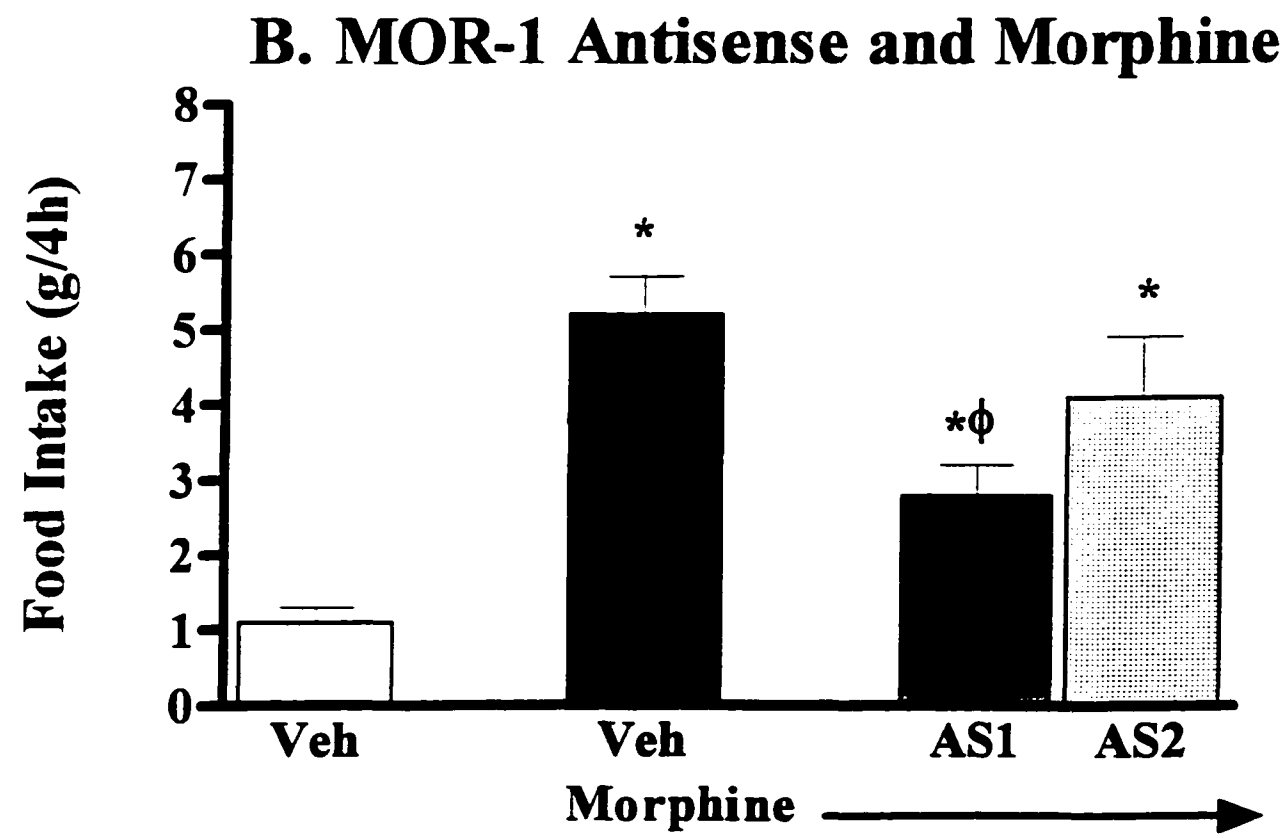


Figure 9. A) Alterations (Mean, ±SEM) in M6G-induced hyperphagia (4h) following central pretreatment with AS ODN probes directed against either exons 1 (AS1), 2 (AS2), 3 (AS3), or 4 (AS4) of the MOR-1 clone, or a missense probe (MS2). B) Alterations (Mean, ±SEM) in morphine (5 μg, i.c.v.)-induced hyperphagia (4h) following central pretreatment with either AS ODN probes directed against exons 1 (AS1) or 2 (AS2) of the MOR-1 clone. Significant differences are denoted relative to either corresponding vehicle control values (*) or significant decreases relative to either Veh/Morphine values (phi) (Tukey comparisons, $p < .05$).





9A).

MOR-1 AS ODN treatment and morphine-induced hyperphagia

Significant differences in intake were observed among AS ODN conditions relative to vehicle and morphine treatment alone ($F(3,27)= 14.79$, $p<.0001$). Morphine-induced hyperphagia was differentially affected by MOR-1 AS ODN pretreatment such that it was significantly reduced by the AS ODN directed against exon 1 (AS1: 59%), but not exon 2 (AS2) of the MOR-1 clone (Figure 9B). This pattern of effects coincides with MOR-1 AS ODN effects upon DAMGO-induced hyperphagia (Leventhal et al., 1997), and is distinct from that pattern observed for M6G-induced hyperphagia.

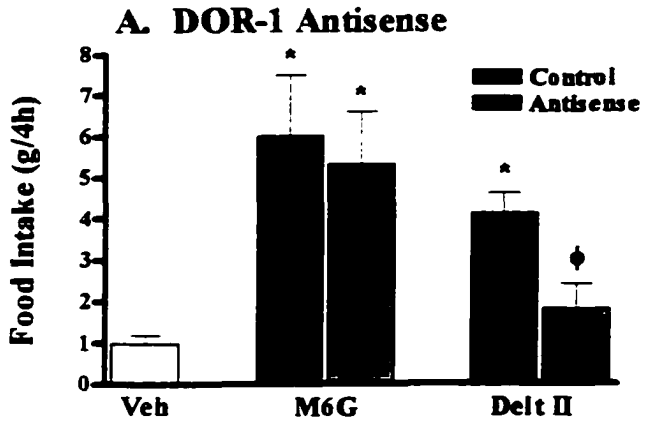
DOR-1 AS ODN treatment, M6G and deltorphin II

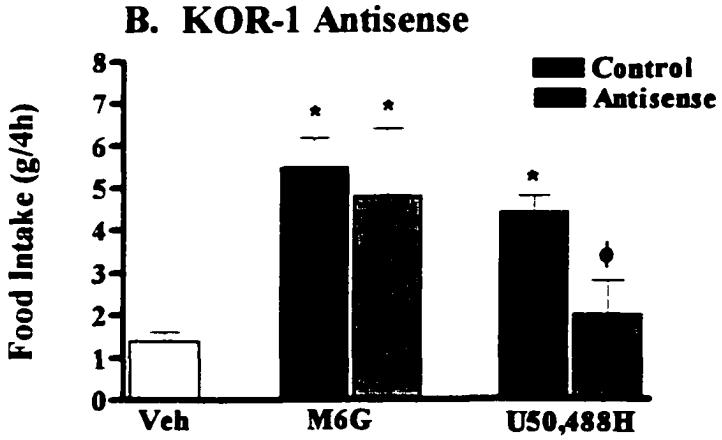
In assessing DOR-1 AS ODN effects upon M6G-induced hyperphagia, significant differences in intake were observed among conditions ($F(2,19)= 9.75$, $p<.0012$). An AS ODN directed against exon 3 of the DOR-1 clone failed to significantly alter M6G-induced hyperphagia (Figure 10A). To establish the activity of this probe, its effects upon deltorphin II-induced hyperphagia were evaluated. The significant difference in intake among conditions ($F(2,15)= 15.47$, $p<.0002$) revealed that deltorphin II-induced hyperphagia was significantly reduced by 77% following the AS ODN probe directed against exon 3 of the DOR-1 clone (Figure 10A).

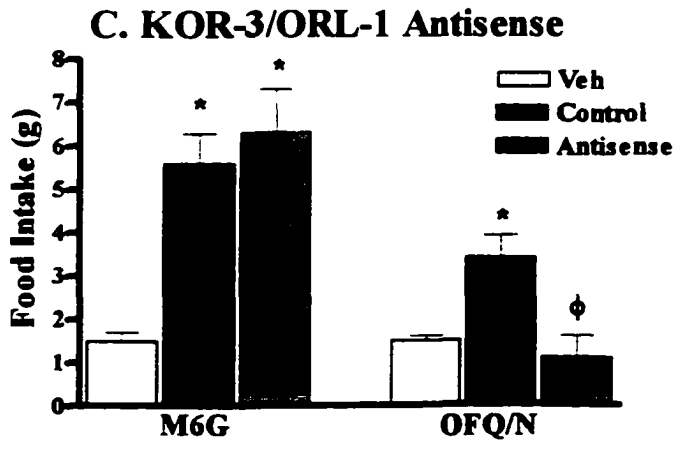
KOR-1 AS ODN treatment, M6G and U50,488H

In assessing KOR-1 AS ODN effects upon M6G-induced hyperphagia, significant differences in intake were observed among conditions ($F(2,17)= 9.29$, $p<.0019$). An AS ODN directed against exon 3 of the KOR-1 clone failed to significantly alter M6G-induced hyperphagia (Figure 10B). To establish the activity of this probe, its effects upon U50,488H-induced hyperphagia were evaluated. The significant difference in intake among conditions

Figure 10. A) Alterations (Mean, ±SEM) in hyperphagia (4 h) induced by M6G (500 ng, i.c.v.) or the selective δ_2 opioid agonist, deltorphin II (Delt II) (20 μg, i.c.v) following central pretreatment with an AS ODN probe directed against exon 3 of the DOR-1 clone. B) Alterations (Mean, ±SEM) in hyperphagia (4 h) induced by M6G or the selective κ_1 opioid agonist, U50,488H (20 μg, i.c.v) following central pretreatment with an AS ODN probe directed against exon 3 of the KOR-1 clone. C) Alterations (Mean, ±SEM) in hyperphagia induced by M6G (4 h) or the selective endogenous peptide for the KOR-3/ORL-1 clone, OFQ/N (18 μg, i.c.v, 2 h) following central pretreatment with an AS ODN probe directed against exon 3 of the KOR-3/ORL-1 clone. Significant differences are denoted relative to either corresponding vehicle control values (*) or significant decreases relative to Veh/agonist values (phi) (Tukey comparisons, $p < .05$).







($F(2,17) = 12.31$, $p < .0005$) revealed that U50,488H-induced hyperphagia was significantly reduced by 82% following the AS ODN probe directed against exon 3 of the KOR-1 clone (Figure 10B).

KOR-3/ORL-1 AS ODN treatment, M6G and OFQ/N

In assessing KOR-3/ORL-1 AS ODN effects upon M6G-induced hyperphagia, significant differences in intake were observed among conditions ($F(2,22) = 30.69$, $p < .0001$). An AS ODN directed against exon 3 of the KOR-3/ORL-1 clone failed to significantly alter M6G-induced hyperphagia (Figure 10C). To establish the activity of this probe, its effects upon OFQ/N-induced hyperphagia were evaluated. The significant difference in intake among conditions ($F(2,24) = 12.96$, $p < .0002$) revealed that OFQ/N-induced hyperphagia was eliminated following the AS ODN probe directed against exon 3 of the KOR-3/ORL-1 clone (Figure 10C).

Discussion

The present findings confirmed that, centrally-administered M6G significantly and dose-dependently increased spontaneous food intake, and this is the first reported observation of a hyperphagic response elicited by this morphine metabolite. The increased intake was gradual, occurring 2 and 4 h after M6G administration, but failing to increase intake after 1 h. This temporal pattern of ingestive effects is commonly observed following administration of such other opiates as heroin, butorphanol, codeine and levorphanol (see review: Gosnell and Levine, 1996). M6G also produced clear dose-dependent actions with low (10 ng) doses failing to increase intake, and higher doses (100-1000 ng) systematically increasing intake. The effective dose range of M6G to induce feeding is more than 10-fold lower than comparable morphine doses following ventricular administration (see review: Gosnell and Levine, 1996). Thus, the relationship between the respective potencies of M6G-induced

hyperphagia relative to morphine-induced hyperphagia is similar to the relationship between their respective potencies to elicit analgesic responses (Abbott and Palmour, 1988; Frances et al., 1992; Paul et al., 1989; Shimomura et al., 1971; Sullivan et al., 1989). These behavioral and functional differences appear to persist even though M6G labels μ receptors with an affinity slightly less than morphine in binding assays (Paul et al., 1989).

Selective opioid antagonists differentially altered the magnitude of M6G-induced hyperphagia. The selective, irreversible μ opioid receptor antagonist, ßFNA, significantly and dose-dependently decreased M6G-induced hyperphagia, and almost blocked its expression following the highest antagonist dose. ßFNA exerted this inhibitory action upon M6G-induced hyperphagia without altering the low levels of spontaneous intake following vehicle treatment. M6G-induced hyperphagia was unaffected by pretreatment with an equimolar dose of the κ_1 opioid receptor antagonist, NorBNI which distinguishes this ingestive response from other forms of μ-mediated hyperphagia (Levine et al., 1990, 1991). Further, an equimolar dose of either δ_1 (DALCE) or δ_2 (NTII) opioid receptor antagonists failed to alter the magnitude of M6G-induced hyperphagia. These data strongly suggest that M6G-induced hyperphagia is acting through selective activation of pharmacologically-characterized μ opioid receptors.

The differences in the mediation of M6G-induced hyperphagia relative to DAMGO-induced and morphine-induced hyperphagia were characterized further by our AS ODN studies. In Specific Aim One, spontaneous intake and body weight were significantly reduced by AS ODNs directed against each of the four exons of the MOR-1 clone. Rossi and co-workers (1995a, 1995b, 1997a) demonstrated that the actions of AS ODNs directed against either exons 2 or 3 of the MOR-1 clone which blocked M6G-induced analgesia were distinct from the actions of AS ODNs directed against either exons 1 or 4 of the MOR-1 clone, which

blocked analgesia elicited by morphine and DAMGO. Specific Aim Two found that DAMGO-induced hyperphagia displays an identical pattern of sensitivity to AS ODNs directed against the MOR-1 clone to that observed for morphine and DAMGO-induced analgesia. The present study found that M6G-induced hyperphagia was reduced by AS ODNs directed against either exon 2 or exon 3 of the MOR-1 clone, while AS ODNs directed against either exons 1 or 4 of this clone were ineffective. The pattern of MOR-1 AS ODN effects upon M6G-induced hyperphagia was specific to the AS ODN sequence since a MS ODN which differed from an effective AS ODN probe by changing the order of three nucleotide bases, failed to alter M6G-induced hyperphagia. The present study further demonstrated that the pattern of MOR-1 AS ODN effects upon morphine-induced hyperphagia was identical to that observed for DAMGO-induced hyperphagia, and distinct from the hyperphagic responses of its active metabolite, M6G.

Despite these important dissociations and the positive controls employed in *vivo* testing, it is important to determine whether there are changes in the transcriptional and translational products of the genes in question. However, there are crucial limitations in discerning whether the binding of DAMGO or morphine relative to M6G *in vitro* are altered by the different AS ODN treatments. The levels of high affinity M6G binding in the brain is only 10% of total μ opioid receptor binding (Brown, Yang, Ouerfelli, Standifer, Byrd and Pasternak, 1997b). Therefore, even in the event that a particular MOR-1 AS ODN treatment (e.g., exon 2 or exon 3) completely eliminated M6G binding, detection of such changes would be difficult as a function of total μ opioid receptor binding. Further, AS ODN administration generally only produces modest (40%) reductions in receptor protein levels for opioid receptors (Pasternak and Standifer, 1995), thus making the changes in M6G binding by AS ODN treatment even more difficult to detect. Another possibility for such differences

may be due to changes in signalling for G-protein coupling for one ligand relative to the other. Although there is differential blockade of opioid analgesia by AS ODNs directed against various G-protein subunits (Standifer et al., 1996), it is not known whether these substrates mediate the observed effects. These provisos need to be considered despite our *in vivo* positive controls. However, these data appear to provide converging evidence for μ (MOR-1) mediation of M6G-induced hyperphagia, and the exons subserving this response are both distinct from traditional μ agonists in both analgesic and hyperphagic assays, yet identical to those exons subserving M6G-induced analgesia.

The involvement of opioid receptor clones in mediating M6G-induced hyperphagia was limited to the MOR-1 clone since AS ODN probes directed against either the DOR-1, KOR-1 or KOR-3/ORL1 clones failed to alter M6G-induced hyperphagia. The present study demonstrated conclusively that the failure of these probes was due to their lack of inherent involvement in M6G-induced hyperphagia, and not because these probes lacked intrinsic activity in hyperphagic assays. Thus, an AS ODN directed against exon 3 of the DOR-1 clone significantly reduced hyperphagia induced by the selective δ_2 opioid agonist, deltorphin II. Such inhibition is of interest because it has been suggested that the DOR-1 clone gene encodes the pharmacologically-characterized δ_2 opioid receptor subtype. This DOR-1 AS ODN effect parallels actions observed in analgesic assays for deltorphin II (Rossi et al., 1997b). Further, an AS ODN directed against exon 3 of the KOR-1 clone significantly reduced hyperphagia induced by the selective κ_1 opioid agonist, U50,488H. This KOR-1 AS ODN effect parallels actions observed in analgesic assays for U50,488H (Chien et al., 1994). Finally, an AS ODN directed against exon 3 of the KOR-3/ORL1 clone significantly reduced hyperphagia induced by the non-traditional opioid peptide, OFQ/N (Meunier et al., 1995; Reinscheid et al., 1995). OFQ/N has little affinity for traditional opioid receptors and its

actions through the KOR-3/ORL1 clone has been confirmed in both hyperphagic and analgesic assays (Leventhal, Mathis, Rossi, Pasternak and Bodnar, 1998; Rossi et al., 1998). It should be noted that whereas effective doses of each of these agonists elicit significant hyperphagia in each animal, the magnitude of some of these agonists were not equivalent to that induced by M6G. Clear dose-response relationships are often problematic for opioid-induced hyperphagia since they produce sharp step-wise functions (Gosnell and Levine, 1996). Yet these data provide novel evidence indicating that hyperphagic responses induced by deltorphin II, U50,488H and nociceptin are mediated respectively by the DOR-1, KOR-1 and KOR-3/ORL1 clones. These data further indicate the selectivity of μ and MOR-1 opioid actions in the mediation of M6G-induced hyperphagia.

It is now clear that a single receptor is not responsible for the common drug actions of morphine and M6G across a number of functional assays. Specifically, the similarity in the AS ODN profile of the hyperphagic and analgesic responses to M6G, relative to the AS ODN profile of the hyperphagic and analgesic responses to traditional μ receptor agonists lend credence to the concept of a novel M6G receptor. The persistence of M6G-induced analgesia in μ-deficient CXBK mice, morphine-tolerant mice, and transgenic mice with disruption of exon 1 of the MOR-1 gene provides further support for the existence of a novel M6G receptor (Brown et al., 1997a, 1997b; Rossi et al., 1996; Schuller et al., 1997). Such a receptor could conceivably result from alternative splice variants of the MOR-1 clone, although a distinct gene cannot be ruled out.

CHAPTER 6: SPECIFIC AIM FOUR.

KOR-3/ORL-1 AS ODN Probes: Effects Upon OFQ/N-induced Hyperphagia.

Introduction

OFQ/N, like other opioid peptides, stimulates naloxone-reversible food intake following ventricular, hypothalamic and accumbens administration (Pomonis et al., 1996; Stratford et al., 1997). The lack of a selective antagonist for the KOR-3/ORL-1 receptor makes it difficult to assert that this receptor mediates OFQ/N-induced hyperphagia. Since OFQ/N-induced analgesia is reduced by AS ODNs targeted against either exons 2 or 3 of the KOR-3/ORL-1 clone in rat (Rossi et al., 1998), the present study, completed and published in European Journal of Pharmacology (1998) 349: R1-R3, examined the profile of OFQ/N-induced hyperphagia following AS ODNs directed against the KOR-3/ORL-1 clone.

Methods

KOR-3/ORL-1 AS ODNs and a MS ODN were synthesized as previously described and purified for the first (AS1), second (AS2) and third (AS3) coding exons (See Table 3). As previously described, spontaneous food intake (6-8 h of the light cycle) was assessed following OFQ/N (0, 0.5, 1, 10 nmol, n=6/8). Rats were evaluated for eating latency (0-15 min), and cumulative intake (0.5, 1, 2 h). Four groups then received AS1 (n=8), AS2 (n=7), AS3 (n=7) or MS3 (n=7) against the KOR-3/ORL-1 clone on days 1, 3, and 5. On day 6, all rats received OFQ/N (10.0 nmol) with intake and latency reassessed over 2 h. Three additional groups received naltrexone (0.1, 1, 10 μg, n=6/8 each) 15 min prior to OFQ/N (10.0 nmol) with intake and latency reassessed over 2 h.

Results

Significant differences were observed among OFQ/N doses after 0.5 ($F(3,33)=17.46$, $p<.0001$), 1 ($F=17.66$, $p<.0001$) and 2 ($F=14.94$, $p<.0001$) h for intake and

latency ($F = 29.71$, $p < .0001$). OFQ/N increased intake 3-5 fold, and decreased latency (3-5 min). Significant differences were observed among vehicle and AS ODN treatments after 0.5 ($F(5,125) = 56.25$, $p < .0001$), 1 ($F = 36.23$, $p < .0001$) and 2 ($F = 28.93$, $p < .0001$) h for intake and latency ($F = 41.44$, $p < .0001$). OFQ/N-induced hyperphagia was significantly reduced by AS ODNs directed against either exons 1 (AS1: 71%), 2 (AS2: 86%) or 3 (AS3: 100%) of the KOR-3/ORL-1 clone, but not against the MS ODN (Figure 11). Significant decreases in eating latency by OFQ/N was blocked by each of the KOR-3/ORL-1 AS ODNs, but not by the MS ODN. Significant differences were observed among vehicle and naltrexone doses after 0.5 ($F(4,48) = 21.81$, $p < .0001$), 1 ($F = 13.02$, $p < .0001$) and 2 ($F = 11.13$, $p < .0001$) h for intake and latency ($F = 27.40$, $p < .0001$). OFQ/N-induced hyperphagia was significantly reduced by the 10, but not the 0.1 or 1.0 μg doses of naltrexone (Figure 12). Decreased eating latency by OFQ/N was blocked by naltrexone.

Discussion

Increased food intake following OFQ/N and its naltrexone sensitivity confirms previous findings (Pomonis et al., 1996; Stratford et al., 1997). AS ODN mapping of the KOR-3/ORL-1 clone revealed that OFQ/N-induced hyperphagia is blocked by probes targeting each of the three exons. These effects are specific given the ineffectiveness of the MS ODN in which the order of three pairs of bases from the AS ODN sequence had been switched. These data suggest that the receptor responsible for OFQ/N-induced hyperphagia

Figure 11. Alterations in food intake (Mean, ±SEM) following i.c.v. administration of OFQ/N in rats receiving AS ODN probes directed against either exons 1 (AS1), 2 (AS2), 3 (AS3), of the ORL-1/KOR-3 clone or a missense probe (MS3). Significant differences are denoted relative to either vehicle (VEH) treatment (#) or relative to OFQ/N treatment (*) (Tukey comparisons, ($p < .01$).

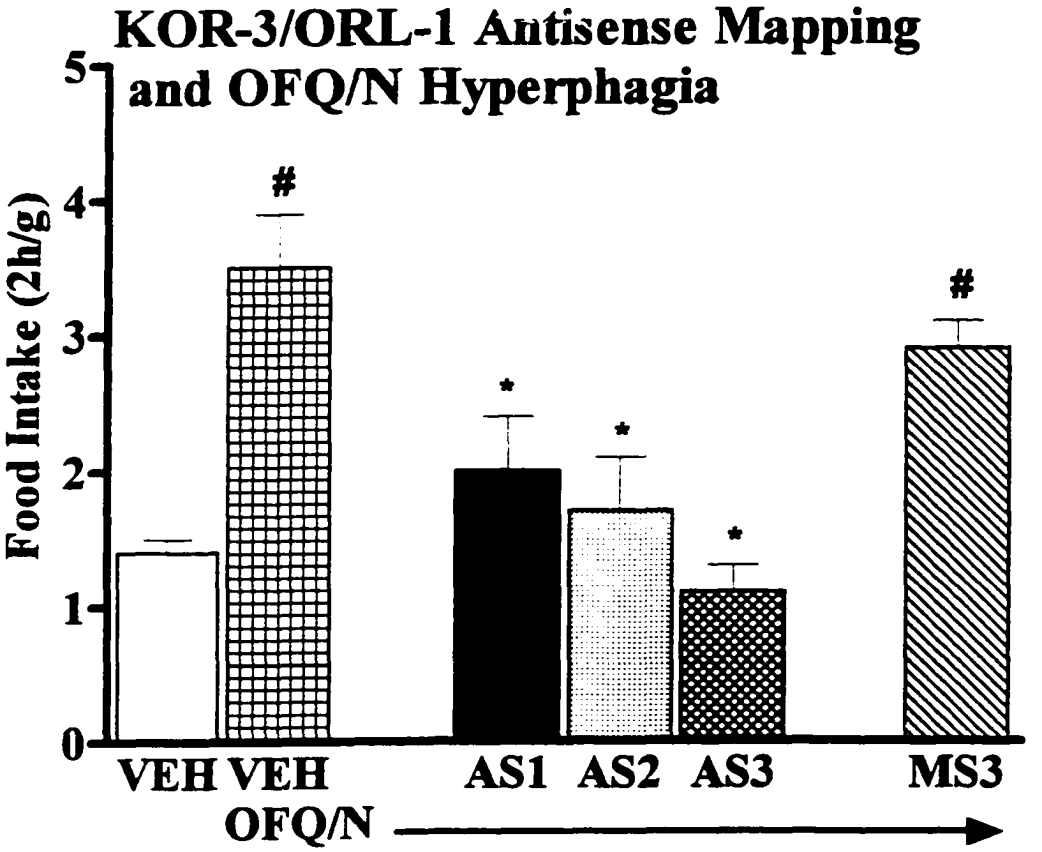
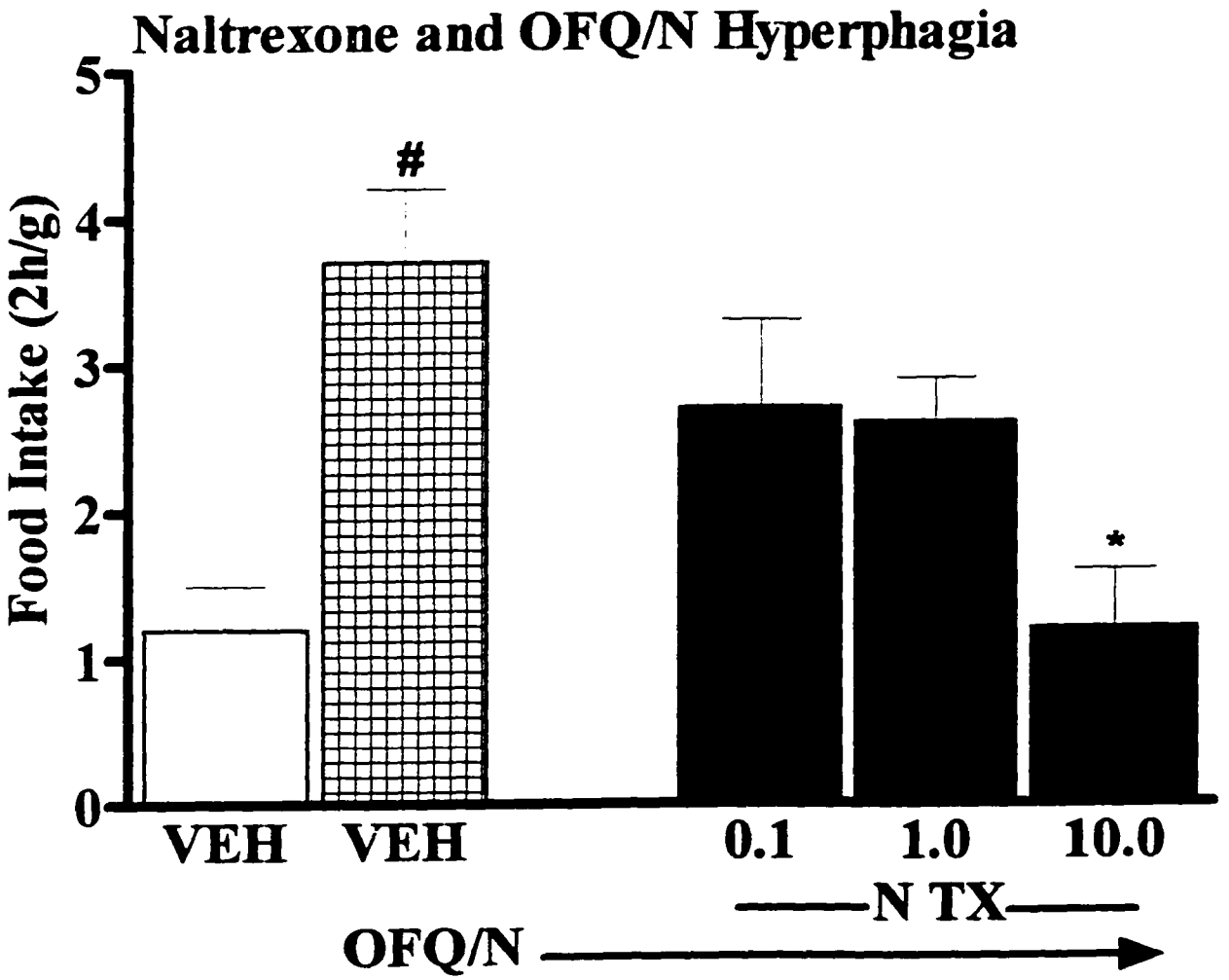


Figure 12. Alterations in food intake (Mean, ±SEM) following i.c.v. administration of OFQ/N in rats receiving i.c.v. naltrexone (NTX) at doses of 0.1, 1 or 10 μg. Significant differences are denoted relative to vehicle (VEH) treatment (#) or relative to OFQ/N treatment (*) (Tukey comparisons, ($p < .01$).



CHAPTER 7. GENERAL DISCUSSION

The present series of studies have provided further insight into the opioid modulation of ingestive behavior. Specifically, the studies have focused primarily on the role of the MOR-1 clone (Table 5) and secondarily on the KOR-3/ORL-1 clone. Results from Chapter 3 revealed that the MOR-1 clone gene encodes the receptor responsible for modulating spontaneous food intake and body weight. Therefore, the clone encoded by MOR-1 gene appears to be involved in maintaining homeostasis. However, it should be noted that AS ODN alterations in spontaneous food intake were operationalized as a suppression of daily food intake. It is possible that other nonspecific factors may be affecting food intake as well such as malaise, or other compensatory transmitter and receptor mechanisms resulting from down regulation of the opioid system. The use of a MS ODN control addresses some of these issues, particularly potential nonspecific effects of injecting nucleotide bases centrally. MS ODN probes do bind to the mRNA strand, but do not do it as well as the AS ODN probe. Therefore, MS ODN do not affect protein levels. Despite these provisos, the MS ODN control constitutes one of the best approaches in assessing specificity of effects since our MS ODNs were identical in composition to their corresponding AS ODN except for minor alterations in the order of nucleotide bases. Such highly specific effects confer this technique with unique advantages relative to the use of antagonists.

Further studies employing the AS ODN technique are needed to confirm results obtained using selective μ antagonists in investigating the role of the MOR-1 clone during regulatory challenges in which an animals homeostasis is compromised. Indeed a recent study utilized AS ODNs directed against opioid receptor clones to characterize hyperphagia induced by the antimetabolic glucose analogue, 2DG (Burdick, Yu, Ragnauth, Moroz, Pan, Rossi, Pasternak and Bodnar, in press). 2DG-induced hyperphagia was significantly reduced by AS

Table 5 Summary of MOR-1 Antisense Mapping Studies Across Ingestive Paradigms

Paradigm Exon	1	2	3	4
Free Feeding	+	+	+	+
DAMGO Hyperphagia	+	-	-	+
MorphineHyperphagia	+	-	?	?
M6G Hyperphagia	-	+	+	-

\+ denotes significant reduction relative to vehicle treatment
\- denotes ineffective relative to vehicle treatment.

ODN probes targeted against each of the four exons of the MOR-1 clone, confirming the effectiveness of μ antagonism (Arjune et al., 1990; Koch and Bodnar, 1994), 2DG-induced hyperphagia was also reduced by AS ODNs directed against the KOR-1 clone, confirming the effectiveness of κ antagonism (Arjune and Bodnar, 1990; Koch and Bodnar, 1994). 2DG-induced hyperphagia was also reduced by AS ODNs directed against the KOR-3/ORL-1 clone, confirming κ_3-agonist-induced enhancements (Yu et al., 1997). Finally, DOR-1 AS ODNs were ineffective in altering 2DG-induced hyperphagia, parallelling the ineffectiveness of δ antagonism (Arjune, Bowen and Bodnar, 1991). In addition to providing confirmatory evidence for antagonists, the AS ODN technique might specify the particular gene product associated with receptors mediating different opioid-sensitive homeostatic responses.

Results from the fourth and fifth Chapter confirmed the specificity of the regions of the MOR-1 clone in mediating agonist-induced hyperphagia. Thus, MOR-1 AS ODN profiles of the analgesic and hyperphagic responses to DAMGO and morphine relative to M6G provide converging results regarding the exons of the MOR-1 clone that are subserving the functional effects of various μ-opioid agonists. It has been suggested that alternative splice variants of the MOR-1 gene may exist or that there may be a distinct gene encoding a novel μ opioid receptor subtype. Any splice variant or novel receptor should resemble other opioid receptor clones in that it would be a G-protein coupled receptor with seven transmembrane spanning domains. Further, any splice variant of the MOR-1 clone should contain four coding exons. It has been hypothesized (Rossi et al., 1997a) that some exons would be conserved from the previously isolated MOR-1 clone while others would be different. However, actual molecular cloning of a splice variant and/or novel receptor will be necessary to confirm these hypotheses and allow further explorations into the functional differences of various μ agonists.

The recently-discovered endomorphins have been proposed to be the endogenous ligand for the μ receptor/MOR-1 clone. Therefore, another issue which must be addressed is that if a splice variant of the MOR-1 clone or a novel μ receptor subtype exist, there should be an additional endogenous μ-acting peptide(s) for this receptor. Thus, if further, μ receptors are cloned, new classes of endogenous opioid peptides may be identified. The AS ODN differences observed for hyperphagia elicited by DAMGO or morphine relative to M6G can however be explained by alternative hypotheses. Pharmacokinetic differences between DAMGO and morphine relative to M6G cannot be ruled out as an explanation for the dissimilarities in the potencies and AS ODN profiles observed in both hyperphagic and analgesic assays. Thus, morphine displays greater hydrophobicity relative to M6G. It is also possible that morphine and M6G are both exerting their activity on the same μ receptor/MOR-1 clone, but differ in their activation of receptor domains. Therefore, further research is necessary to rule out pharmacokinetic differences between morphine and M6G.

Results from Chapter 6 extended the AS ODN technique to the recently-discovered KOR-3/ORL-1 clone and endogenous peptide, OFQ/N. This study revealed that the KOR-3/ORL-1 clone gene encodes the receptor mediating OFQ/N-induced hyperphagia. However, the pre-pro-orphanin/nociceptin gene encodes other biologically active peptides (e.g. nocistatin). Further, the heptadecapeptide, OFQ/N may be susceptible to post-translational processing into either OFQ/$N_{1\text{-}11}$ or OFQ/$N_{1\text{-}7}$, which may have their own respective biological and receptor activity. Additional AS ODN mapping studies might reveal whether each of these compounds are functionally mediated by the KOR-3/ORL-1 clone. Like M6G, if these endogenous peptides are not mediated by one of the cloned opioid receptors, it could raise the possibility of more novel opioid receptor subtypes.

It is known that AS ODNs produce a 30% reduction in opioid receptor mRNA when

injected into the central nervous system (Standifer et al., 1994), however in the present studies it is unclear exactly where the AS ODN are exerting their effects. Since all the experiments used an injection protocol in which the AS ODNs were injected into the lateral ventricle it is likely that the AS ODNs are affecting periventricular sites. Potential sites of action are those areas known to modulate opioid-induced feeding such as the PVN, VMH, nucleus accumbens, NTS or parabrachial nucleus, however more distant sites of action such as the amygdala, VTA and lateral hypothalamus can not be ruled out. AS ODN mapping studies of intracerebral sites should be performed in order to determine more precisely which sites are mediating the observed changes. Further, it is also possible that the AS ODN are exerting their effects on multiple sites, but in an unequal manner such that as you move farther away from the lateral ventricle the effects of the AS ODN diminish. Diffusion studies in which animals are double cannulated in the lateral ventricle and in the various opioid feeding sites, both periventricular and non-periventricular, would be able to determine the diffusion of the AS ODNs as well as the potency of the effects as you move to more distal sites from the ventricle. These studies would provide important information regarding the anatomical location and distribution of AS ODN effects.

One concern for the current dissertation is its reliance on the assumption that the AS ODN technique works according to the proposed mechanisms of action. This might be a risky assumption if an alternative mechanism of action is subsequently discovered, or if the observed effects are determined to result from heretofore undefined nonspecific actions. However, other lines of evidence appear to support the validity of the present data and provide converging evidence. In all but the first experiment, corroborating antagonist data was obtained to confirm receptor mediation of the agonists employed. Further, the pattern of AS ODN effects upon ingestive behavior corresponded with the pattern of effects observed in

analgesic assays. Finally our findings employing the AS ODN knockdown technique have recently been corroborated in analgesic studies employing MOR-1 knockout mice (Schuller et al., 1997). Specifically, MOR-1 knockout mice exhibit M6G-induced, but not morphine-induced analgesia, further distinguishing M6G from morphine. Future studies employing knockout mice in hyperphagic assays will hopefully provide further converging evidence for antagonist and AS ODN effects upon ingestion.

The opioids are one of a number of transmitter and peptide systems which mediate ingestive behavior. Therefore, one important issue that is beyond the scope of the current dissertation is potential interaction between the opioid system and other neurotransmitter and peptide systems. Any alteration in opioid receptor and peptide levels resulting from the AS ODN treatment does not occur in isolation, but may produce subtle or overt changes in other endogenous systems. The opioids are known to interact with both orexigenic (NPY, dopamine and norepinephrine) and anorexigenic (serotonin) transmitters and peptides. Specifically, central and peripheral injections of naloxone decrease NPY-induced food intake (Levine, Grace and Billington, 1990). Chronic treatment with neuroleptics increases morphine-induced hyperphagia (Vaccarino, 1996). ß-endorphin-induced hyperphagia in the PVN and VMH is blocked by α-adrenergic receptor antagonism (Leibowitz and Hor, 1982). Pretreatment with serotonin receptor antagonists potentiates the hypophagic effects of naltrexone in food-deprived rats (Beczkowska and Bodnar, 1991). Thus any alterations in food intake and bodyweight resulting from AS ODN treatment observed in the current dissertation need to consider the effects on other transmitter systems and may provide a fruitful area for future research.

The data obtained in this dissertation has implications for rational drug design. Specifically, the observations that the AS ODN hyperphagia profiles of DAMGO and

morphine relative to M6G were identical to profiles observed in analgesic assays suggest that common exons of the MOR-1 clone are subserving the functional activity of these agonists. Analgesic studies employing AS ODNs have observed that heroin, fentanyl and other abused drugs have a similar AS ODN profile to M6G (Rossi, et al., 1996). If future studies investigating the AS ODN profiles of these agents in hyperphagic assays were to find similar AS ODN profiles as those observed in analgesic assay it would provide compelling evidence implicating exon 2 of the MOR-1 clone in the addictive properties of opiates. Therefore, this region of the receptor clone may be a target in which to develop drugs to treat addictive disease or a region of the receptor to avoid in developing novel analgesics that lack addictive properties.

Addiction is usually associated with narcotic agents, however addiction to food is also a serious problem in our society. Using microdialysis and self-administration approaches, the rewarding properties of both opiate drugs and food have been linked to the nucleus accumbens (see reviews: Salamone, 1994; Self and Nestler, 1995). Specifically, both stimuli have been reported to exert their rewarding effects via extracellular dopamine release in the nucleus accumbens. Dopamine release in the nucleus accumbens has also been connected with other substances such as cocaine, caffeine and amphetamines as well as other natural reinforcers such as sex, all of which have the propensity to be abused, and result in addiction. Future microdialysis and self-administration studies might employ AS ODN mapping strategy of opioid receptor clones, especially the MOR-1 clone, in ingestive assays and in response to opioid self-administration. One could then examine whether differential effects in extracellular dopamine release in the nucleus accumbens in response to these manipulations are altered by different AS ODN probes. Such data would be of great importance for aiding pharmacological treatment of addictive diseases.

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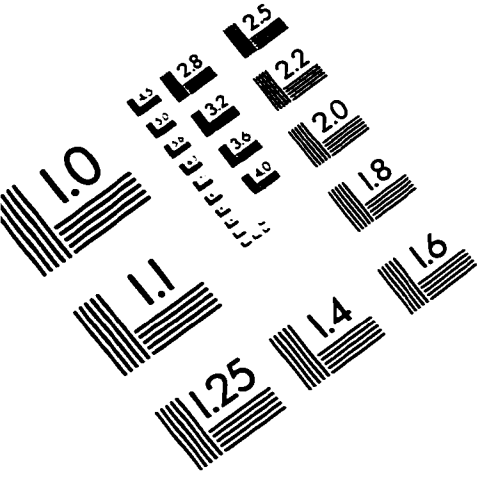
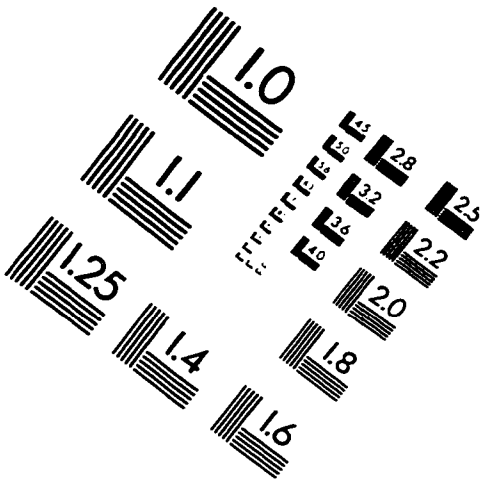
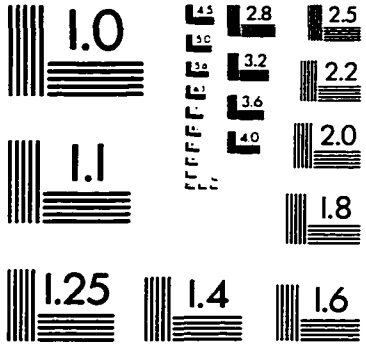
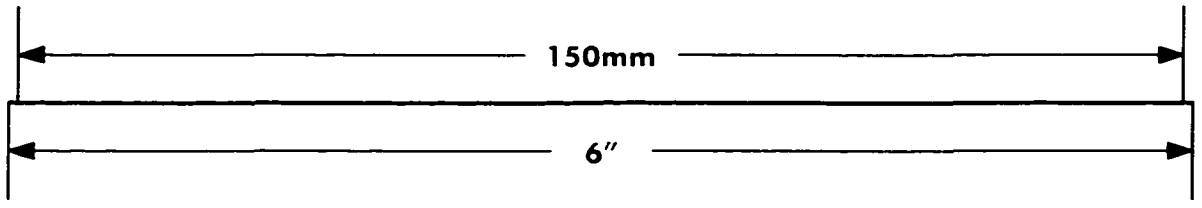
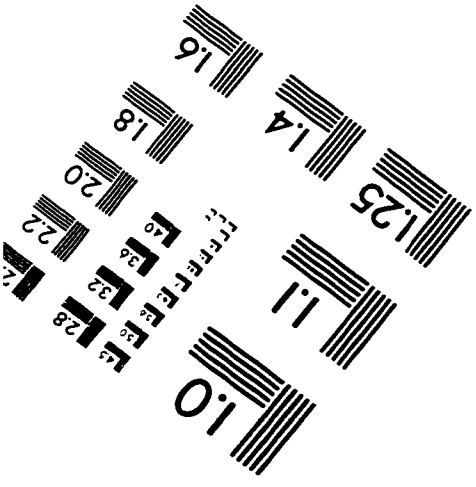
IMAGE EVALUATION
TEST TARGET (QA-3)











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