PHARMACOLOGICAL, MOLECULAR AND NEUROANATOMICAL ANALYSIS OF THE ROLE OF OPIOID RECEPTOR SUBTYPES AND GENES IN REGULATORY CHALLENGES

by

Maria M. Hadjimarkou

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

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Abstract

PHARMACOLOGICAL, MOLECULAR AND NEUROANATOMICAL ANALYSIS OF THE ROLE OF OPIOID RECEPTOR SUBTYPES AND GENES IN REGULATORY CHALLENGES

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Maria M. Hadjimarkou

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Increases in food intake following 24 h of food deprivation are reduced by systemic and central administration of general opioid antagonists. Mu-selective antagonists are more effective than κ-selective antagonists in reducing deprivation-induced intake, whereas δ-selective antagonists are minimally effective. Antisense oligodeoxynucleotide (AS ODN) probes directed against different exons of the mu (MOP), delta (DOP), kappa (KOP) and nociceptin (NOP) opioid receptor genes have been able to differentially alter feeding responses elicited by glucoprivation, lipoprivation and by different opioid peptides and receptor agonists. The dissertation examined whether ventricular administration of AS ODN probes directed against different exons of the opioid receptor genes or against the α-subunit of different G-proteins ($G_i\alpha_1$, $G_i\alpha_2$, $G_i\alpha_3$, $G_s\alpha$, $G_o\alpha$, $G_q\alpha$ or $G_{x/z}\alpha$) caused changes in deprivation-induced intake in rats. The third study evaluated changes in MOR-1 and MOR-1C immunoreactivity in hypothalamic and extra-hypothalamic sites implicated in feeding behavior, in rats exposed to either food restriction of various durations (2, 7, 14 or 14 days followed by a 7-day recovery period) or food deprivation (24, 48 or 48 h followed by a 7-day recovery period), in addition to an *ad libitum* group. The fourth study used AS ODN probes directed against exons of the opioid receptor

genes in mice, in addition to the general opioid receptor antagonist naltrexone and the selective opioid receptor antagonists βFNA, NBNI and NTI, in both species. Antisense probes against exons of the MOP gene moderately reduce deprivation-induced intake whereas βFNA produces great reductions in both rats and mice. MOR-1-LI remained unchanged in rats that were either food-deprived or food-restricted but MOR-1C-LI was significantly increased in the parvocellular PVN following food restriction of 14 days. Thus the data from the studies comprising this dissertation suggest that the regulatory challenge of food deprivation is mediated by all opioid receptors in the mouse and by mu and kappa in the rat. Moreover, the mu receptor antagonist effects seem to be a reflection of effects on MOP splice variants recently identified in the mouse.

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Table of Contents

List of Tables

List of Figures

CHAPTER 1. INTRODUCTION

The endogenous opioid peptide and receptor systems appear to play multiple modulatory roles rather than direct (e.g. sensory or motor) roles in ingestive responses (see reviews: Morley et al., 1983; Levine et al., 1985; Cooper et al., 1988). There are three general classes of mechanisms by which the endogenous opioid system modulates ingestive behavior. Opioids can affect sensory integration, including responsivity to the taste, smell, texture of food stimuli and salient signals accompanying these stimuli as well as metabolic integration, including post-ingestive signals related to further food intake and regulation of body weight. A second major role for opioids involves the emotional integration of food, including its palatable, hedonic and incentive salient characteristics. A third mechanism by which opioids alter food intake is by interacting with energy needs, and includes the types of macronutrients ingested, body adiposity and obesity responses, and the homeostatic state of the organism such as deprivation, lipoprivation or glucoprivation, (e.g., see reviews: Bodnar, 2004; Glass et al., 1999). Indeed, site-specific actions for these three classes have been proposed: whereas opioids in the hindbrain are presumably involved in the sensory and metabolic integration regulating food intake, and opioids in the amygdala may regulate "emotional" processing of food intake, opioids in the hypothalamus are thought to regulate energy needs (Glass et al., 1999).

Opioid modulation of the orosensory and/or hedonic properties of the ingested stimulus putatively increase its palatable properties. This statement is supported by the fact that opioid antagonists decrease palatable intake and opioid agonists increase palatable intake primarily by altering the maintenance of intake rather than the initiation

(e.g., Beczkowska et al., 1992; Kirkham and Cooper, 1988; Leventhal et al., 1995). Alternatively, opioid agonists were proposed to stimulate intake of either a particular class of macronutrients (e.g., fat: Marks-Kaufman, 1982) or those particular macronutrients, which are consistent with the individual animal's baseline diet preferences (Gosnell et al., 1990). An additional, and not necessarily antagonistic hypothesis is that the opioid system is intimately involved in the monitoring of energy homeostasis, and as such, is involved in the detection of perturbations in the survival process (Morley et al., 1983; Bodnar, 1996). A classic paradigm that has typically been employed to study such perturbations is in the food-deprived or food-restricted rodent.

Studies from our and other laboratories have addressed questions on the role of the opioid receptor subtypes in pharmacologically induced feeding following administration of opioid receptor agonists. To address receptor specificity, studies over the past 15 years have made use of highly selective opioid receptor subtype antagonists. These studies suggest that μ, and to a lesser degree κ, but not δ opioid receptors are involved in deprivation-induced feeding (see review: Bodnar, 1996). Further, over the past decade, molecular strategies including the development of opioid and G-protein AS ODN probes have proven useful in examining the precise pharmacological role of the opioid system role in feeding behavior as well as its role in other homeostatic challenges like glucoprivation and lipoprivation (see review: Silva and Bodnar, 2003). Finally, an emerging series of studies (see review: Bodnar, 2004) have demonstrated that alterations in ingestive states, including food restriction, food deprivation and palatability, can change opioid gene expression in nuclei relevant for the mediation of ingestive behavior. However, many of these studies have focused on specific aspects of feeding challenges

(e.g., lipoprivation and glucoprivation), or focused on agonist and antagonist treatment in specific sites, and have not addressed a role for specific opioid mechanisms in the most basic regulatory challenge: food deprivation. Therefore, the focus of this dissertation is to examine the role of opioids in food deprivation through the following four specific aims.

1. **To evaluate the roles of specific opioid receptor genes in the regulation of food intake following 24 h of food-deprivation in rats following ventricular administration of opioid AS ODN probes targeted against the coding exons of the MOP, DOP, KOP and NOP genes.**
2. **To evaluate the roles of specific G-protein-mediated effects in the regulation of deprivation-induced intake in rats following ventricular administration of AS ODN probes directed against the alpha subunits of Gi_1, Gi_2, Gi_3, Gs, Gq, Go and Gx/z proteins.**
3. **To evaluate site-specific changes in immunoreactivity of the MOP (MOR-1) gene and one of its isoforms, the MOR-1C splice variant, in hypothalamic and extra-hypothalamic brain sites implicated in feeding behavior following different durations of either food deprivation or food restriction in rats.**
4. **To generalize the opioid receptor subtype mediation of deprivation-induced intake from the rat to the mouse through systematic analyses of opioid receptor subtype antagonist effects as well as ventricular administration of opioid AS ODN probes targeted against the coding exons of the MOP, DOP, KOP and NOP genes and the extended exons of the Opr*m* gene, in mice.**

The following sections will provide background information regarding I) Opioid Peptides, II) Opioid Receptors, III) Opioid Receptor Genes, IV) Opioid AS ODN

Probes and Feeding Behavior, V) Opioids and Palatable Intake, VI) Opioid Antagonists and Regulatory Challenges, VII) Opioid AS ODN Probes and Regulatory Challenges, VIII) Regulatory Challenges Alter Opioid Gene Expression, IX) G-Protein Alpha Subunit AS ODN Probes and Feeding Behavior. The background will be followed by a Rationale for the present studies.

I. Opioid Peptides.

Opioid peptides derive from four precursor prohormone genes, proenkephalin (PENK), proopiomelanocortin (POMC), prodynorphin (PDYN) and proorphanin OFQ/nociceptin (e.g., Akil et al., 1984; Mansour et al., 1995; Meunier et al., 1995). These prohormones are subsequently cleaved by proteases at specific sites, to give rise to a number of smaller, active peptides (e.g., Marx, 1987). Proopiomelanocortin gives rise to the melanocortin-stimulating hormones (α, β, and γ), ACTH and β-endorphin. Proenkephalin is cleaved to give rise to both pentapeptide (Met- and Leu-enkephalin) and longer-chained enkephalins. Prodynorphin gives rise to dynorphin $A_{(1-17)}$ and $B_{(1-8)}$ as well as $AB_{(1-32)}$. The most recently discovered hectapeptide, nociceptin/orphanin FQ (N/OFQ), is a product of the pronociceptin gene, which also gives rise to nocistatin, which is structurally similar to dynorphin A (Lapalu et al., 1997; Reinscheid et al., 1995; Reinscheid et al., 1998).

Proopiomelanocortin is found specifically in the arcuate nucleus of the hypothalamus and the dorsal medulla as well as in the pituitary gland, gastrointestinal tract and adrenal medulla. Proenkephalins and prodynorphins are secreted by short interneurons, which are widely distributed in the CNS, particularly in the spinal cord, limbic system, periaqueductal gray, locus coeruleus, cerebral cortex and medulla as well

as in the gastrointestinal tract. Met-Enkephalin and Leu-enkephalin neurons are found in hypothalamic sites important in feeding behavior such as the ARC, VMH and DMH and the PVN (Morley, 1987; Finley et al., 1981; Khachaturian et al., 1985). Dynorphin cells are found in the hypothalamus, especially in the ARC and PVN (Morley, 1987; Khachaturian et al., 1985; Levine and Billington, 1989; Lambert et al., 1993). Thus, feeding is stimulated following direct injection of β-endorphin (e.g., Grandison and Guidotti, 1977), enkephalins (e.g., Gosnell et al., 1986; Jackson and Sewell, 1985), dynorphin (e.g., Morley and Levine, 1981) and OFQ/N (e.g., Olszewski et al., 2000, 2002).

II. Opioid Receptors.

Opium has been used in many cultures over the centuries for its psychotropic and analgesic properties. Its potent effects, together with its strong addictive properties as measured by tolerance and withdrawal, led to an intense effort to identify its specialized receptors. In 1973, with the advent of radioreceptor assay, three different research teams simultaneously isolated the endogenous opioid receptors (Pert and Snyder, 1973; Simon, 1973; Terenius, 1973). This breakthrough was followed by the discovery of the first endogenous opioid peptide family, the enkephalins (Hughes et al., 1975). Opioid peptides and receptors were subsequently implicated in a number of functions including analgesic, autonomic, reward and ingestive mechanisms. Since multiple opioid ligands were discovered, the possibility of multiple opioid receptors became more plausible. Strong evidence for the existence of multiple opioid receptor families was based on incomplete cross-tolerance studies across known agonists (Martin et al., 1976) and based on those agonists were given the nomenclature: μ (morphine), κ (ketocyclazosine), and σ (SKF10,

047). Subsequent differential biochemical and bioassay results in the guinea pig ileum and mouse vas deferens supported an additional opioid receptor for enkephalins (δ: Lord et al., 1977). A recently-described novel receptor alternatively named kappa-3 or ORL-1 (opioid-related) (Pan et al., 1994, 1995; Bunzow et al., 1994; Keith et al., 1994; Mollereau et al., 1994; Chen et al., 1994) displays low affinity for the classical opioid agonists and strong binding with the endogenous heptadecapeptide nociceptin or orphanin FQ (Reinscheid et al., 1995; Meunier et al., 1995). All of the above opioid receptor families are 7-transmembrane domain G-protein coupled receptors with great similarity in the transmembrane domains (73-76%) and intracellular loops (86-100%). The distinction among the receptors seems to lie in the N and C terminals and the extracellular loops (9-10%, 14-20%, 14-72% similarity, respectively) (Law et al., 2000). Once activated, the opioid receptors can inhibit adenylyl cyclase and stimulate phospholipase C, N- and L-type Ca^{2+} channels as well as K^{+} channels and mitogen–activated protein kinases ERK1 and ERK2 (Evans et al., 1992; Kieffer et al., 1992; Chen et al., 1993a,b; Fukuda et al., 1993, 1994, 1996; Yasuda et al., 1993; Meng et al., 1993; Li et al., 1993; Tallent et al., 1994; Piros et al., 1995, 1996; Johnson et al., 1994; Spencer et al., 1997; Henry et al., 1995; Li and Chang, 1996).

Opioid receptors are abundant in the central and peripheral nervous systems in varying densities. The mu opioid receptor is found primarily in the cerebral cortex, thalamus and hypothalamus, the periaqueductal gray, the interpeduncular nucleus and the median raphe. In the periphery it is distributed in the myenteric plexus and vas deferens. The delta opioid receptor is also found in the cerebral cortex, the amygdala, nucleus accumbens, olfactory tubercle and pontine nucleus. Finally, the kappa opioid receptor is

found in cerebral cortex, substantia nigra, interpeduncular nucleus, striatum and hippocampus. In the periphery it is found in the myenteric plexus of the guinea pig ileum and in certain smooth muscles (Watson and Arkinstall, 1994). Binding studies with a wide range of opioid agonists provided more support for the idea that mu and delta receptors have greater similarities in their affinity patterns whereas the kappa receptor is more distinct (Akil et al., 1998; Mansour et al, 1995; Brownstein et al, 1993).

a) Mu Opioid Receptor:

The mu receptor gene is located on the distal part of the long arm of chromosome 6 (Zaki et al., 1996). It seems to be the primary binding site of morphine and its metabolite M6G, as well as of the peptide β-endorphin (Silva et al., 2001). The existence of selective opioid agonists and antagonists such as DAMGO and βFNA respectively, allowed extensive characterization of this receptor family. Even though selective binding is achieved through the availability of these selective agonists at low concentrations, in natural situations, opioid peptides may be less selective in their binding, probably due to the great degree of similarity among the opioid receptors (Mansour, 1995). For example Met- and Leu-enkephalin seem to have greater affinity for the δ receptor whereas proenkephalin has greater affinity for the μ receptor.

Based on pharmacological and biochemical studies, it is suggested that there are multiple opioid receptor subtypes. The mu opioid receptor pharmacological actions suggested the existence of two receptor subtypes, μ_1 and μ_2. The μ_1 receptor subtype binds naloxonazine with high affinity, whereas μ_2 seems to be insensitive to naloxonazine administration but is associated to low-affinity actions of morphine, such as respiratory depression and inhibition of gastrointestinal transit (Bodnar, 1998; Pasternak and Wood,

1986; Hahn et al., 1982). Blockade of both μ_1 and μ_2 receptor subtypes occurs following administration of β-funaltrexamine (βFNA). Moreover, the morphine metabolite M6G, which seems to have great potential in the suppression of pain due to the fact that it is twice as potent as morphine when administered systemically and 100-fold more potent when given centrally, seems to bind at distinct sites. Evidence for this possibility came from the fact that CXBK mice, which are unresponsive to morphine administration in analgesic assays, showed analgesia following treatment with M6G (see review: Rossi and Pasternak, 1997). Moreover, a selective antagonist of M6G, 3-O-methylnaltrexone, blocks M6G but not morphine analgesia. Even more interesting is the fact that this same antagonist blocked analgesia induced by heroin, which strongly suggests that morphine has a distinct binding site from that of M6G or heroin (Brown et al., 1997). This implication seems to be in agreement with the fact that heroin seems to be the preferred drug of abuse and not morphine and the ability of drug users to distinguish between heroin or morphine administration, thus providing further support that the two opiates are acting through distinct mechanisms and most likely distinct binding sites (Pasternak, 2001a,b; Rossi, et al., 1996; Martin and Fraser, 1961).

Another source of supporting pharmacological evidence for multiple mu opioid receptor subtypes variants came from both clinical and sub-clinical evidence (Pasternak, 2001a,b,c). Patients who regularly receive a certain mu opioid receptor agonist to suppress pain soon develop tolerance, which means that increasingly greater doses of the analgesic are needed. A way to account for this problem turned out to be "opioid rotation" according to which patients are switched to a different mu-sensitive opioid analgesic. Surprisingly, patients show incomplete cross-tolerance to the second mu-

sensitive opioid, meaning that they need less of the initial analgesic dose (~50%) to achieve sufficient levels of analgesia.

b) Delta and Kappa$_1$/Kappa$_3$ Opioid Receptors:

The delta opioid receptor gene is located on the distal part of the short arm on chromosome 1 (Zaki et al., 1996). The δ receptor has been subdivided into δ_1 and δ_2 receptor subtypes based on the binding affinity and pharmacological activity of the antagonists, D-Ala^2,Leu^5,Cys^6-enkephalin (DALCE) to δ_1 and naltrindole isothiocyanate (NTII) to the δ_2 receptor subtypes in analgesic and ingestive studies (see reviews: Bodnar, 1996, 2000, 2004). The κ receptor is located on the proximal long arm of chromosome 8 (Simonin et al., 1995). At least two subtypes have been identified, κ_1 and κ_3. The κ_1 has been distinguished based on its ability to bind nor-binaltorphimine (nor-BNI) and κ_3 through binding to naloxone benzoylhydrazone (Clark et al., 1989; Bodnar, 1996). The orphan receptor kappa 3 or ORL-1 is the binding site of the peptide orphanin or nociceptin.

III. Opioid Receptor Genes.

Following the successful cloning of the delta opioid receptor (Evans et al., 1992; Kieffer et al., 1992), all of the opioid receptor genes were identified and initially termed MOR-1, DOR-1, KOR-1 and ORL/OFQ for the expression of the μ, δ, κ_1 and κ_3 receptors, respectively (Reisine and Bell, 1993; Uhl et al., 1994). A newer adopted nomenclature is MOP for the μ, DOP for the δ, KOP for κ_1 and NOP for κ_3 receptors. A number of molecular approaches could be used to study their ingestive effects.

One approach that became possible following the cloning of the opioid receptors is the "knockout" technique, in which a target receptor gene is deleted from the genome of an

embryo. This approach results in the development of an animal, which is lacking that particular receptor allowing the study of that particular gene, through the absence or overexpression of a particular behavior, which is normally coded by that gene. One limitation of this approach is the development of possible compensatory mechanisms that the organism might engage in, in order to account for that particular "loss" in its genome. For example, POMC-deficient mice are hyperphagic and obese suggesting a role for that particular prohormone in energy homeostasis (Yaswen et al., 1999). A study by Appleyard et al., (2003) used β-endorphin knockout mice to study the role of the orexigenic peptide β-endorphin, a POMC-derived peptide. These mice were also found to be obese not because of failure in energy balance regulation but due to changes in caloric intake. This hyperphagia was reversed by ventricular administration of the peptide. Moreover, these effects seemed to be independent of the effects of α-MSH knockout mouse, which also results in an obese phenotype. Thus, a novel anorectic role of peptide β-endorphin is revealed in this study through the knockout approach.

An alternative technique, which also allows great specificity at the gene level, is the "knockdown" approach in which the target gene is inactivated via a number of possible ways, most likely prevention of translation or rapid degradation of the targeted sequence (Myers and Dean, 2000). This inactivation is accomplished through the introduction of a DNA sequence, which hybridizes with the complementary mRNA that carries the codes for the target gene. The antisense sequence of around 20 bases long is effective in downregulating the expression of the opioid receptors by a maximum of 50%, depending on the abundance of the mRNA targeted at each particular site (Rossi and Pasternak, 1997). This technique is widely known as the antisense approach, since the

sequence of oligodeoxynucleotides introduced in the organism is complementary to the "sense", the already existing mRNA sequence that codes for that gene. This technique makes it possible to study individual exons of that particular gene, corresponding to different parts of the receptor of interest. Studies using this novel technique have offered more insight and complemented the findings from the more traditional pharmacological studies.

IV. Opioid Antisense ODN Probes and Feeding Behavior

The antisense probe approach provides converging evidence with pharmacological studies and aids in the clarification of the role of opioid receptors in the regulation of feeding behavior. This complementation of findings was accomplishment by examining feeding responses induced by selective opioid agonists, and by comparing them with studies using the same techniques in analgesic assays.

Antisense probes targeted against either coding exons 1 or 4 of the MOP gene significantly reduced feeding elicited by the highly-selective mu agonist, DAMGO (Leventhal et al., 1997). This effect was highly specific for two reasons. First, antisense probes directed against exons 2 or 3 of the MOP gene were ineffective, and second, a missense probe that differed from an effective antisense probe by sequence reversal of two pairs of bases also failed to affect DAMGO-induced feeding. Further, as observed in analgesic studies (see review: Rossi and Pasternak, 1997), the mu-selective (reversed by beta-funaltrexamine) feeding responses elicited by morphine and M6G, were differentially altered by antisense probes directed against the MOP gene (Leventhal et al., 1998b). Thus, morphine-induced feeding was effectively reduced by AS ODN probes targeted against exons 1 and 4, but not exons 2 or 3, and M6G-induced feeding was

significantly reduced by AS ODN probes targeted against exons 2 or 3, but not 1 or 4. Importantly, neither delta, nor kappa opioid antagonists altered M6G-induced feeding, and correspondingly antisense probes directed against the different exons of the DOP, KOP or NOP genes failed to alter M6G-induced feeding. Since the MOP antisense probes targeting these exons were effective for one agonist class (e.g., morphine, DAMGO) but not the other agonist class (e.g., M6G) of ingestive and analgesic responses, it was concluded that the actions of morphine and M6G are occurring through binding to distinct splice variants of the Opr*m* gene (Rossi and Pasternak, 1997).

This approach was also used to delineate feeding responses induced by the endogenous opioid peptides, beta-endorphin and dynorphin. Beta-endorphin-induced feeding was maximally reduced by mu antagonists and antisense probes directed against the MOP gene, moderately reduced by kappa antagonism, and minimally reduced by delta antagonism and antisense probes directed against the DOP, KOP or NOP genes (Silva et al., 2001). In contrast, dynorphin-induced feeding was maximally reduced by kappa antagonists and antisense probes directed against the KOP or NOP genes, moderately reduced by mu antagonism and antisense probes directed against the MOP gene, and minimally reduced by delta antagonism and antisense probes directed against the DOP gene (Silva et al., 2002). Interestingly, AS probes directed against the three exons of the NOP gene resulted in significant reductions in food intake induced by OFQ/N, suggesting that the NOP gene fully encodes OFQ/N-induced feeding (Leventhal et al., 1998a).

V. Opioids and Palatable Intake:

As indicated earlier, opioid antagonists decrease intake of palatable diets by altering the maintenance and not the initiation of intake (e.g., Kirkham and Cooper, 1988; Beczkowska et al., 1992; Leventhal et al., 1995). Hedonic hypotheses of opioid mediation of ingestive behavior have come from two lines of evidence: a) pharmacological manipulation of the opioid system alters palatable intake, and b) palatable intake alters opioid receptor genes.

The inhibitory effects of naloxone and naltrexone are most apparent for intake of sucrose and saccharin solutions and high-fat or high-sugar diets (Apfelbaum and Mandenoff, 1981; Cooper, et al., 1985; Lynch and Libby, 1983; Mandenoff et al., 1982), and saccharin intake is reduced in opioid-deficient CXBK mice (Yirmaya et al., 1988). Opioid receptor subtype antagonists differentially decrease palatable intake as well, in both real-feeding (Beczkowska et al., 1992, 1993) and sham-feeding (Leventhal et al., 1995; Leventhal and Bodnar, 1996) animals, suggesting that it is the orosensory component of the palatable substrate that is affected by selective opioid antagonism. A summary of an extensive literature examining opioid receptor subtype antagonism reveals that mu and kappa antagonists are more effective in reducing intake of simple carbohydrates and fats, whereas delta antagonists are more effective in reducing intake of saccharin, an effect related to delta effects upon alcohol intake (see review: Bodnar, 1996). Relationships between homeostasis and palatability reveal that animals made obese by long exposure to a palatable diet are more sensitive to naltrexone, implicating the opioid system in energy balance regulation (Karanek et al., 1997; Mandenoff, et al., 1982). Moreover, food-restricted animals reduce their consumption of sucrose or

polycose diet after naltrexone infusions but not after a less-preferred starch-based diet (Weldon et al., 1996). Levine and co-workers (2002) demonstrated that chronic naltrexone infusion could suppress the expression of an already developed preference for a specific nutrient only if the exposure to this particular nutrient has been briefly interrupted. It should be noted however that naltrexone fails to affect a conditioned flavor preference for sucrose in either sham-feeding animals (flavor-flavor conditioning: Yu et al., 1999) or in animals receiving intragastric infusions (flavor-nutrient conditioning: Azzara et al., 2000).

Palatable intake affects opioid receptor genes. Fos-LI induced by palatable food was significantly altered by naltrexone administration in the BNST and CeA with smaller decreases in the NAC shell and VTA (Park and Carr, 1998). Consumption of palatable food affects turnover of μ and κ receptors and peptides in nuclei involved in feeding behavior (Dum et al., 1984; Welch, et al., 1996), and increases opioid receptor binding in homogenates of midbrain and cortex (Tsujii et al., 1986). Animals on palatable diets were divided into either high- or low-weight gain (HWG/LWG) groups and mu receptor binding levels were assessed (Smith et al., 2002). Mu receptor binding in both HWG and LWG animals increased in the NTS, dorsal endopiriform nucleus, fundus striati and medial preoptic area. Specific increases in mu binding in HWG animals were observed in the medial habenula and amygdala. Obesity was not a prerequisite condition since non-obese animals consuming a 25% glucose solution displayed increased forebrain mu receptor binding (Colantouoni et al., 2001). POMC mRNA levels selectively increased in the arcuate nucleus of obesity-sensitive, but not obesity-resistant animals placed on long-term cafeteria diets (Torri et al., 2002). Intake of a palatable food (Fonzies) stimulates

dopamine release in the NAC and medial prefrontal cortex; mu-1 antagonist pretreatment with naloxonazine blocked this response in the former, but not latter structure (Tanda and DiChiara, 1998). Long-term exposure to a high fat diet increased mu opioid receptors in the hypothalamus (Barnes et al., 2003). Moreover, long-term exposure to an Ensure diet reduced enkephalin gene expression in striatal, and particularly ventral striatal regions (Kelley et al., 2003).

VI. Opioid Antagonists and Regulatory Challenges:

The involvement of opioids in energy homeostasis can be demonstrated by situations in which food is either unavailable (food deprivation) or limited (food restriction) for various durations. Varying these parameters produces comparable alterations in body weight as well as subsequent intake and weight levels following reintroduction of *ad libitum* intake. Additionally, the homeostatic challenge can be specific to a certain macronutrient, such as glucose or fats. Glucoprivation can be induced by systemic administration of the anti-metabolic glucose analogue, 2-deoxy-D-glucose (2DG), which causes intracellular hypoglycemia and extracellular hyperglycemia or by insulin, which causes both intracellular and extracellular hypoglycemia. These glucoprivic states induce the animal to eat in order to regain homeostasis. Lipoprivation can be induced by systemic administration of the free fatty acid mercaptoacetic acid, an oxidation inhibitor that prevents the animal from metabolizing lipids, again causing increases in food consumption. It is very clear that multiple neurochemical ligand and receptor systems are involved in each of these regulatory challenges, and our review of opioid involvement in each of these responses in no way suggests either exclusive or primary opioid mediation.

The initial study clearly demonstrating that opioids play a role in ingestive behavior was performed using food deprivation as the stimulus (Holtzman, 1974), and demonstrated that the general opioid antagonist naloxone significantly reduced deprivation-induced food intake in rats with the same potency and magnitude as the classic anorectic agent, amphetamine. General systemic opioid antagonism reduced both food and water intake in normophagic rats and mice (Brown and Holtzman, 1979; Cooper, 1980; Frenk and Rogers, 1979; Holtzman, 1975; Maickel et al., 1977), in obese Zucker rats (Thornhill et al., 1982) and in a wide number (wolves, tigers, woodchucks), but not all (Chinese hamsters, raccoons) mammalian species (Billington et al., 1984, 1985; Morley et al., 1983; Nizielski et al., 1985). General opioid antagonism also decreased glucoprivation-induced feeding responses elicited by either 2DG or insulin (Lowy et al., 1980; Beczkowska and Bodnar, 1991), or lipoprivic feeding responses elicited by mercaptoacetate (Stein et al., 2000). All three types of homeostatically driven intake were significantly reduced by ventricular administration of selective mu and kappa, but not delta or mu-1 antagonists (Arjune and Bodnar, 1990; Arjune et al., 1990, 1991; Koch and Bodnar, 1994; Levine et al., 1990; Simone et al., 1986; Stein et al., 2000). Moreover, new and potent kappa (GNTI: Jones et al., 1998; Jones and Porthoghese, 2000) and ORL-1 ([Nphe^1]NC(1-13)NH_2) antagonists significantly reduced deprivation-induced feeding (Jewett et al., 2001; Polidori et al., 2000).

β-Endorphin stimulates food intake in mildly deprived (6 h) rats following direct administration in the ventromedial hypothalamus or lateral ventricle (McKay et al., 1981; Grandison and Guidotti, 1977). Naloxone and naltrexone decreased deprivation-induced intake following direct injections into the VMH and LH (Thornhill and Saunders, 1984).

Deprivation-induced intake was equally reduced following general (naltrexone) or mu (βFNA) antagonist pretreatment into the hypothalamic PVN and this response was moderately affected by kappa (NBNI) and minimally affected by delta (naltrindole) antagonists. Similarly, 2DG-induced feeding was reduced following hypothalamic PVN pretreatment with general, mu and kappa, but not delta opioid antagonists (Koch et al., 1995). Interestingly, although the same pattern of selective antagonist effects in the PVN was observed for reductions in intake of a palatable sucrose solution, the magnitude of the antagonist effects were much smaller. This fits in nicely with the proposal that opioid modulation of intake in hypothalamic sites is more closely related to energy needs than palatability (Glass et al., 1999). The PVN is also an important site in the emerging systems neuroscience of opioid control of ingestion. Thus, DAMGO-induced feeding elicited from the central nucleus of the amygdala was blocked by naltrexone pretreatment in the PVN, but DAMGO-induced feeding elicited from the PVN was not blocked by naltrexone pretreatment in the central nucleus of the amygdala (Giraudo et al., 1998). A bidirectional opioid-opioid pathway is observed between the VTA and PVN such that DAMGO-induced feeding elicited from one site is blocked by naltrexone pretreatment in the other site (Quinn et al., 2003). NPY-induced feeding elicited from the PVN is reduced by naltrexone pretreatment into the NTS (Kotz et al., 1997; Kotz et al., 1998; Kotz et al., 2000). Furthermore, PVN lesions enhance galanin-induced feeding elicited in the NTS, whereas peripheral administration of the mu and kappa agonist butorphanol induced c-Fos immunoreactivity in the PVN, the NTS and central amygdala (Koegler and Ritter, 1998; Kim et al., 2001).

Extra-hypothalamic sites like the nucleus accumbens seem to be involved in opioid mediation of deprivation-induced intake since pretreatment with general and mu opioid receptor antagonists reduce this response to a greater degree than the PVN (Bodnar et al., 1995; Kelley et al., 1996). Kappa antagonists administered into the nucleus accumbens produce lesser effects upon deprivation-induced intake. However, general, mu and kappa antagonists produce pronounced reductions in glucoprivic intake and mild reductions in sucrose intake following administration into the nucleus accumbens (Bodnar et al., 1995). Thus, it appears that mu and kappa receptors each play a role in these responses in this nucleus. It is important to note that feeding responses elicited by either mu, delta-1 or delta-2 opioid agonists administered into the nucleus accumbens are each dependent upon multiple (mu, kappa and delta) opioid receptors given the ability of multiple opioid antagonists to block a given agonist-induced feeding response (Ragnauth et al., 2000).

Even though the nucleus accumbens and the VTA have strong reciprocal connections (e.g., Meredith et al., 1993; VanBockstaele et al., 1995; Zahm et al., 1985), the VTA seems to play a minor role in deprivation-induced feeding since selective mu, kappa and delta opioid antagonists are generally ineffective in reducing this type of intake, and naltrexone is effective only at a high dose (Ragnauth et al., 1997). Similarly, glucoprivation-induced intake was significantly reduced by a high dose of naltrexone and a δ_2 antagonist but not by mu or kappa antagonists (Ragnauth et al., 1997). This is in contrast to the ability of general, mu and kappa, but not delta opioid antagonist pretreatment in the VTA to reduce DAMGO-induced feeding elicited from the same site (Lamonte et al., 2002), and the ability of general opioid antagonists administered to one

site (VTA or accumbens) to block DAMGO-induced feeding elicited from the second site (MacDonald et al., 2003).

VII. Opioid AS ODN Probes and Regulatory Challenges:

The use of antisense probes to evaluate normal ingestive responses initially involved analysis of effects upon body weight and food intake. Thus, antisense probes directed against each of the four exons of the MOP gene produced comparable reductions in food intake and body weight during the time course of treatment; a missense control failed to exert effects (Leventhal et al., 1996). Antisense oligodeoxynucleotides targeted against the various exons of the opioid genes differentially altered glucoprivation-induced feeding as a function of the 2DG dose employed. Thus, the probes were active against food intake elicited by a 200-mg/kg but not 500-mg/kg dose of 2-deoxyglucose (Burdick et al., 1998; Leventhal et al., 1996). 2DG-induced feeding was potently reduced by AS ODN probes against exons 1, 2 and 4 of the MOP gene and was moderately reduced by AS ODN probes directed against exon 2 of the KOP gene. In contrast, AS ODN probes targeting the three exons of the DOP and NOP genes either failed to produce effects or did so at a marginal level (Burdick et al., 1998). These glucoprivic effects mirrored the selective opioid antagonist effects described earlier. Lipoprivic feeding induced by mercaptoacetate administration also displayed converging sensitivity to selective antagonists and AS probes (Stein et al., 2000). Thus, antisense probes directed against exons 1 or 2 of the MOP gene significantly reduced lipoprivic feeding in the same manner as mu opioid receptor antagonists. AS probes directed against exon 3 of the KOP gene and exons 1 and 2 of the NOP gene reduced lipoprivic feeding relative to kappa

antagonists, and an AS probe directed against exon 1 of the DOP gene reduced lipoprivic feeding like delta antagonists (Stein et al., 2000).

VIII. Regulatory Challenges Alter Opioid Gene Expression:

Many studies have looked at the role of opioid peptides and potential changes in their productions dependent on homeostatic challenges such as food deprivation, food restriction and streptozotocin-induced diabetes. *In situ* hybridization demonstrated that food deprivation resulted in decreases of NOP mRNA expression in the PVN, ceAMY and LH, suggesting that this peptide is modulating deprivation-induced feeding in these forebrain structures (Rodi et al., 2002). Moreover, the naturally-occurring NOP antagonist, nocistatin, significantly decreased 18 h deprivation-induced intake when injected into the lateral ventricle at doses of 1 or 3 nmol for up to 2 hours following treatment but not longer term (Olszewski et al., 2000). It is important to note that nocistatin also significantly reduced N/OFQ-induced food intake. The 3 nmol dose of nocistatin did not produce any changes in c-Fos immunoreactivity in the PVN or the SON, which argues that this peptide does not exert its effects through oxytocin or vasopressin connections.

Bertile et al., (2003) examined gene expression of a number of peptides in long-term fasted rats. Three phases of deprivation were used in which the animals were deprived for 24 h (adaptation), 4 days (starving and utilizing lipid oxidization but sparing proteins) or 5-7 days (strong metabolizing of protein is taking place as a substitute for the diminishing fat stores). Following deprivation, the animals were either sacrificed or re-fed for up to 3 days and levels of orexigenic or anorectic peptide gene expression were assessed. Typically, short-term food deprivation results in increases in orexigenic and

decreases in the anorexigenic gene expression by northern blot analysis (Korner et al., 2000; Ahima et al., 1999; Presse et al., 1996; Brady et al., 1990). This study (Bertile et al., 2003) confirmed this concept given that increased NPY gene expression positively correlated with deprivation duration and re-feeding status, effects consistent with increased NPY levels following prolonged (3-4 days) food deprivation (Ahima et al., 1999; Kaneda et al., 2001). POMC gene expression was time-dependently decreased as a function of deprivation with longer (5-7 days) producing greater effects than shorter (4-days). Re-feeding of the animals reversed the inhibitory effects of deprivation on POMC gene expression.

Some laboratories used quantitative *in vitro* autoradiography to compare μ and κ receptor binding in *ad libitum* and food-restricted animals. Although chronic food restriction increased [3H] naloxone binding in the midbrain, it decreased mu opioid binding in the basal amygdala, parabrachial nucleus (PBN) and habenula. Kappa opioid binding was respectively decreased (habenula) and increased (bed nucleus of the stria terminalis (BNST), ventral pallidum, medial preoptic area and PBN) following food restriction (Tsujii et al., 1986a; Wolinsky et al., 1994, 1996b). Dynorphin (DYN) A1-17 is increased by chronic food restriction in the dorsomedial, ventromedial and paraventricular hypothalamic nuclei, and decreased in the central amygdala. The shorter DYN A1-8 peptide is increased in the nucleus accumbens (NAC), BNST, cortex, striatum, midbrain and lateral hypothalamus (LH) (Berman et al., 1994, 1997; Tsujii et al., 1986b). Whereas chronic food restriction decreases hypothalamic arcuate beta-endorphin (BEND) and DYN (Brady et al., 1990; Kim et al., 1996), its combination with exercise increases hypothalamic BEND and DYN (Aravich et al., 1993). A similar study

(Carr et al., 1998) examined c-Fos immunoreactivity in either food restricted or *ad lib* fed animals following a naltrexone injection before sacrifice. Food-restricted animals showed greater Fos-LI in certain brain areas (BNST and CeAMY) than *ad lib* controls, regardless of whether they received a saline or a naltrexone injection, probably indicating that these neurons in these particular areas are normally activated under food-restriction situations. This activation can be attributed to stress associated with food restriction or to the daily feeding pattern. Contrary to their hypothesis, the authors found that there were no changes in c-Fos-LI in food-restricted animals. This could be explained by either the fact that naltrexone is minimally effective in blocking food restriction-induced feeding if the kind of food involved is not palatable. However, administration of naltrexone produced significant Fos-LI in the BSTLD, NAC shell and CeAmy, but not the VTA. Pairing general opioid antagonists with food restriction also increases arcuate NPY mRNA, while decreasing brown fat uncoupling protein (Kotz et al., 1996).

Carr (Carr et al., 1999) hypothesized that the actions of mu receptors played a greater role in reward mechanisms of feeding, whereas kappa receptors played a greater role in motivation to feed. To distinguish whether naltrexone-induced changes in c-Fos-LI are due to reward mechanisms and/or feeding motivation, animals received CTAP or NBNI followed by a naltrexone injection and c-Fos immunoreactivity was measured subsequently. Interestingly, the kappa-selective antagonist NBNI markedly increased Fos-LI in the BSTLD, while the mu-selective antagonist CTAP did not. Yet both NBNI and CTAP increased Fos-LI in the CeAmy. In contrast, CTAP, but not NBNI significantly increased Fos-LI in the NAC, whereas neither antagonist affected Fos-LI in the VTA (Carr et al., 1999). These latter findings provide support for the differential

mediation of specific opioid receptor subtypes in these structures as indicated earlier by the Fos-LI following a systemic injection of naltrexone.

The mu opioid receptor seems to be directly implicated in glucoprivation as demonstrated by Briski and Sylvester (2001). Animals in 2DG-induced glucoprivic state were injected with the mu selective antagonist CTAP or saline and double labeling immunocytochemistry was performed to investigate whether the Fos immunoreactive cells that result from this glucoprivic state would be colocalized with mu immunoreactive cells and furthermore, to examine whether this pattern of immunostaining would be altered following treatment with the mu-selective antagonist CTAP. This study showed that CTAP decreased Fos expression by receptor immunoreactive positive cells in several but not all of these loci, allowing us to conclude that the mu receptors may play a facilitatory role in the homeostatic modulation that goes on during this glucoprivic state. Mu-receptor immunoreactivity was detected in the LS, MS, MPOA, BNSTa, parastrial nucleus (PS), PVAa and LPO. Glucoprivic animals showed co-labeling of Fos and mu-receptor-LI in the LS, MS, MPOA, BSTa and PVAa and LPO. CTAP did not alter mean numbers of mu-LI in any structure but decreased staining of these cells for Fos LI within the LS, MS MEPO and BNST. A possible limitation of the study was the fact that CTAP was injected intracerebroventricularly so it might have affected some brain areas (i.e. that are closer to the ventricles) more than others.

In a quantitative solution hybridization assay, mRNA levels of prodynorphin (pg) were assessed in STZ-induced diabetic animals and food restricted animals (1 h meal a day of 10 g maximum of rat chow), relative to control (*ad lib*). Several nuclei were assessed but significant changes in prodynorphin mRNA occurred in dorsomedial,

ventromedial and lateral hypothalamus, where mRNA levels were higher in the food-restricted group. In both diabetic and food-restricted animals significant increases in prodynorphin mRNA were found in the central AMY as well. No changes were observed in the Caudate Nucleus, the N. Accumbens or the BNST (Berman et al., 1995, 1997). In contrast, diabetes decreased hypothalamic BEND (Kim et al., 1999; Locatelli et al., 1986). A similar study looked at STZ-treated diabetic animals, which showed increased κ binding in MPOA and decreases in μ binding in the lateral habenula (Wolinsky et al., 1996b).

IX. G-Protein Alpha Subunit AS ODN Probes and Feeding Behavior:

Opioid and other 7-transmembrane domain receptors mediate their effects through the putative activation of guanine nucleotide binding proteins (G-proteins) with which they are coupled (Chance et al, 1989; Panchalingam and Undie, 2000; Plata-Salaman, 1995; Raffa et al., 1996; Seeley et al., 1996; Standifer and Pasternak, 1997). *In vitro* studies have also suggested that opioid receptors can activate multiple effectors through different G-protein -subunits to modulate the inhibition of cAMP (see review: Standifer and Pasternak, 1997). The G proteins are heterotrimeric proteins with α, β, and γ subunits. The α-subunit has been identified as the pharmacologically relevant subunit due to its intrinsic GTPase activity, although the β and γ subunits can also modulate the activity of various effectors (see review: Standifer and Pasternak, 1997). Following binding of a ligand the receptor gets activated and the alpha subunit dissociates from the trimer. The α-subunit also seems to establish the identity of G-protein-mediated neuropeptide receptors. For example, whereas pertussis toxin (PTX) irreversibly inactivates a number of G-proteins, including $G_i\alpha$ and $G_o\alpha$, but not $G_s\alpha$, cholera toxin

(CTX) selectively activates the $G_s\alpha$ subtype (e.g., Roerig, 1998; Stryer and Bourne, 1986). PTX pretreatment selectively blocks the analgesic responses to opioid agonists including morphine, DAMGO, and sufentanil (Bodnar et al., 1990; Goode and Raffa, 1997), and also inhibits neuropeptide Y-induced feeding (Chance et al., 1989). However, administration of PTX and CTX produces non-specific weight loss and reductions in baseline food intake, presumably induced by malaise (Bodnar et al., 1990; Chance et al., 1989).

The use of intracerebroventricular G-protein AS ODN probes produces significant down-regulation (50-70%) of G-protein α-subunits (Sanchez-Blanquez et al., 1995; Standifer et al., 1996). Administration of AS ODN probes against either $G_i\alpha_2$ or $G_{x/z}\alpha$ significantly reduce the analgesic activity of mu and delta opioid agonists (Raffa et al., 1996; Sanchez-Blanquez et al., 1995). Morphine and M6G analgesia can be distinguished from one another on the basis of their individual G-protein activation profile such that AS ODN probes directed against either the G_{i2} or G_o α-subunits reduced morphine, but not M6G-induced analgesia, whereas AS ODN probes directed against the G_{i1} or the $G_{x/z}$ α-subunits reduced M6G, but not morphine analgesia. AS ODN probes directed against the G_s α-subunit reduced both analgesic responses in this study (Rossi et al., 1995; Standifer et al., 1996).

Analysis as to whether AS probes directed against different G-protein alpha-subunits differentially altered feeding responses elicited by morphine and M6G indicated that morphine-induced feeding was significantly reduced by an AS probe directed against $G_i\alpha_2$, but was unaffected by AS probes directed against $G_i\alpha_1$, $G_i\alpha_3$, $G_o\alpha$, $G_{x/z}\alpha$ or $G_q\alpha$. An AS probe directed against $G_s\alpha$ actually enhanced morphine-induced feeding. In

contrast, M6G-induced feeding was significantly reduced by AS probes directed against either $G_i\alpha_1$, $G_i\alpha_3$ or $G_{x/z}\alpha$ but was unaffected by AS probes directed against $G_i\alpha_2$, $G_o\alpha$, $G_s\alpha$ or $G_q\alpha$ (Silva et al., 2000). Moreover, feeding responses elicited by both BEND and DYN were significantly reduced by an AS probe directed against $G_i\alpha_1$, indicating some similarity with that of M6G. However, an AS probe directed against $G_o\alpha$ also reduced DYN-induced feeding, whereas AS probes directed against $G_i\alpha_2$ and $G_i\alpha_3$ significantly increased BEND-induced feeding. Therefore, the $G_i\alpha_1$ subunit appears to be implicated in the feeding responses elicited by either BEND or DYN since reductions are observed following pretreatment with an AS ODN probe directed against it (Silva et al., 2002). Therefore, the present data suggest that BEND may act through particular mu opioid receptor splice variants that are also sensitive to M6G. However, since DYN appears to act primarily through kappa opioid receptors (Silva et al., 2002), it is presumably activating this receptor by direct activation of its coupled $G_i\alpha_1$ subunit, which is supported by *in vitro* evidence (Kohno et al., 2000; Misawa et al., 1995; Ueda et al., 1996). G-protein AS ODN effects appear to have functional significance beyond agonist-induced effects. AS probes directed against $G_o\alpha$ and $G_{oA}\alpha$ significantly reduced the short-term increases in food intake at the beginning of the dark cycle (e.g., nocturnal feeding) when rats typically ingest most of their daily food ration. In contrast, either AS probes directed against $G_s\alpha$, $G_q\alpha$ or $G_i\alpha$ or a nonsense control probe failed to alter nocturnal intake (Plata-Salaman et al., 1995).

CHAPTER 2: RATIONALE

Specific Aim One: Deprivation-Induced Feeding: Evaluation of the opioid receptor genes following administration of opioid receptor AS ODN in rats.

The ability of systemic naloxone and other general opioid antagonists like naltrexone to reduce deprivation-induced feeding was the first physiological and pharmacological evidence implicating the endogenous opioid system in feeding behavior (Brown and Holtzman, 1979; Cooper, 1980; Frenk and Rogers, 1979; Holtzman, 1974). General opioid antagonists also reduced deprivation-induced feeding following intracerebral administration into the hypothalamic paraventricular nucleus, nucleus accumbens, and to a lesser degree, the ventral tegmental area (Bodnar et al., 1995; Kelley et al., 1996; Koch et al., 1995; Ragnauth et al., 1997). The ability of selective μ, κ and δ opioid receptor subtype antagonists to alter deprivation-induced feeding suggests differential opioid receptor subtype mediation. Thus, ventricular pretreatment with either the μ-selective opioid antagonist, β-funaltrexamine (β-FNA), or the μ_1-selective opioid antagonist, naloxonazine, produces a 50-75% reduction in deprivation-induced intake, effects equal in magnitude to general opioid antagonism (Arjune et al., 1990; Koch and Bodnar, 1994; Levine et al., 1991; Simone et al., 1986). In contrast, ventricular pretreatment with the κ-selective opioid antagonist nor-binaltorphamine (Nor-BNI) significantly, but moderately reduced (~30%) deprivation-induced feeding (Koch and Bodnar, 1994; Levine et al., 1990). Finally, ventricular pretreatment with the δ-opioid receptor antagonists, naltrindole or DALCE, failed to significantly alter deprivation-induced intake (Arjune et al., 1991; Koch and Bodnar, 1994). Intracerebral microinjection studies indicate that deprivation-induced intake is markedly reduced by μ

antagonists in the hypothalamic paraventricular nucleus and nucleus accumbens, and to a lesser degree in the ventral tegmental area. Deprivation-induced feeding is also modestly reduced by κ antagonists in each of the three sites, and modestly reduced by δ antagonists in only the ventral tegmental area (Bodnar et al., 1995; Kelley et al., 1996; Koch et al., 1995; Ragnauth et al., 1997). Therefore, based on these data, it would appear that opioid receptor mediation of deprivation-induced feeding occurs primarily through the μ receptor, secondarily through the κ receptor, and minimally through the δ receptor.

The AS ODN technique has been used in ingestive studies to correlate the molecular biology of the opioid receptors with their *in vivo* functional effects, and to provide converging and complementary lines of evidence to those supplied in antagonist studies. Specifically, the AS ODN technique has been used to investigate opioid receptor subtype involvement in ingestive responses to glucoprivic and lipoprivic regulatory challenges. Thus, confirming the inhibitory actions of specific μ and κ_1, but not δ opioid antagonists, 2DG-induced feeding was potently reduced by AS ODN probes directed against exons 1 and 2 of the MOP gene across a 4-hour time course and by AS ODN probes against exons 3 and 4 of the MOP gene over a limited 1-2 h duration (Burdick et al., 1998). Moreover, an AS ODN probe directed against exon 2 of the KOP gene also significantly reduced 2DG-induced feeding, whereas AS ODN probes directed against either the NOP or DOP genes were minimally effective (Burdick et al., 1998). Furthermore, confirming the antagonist actions of all three opioid receptor subtypes, MA-induced feeding was significantly reduced by AS ODN probes directed against exons 1, 2, or 3 of the MOP gene, exon 3 of the KOP gene, exons 1 or 2 of the NOP gene, or exon 1 of the DOP gene (Stein et al., 2000). Given the convergence of effects between

selective opioid antagonists and specific opioid AS ODN probes for such homeostatic challenges as glucoprivation and lipoprivation, one would expect the same pattern of effects for deprivation-induced feeding in the rat.

Thus, the **first specific aim** of this dissertation is to examine the role of opioid receptor genes in deprivation-induced intake in rats, following administration of AS ODN targeted against the coding exons of the opioid receptor genes, MOP, DOP, KOP, and NOP and establish whether there is convergence of these findings with studies examining the role of the opioid system in food deprivation using the pharmacological approach. If convergence indeed occurs, the following three hypotheses would be supported.

1. Based on the prior antagonist findings that the receptor most strongly involved in deprivation-induced feeding in rats is the μ opioid receptor, it is hypothesized that intraventricular administration of the MOP antisense probes will result in the most potent reductions in deprivation-induced intake compared to the other probes in rats.
2. Based on the moderate effectiveness of κ opioid receptor antagonists to reduce deprivation-induced intake in rats, it is hypothesized that moderate reductions would be observed following KOP and NOP antisense probes in rats.
3. Since δ opioid receptor antagonists failed to affect deprivation-induced feeding in rats, it is hypothesized that DOP antisense probes will minimally alter deprivation-induced intake in rats.

<u>Specific Aim Two:</u> Deprivation-Induced Feeding: Evaluation of G-protein alpha subunit role in deprivation-induced intake in rats.

As indicated earlier, highly similar patterns of effects were observed for G-protein AS ODN-mediated effects upon feeding and analgesic responses elicited by morphine and M6G (Rossi et al., 1995; Silva et al., 2000; Standifer et al., 1996). Morphine-induced feeding was significantly and selectively reduced by an AS ODN probe directed against $Gi\alpha_2$, and enhanced by pretreatment with a $G_s\alpha$ probe (Silva et al., 2000). In contrast, M6G-induced feeding was significantly and selectively reduced by AS ODN probes against $G_i\alpha_1$, $G_i\alpha_3$ or $G_{x/z}\alpha$. Since control nonsense ODNs did not alter either morphine- or M6G-induced feeding, these effects could not be attributed to nonspecific AS ODN effects. Importantly, these effects occurred independently of any G-protein AS ODN-mediated effect upon either body weight or *ad libitum* food intake (24 h) per se (Silva et al., 2000).

Plata-Salaman and coworkers (1995) found highly selective and specific G-protein AS ODN probe effects upon food intake during the onset of the dark cycle when rats typically ingest most of their daily food ration. An AS ODN probe directed against $G_o\alpha$ and $G_{oA}\alpha$ in particular significantly decreased nocturnal food, but not water intake. Importantly, AS ODN probes directed against $G_s\alpha$, $G_q\alpha$, or $G_i\alpha$ as well as a sense control failed to produce these effects. As indicated earlier, the ingestive response to food deprivation is a commonly used regulatory challenge to assess the roles of putative physiological and pharmacological systems in mediating food intake (e.g., Cooper and Clifton, 1996).

Therefore, the **second specific aim** of this dissertation is to examine the role of ventricularly administered antisense ODN probes directed against either the G_{i1}, G_{i2}, G_{i3}, G_s, G_o, $G_{x/z}$ or G_q α-subunits as well as a control nonsense probe upon food intake following 24 h of food deprivation in rats. Based upon previous studies, we have the following hypotheses.

1. Since all opioid receptors are G protein-coupled receptors working primarily through the Gi and Go alpha subunits, it is hypothesized that depending on the magnitude of the opioid receptor implication in deprivation-induced intake, more potent reductions in deprivation-induced intake will be observed following treatment by these particular probes and not others.
2. Since nocturnal feeding is significantly reduced by antisense probes targeted against $G_o\alpha$ but not Gs, Gq or Gi alpha subunits, and since opioid antagonists act similarly on nocturnal and deprivation-induced intake, it is hypothesized that similar G-protein alpha subunits will be effective in reducing deprivation-induced intake.

<u>Specific Aim Three:</u> Food Deprivation and Food Restriction: Homeostatic challenges alter immunoreactivity in opioid receptor gene expression.

Manipulations of the endogenous opioid system significantly alter feeding behavior so that opioid agonists typically stimulate intake, and opioid antagonists typically inhibit intake (see reviews: Bodnar, 2004; Cooper et al., 1988; Gosnell et al., 1996; Levine et al., 1985; Morley et al., 1983). In turn, behavioral states related to ingestive behavior alter levels of opioid peptides, receptors and genes. These behavioral states include food restriction, food deprivation, streptozotocin-induced diabetes and

exposure to palatable diets. Although chronic food restriction increased [3H] naloxone binding in the midbrain, it decreased mu opioid binding in the basal amygdala, parabrachial nucleus (PBN) and habenula. Kappa opioid receptor binding was respectively decreased (habenula) and increased (bed nucleus of the stria terminalis (BNST), ventral pallidum, medial preoptic area and PBN following food restriction (Tsujii et al., 1986a; Wolinsky et al., 1994, 1996b). Dynorphin (DYN) A1-17 is increased by chronic food restriction in the dorsomedial, ventromedial and paraventricular hypothalamic nuclei, and decreased in the central amygdala. The shorter DYN A1-8 peptide is increased in the nucleus accumbens (NAC), BNST, cortex, striatum, midbrain and lateral hypothalamus (LH) (Berman et al., 1994, 1997; Tsujii et al., 1986b). Whereas chronic food restriction decreases arcuate beta-endorphin (BEND) and DYN (Brady et al., 1990; Kim et al., 1996), its combination with exercise increases hypothalamic BEND and DYN (Aravich et al., 1993). Food-restricted rats display increased c-Fos immunoreactivity in the BNST, central nucleus of the amygdala and NAC following naltrexone pretreatment, in the BNST and amygdala following kappa antagonism, and in the NAC following mu antagonism (Carr et al., 1998, 1999). Pairing general opioid antagonists with food restriction also increases arcuate NPY mRNA, while decreasing brown fat uncoupling protein (Kotz et al., 1996). In addition to food restriction, streptozotocin-induced diabetes similarly increased DYN A1-17 in the dorsomedial and ventromedial hypothalamus, and Dynorphin A (1-8) in the LH (Berman et al., 1995, 1997). In contrast, diabetes decreased hypothalamic BEND (Kim et al., 1999; Locatelli et al., 1986). Both diabetes and food restriction increase kappa binding in the medial preoptic area and decrease mu binding in the lateral habenula (Wolinsky et al., 1996a).

Moreover, glucoprivation selectively decreases mRNA levels of pro-dynorphin, but not pro-opiomelanocortin or pro-enkephalin (Giraudo et al., 1998). Further, food deprivation lowers mRNA levels for the NOP receptor in the PVN and LH as well as the central nucleus of the amygdala, and lowers pro-OFQ/N mRNA levels in the central nucleus of the amygdala (Rodi et al., 2002).

Long-term exposure to palatable solutions, but not the accompanying weight gain increases hypothalamic DYN protein and mRNA levels (Welch et al., 1996). Sucrose consumption significantly enhances the ability of naloxone to increase c-Fos activity in the lateral hypothalamus, ventral tegmental area, central nucleus of the amygdala, and medial preoptic area (Park and Carr, 1998; Pomonis et al., 2000). Antagonism of mu-1 opioid receptors blocks palatability-induced stimulation of dopamine release in the NAC (Tanda et al., 1998). Long-term exposure to a high fat diet increased mu opioid receptors in the hypothalamus (Barnes et al., 2003), but reduced enkephalin gene expression in striatal, and particularly ventral striatal regions (Kelley et al., 2003). Although BEND levels were initially associated with overeating in genetically obese ob/ob mice and fa/fa rats (Margules et al., 1978), genetically-obese Zucker rats display reductions in POMC mRNA levels that correspond to reductions in alpha-melanocyte stimulating hormone, yet no changes in BEND (Kim et al., 2000). Further, DYN levels are increased and [3H]-naloxone binding is decreased in obese Zucker rats (Roane et al., 1988).

The effects of general and selective opioid receptor subtype antagonists upon deprivation-induced intake have been extensively reviewed in previous sections as well as the effects of opioid AS ODN probes upon feeding responses associated with opioid mediation. Although AS ODN probes directed against each of the four exons of the MOP

gene are very effective in reducing feeding and body weight under spontaneous intake conditions (Leventhal et al., 1996), feeding responses elicited by the mu-selective opioid agonists, morphine and DAMGO, as well as the opioid peptides, BEND and DYN, are differentially altered by different exons of the MOP, DOP, KOP and NOP genes (Leventhal et al., 1997, 1998; Silva et al., 2001, 2002). These data suggest the existence of isoforms or splice variants of these identified opioid receptor genes. Thus, identified MOP isoforms (Bare et al., 1994; Pan et al., 1999, 2000, 2001; Pasternak and Pan, 2000; Zimprich et al., 1995) demonstrate important site-specific differences in density and distribution (Abbadie and Pasternak, 2001; Abbadie et al., 2000a, 2000b; 2001; Ding et al., 1996). Importantly, the MOR-1 gene, and particularly its MOR-1C isoform, is differentially localized in sites intimately implicated in the opioid mediation of ingestive behavior (see reviews: Bodnar, 2004; Glass et al., 1999; Gosnell and Levine, 1996). Therefore, given that food deprivation and food restriction alter opioid receptor binding and early oncogene activity as reviewed above, it is important to determine whether these homeostatic challenges specifically alter the immunoreactivity of these important opioid genetic markers.

Thus, the **third specific aim** will evaluate changes in MOR-1 and MOR-1C immunoreactivity in hypothalamic and extra-hypothalamic sites implicated in feeding behavior, in rats exposed to either food restriction of various durations (2, 7, 14 or 14 days followed by a 7-day recovery period) or food deprivation (24, 48 or 48 h followed by a 7-day recovery period), in addition to an *ad libitum* group. Based on the data just reviewed, the following hypotheses are made.

1. Strong homeostatic challenges result in alterations in opioid gene expression. Since the μ opioid receptor is intimately involved in feeding induced by food deprivation, it is hypothesized that rats that are food deprived or food restricted will display alterations in immunoreactivity of the MOR-1 receptor in brain sites associated with mu-mediated regulation of intake.
2. Several Opr*m* receptor splice variants have been identified. The MOR-1C receptor is distributed in brain sites implicated in feeding behavior, thus it is hypothesized that food deprivation or food restriction will alter the expression of this particular gene, and thus immunoreactivity levels, in hypothalamic and extra-hypothalamic sites.
3. Changes in immunoreactivity suggest that the opioid receptor gene expression has been altered. Brief homeostatic challenges may be insufficient to produce changes in protein levels of these particular receptors, and thus it is hypothesized that the longer durations of food deprivation or food restriction will result in greater changes in immunoreactivity for both the MOR-1 and MOR-1C receptors.

Specific Aim Four: Deprivation-Induced Feeding: Evaluation of opioid receptor genes through ventricular administration of opioid receptor AS ODN probes in mice.

General opioid antagonists like naltrexone reduce deprivation-induced feeding and body weight following systemic or ventricular administration, in both rats and mice (Brown and Holtzman, 1979; Cooper, 1980; Frenk and Rogers, 1979; Holtzman, 1974). These effects persist following intracerebral administration of general opioid antagonists

into the hypothalamic paraventricular nucleus, nucleus accumbens and to a lesser degree, the ventral tegmental area of rats ((Bodnar et al., 1995; Kelley et al., 1996; Koch et al., 1995; Ragnauth et al., 1997). The ability of selective μ, κ and δ opioid receptor subtype antagonists to alter deprivation-induced feeding in rats suggests differential opioid receptor subtype mediation, and the studies reviewed earlier suggest that opioid receptor mediation of deprivation-induced feeding in rats occurs primarily through the μ receptor, secondarily through the κ receptor, and minimally through the δ receptor. The previous sections have also reviewed converging and complementary lines of evidence between opioid AS ODN probes and opioid receptor subtype antagonist studies. It is conceivable that the hypothesized convergence between these two techniques may not occur for deprivation-induced feeding in the rat or the mouse, since splice variants or isoforms of the MOP gene that were recently identified in the mouse (Bare et al., 1994; Pan et al., 1999, 2000, 2001; Pasternak, 2000, 2001; Zimprich et al., 1995) may be responsible for mediating the opioid component of deprivation-induced feeding. One could evaluate whether isoforms of the MOP (MOR-1) gene are responsible for μ opioid-mediated actions by using AS ODN probes directed against additional exons (5, 6, 7, 8, 9, 10, 12 and 13). However, these exons and their AS ODN probes have only been identified in the mouse, and this animal would have to be used in order to test this hypothesis. Yet, although the mouse displays the same pattern of naloxone-induced inhibition of deprivation-induced feeding as the rat (e.g., Brown and Holtzman, 1979; Cooper, 1980; Frenk and Rogers, 1979; Holtzman, 1974) and indeed other species (e.g., wolves, tigers, woodchucks and deer, but not Chinese hamsters or raccoons: see review: Bodnar, 2004), studies using selective opioid receptor subtype antagonists have been exclusively carried

out in the rat. Thus, a systematic comparison of selective opioid antagonist effects upon deprivation-induced feeding in the mouse and the rat is warranted.

Therefore, the **fourth specific aim** will attempt to determine whether splice variants of the Opr*m* gene and their coding exons are implicated in deprivation-induced feeding in the mouse, since based on pharmacological studies, this particular receptor is very important in modulating this homeostatic challenge. Thus, AS ODN probes directed against each of the exons of the mouse MOP (exons 1, 2, 3, 4, 5a, 6, 7, 8, 9, 10, 12, 13), DOP (exons 1, 2, 3), KOP (exons 1, 2, 3) and NOP (exons 1, 2) genes will be administered and their effects on deprivation-induced intake will be assessed. In addition, we will evaluate the level of the opioid receptor involvement in deprivation-induced intake in both rats and mice, by administering general (naltrexone) and selective opioid receptor antagonists (β-funaltrexamine (μ), nor-binaltorphamine (κ) and naltrindole (δ)), in both species.

1. Based on pharmacological studies in the rat, the mu opioid receptor is very important in the regulation of deprivation-induced intake. Since the mouse shares a great degree of its genome with the rat, it is hypothesized that deprivation-induced intake in the mouse will be maximally reduced by mu opioid receptor selective antagonists, moderately reduced by kappa selective antagonists, and minimally reduced by delta receptor antagonists.
2. Since mice are significantly smaller than rats, it is of importance to examine whether a milder homeostatic challenge of 12 h of food deprivation would produce a similar or differential pattern of results, following general and selective opioid antagonists in mice.

3. Antisense ODN probes targeting the exons of the MOP gene are expected to produce the most potent reductions in deprivation-induced intake, the probes targeting the KOP and NOP genes producing moderate reductions in deprivation-induced intake, whereas the probes targeting the DOP exons are expected to be less effective in altering deprivation-induced intake, in the mouse.

CHAPTER 3. GENERAL METHODS

1. Subjects, Housing, Surgery, Injections.

A. Rats:

Male albino Sprague-Dawley rats (90-100 days old, Charles River Laboratories, Kingston, NY) were housed individually in wire mesh cages and maintained on a 12-h light/dark cycle with water and food (Rat LabDiet, 1500) available *ad libitum*. Each animal 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 structure, 1.3 mm lateral to the sagittal suture and 3.6 mm from the top of the skull. Cannulae were 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 begins. After completion of behavioral testing, all animals were sacrificed with an overdose of anesthetic (Euthasol: Del Marva Laboratories, Henry Schein, NY), and the cannulae placements were verified histologically.

All injections were administered through a stainless steel internal cannula (28-gauge, Plastics One), which extended 0.5-1.0 mm beyond the tip of the cannula, attached to a Hamilton syringe by polyethylene tubing. The microinjections were done over a 30 s-period after which the internal cannula was replaced by a dummy cannula in order to prevent efflux. During each test phase in the AS ODN protocol, rats would receive three microinjections of the particular probe on days 1, 3 and 5, since this time course of treatment is presumed to downregulate existing receptors as well as the synthesis of new

receptors. Central antagonists were infused with a single microinjection whereas systemic naltrexone injections were administered subcutaneously in a 1ml/kg volume.

B. Mice:

Male CD-1 mice (25-30 g, Charles River Laboratories, Wilmington, MA) were initially housed in groups (n=4-5) in clear polyethylene cages and maintained on a 12-h light/dark cycle, with water and standard mouse chow pellets (Mouse LabDiet, 5015) available *ad libitum*. Before behavioral testing begins, mice were housed individually in polyethylene cages equipped with metal grids placed at the floor of each cage, and allowed at least three days to acclimate. The presence of the grid floor allowed for the placement of paper towels for spillage collection (see Intake procedures). On the day(s) of the injections, mice were exposed to an isoflurane and oxygen combination until full anesthesia was observed. An incision along the midline was made to expose the underlying sutures, and freehand injections were administered in the lateral ventricle at 2 mm anterior to the lambda and 3-3.5 mm lateral to midline, through an internal cannula (28-gauge) that extended 2mm below the surface of the skull. This procedure was periodically verified in control mice using luxol fast blue injections to visualize entry into the ventricles. Systemic naltrexone injections were administered subcutaneously in a 10 ml/kg volume.

2. AS ODN Probes

All phosphodiester AS ODN probes were purchased (Midland Certified Reagent Company, Midland, TX) and purified by ethanol precipitation and diluted in 0.9% normal saline at 5 μg/μl. Each animal received three injections of 2 μl each. This dose has been determined to be effective in feeding studies, without causing any nonspecific effects.

The AS ODN sequences (19-22 bases long) directed against the individual exons of either the MOP, DOP, KOP or NOP opioid receptor genes were found to be specific to the rat and the mouse clone respectively, and this was verified by a Gen Bank search (Table 1).

To assess the specificity of AS ODN probe effects, two controls were used in addition to a saline treatment. The first was a missense (MS) ODN control in which the order of two pairs of nucleotide bases is reversed. The second control was a "nonsense" (NS) ODN probe of comparable size, consisting of completely scrambled nucleotide bases.

3. Drugs.

The general opioid receptor antagonist naltrexone as well as the opioid receptor selective antagonists βFNA, NBNI and NTI was purchased from Sigma Chemical Company (St. Louis, MO) and dissolved in distilled water. All antagonists were infused in the lateral ventricle in both mice and rats via microinjections of 5 μl volumes over at least 30 sec.

4. General Testing Protocol-Antisense ODN Probes and Opioid Antagonists

Following recovery from surgery (rats) and acclimation in the grid-equipped cages (mice), animals participating in the antisense probes treatment entered a 7- or 8-day testing paradigm. On days 1, 3 and 5 at 2-4 h into the light cycle, animals received a microinjection of an antisense probe. Following the third microinjection on day 5, the animals were food-deprived for either 12 or 24 h with water available *ad libitum*. On day 6, food was reintroduced, and short-term cumulative intake was assessed after 0.5, 1, 2 and 4 h. Longer-term intake was assessed on days 7 (24 h) and 8 (48 h). Body weight

Table 1. Sequence of opioid antisense oligodeoxynucleotides (AS ODN) common for the rat and mouse.

Probe	Sequence
MOP Gene:	
Exon 1 AS	CGC CCC AGC CTC TTC CTC T
Exon 2 AS	TTG GTG GCA GTC TTC ATT TTG G
Exon 3 AS	TGA GCA GGT TCT CCC AGT ACC A
Exon 4 AS	GGG CAA TGG AGC AGT TTC TG
DOP Gene:	
Exon 1 AS	TGT CCG TCT CCA CCG TGC
Exon 2 AS	ATC AAG TAC TTG GCG CTC TG
Exon 3 AS	AAC ACG CAG ATC TTG GTC AC
KOP Gene:	
Exon 1 AS	GCT GCT GAT CCT CTG AGC CCA
Exon 2 AS	CCA AAG CAT CTG CCA AAG CCA
Exon 2 MS	CCA **AGA** CAT CTG C**AC** AAG CCA
Exon 3 AS	GGC GCA GGA TCA TCA GGG TGT
NOP Gene:	
Exon 1 AS	GGG GCA GGA AAG AGG GAC TCC
Exon 2 AS	GAC GAG GCA GTT CCC CAG GA
Exon 3 AS	GGG CTG TGC AGA AGC CGA GA
Nonsense	GGG GGA AGT AGG TCT TGG

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

was monitored throughout the paradigm as noted in specific procedures. Animals receiving the opioid receptor antagonists entered a 4-day paradigm, in which antagonists were administered either on the deprivation day (mu antagonist) or on the day of food reintroduction (naltrexone, kappa or delta antagonist). Food was reintroduced 24 h after deprivation, and intake was assessed after 0.5, 1, 2, 4, 24 and 48 h. Body weight was monitored daily as described in the protocols.

5. Statistical Analysis

A randomized-block two-way analysis of variance was performed on cumulative food intake data with the between-subject variable of vehicle and the different ODN or antagonist conditions, the within-subject variable of post-deprivation intake time (0.5-4 and 24-48 h), and for the interaction between condition and time. Tukey comparisons ($P< 0.05$) were used to assess significant AS ODN or antagonist effects relative to vehicle control treatment.

Deprivation-induced weight loss scores were assessed for each animal by subtracting the Post-Deprivation weight from its corresponding Pre-Deprivation weight, and recovery-induced weight gain scores were assessed for each animal by subtracting the Recovery weight from its corresponding Post-Deprivation weight. Separate one-way analyses of variance were performed on Deprivation-Induced weight loss and randomized-block two-way analysis of variance for the Recovery-Induced weight gain for each AS, MS and NS ODN treatments relative to vehicle control values with Tukey comparisons ($P< 0.05$) indicating individual significant effects relative to vehicle control treatment.

CHAPTER 4. SPECIFIC AIM ONE.

Opioid Antisense Probes and Deprivation-Induced Feeding in the Rat

Introduction

The premise that the endogenous opioid peptide and receptor system is intimately involved in the mediation of food intake was based on an initial study by Holtzman (1974) which showed that systemic administration of the general opioid antagonist, naloxone, significantly reduced food intake elicited by food deprivation. The ability of systemic naloxone and other general opioid antagonists like naltrexone to reduce deprivation-induced feeding was subsequently confirmed (Brown and Holtzman, 1979; Cooper, 1980; Frenk and Rogers, 1979), and general opioid antagonists also reduced deprivation-induced feeding following intracerebral administration into the hypothalamic paraventricular nucleus, nucleus accumbens and to a lesser degree, the ventral tegmental area (Bodnar et al., 1995; Kelley et al., 1996; Koch et al., 1995; Ragnauth et al., 1997). The ability of selective μ, κ and δ opioid receptor subtype antagonists to alter deprivation-induced feeding suggests differential opioid receptor subtype mediation. Thus, ventricular pretreatment with either the μ-selective opioid antagonist, β-funaltrexamine (β-FNA), or the μ_1-selective opioid antagonist, naloxonazine, produces a 50-75% reduction in deprivation-induced intake, effects equal in magnitude to general opioid antagonism (Arjune et al., 1990; Koch and Bodnar, 1994; Levine et al., 1998; Simone et al., 1985). In contrast, ventricular pretreatment with the κ-selective opioid antagonist nor-binaltorphamine (Nor-BNI) significantly, but moderately reduced (~30%) deprivation-induced feeding (Koch and Bodnar, 1994; Levine et al., 1990). Finally, ventricular pretreatment with the δ-opioid receptor antagonists, naltrindole or DALCE,

failed to significantly alter deprivation-induced intake (Arjune et al., 1991; Koch and Bodnar, 1994). Intracerebral microinjection studies indicate that deprivation-induced intake is markedly reduced by μ antagonists in the hypothalamic paraventricular nucleus and nucleus accumbens, and to a lesser degree in the ventral tegmental area. Deprivation-induced feeding is also modestly reduced by κ antagonists in each of the three sites, and modestly reduced by δ antagonists in only the ventral tegmental area (Bodnar et al., 1995; Kelley et al., 1996; Koch et al., 1995; Ragnauth et al., 1997). Therefore, based on these data, it would appear that opioid receptor mediation of deprivation-induced feeding occurs primarily through the μ receptor, secondarily through the κ receptor, and minimally through the δ receptor. This pattern of effects can be compared with feeding responses induced by other regulatory challenges such as those elicited by 2-deoxy-D-glucose (2DG)-induced glucoprivation or mercaptoacetate (MA)-induced lipoprivation. 2DG-induced feeding is significantly and potently reduced by ventricular and intracerebral pretreatment with μ and κ, but not δ opioid antagonists (Arjune and Bodnar, 1990; Arjune et al., 1990, 1991; Bodnar et al., 1995; Koch and Bodnar, 1994; Koch et al., 1995; Ragnauth et al., 1997), whereas MA-induced feeding is potently reduced by all three opioid subtype antagonists (Stein et al., 2000).

Following the cloning and identification of opioid receptor genes of MOP (originally MOR-1), DOP (originally DOR-1) and KOP (originally KOR-1) as well as the genomically identified NOP (originally KOR-3/ORL-1) (see reviews: Kieffer, 1995; Uhl et al, 1994), a "knockdown" technique involving temporary elimination of receptor protein expression was developed using AS ODNs (see reviews: Pasternak and Standifer, 1995; Rossi and Pasternak, 1995). The AS ODN technique has been used in analgesic

and ingestive studies to correlate the molecular biology of the opioid receptors with their *in vivo* functional effects, and to provide converging and complementary lines of evidence to those supplied in antagonist studies. Specifically, the AS ODN technique has been used to investigate opioid receptor subtype involvement in ingestive responses to glucoprivic and lipoprivic regulatory challenges. Thus, confirming the inhibitory actions of specific μ and κ_1, but not δ opioid antagonists, 2DG-induced feeding was potently reduced by AS ODN probes directed against exons 1 and 2 of the MOP gene across a 4-hour time course and by AS ODN probes against exons 3 and 4 of the MOP gene over a limited 1-2 h duration (Burdick et al., 1998). Moreover, an AS ODN probe directed against exon 2 of the KOP gene also significantly reduced 2DG-induced feeding, whereas AS ODN probes directed against either the NOP or DOP genes were minimally effective (Burdick et al., 1998). Furthermore, confirming the antagonist actions of all three opioid receptor subtypes, MA-induced feeding was significantly reduced by AS ODN probes directed against exons 1, 2, or 3 of the MOP gene, exon 3 of the KOP gene, exons 1 or 2 of the NOP gene, or exon 1 of the DOP gene (Stein et al., 2000). The AS ODN technique has also differentiated among feeding responses elicited by μ-selective opioid agonists such that AS probes directed against exons 1 and 4 of the MOP gene decrease feeding elicited by morphine or DAMGO, whereas AS probes directed against exons 2 and 3 of the MOP gene decrease feeding elicited by the morphine metabolite, morphine-6β-glucuronide (M6G) (Leventhal et al., 1997; Leventhal et al., 1998). This pattern of AS effects is identical to that observed in analgesic assays (Rossi et al., 1997; Rossi et al., 1995; Rossi et al., 1995). Further, the use of selective antagonists and the AS ODN technique has yielded converging and complementary evidence implicating the μ

receptor and MOP gene in mediating feeding elicited by β-endorphin, and the κ receptor and KOP/NOP genes in mediating feeding elicited by dynorphin A_{1-17} (Silva et al., 2002; Silva et al., 2001).

The present study is aiming to establish whether the knockdown AS ODN approach will provide converging and complementary information with previously determined selective opioid receptor antagonist data in assessing selective opioid receptor involvement in deprivation-induced feeding. Specifically, the present study will examine whether AS ODN probes directed against each of the exons of the MOP, DOP, KOP and NOP opioid receptor genes alter food intake and body weight changes elicited by food deprivation over a 24 h period. These experiments were published in Brain Research (987: 223-232, 2003).

Methods

Surgeries: see General Methods, Chapter 3.

Procedure

Following recovery from surgery and approximately one month after arrival in the vivarium, food intake and body weight of each acclimated rat was assessed over 48 h to verify normal feeding responses. Before receiving an AS probe, animals entered the following 7-day control paradigm: On Days 1 and 3, at 2-4 h into the light cycle, all 65 rats were individually weighed, then received a microinjection (2 μl, i.c.v.) of 0.9 % normal saline (0.9% NaCl in H_2O) with standard rat chow pellets and water provided *ad libitum*. On Day 5, the animals were weighed (pre-deprivation weight), received a third microinjection of 0.9% normal saline, and returned to their home cage without their food bins. The animals remained food-deprived with water available for 24 h. On Day 6, the

animals were re-weighed (post-deprivation weight) to assess body weight loss as a result of food deprivation. Pre-weighed standard chow pellets were reintroduced on the floor of the cage with water available in a manner identical to our previous studies with opioid agonists and other regulatory challenges (Burdick et al., 1998; Leventhal et al., 1997, 1998; Stein et al., 2000). Food intake (±0.1 g) was assessed by weighing food pellets adjusted for spillage collected by brown paper towels beneath the wire mesh cage at 0.5, 1, 2, and 4 h following food reintroduction. Next, a pre-weighed food bin was placed inside the cage with water available, and a brown paper towel beneath the cage to collect spillage. Lastly, on Day 7, cumulative intake was assessed after 24 h by measuring the food bin and adjusting for spillage. The animals were re-weighed (recovery weight) to assess food reintroduction-induced body weight gain.

All animals were allowed two weeks to fully recover from the initial deprivation paradigm that was confirmed by full recovery of body weight and normal food intake. Then during the third week, the paradigm was repeated with subgroups of animals (n= 5-9) matched for deprivation-induced intake values under vehicle control conditions. These rats received microinjections of the opioid AS ODN directed against each of the four exons of the MOP gene, each of the three exons of the DOP gene, each of the three exons of the KOP gene, each of the three exons of the NOP gene, a KOP exon 2 MS ODN probe (that differed by two pairs of bases from its corresponding AS ODN probe), and a completely-scrambled NS ODN probe that consisted of 18 bases not related to any sequence in Gen Bank. The probes were administered on days 1, 3 and 5, with the animals food-deprived for 24 h, and tested for food intake and body weight on days 6 and 7 as described above.

Results

Opioid AS ODN probes and deprivation-induced food intake: Significant differences in the magnitude of deprivation-induced food intake were observed among vehicle control and ODN conditions ($F(15,143)= 3.31$, $P<0.0001$), among post-deprivation intake times ($F(4,572)= 2580.29$, $P<0.0001$), and for the interaction between conditions and times ($F(60,572)= 2.72$, $P<0.0001$). An identical pattern of effects occurred for the rate of deprivation-induced food intake with significant differences observed among vehicle control and ODN conditions ($F(15,143)= 2.22$, $P<0.008$), among post-deprivation intake times ($F(4,572)= 1017.08$, $P<0.0001$), and for the interaction between conditions and times ($F(60,572)= 1.61$, $P<0.004$). AS ODN probes directed against the different exons of the opioid receptor genes differentially and significantly altered deprivation-induced feeding and the rate of feeding across the time course of testing relative to corresponding control vehicle treatment.

Thus, for the MOP gene, AS ODN probes directed against exons 2 and 4 significantly reduced deprivation-induced feeding after 1, 2, 4 and 24 h (Figures 1A and 2A), whereas the AS ODN probe directed against exon 3 significantly reduced deprivation-induced feeding after 2, 4 and 24 h (Figures 1A and 2A). An identical pattern of significant effects was observed for the rate of deprivation-induced intake (Table 2). In contrast, the AS ODN probe directed against exon 1 of the MOP gene failed to alter deprivation-induced feeding at any time point. Peak effects in reductions occurred after 4 h for three MOP AS ODN probes. These significant reductions were seen by probes against exons 2 (28%), 3 (20%) and 4 (16%) with persistent 12-15% significant decreases noted after 24 h.

Figure 1. Alterations (Mean ± S.E.M.) in food intake 0.5-4 h following reintroduction of food in food-deprived (24 h) rats pretreated with either a vehicle control or antisense oligodeoxynucleotide (AS ODN) probes directed against either exons 1, 2, 3 or 4 of the MOP gene (Panel A), exons 1, 2 or 3 of the DOP gene (Panel B), exons 1, 2 or 3 of the KOP gene (Panel C), or exons 1, 2 or 3 of the NOP gene (Panel D). Alterations in food intake are also presented following either a missense (MS) ODN identical to the AS ODN directed against exon 2 of the KOP gene except for the reversal of two pairs of nucleotide bases or a nonsense (NS) ODN. The crosses denote significant alterations in food intake relative to vehicle control treatment (Tukey comparisons ($P<0.05$), and the asterisks denote significant alterations in food intake relative to the KOP exon 2 AS ODN (Fisher comparisons, $P<0.05$).

Figure 1.

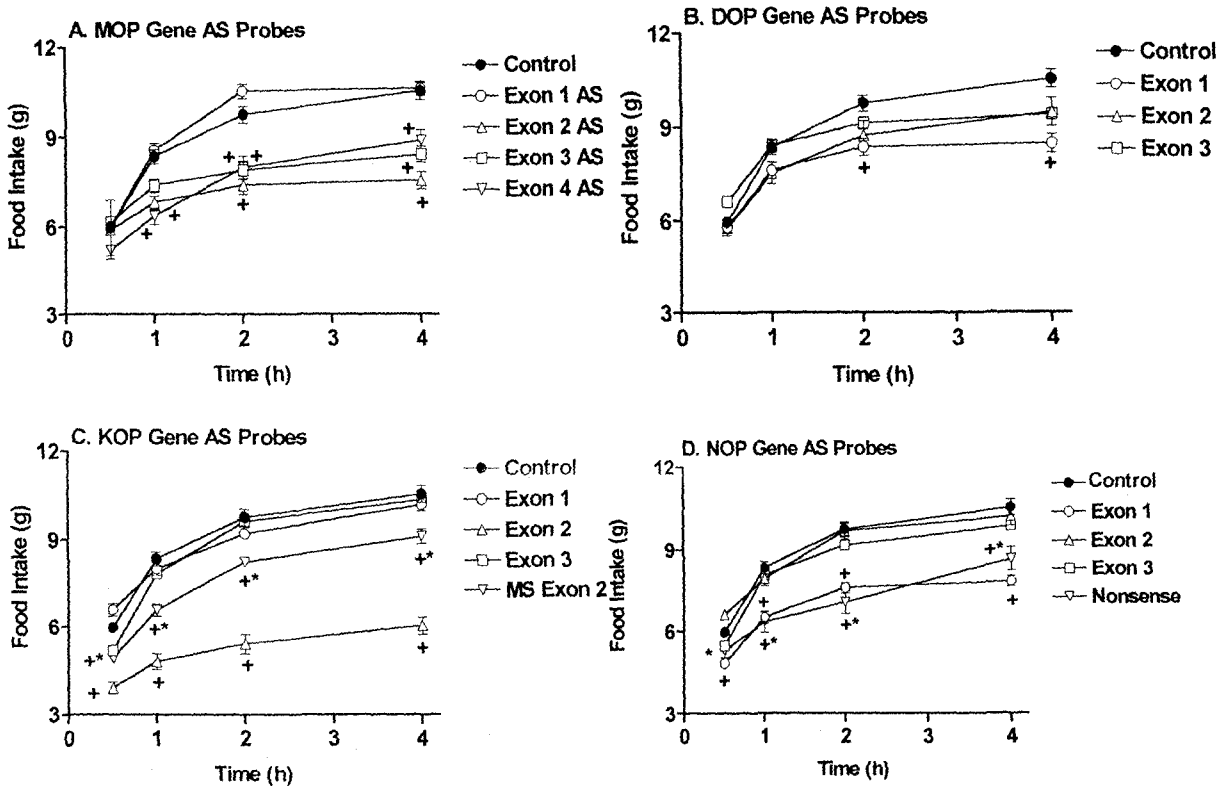


Figure 2. Alterations (Mean ±S.E.M.) in either food intake 24 h following reintroduction of food (Panel A), weight loss during deprivation (Panel B) or weight recovery after deprivation (Panel C) in food-deprived (24 h) rats pretreated with either a vehicle control or AS ODN probes directed against either exons 1, 2, 3 or 4 of the MOP gene, exons 1, 2 or 3 of the DOP gene, exons 1, 2 or 3 of the KOP gene, or exons 1, 2 or 3 of the NOP gene. The crosses denote significant alterations in food intake relative to vehicle control treatment (Tukey comparisons ($P<0.05$), and the asterisks denote significant alterations in food intake relative to the KOP exon 2 AS ODN (Fisher comparisons, $P<0.05$).

Figure 2.

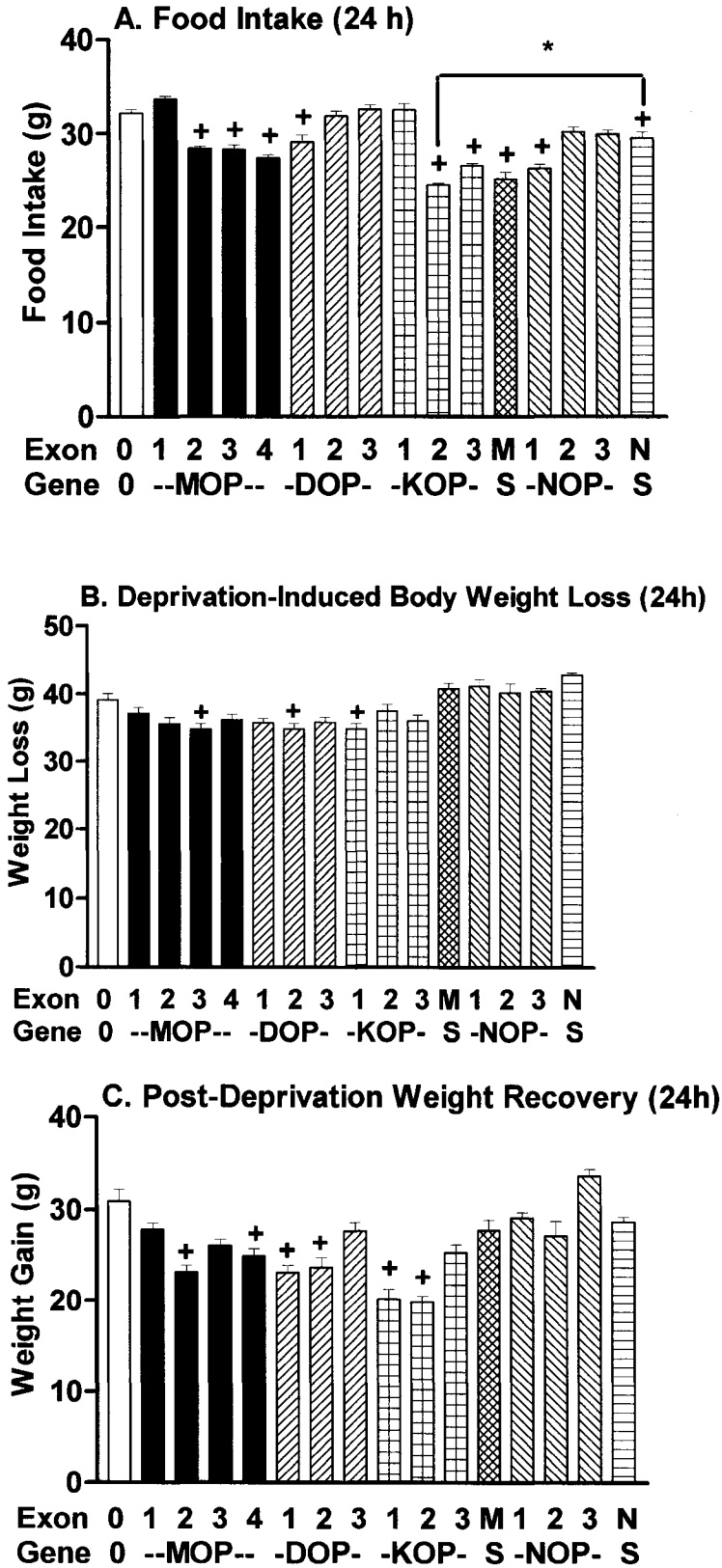


Table 2. Alterations in the rate (g/h) of food intake across a 24 h time course of food reintroduction in food-deprived (24 h) rats following vehicle control or antisense (AS), missense (MS) or nonsense oligodeoxynucleotide probes directed against the MOP, DOP, KOP and NOP genes.

Treatment	0.5 h	1.0 h	2.0 h	4.0 h	24.0 h
	M (±SEM)	*M (±SEM)*	*M (±SEM)*	*M (±SEM)*	*M (±SEM)*
Control	11.94(0.34)	8.34(0.24)	4.87(0.14)	2.63(0.07)	1.34(0.06)
MOP AS Ex 1	12.00(0.28)	8.55(0.24)	5.27(0.12)	2.66(0.06	1.41(0.02)
MOP AS Ex 2	11.80(0.38)	6.82(0.22) +	3.70(0.15) +	1.89(0.07) +	1.18(0.01) +
MOP AS Ex 3	12.33(0.28)	7.38(0.22)	3.95(0.14) +	2.11(0.06) +	1.18(0.02) +
MOP AS Ex 4	10.4(0.37)	6.37(0.29) +	3.18(0.15) +	2.22(0.09) +	1.14(0.01) +
DOP AS Ex 1	11.63(0.35)	7.61(0.29)	4.18(0.14) +	2.12(0.08) +	1.21(0.04) +
DOP AS Ex 2	11.53(0.50)	7.52(0.34)	4.37(0.24)	2.37(0.12)	1.33(0.02)
DOP AS Ex 3	13.23(0.27)	8.40(0.21)	4.57(0.10)	2.35(0.04)	1.36(0.02)
KOP AS Ex 1	13.17(0.43)	8.00(0.23)	4.60(0.06)	2.55(0.06)	1.36(0.04)
KOP AS Ex 2	7.83(0.41) +	4.80(0.27) +	2.70(0.16) +	1.51(0.07) +	1.02(0.01) +
KOP AS Ex 3	10.36(0.16)	7.82(0.15)	4.80(0.08)	2.59(0.03)	1.11(0.01) +
KOP MS Ex 2	9.82(0.28)+*	6.57(0.21) +*	4.12(0.08) +*	2.28(0.06) +*	1.05(0.04) +
NOP Exon 1	9.66(0.27) +	6.51(0.23) +	3.80(0.09) +	1.96(0.05) +	1.10(0.02) +
NOP Exon 2	13.24(0.27)	7.94(0.27)	4.84(0.15)	2.56(0.08)	1.26(0.02)
NOP Exon 3	10.93(0.32)	8.07(0.18)	4.58(0.17)	2.47(0.03)	1.25(0.02)
Nonsense	10.60(0.56) *	6.34(0.38) +*	3.54(0.21) +*	2.17(0.11) +*	1.23(0.02) +*

Note 1: An additional measure that is independent of cumulative intake, the rate of consumption, was assessed during each of the post-deprivation intake periods in which intake was divided by the particular time point (h) during which it was assessed. Then, a second randomized-block two-way analysis of variance was performed on the rate of food intake data for the between-subject variable (vehicle and the 15 different ODN conditions), the within-subject variable (post-deprivation intake time), and for their interaction.

Note 2: Deprivation-induced food intake (g) as a function of AS ODN probe treatment and controls over 24 hours. Crosses indicate differences compared to control, $P < 0.05$. Asterisks indicate difference among KOP Exon 2, MS and Nonsense, $P < 0.05$.

For the DOP gene, deprivation-induced feeding was significantly, but modestly reduced by the exon 1 AS ODN probe only after 2 (14%), 4 (20%) and 24 (10%) h (Figures 1B and 2A). Again, an identical pattern of significant effects was observed for the rate of deprivation-induced intake (Table 2). In contrast, AS ODN probes directed against exons 2 or 3 of the DOP gene failed to alter deprivation-induced feeding at any time point.

For the KOP gene, deprivation-induced feeding was markedly and significantly reduced by the AS ODN probe directed against exon 2 across the time course with effects noted after 0.5 (35%), 1 (43%), 2 (45%), 4 (43%) and to a lesser degree, 24 (24%) h (Figures 1C and 2A). Although an AS ODN probe directed against exon 3 of the KOP gene failed to affect deprivation-induced feeding after 0.5-4 h, it produced a modest significant reduction (17%) after 24 h (Figure 2A). Again, an identical pattern of significant effects was observed for the rate of deprivation-induced intake (Table 2). In contrast, an AS ODN probe directed against exon 1 of the KOP gene failed to alter deprivation-induced feeding at any time point. Although deprivation-induced intake was significantly reduced by either the MS ODN (0.5-4 h, Figure 1C) or NS ODN (1-4 h, Figure 1D) probes, the magnitude of these effects were significantly less than that observed following the AS ODN probe directed against exon 2 of the KOP gene.

Moreover, the significant reductions in deprivation-induced intake after 24 h (Figure 2A) following the NS, but not the MS ODN was also significantly less than that observed following the AS ODN probe directed against exon 2 of the KOP gene. This same pattern of MS and NS ODN effects relative to the AS ODN probe directed against

exon 2 of the KOP gene was observed for the rate of deprivation-induced intake as well (Table 2).

For the NOP gene, deprivation-induced feeding (Figures 1D, 2A) as well as the rate of intake (Table 2) was modestly (19-26%) but significantly reduced by the AS ODN probe directed against exon 1 across the time course. In contrast, AS ODN probes directed against either exon 2 or 3 of the NOP gene failed to affect deprivation-induced feeding at any time point.

Opioid AS ODN probes and deprivation-induced weight changes: Significant differences in the magnitude of deprivation-induced weight loss were observed among vehicle control and ODN conditions ($F(15,945)= 57.25$, $P<0.0001$). Deprivation-induced weight loss was modestly (11%), but significantly reduced in rats receiving AS ODN probes directed against exon 3 of the MOP gene, exon 2 of the DOP gene, and exon 1 of the KOP gene relative to vehicle control values (Figure 2B). However, these weight loss differences did not appear to explain ODN-induced changes in deprivation-induced intake. Whereas deprivation-induced food intake was subsequently reduced in animals receiving AS ODN probes directed against exon 3 of the MOP gene (see above), deprivation-induced food intake was unaffected in the other two ODN groups.

Significant differences in the magnitude of food reintroduction-induced weight gain were observed among vehicle control and AS ODN conditions ($F(15,945)= 76.26$, $P<0.0001$). Significant reductions in the magnitude of body weight recovery were noted for AS ODN probes directed against exons 2 (27%), 3 (19%) or 4 (22%) of the MOP gene, exons 1 (28%) or 2 (26%) of the DOP gene, or exons 1 (37%) or 2 (38%) of the KOP gene relative to the vehicle control condition (Figure 2C). In contrast, weight

recovery changes failed to occur following AS ODN probes directed against exon 1 of the MOP gene, exon 3 of the DOP or KOP genes, or exons 1, 2 or 3 of the NOP gene. It should be noted that neither the MS ODN for exon 2 of the KOP gene nor the NS ODN significantly altered either deprivation-induced weight loss or food reintroduction-induced weight gain relative to vehicle control treatment. All animals recovered their full body weight within two weeks after treatment (data not shown).

Discussion

The present study demonstrated that AS ODN probes directed against different exons of each of the opioid receptor genes selectively altered the magnitude of deprivation-induced food intake and body weight recovery. Thus, AS ODN probes directed against particular exons of the MOP (exons 2, 3, 4), KOP (exon 2), NOP (exon 1) or DOP (exon 1) genes reduced deprivation-induced feeding, AS ODN probes directed against particular exons of the MOP (exons 2, 3, 4), KOP (exons 1, 2, 3) or DOP (exons 1, 2), but not NOP genes reduced food reintroduction-induced weight gain. In contrast, none of the AS ODN probes exacerbated the weight loss noted during the deprivation period. Some points relative to the data should be considered. First, although significant in both (cumulative and rate) measures of intake, the size of some of the effects was relatively small. Relative to some other published reports (e.g., Rowland and Carlton, 1988), the inter-individual variability of deprivation-induced feeding under vehicle control conditions was relatively small, probably due to the extensive acclimation (~1 month) of the animals to the environment as well as the handling of animals before testing. The large number (65) of animals reduced variance in the control group, and the matching of animals based on their vehicle values reduced variance in the experimental

conditions. Second, the use of standard food chow pellets suggests that any effects of the antisense probes were probably due to changes in energy-related relative to palatability-related aspects of the food source. Third, although water intake was not explicitly measured in this study, its contribution to AS ODN-induced changes in body weight recovery in food-deprived animals is probably not a prevailing factor in the body weight changes. Whereas KOP AS ODN probes were more effective in reducing body weight recovery than MOP AS ODN probes, selective μ, but not κ opioid antagonists significantly reduce water intake following water deprivation (Beczkowska et al., 1992). The following sections examine these effects in terms of opioid receptor subtype specificity in mediating deprivation-induced feeding.

DOP gene, the δ opioid receptor and deprivation-induced feeding: Ventricular pretreatment with such δ opioid antagonists as DALCE and naltrindole failed to alter deprivation-induced feeding (Arjune et al., 1991; Koch and Bodnar, 1994), whereas intracerebral administration of the δ_2 opioid antagonist, naltrindole isothiocyanate, into the ventral tegmental area produces small, but significant reductions in deprivation-induced feeding (Ragnauth et al., 1997). The present study found that only the AS ODN probe against exon 1 of the DOP gene produced significant, though minimal (10-20%) reductions in deprivation-induced feeding, an effect consistent with the previous opioid antagonist data. This small reduction in deprivation-induced feeding was accompanied by a 26-28% reduction in weight gain observed 24 h following reintroduction of food, suggesting that the reduction in weight recovery was not entirely accounted for by intake reductions. This dichotomy between intake and weight reductions has been observed previously for small chronic delta antagonist effects (Cole et al., 1995, 1997). A similar

correspondence between δ antagonist and DOP AS ODN effects has been observed for the feeding responses elicited by glucoprivic and lipoprivic regulatory challenges (Arjune et al., 1991; Bodnar et al., 1995; Burdick et al., 1998; Koch and Bodnar, 1994; Koch et al., 1995; Ragnauth et al., 1997; Stein et al., 2000).

NOP gene and deprivation-induced feeding: The AS ODN probe against exon 1 of the NOP gene modestly (19-26%), but significantly reduced deprivation-induced feeding without affecting body weight recovery. The presumed endogenous ligand for the ORL-1 receptor and the NOP gene, orphanin FQ/nociceptin, elicits feeding following central administration (Pomonis et al., 1996; Stratford et al., 1997), and this agonist effect is blocked by AS ODN probes directed against each of the three exons of the NOP gene (Leventhal et al., 1998), suggesting that the receptor responsible for these ingestive effects is completely encoded by the gene. The modest effect of NOP AS ODN probes upon deprivation-induced feeding is similar to that observed following glucoprivic and lipoprivic regulatory challenges (Burdick et al., 1998; Stein et al., 2000).

KOP gene, the κ opioid receptor and deprivation-induced feeding: Either ventricular or intracerebral pretreatment with the κ_1 opioid antagonist Nor-BNI produces significant (~30%) reductions in deprivation-induced feeding (Bodnar et al., 1995; Kelley et al., 1996; Koch and Bodnar, 1994; Koch et al., 1995; Levine et al., 1990; Ragnauth et al., 1997). The present study found that only the AS ODN probe directed against exon 2 of the KOP gene produced significant and consistent short-term (4 h: 25-45%) and longer-term (24 h: 25%) reductions in deprivation-induced feeding. Body weight gain was reduced (21-38%) following reintroduction of food by AS ODN probes against all three exons of the KOP gene. The specificity of the KOP exon 2 AS ODN probe effect

was examined using both a MS ODN probe, which differed by the sequence reversal of two pairs of bases, and a completely scrambled NS ODN probe. Although both the MS ODN and the NS ODN probes produced small and significant reductions in deprivation-induced intake, the magnitude of these effects were in turn significantly smaller than the KOP exon 2 AS ODN probe effect. Moreover, this KOP AS ODN probe, but not the MS or NS ODN probes, significantly retarded body weight recovery.

Mismatch controls typically establish the specificity of response. Because even nonspecific effects can be sequence-specific, we try to keep our mismatch probes closely matched to the antisense probe. Switching the sequence of four bases out of 20 is usually sufficient to completely eliminate activity in most situations, and thereby serve as an effective missense control (see review: Pasternak and Pan, 2000). However, a number of previous studies have observed missense probe effectiveness for KOP (Pasternak et al., 1999), DOP (Rossi et al., 1997), MOP (Abbadie et al., 2002; Rossi et al., 1997) and NOP (Pan et al., 1995; Rossi et al., 1997) probes, particularly in analgesic assays. Of consequence for the present results, a KOP missense probe significantly reduced analgesia elicited by the κ_1 agonist, U50488H by 15%, yet this effect was significantly smaller than the reduction observed by the proper AS ODN (Pasternak et al., 1999), effects that parallel the present findings.

Therefore, it appears that both κ_1 opioid antagonist effects and KOP AS ODN effects consistently reduce deprivation-induced feeding, complementing similar strong κ_1 antagonist and KOP AS ODN effects upon glucoprivic or lipoprivic regulatory challenges (Burdick et al., 1998; Stein et al., 2000). Similarly, a combined opioid antagonist and opioid AS ODN approach has also been used to distinguish the receptor

responsible for feeding elicited by dynorphin A_{1-17}, indicating a primary role for the κ_1 opioid receptor and KOP opioid receptor gene (Silva et al., 2002).

MOP gene, the μ opioid receptor and deprivation-induced feeding: The ability of selective μ and μ_1 opioid antagonists to produce the most marked degree of inhibition of deprivation-induced intake following ventricular (Arjune et al., 1990; Koch and Bodnar, 1994; Levine et al., 1991; Simone et al., 1985) and intracerebral (hypothalamic paraventricular nucleus, nucleus accumbens: (Bodnar et al., 1995; Kelley et al., 1996; Koch et al., 1995; Levine et al., 1991) administration would suggest that AS ODN probes directed against particular exons of the MOP gene would produce the most potent and marked reductions in deprivation-induced intake and weight recovery. Yet, the marginal short-term (4 h: 16-27%) and longer-term (24 h: 12-15%) reductions in deprivation-induced feeding by MOP AS ODN probes against either exons 2, 3 or 4 fall far short of the 50-70% reductions noted over the same time course by βFNA or naloxonazine. To minimize the possibility of a false negative, we routinely examine multiple AS probes targeting each exon, as well as its mismatch complement. It is not clear why some AS ODNs are active against deprivation-induced feeding while others are not. However, at least two potential factors may explain this effect: a) downregulation limitations and efficiency of hybridization for the AS probes and mRNA abundance induced by the manipulation, and b) isoforms of the MOP gene.

Part of this inability of the AS ODN probes to produce more potent effects may be a reflection of the fact that the downregulation of mRNA and protein following AS treatment is limited, oftentimes by 30-50% (Pasternak and Pan, 2000; Standifer et al., 1994; Standifer et al., 1995; Standifer et al., 1996). The ability of these probes to actively

downregulate other functions (see review: Rossi and Pasternak, 1997) makes technical problems related to the AS unlikely. Thus, the AS ODN probes used against each of the exons of the MOP gene displayed efficiency of hybridization since they differentially affected equi-effective opioid-induced feeding (as well as analgesic) responses: exon 1 (DAMGO, morphine, β-endorphin, dynorphin), exon 2 (M6G, beta-endorphin), exon 3 (M6G) and exon 4 (DAMGO, β-endorphin) (Leventhal et al., 1997, 1998; Silva et al., 2001, 2002). It also seems likely that the effectiveness of AS treatment is dependent on the abundance of the mRNA being targeted. The more abundant the message, the more difficult it may be to achieve greater levels of downregulation. The low abundance of opioid receptor mRNA may typically enhance their suitability in AS studies such as the potent and similar magnitudes by βFNA and MOP (especially exon 1) AS ODN probes in reducing glucoprivic and lipoprivic regulatory challenges (Arjune and Bodnar, 1990; Arjune et al., 1990, 1991; Bodnar et al., 1995; Burdick et al., 1998; Koch and Bodnar, 1994; Koch et al., 1995; Ragnauth et al., 1997; Stein et al., 2000) as well as feeding elicited by opiates, opioid agonists and the opioid peptides, β-endorphin and dynorphin $A_{1\text{-}17}$ (Leventhal et al., 1997, 1998; Silva et al., 2001, 2002). In contrast, food deprivation may increase the abundance of opioid receptor mRNA in the same way as it profoundly alters both opioid peptide and opioid receptor levels (Aravich et al., 1993; Berman et al., 1994, 1997; Kim et al., 1996; Wolinsky et al., 1994, 1996a, 1996b). Therefore, the smaller magnitudes of effects may be due to the lesser ability of these probes to downregulate the increased receptor messages produced by this profound homeostatic challenge.

A second possibility for the smaller magnitude of AS effects is that different coding regions, isoforms, or splice variants of the MOP gene may mediate these feeding responses. Indeed, the pattern of these MOP AS ODN effects upon deprivation-induced feeding resembles the ability of AS ODN probes against exons 2 or 3, but not exons 1 or 4 of the MOP gene to reduce feeding elicited by the morphine metabolite M6G (Leventhal et al., 1998). Although there is only one identified MOP gene (Chen et al., 1993; Wang et al., 1993), the existence of an alternative spliced exon 1 (corresponding to the N-terminus) was strongly suggested by *in vivo* AS mapping studies. An alternative upstream exon homologous to the mouse exon 11 had been identified through a rat genome database search (unpublished data). However, the actual exon and associated variants have not been isolated so far. Most of the multiple isoforms or splice variants of the MOP gene are clones with different coding exons after the third exon of the MOP [MOR-1] clone in the mouse (Bare et al., 1994; Pan et al., 1999, 2000, 2001; Pasternak et al., 2001; Pasternak and Pan, 2000; Zimprich et al., 1995). Recently, six additional splice variants from mouse, five from rat and six from human MOP gene have been identified with homologous exon compositions across the species (unpublished data), suggesting that alternative splicing of the MOP gene is conserved across species. For instance, the MOR-1C clone has coding regions of exons 7, 8 and 9 instead of exon 4 in addition to the common exons 1, 2 and 3 (Pan et al., 1999), and antibodies directed against these coding regions have allowed anatomical comparisons with the MOP gene itself (antibody against exon 4) (Abbadie et al., 2000a, 2000b, 2001a, 2001b). Thus, hypothalamic areas associated with ingestive responses show sparse immunoreactivity to MOP exon 4 antibodies, and dense immunoreactivity to MOR-1C exons 7-9 antibodies. Further,

whereas MOP exon 4-LI was minimally affected following food deprivation and food restriction, these manipulations significantly increased MOR-1C-LI in the hypothalamic paraventricular nucleus (Abbadie et al., 2001), a site at which microinjection of the μ opioid antagonist, βFNA, produced some of the most marked reductions in deprivation-induced feeding (Koch et al., 1995). Whereas these techniques may provide evidence for receptor isoforms of the opioid receptor gene, definitive proof of this concept will require the isolation of cDNA encoding the splice variants.

In conclusion, opioid antagonist analyses of deprivation-induced feeding suggested a primary role for μ, and lesser roles for κ_1 and δ opioid receptors in this response. The present AS study clearly complemented the significant κ_1 opioid antagonist effects, and indicated that the δ opioid receptor is minimally involved in deprivation-induced feeding. MOP AS probes were clearly less effective in reducing deprivation-induced feeding than μ opioid antagonists. AS mapping approaches can be used to target sites anywhere along an mRNA as well as demonstrate the existence of multiple receptors and or receptor subtypes, even though evidence from many laboratories describe a single MOP gene (see review: Pasternak and Pan, 2000). The relatively small effects induced by the traditional (exons 1-4) MOP AS probes suggest additional intriguing potential targets (MOP, DOP and KOP splice variants) involved in mediating feeding responses to deprivation and other manipulations. Although many questions remain regarding the opioid mechanisms mediating deprivation-induced feeding, it does appear that more than the traditional μ opioid receptor controls this behavior.

CHAPTER 5. SPECIFIC AIM TWO.

Deprivation-induced feeding: The Role of G-Protein Alpha Subunits in Deprivation-Induced Feeding Through Administration of Antisense ODN Probes

Introduction

The antisense oligodeoxynucleotide (AS ODN) technique has been used to functionally characterize specified targets by correlating the molecular biology of the target with its *in vivo* pharmacology (see review: Myers and Dean, 2000). The ability of AS ODN treatment to selectively disrupt protein function is based upon its presumed ability to specifically anneal with mRNA sequences of complementary bases resulting in protein down-regulation through either translational blockade, splicing arrest, and/or RNase-H degradation of mRNA (see review: Myers and Dean, 2000). Recently, this technique has been found to be useful in examining receptor-selective effects for particular neuropeptide systems (e.g., opioids, neuropeptide Y and galanin) involved in the elicitation of feeding behavior. Although DAMGO, morphine and morphine's active metabolite, morphine-6beta-glucuronide (M6G) each elicit feeding that was blocked by pretreatment with mu-selective opioid antagonists (Leventhal, 1998), the AS ODN technique could differentiate these responses further yet provide converging evidence with antagonist effects. Thus, a detailed mapping study of the four exons of the mu opioid receptor (MOR-1) clone indicated that AS ODN probes targeted against either exons 1 or 4 of the MOR-1 gene (Opr*m*1) blocked feeding elicited by either morphine or the mu-selective opioid agonist, DAMGO, yet had no effect on feeding induced by M6G (Leventhal et al., 1998; Rossi et al., 1997). In contrast, AS ODN probes targeted against either exons 2 or 3 of Opr*m*1 blocked M6G-induced feeding, yet were ineffective against

morphine-induced feeding. AS ODN probes directed against the DOR-1, KOR-1 and KOR-3/ORL-1 opioid receptor clones reduce feeding elicited by delta, kappa and ORL-1 opioid agonists respectively (Leventhal et al., 1998a, 1998b). Moreover, these opioid AS ODN probes selectively and differentially reduced food intake under normal, glucoprivic and lipoprivic conditions (Leventhal et al., 1996; Burdick et al., 1998; Stein et al., 2000), indicating that they are active under normal homeostatic conditions, and not just against opioid agonist-induced effects. AS ODN probes directed against either neuropeptide Y itself or the Y_5 receptor decrease food intake (Schaffhauser, 1997), whereas AS ODN probes directed against galanin decrease fat intake and body weight (Akabayashi et al., 1994). Other studies have shown the usefulness of AS ODN probes in identifying G-protein involvement as well (e.g., (Plata-Salaman et al., 1995; Silva et al., 2000, 2002).

Opioid and other neuropeptide receptors mediate their effects through the putative activation of guanine nucleotide binding proteins (G-proteins) with which they are coupled (Childers, 1988; Pasternak and Standifer, 1995; Reisine and Bell, 1993; Standifer et al., 1996; Uhl et al., 1994). *In vitro* studies have also suggested that opioid receptors can activate multiple effectors through different G-protein -subunits to modulate the inhibition of cAMP (see review: Roerig, 1998). G-proteins are composed of three distinct subunits (α, β, γ) that couple the receptors with their effectors. The α-subunit has been identified as the pharmacologically relevant subunit due to its intrinsic GTPase activity, although the β and γ subunits can also modulate the activity of various effectors (see review: Standifer and Pasternak, 1995). The α-subunit also seems to establish the identity of G-protein-mediated neuropeptide receptors. For example, whereas pertussis toxin (PTX) irreversibly inactivates a number of G-proteins, including $G_i\alpha$ and $G_o\alpha$, but not

$G_s\alpha$, cholera toxin (CTX) selectively blocks the $G_s\alpha$ subtype (e.g., Gilman, 1987; Goode and Raffa, 1997; Hildebrandt et al., 1983; Kadata and Ui, 1981; Stryer and Bourne, 1986). PTX pretreatment selectively blocks the analgesic responses to opioid agonists including morphine, DAMGO, and sufentanil (Bodnar et al., 1990; Goode and Raffa, 1997), and also inhibits neuropeptide Y-induced feeding (Chance et al., 1989). However, administration of PTX and CTX produces non-specific weight loss and reductions in baseline food intake, presumably induced by malaise (Bodnar et al., 1990; Chance et al., 1989).

The use of intracerebroventricular G-protein AS ODN probes produces significant down-regulation (50-70%) of G-protein α-subunits (Sanchez-Blasquez et al., 1995; Standifer et al., 1996). Administration of AS ODN probes against either $G_i\alpha_2$ or $G_{x/z}\alpha$ significantly reduces the analgesic activity of mu and delta opioid agonists (Raffa et al., 1996; Sanchez-Blasquez et al., 1995). Morphine and M6G analgesia can be distinguished from one another on the basis of their individual G-protein activation profile such that AS ODN probes directed against either the G_{i2} or G_o, α-subunits reduced morphine, but not M6G-induced analgesia, whereas AS ODN probes directed against the G_{i1} or the $G_{x/z}$ α-subunits reduced M6G, but not morphine analgesia. AS ODN probes directed against the G_s α-subunit reduced both analgesic responses in this study (Rossi et al., 1995; Standifer et al., 1996). A highly similar pattern of effects was observed for G-protein AS ODN-mediated effects upon feeding responses elicited by morphine and M6G. Morphine-induced feeding was significantly and selectively reduced by an AS ODN probe directed against $Gi\alpha_2$, and enhanced by pretreatment with a $G_s\alpha$ probe (Silva et al., 2000). In contrast, M6G-induced feeding was significantly and selectively reduced by AS ODN

probes against $G_i\alpha_1$, $G_i\alpha_3$ or $G_{x/z}\alpha$. Since control nonsense ODNs did not alter either morphine- or M6G-induced feeding, these effects could not be attributed to nonspecific AS ODN effects. Importantly, these effects occurred independently of any G-protein AS ODN-mediated effect upon either body weight or ad libitum food intake (24 h) per se (Silva et al., 2000).

Plata-Salaman and coworkers (1995) found highly selective and specific G-protein AS ODN probe effects upon food intake during the onset of the dark cycle when rats typically ingest most of their daily food ration. An AS ODN probe directed against $G_o\alpha$ and $G_{oA}\alpha$ in particular significantly decreased nocturnal food, but not water intake. Importantly, AS ODN probes directed against $G_s\alpha$, $G_q\alpha$, or $G_i\alpha$ as well as a sense control failed to produce these effects. The ingestive response to food deprivation is a commonly used regulatory challenge to assess the roles of putative physiological and pharmacological systems in mediating food intake (e.g., Cooper and Clifton, 1996). Therefore, to evaluate whether these probes produced similar or dissimilar effects upon deprivation-induced feeding relative to nocturnal intake, the present study evaluated the effects of ventricularly-administered AS ODN probes directed against either G_{i1}, G_{i2}, G_{i3}, G_s, G_o, $G_{x/z}$ or G_q α-subunits as well as a control nonsense probe upon food intake following 24 h of food deprivation. Although AS ODN probes against G-proteins fail to affect baseline 24 h food intake and body weight (Silva et al., 2000), alterations in deprivation-induced weight loss and in subsequent body weight recovery were assessed following administration of each G-protein AS ODN probe as well.

Moreover, it was important to examine whether the actions of these probes were specific to feeding or nonspecific effects (malaise, aversion to food) that may have been

associated with the treatment of the probe. Thus, a flavor-flavor conditioned flavor preference test was used in which one novel taste in a single bottle (either cherry- or grape-flavored saccharin solution) was paired to a vehicle or a G-protein antisense probe injection in deprived rats. Following a period of recovery, all rats were deprived again but this time without the injection treatment and presented with two bottles (cherry and grape-flavored saccharin). The drinking amount of the flavored-saccharin solution was recorded for a 24 h time course. If the rats displayed conditioned taste aversion to any of the probes, the aversion should be reflected in the avoidance of the flavor that was previously paired with those probes. This experiment was published in Brain Research (955: 45-54, 2002).

Methods

Surgeries: See General Methods, Chapter 3.

Procedure

All protocols were designed based on the Queens College Institutional Animal Care and Use Committee. To assess the effects of food deprivation upon baseline intake and body weight, all rats underwent an initial 4-day protocol beginning at approximately 3 h into the light cycle. On Day 1, all animals were weighed (Pre-Deprivation Weight 1), and pre-weighed food bins and water were made available to the animals for 24 h. Food intake (Pre-Deprivation Intake) was measured after 24 h with spillage collected beneath each cage by paper towels. On Day 2, all animals were re-weighed (Pre-Deprivation Weight 2), and the rats were then food-deprived (water continuously available) for the next 24 h. On Day 3, all animals were re-weighed (Post-Deprivation Weight) again, and food was reintroduced by placing pre-weighed pellets on the floor of the wire mesh

cages. Cumulative intakes, adjusted for spillage collected beneath each cage by paper towels, were assessed 0.5, 1, 2, 4 h thereafter. Following this procedure, pre-weighed food bins and water were again made available for the next 20 h, allowing for the overall 24 h intake (Post-Deprivation Intake) to be measured at the beginning of Day 4. Rats were re-weighed again on Day 4 (Recovery Weight).

After a two week interval in which all rats were observed to completely recover normal body weights, subgroups of seven animals each received a single G-protein α-subunit AS ODN probe (25:g, 5:l, i.c.v.) directed against either G_{i1}, G_{i2}, G_{i3}, G_s, G_o, $G_{x/z}$, G_q, or a nonsense (NS) ODN on Day 2 of the protocol (Table 3). The subgroups of rats were matched for their deprivation-induced intake responses collected under baseline control procedures. Two weeks following the first AS ODN treatment, rats with patent cannulae were retested with a second single AS ODN or nonsense probe on Day 2 of another 4-day food deprivation protocol to fill out treatment groups.

Conditioned Taste Aversion Paradigm

In order to ensure that the effects of the antisense probes were specific to the treatment itself and not due to malaise or possible taste aversions, a conditioned taste aversion paradigm was performed. The probes that most potently reduced deprivation-induced intake (G_s and G_q) were selected as the most appropriate probes for this protocol. Twenty-one cannulated rats received a single calibrated (100 ml, 1 ml gradations) bottle of an unflavored 0.2% saccharin solution for 2 h daily over three days, and on the basis of their saccharin intake, were matched into three groups of seven rats each, for the following six-day paradigm. Saccharin was used as the unconditioned taste because it increases intake, yet has no intrinsic nutritive value to interfere with deprivation-induced

food intake. The first phase of the paradigm allowed the exposure of a particular flavored saccharin solution to be paired with a control or AS ODN condition in deprived rats. On Day 1, each of the rats was weighed (Pre-Deprivation Weight) and received one of the following three microinjection conditions: vehicle, the $G_s\alpha$ AS ODN or the $G_q\alpha$ AS ODN (Table 3). Following these injections, rats were then food-deprived (water continuously available) for the next 24 h. On Day 2, each of the rats was re-weighed (Post-Deprivation Weight) again to assess deprivation-induced weight loss, and food was reintroduced by placing pre-weighed pellets on the floor of the wire mesh cages. Half of the rats in each injection group received a grape-flavored (unsweetened Kool-Aid, 0.05%) saccharin (0.2%) solution in a calibrated bottle on the front of the cage, while the remaining half of the rats in each injection group received a cherry-flavored (unsweetened Kool-Aid, 0.05%) saccharin (0.2%) solution. Cumulative deprivation-induced food intakes, adjusted for spillage collected beneath each cage by paper towels, were assessed 0.5, 1, 2 and 4 h thereafter on Day 2. Cumulative deprivation-induced flavored saccharin intakes were also assessed over the same time period. Following this procedure, pre-weighed food bins, water and the flavored saccharin solution were again made available for the next 20 h, allowing for the overall 24 h food and flavored saccharin intakes (Post-Deprivation Intake) to be measured at the beginning of Day 3. Rats were re-weighed again on Day 3 (Recovery Weight) to assess recovery of body weight following reintroduction of food, and continued to have *ad libitum* access to food and water (but not the flavored saccharin solution). The second phase of the paradigm allowed the evaluation of conditioned flavor preferences (or aversions) to the particular flavored saccharin solution previously paired with a control or AS ODN condition in

Table 3. Base sequence of G-protein antisense oligodeoxynucleotide probes

Target protein	**Sequence (5' - 3')**
$G_{i\alpha 1}$	AGA CCA CTG CTT TGT A
$G_{i\alpha 2}$	CTT GTC GAT CAT CTT AGA
$G_{i\alpha 3}$	AAG TTG CGG TCG ATC AT
$G_{o\alpha}$	CGC CTT GCT CCG CTC
$G_{s\alpha}$	TTG TTG GCC TCA CGC TG
$G_{q\alpha}$	GCT TGA GCT CCC GGC GGG CG
$G_{x/z\alpha}$	GGG CCA GTA GCC CAA TGG G
Nonsense	GGG GGA AGT AGG TCT TGG

deprived rats. At the beginning of Day 4, the rats were re-weighed, were not injected, but were again food-deprived (water continuously available) for the next 24 h. On Day 5, the rats were again re-weighed to assess deprivation-induced weight loss. All rats then received grape-flavored and cherry-flavored saccharin solutions in two calibrated bottles on the front of the cage in the presence of water. Cumulative deprivation-induced saccharin intakes of each flavor in the two bottles were assessed 5, 15, 30 and 60 min thereafter to assess preferences or aversions. Following the 1 h saccharin intake measure, pre-weighed food bins, water and the two bottles of the flavored saccharin solutions were again made available for the next 23 h, allowing for assessment of food and 2-bottle flavored saccharin intakes to be measured after 4 and 24 h. Rats were re-weighed at the beginning of Day 6 to assess recovery of body weight following reintroduction of food.

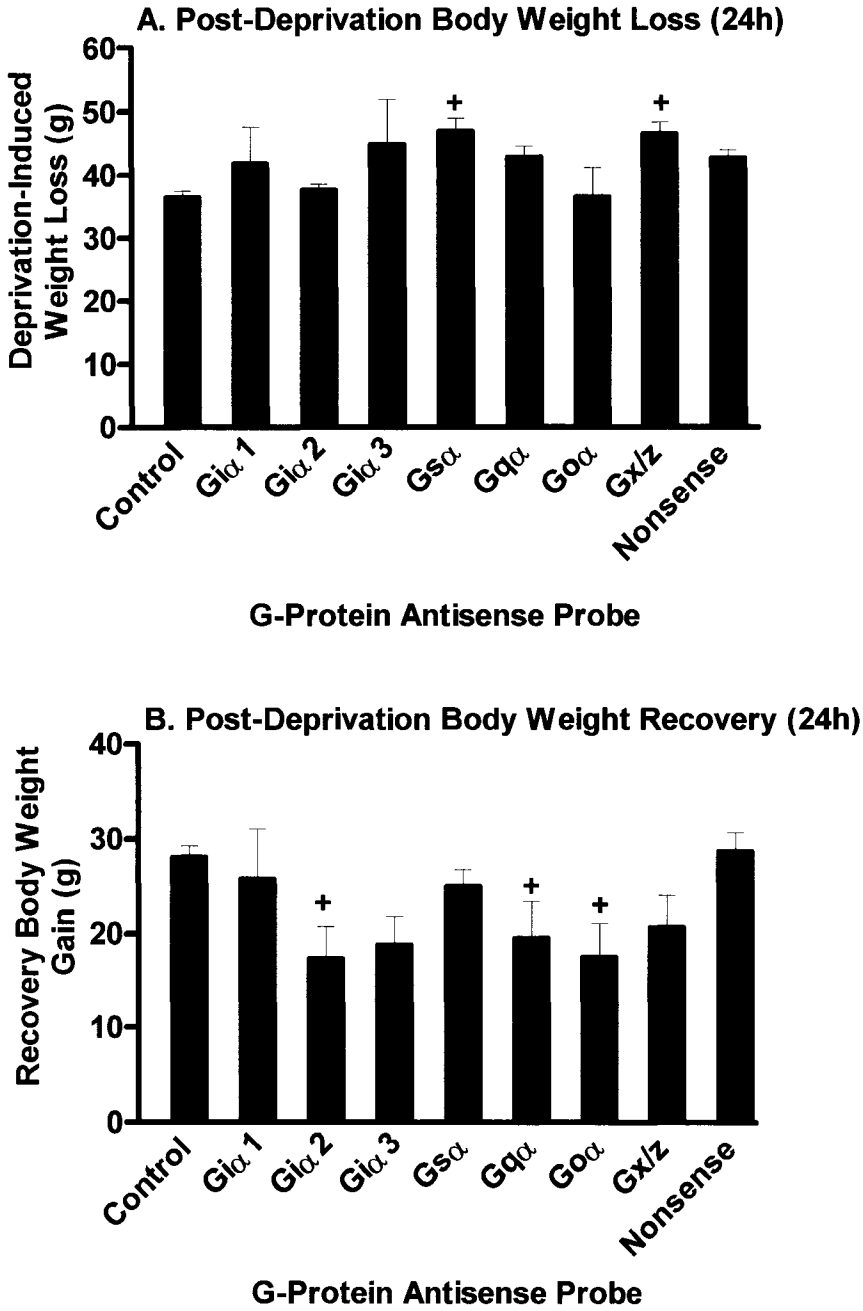
Results

G-Protein AS ODN probes and deprivation-induced weight loss: Significant differences in the magnitude of weight loss following 24 h of food deprivation were observed among treatment conditions ($F(8,105)= 3.30$, $P< 0.0021$). Rats pretreated with the AS ODN probes directed against either the $G_s\alpha$ or the $G_{x/z}\alpha$ subunits displayed significantly greater weight loss following 24 h of food deprivation than control treatment (Figure 3, Panel A). In contrast, none of the other AS ODN probes or the NS ODN probe significantly altered the magnitude of deprivation-induced weight loss.

G-Protein AS ODN probes and recovery weight gain: Significant differences were observed among treatment conditions ($F(8,105)= 2.91$, $P< 0.0056$) in the magnitude of weight gain 24 h following reintroduction of food after food deprivation. Rats pretreated with AS ODN probes directed against either the $G_i\alpha_2$, $G_q\alpha$ or $G_o\alpha$ subunits displayed

Figure 3. Alterations (Mean, $\pm$ SEM) in body weight 24 h following food deprivation (Panel A) and 24 h following food reintroduction (Panel B) in rats pretreated with control treatment (n=37), AS ODN probes (n=7/each) directed against either the $G_i\alpha_1$, $G_i\alpha_2$, $G_i\alpha_3$, $G_s\alpha$, $G_q\alpha$, $G_o\alpha$, $G_{x/z}\alpha$, or a nonsense ODN probe. The crosses denote significant alterations in body weight relative to control treatment.

Figure 3.



significantly less recovery of body weight than control treatment (Figure 3, Panel B). In contrast, none of the other AS ODN probes or the NS ODN probe significantly altered the magnitude of recovery weight gain.

G-Protein AS ODN probes and deprivation-induced food intake: Significant differences in the magnitude of deprivation-induced food intake were observed among control and AS ODN conditions after 0.5 ($F(8,84)= 3.20$, $P< 0.0032$), 1 ($F= 5.38$, $P< 0.0001$), 2 ($F= 5.25$, $P<0.0001$), 4 ($F= 4.18$, $P< 0.0003$) and 24 ($F= 4.16$, $P< 0.0003$) h. AS ODN probes directed against G-protein α-subunits differentially and significantly altered deprivation-induced feeding across the time course of testing relative to control vehicle treatment (Figure 4). Thus, deprivation-induced intake was significantly reduced across the entire initial 4 h time course following AS ODN probes directed against either directed against either $G_{x/z}\alpha$ (47-52% reductions) or to a lesser extent, $G_i\alpha_3$ (37% reductions). Deprivation-induced intake was significantly reduced only between 1-2 h following an AS ODN probes directed against $G_i\alpha_2$ (40-43% reductions). These five AS ODN probes directed against G-protein αsubunits produced significant though more modest (19-25%) reductions in deprivation-induced intake after 24 h (Figure 5). Interestingly, two AS ODN probes directed against either $G_i\alpha_1$ or $G_o\alpha$ failed to significantly alter deprivation-induced feeding across the entire 24 h time course. Finally, and perhaps importantly, a nonsense probe, equal in length to the effective antisense probes, failed to significantly alter deprivation-induced feeding across the entire 24 h time course, indicating that non-specific actions of nucleotide treatment per se were not responsible for the reductions in intake following food deprivation.

Figure 4. Alterations (Mean, ± SEM) in food intake 0.5 (Panel A), 1 (Panel B), 2 (Panel C) and 4 (Panel D) h following reintroduction of food in food-deprived (24 h) rats pretreated with control treatment, AS ODN probes directed against either the $G_i\alpha_1$, $G_i\alpha_2$, $G_i\alpha_3$, $G_s\alpha$, $G_q\alpha$ $G_o\alpha$, $G_{x/z}\alpha$ or a nonsense ODN probe. The crosses denote significant alterations in food intake relative to control treatment.

Figure 4.

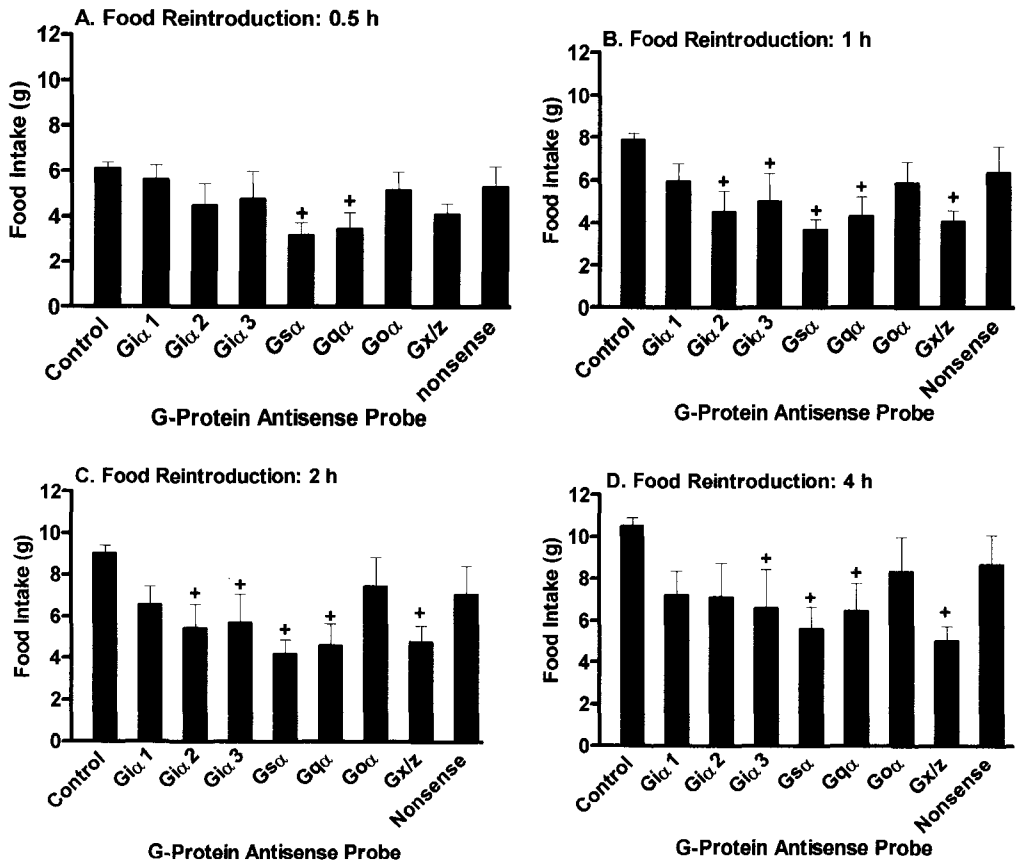
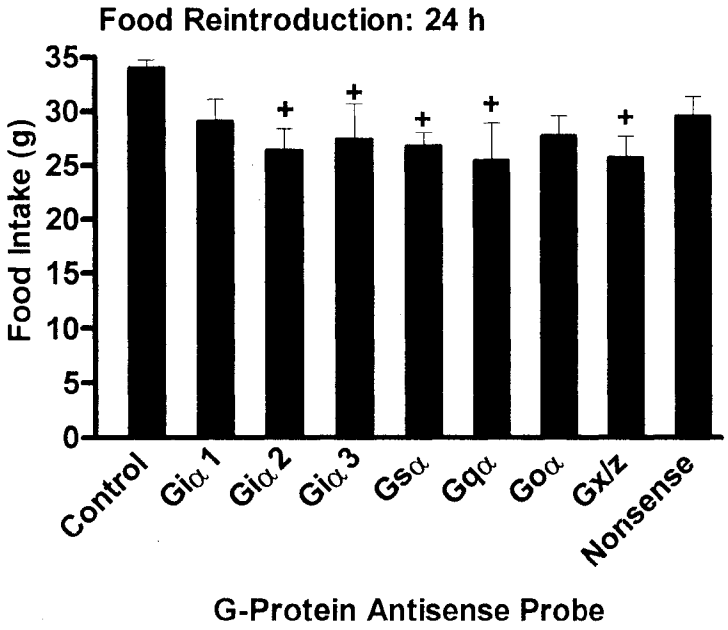


Figure 5. Alterations (Mean, $\pm$ SEM) in food intake 24 h following reintroduction of food in food-deprived (24 h) rats pretreated with control treatment, AS ODN probes directed against either the $G_i\alpha_1$, $G_i\alpha_2$, $G_i\alpha_3$, $G_s\alpha$, $G_q\alpha$, $G_o\alpha$, $G_{x/z}\alpha$ or a nonsense ODN probe. The crosses denote significant alterations in food intake relative to control treatment.

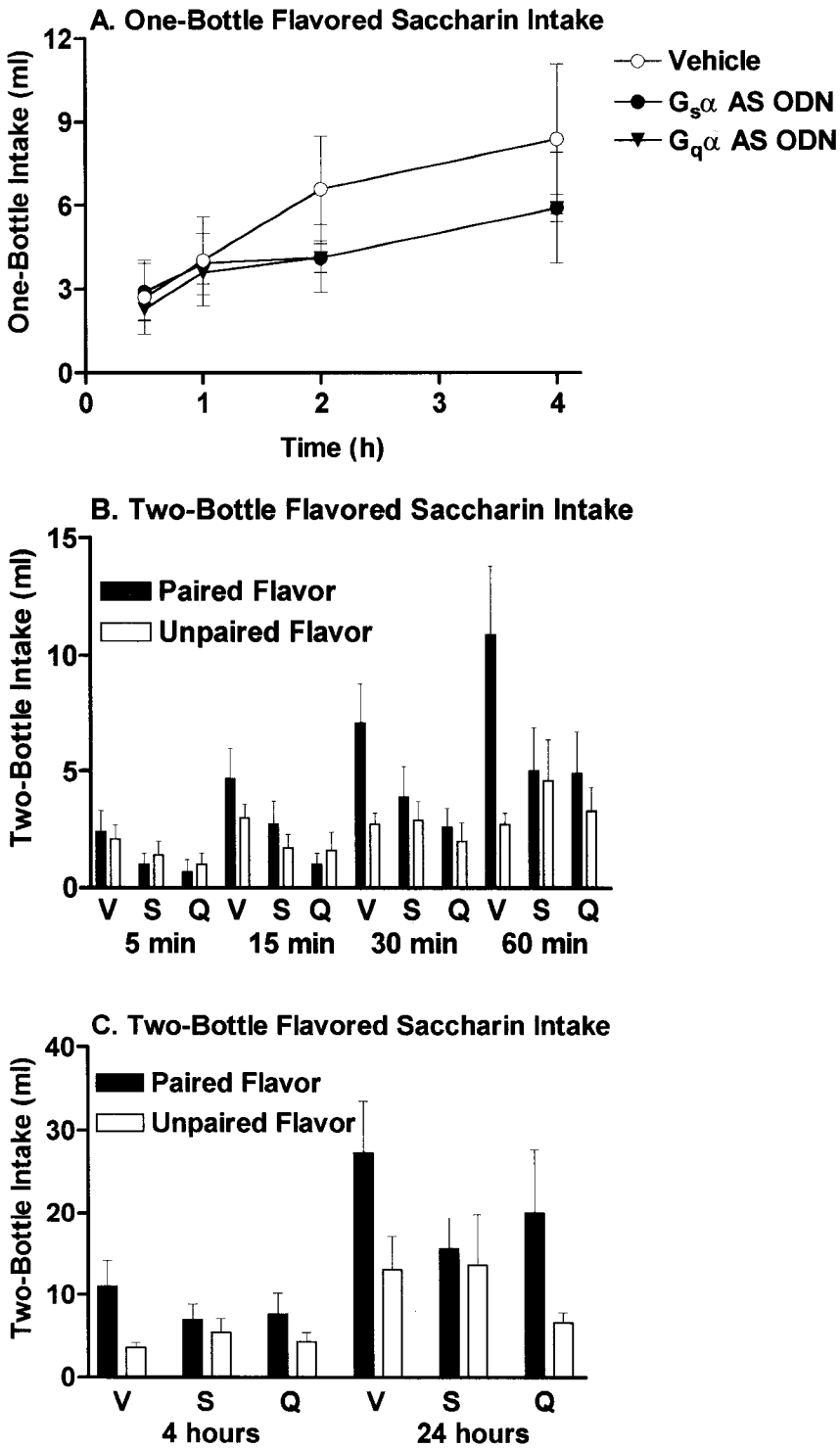
Figure 5.



G-Protein AS ODN probes, conditioned flavor preferences and deprivation-induced food intake: As expected, significant differences in deprivation-induced food intake were observed across groups after 2 ($F(2,18)= 3.63$, $P<0.04$), 4 ($F= 6.56$, $P<0.007$) and 24 ($F= 4.4$, $P<0.03$) h with rats treated with AS ODN probes directed against either $G_s\alpha$ or $G_q\alpha$ displaying significantly less intake than vehicle-treated rats (data not shown). Intake of the one-bottle flavored saccharin solutions failed to differ among the three treatment groups especially after 0.5 ($F(2,18)= 0.09$, n.s.) and 1 ($F= 0.04$, n.s.) h, indicating that the three groups received equal short-term exposure to their respective paired solutions. Although these effects failed to achieve significance, one-bottle flavored saccharin intake tended to be lower after 2 ($F= 1.12$, n.s.) and 4 ($F= 0.57$, n.s.) h in the G-protein AS ODN groups (Figure 6, upper panel). Indeed, one-bottle saccharin intake was significantly less ($F(2,18)= 4.50$, $P<0.026$) after 24 h in the groups receiving AS ODN probes directed against either $G_s\alpha$ (21.3 (+5.3) ml) or $G_q\alpha$ (16.0 (+3.4) ml) relative to vehicle (45.0 (+10.9) ml). Analysis of the two-bottle preference tests failed to reveal significant differences at any of the time points among the three groups (5 min: $F(2,12)= 2.18$, n.s.; 15 min: $F= 3.69$, n.s.; 30 min: $F= 3.80$, n.s.; 60 min: $F= 1.42$, n.s.; 4 h: $F= 0.33$, n.s.; 24 h: $F= 0.98$, n.s.), between paired and unpaired flavors (5 min: $F(1,6)= 0.20$, n.s.; 15 min: $F= 1.91$, n.s.; 30 min: $F= 4.85$, n.s.; 60 min: $F= 3.68$, n.s.; 4 h: $F= 3.86$, n.s.; 24 h: $F= 4.96$, n.s.), or for the interaction between groups and flavors (5 min: $F(2,12)= 0.46$, n.s.; 15 min: $F= 2.07$, n.s.; 30 min: $F= 2.95$, n.s.; 60 min: $F= 3.33$, n.s.; 4 h: $F= 1.48$, n.s.; 24 h: $F= 1.15$, n.s.). Figure 6 (Panels B & C) indicates that the two-bottle intakes of rats receiving AS ODN probes directed against either $G_s\alpha$ or $G_q\alpha$ displayed similar

Figure 6. Panel A: Intake (Mean, ±SEM) of one-bottle flavored (half grape, half cherry, 0.05%) saccharin (0.2%) solutions in food-deprived (24 h) rats receiving vehicle AS ODN probes directed against either $G_s\alpha$ or $G_q\alpha$. Panels B & C: Intakes (Mean, ±SEM) of either a flavor paired [dark bars] with vehicle (V), $G_s\alpha$ (S) or $G_q\alpha$ (Q) AS ODN probes or a novel unpaired [clear bars] flavor in two bottles of saccharin (0.2%) solutions in food-deprived (24 h) rats. The two-bottle intakes of the solutions occurred in the absence of food for the first 60 min and in the presence of food 4 and 24 h later.

Figure 6.



intake patterns of the paired and unpaired flavored saccharin solutions to that of vehicle-treated rats, suggesting that these AS ODN groups did not display conditioned aversions to the particular paired flavor. Finally, measurement of 24-h food intake during this two-bottle preference testing revealed that the three groups failed to differ from each other ($F(2,18)= 0.13$; data not shown).

Discussion

The present study indicated that a number of G-protein AS ODN probes selectively interfered with the full expression of the increased food intake observed following 24 h of food deprivation with $G_{s\alpha}$ or $G_q\alpha$ AS ODN probes producing the most pronounced effects, and AS ODN probes directed against $G_{x/z}\alpha$, $G_i\alpha_2$ and $G_i\alpha_3$ producing smaller effects.

Such a pattern of reductions could not be attributed to nonspecific factors for the following three reasons. First, two active G-protein AS ODN probes directed against either $G_i\alpha_1$ or $G_o\alpha$ failed to significantly alter deprivation-induced intake across any time point over the 24 h intake time course. It is important to note that an equal dose of the AS ODN probe directed against $G_i\alpha_1$ significantly and potently reduced by 50% feeding responses induced by either M6G, β-endorphin or dynorphin (Silva et al., 2000, 2002) over the identical short-term time course (1-4 h) examined in the present study. Further, AS ODN probes directed against $G_o\alpha$ and infused into the third ventricle significantly and selectively reduced nocturnal intake (Plata-Salaman et al., 1995). It should be noted however that the magnitude of deprivation-induced feeding (e.g., ~9 g in 2 h) was significantly higher than comparable responses following opioid agonists (e.g., ~3 g in 2 h) or nocturnal intake (~4 g in 2 h: (Arjune and Bodnar, 1990). Therefore, these G-

protein AS ODN probes appear active in other feeding paradigms, but not against deprivation-induced feeding.

Second, a nonsense probe that was equal in length, but not putatively active against any segment of G-protein α-subunit mRNA, failed to significantly alter either deprivation-induced food intake at any time point, deprivation-induced weight loss, or post-deprivation-induced weight recovery relative to vehicle control animals. Therefore, unlike PTX and/or CTX administration which interferes with specific G-proteins and with body weight and food intake in a non-specific manner (Bodnar et al., 1990; Chance et al., 1989), the use of nucleotide probes per se (as represented by the nonsense control) fails to reduce weight and intake following food deprivation as well as intake and weight under normal conditions (Silva et al., 2000).

The third and perhaps most compelling reason against nonspecific factors mediating G-protein AS ODN probe effects upon deprivation-induced feeding was the failure of the two most effective probes ($G_s\alpha$ and $G_q\alpha$) to produce conditioned taste aversions. A typical procedure to assess taste aversions is to pair a novel salient taste with the potentially aversive stimulus and assess subsequent short-term intake of that taste. In this paradigm however, it is conceivable that the G-protein AS ODN probe might exert its aversive effects over the 24 h of deprivation and over the 24 h of subsequent recovery. Therefore, it was necessary to assess the presence of an aversion over this longer period, and the conditioned flavor preference paradigm (e.g., Yu et al., 1999, 2000) allows for the analysis of the development of either preferences (greater intake of the paired relative to an unpaired flavor) or aversions (decreased intake of the paired relative to an unpaired flavor) of typically-reinforcing saccharin solutions. In the one-bottle exposure test pairing

each of the two G-protein AS ODN probes (or vehicle treatment) with each of the cherry (half) and grape (half) flavors, all groups consumed comparable amounts (~6-8 ml) of their paired flavors after 4 h. As noted, the G-protein AS ODN probes decreased food intake after 24 h, and also decreased flavored saccharin intake in the AS ODN groups (16-21 ml) as compared to the vehicle group (~45 ml). When the rats were re-deprived and given exclusive access (60 min) to the unpaired and paired flavors, all three groups displayed similar intakes towards the two flavors which persisted 4 and 24 h thereafter. Indeed, if any trend emerged, it was that the rats in all groups consumed more of the flavors paired with injections and deprivation. At no time point was an aversion (decreased intake of the paired flavor) observed in rats receiving the $G_s\alpha$ and $G_q\alpha$ AS ODN probes. It is also important to point out that although there were no significant differences in preference between the paired and unpaired solutions in the vehicle and probe treated animals, the vehicle-treated animals consumed more of the paired solution at all time points, suggesting that a hedonic aspect of the solution may be suppressed in the antisense-treated animals.

Another interesting point was that the reductions in deprivation-induced intake produced by specific G-protein AS ODN probes were differentially related to weight changes. In the cases of G-protein AS ODN probes directed against $G_i\alpha_2$ and $G_i\alpha_3$, the significant reductions in food intake were not associated with any significant changes in either deprivation-induced body weight loss or post-deprivation body weight recovery. AS ODN probes directed against $G_s\alpha$ and $G_{x/z}\alpha$ produced significantly greater weight loss during the 24 h of food deprivation, and would therefore presumably create a greater deprivation state. Yet animals receiving these AS ODN probes showed among the most

pronounced reductions in deprivation-induced intake. AS ODN probes directed against $G_i\alpha_2$, Gqα and $G_o\alpha$ produced significantly less weight gain during the 24 h intake recovery period yet these groups showed respective mild reductions, one of the most pronounced reductions and no effect on deprivation-induced intake. Why would particular G-protein AS ODN probes affect weight, but not intake, or alternatively, intake, but not weight. Pharmacological manipulations that affect weight can produce such effects by altering appetitive and/or satiety controls over intake, by altering energy expenditure and balance, by altering metabolism, and/or by altering gastrointestinal transit and/or excretory output (see review: Blundell and King, 1996). Furthermore, non-specific variables such as malaise or illness could have greater effects on weight than intake (see reviews: Halford and Blundell, 2000). Thus, this allows for the possibilities of decreases in intake without decreases in weight and vice-versa. These different patterns are best exemplified by the differential actions of serotonin and leptin in appetite and weight control respectively (see review: Halford and Blundell, 2000). Thus, serotonin decreases short-term intake dramatically without producing major weight loss (e.g., Blundell et al., 1995; Currie, 1996; Halford and Blundell, 2000), particularly within the hypothalamic paraventricular nucleus (Leibowitz et al., 1989; Shor-Posner et al., 1986). In contrast, leptin profoundly decreases body weight by primarily affecting adiposity-linked drive signals arising from metabolic energy demands (Blundell et al., 2001; Campfield et al., 1995; Halaas et al., 1995; Halford and Blundell, 2000; Pelleymounter et al., 1995), yet has far less dramatic effects upon short-term intake (Kahler et al., 1998; Seeley et al., 1996). Although PTX and CTX have been shown to reduce both body weight and food intake (Bodnar et al., 1990; Chance et al., 1989), the G-protein AS ODN

probes produce minimal alterations in baseline weight and intake in non-deprived conditions (Silva et al., 2000). There are no published studies examining G-protein manipulations upon deprivation-induced weight and intake changes, and further work should evaluate the relative contributions of appetitive mechanisms, satiety mechanisms, energy balance and expenditure changes and metabolic changes following these manipulations.

What candidate neurotransmitter and neuropeptide receptor systems that are also coupled to G-proteins could be responsible for these effects? Such orexigenic neurotransmitters and neuropeptides as norepinephrine, opioids, neuropeptide Y, galanin and dopamine each have receptors coupled to G-proteins. The α_2-adrenergic receptor, which has been implicated in mediating the stimulatory ingestive actions of norepinephrine (see review: Leibowitz et al., 1987), acts through the $G_i\alpha$ and $G_o\alpha$ subunits (Coupry et al., 1992; Duzic et al., 1992; Kurose et al., 1991). Opioid receptors, and particularly the : subtype, primarily act through the Giα subunit (see reviews: Roerig, 1998; Standifer and Pasternak, 1997). The orexigenic peptides, NPY and galanin, act through their respective receptors that are linked to the $G_i\alpha$ and secondarily the $G_o\alpha$ subunits (Brown et al., 1995; Freitag et al., 1995; Michel et al., 1995; Wang et al., 1999). The $5HT_{1A}$ receptor subtype, which has been associated with autoreceptor-induced stimulation of feeding, is also associated with the $G_i\alpha$ subunit (Liu et al., 1999). Whereas dopamine D_2 receptors act through the $G_i\alpha$ and $G_o\alpha$subunits (Ghahremani et al., 1999; Gregerson et al., 2001; Neusch et al., 2000), the D_1 receptor acts through the $G_s\alpha$ and $G_q\alpha$ subunits (Panchalinjam and Undie, 2000). Therefore, since it was the $G_s\alpha$ and $G_q\alpha$ AS ODN probes that were the most effective in reducing deprivation-induced

feeding, and the $G_i\alpha$ and $G_o\alpha$ AS ODN probes that were either the least effective or indeed ineffective in reducing deprivation-induced feeding, it would appear that the D_1 receptor antagonist-induced reductions in deprivation-induced feeding (see review: Terry, 1996) match best with the potent effects of the $G_s\alpha$ and $G_q\alpha$ AS ODN effects on this identical response. This possibility offers an explanatory framework for the fact that the vehicle-treated animals consumed greater amounts of the paired solution during the two-bottle test (although not statistically significant) whereas the animals receiving the G_s and G_q antisense probes failed to show this preference. The possibility that the D_1 receptor is implicated in this mechanism can be supported further by the fact that D_1 receptor selective antagonism has effectively blocked flavor-preference conditioning by fructose or sucrose (Baker et al., 2003; Yu et al., 2000).

Interestingly, another receptor that acts selectively through $G_s\alpha$ and $G_q\alpha$ subunits is the MC-4 receptor (Chen et al., 1995; Lee et al., 2001). However, if stimulation of the MC-4 receptor by either α–melanocyte stimulating hormone or the potent MC-4 receptor agonist, MT II, produces potent reductions in feeding responses (e.g., Grill et al., 1998; Rossi et al., 1998; Williams et al., 2000; Wirth et al., 2001) by activating $G_s\alpha$ and $G_q\alpha$ subunits, this would be inconsistent with the present findings of $G_s\alpha$ and $G_q\alpha$ AS ODN probes producing reductions in deprivation-induced feeding as well. All of these possible mechanisms assume direct receptor-effector interactions presumably within the same neuron.

An alternative explanation for the loss of function associated with α–subunit AS ODN treatment may involve a trans-neuronal network in which behavioral effects are mediated through the interaction of multiple signals produced by the agonist (Ross,

1989). In this model, signals can diverge or converge at different levels of the network. For example, agonist-induced feeding may be attenuated by the down-regulation of receptor α-subunits and/or down-regulation occurring further downstream in the signaling pathway. Downstream synapses may contain receptors that are sensitive to down-regulation by α-subunit AS ODN probes, but have no direct involvement in agonist binding. This model proposes that loss of function can occur as a result of disruption at any level of the trans-neuronal network. Thus, the ability of a given G-protein α-subunit AS ODN to alter agonist-induced feeding does not imply that the proposed transmitter or peptide receptor actually couples to the specific G-protein being targeted. This suggests that the G-protein sensitivity profiles of different types of feeding paradigms may not only represent interactions at relevant receptors, but may also comprise an intricate functional network composed of multiple types of G-protein receptor complexes including receptors for other transmitter systems located downstream of that particular receptor.

The use of G-protein AS ODN probes clearly differentiates between two different ingestive states, nocturnal feeding and deprivation-induced feeding. Whereas deprivation-induced feeding is most effectively reduced by AS ODN probes directed against the $G_s\alpha$, $G_q\alpha$, $G_{x/z}\alpha$ and to a lesser extent, $G_i\alpha_2$ and $G_i\alpha_3$ subunits, it is unaffected by AS ODN probes directed against the $G_o\alpha$ and $G_i\alpha_1$ subunits. In contrast, nocturnal feeding is most effectively reduced by AS ODN probes directed against the $G_o\alpha$ subunit, but not by AS ODN probes directed against $G_s\alpha$, $G_q\alpha$ and $G_i\alpha$ subunits (Plata-Salaman et al., 1995). Thus, these dissociations suggest that different effector signaling pathways are employed

in mediating the feeding responses elicited under natural (e.g., nocturnal feeding) and regulatory challenge (e.g., food deprivation) conditions.

CHAPTER 6. SPECIFIC AIM THREE.

Food Deprivation and Food Restriction: Assessment of Opioid Receptor Immunoreactivity Following Food Restriction or Food Deprivation

Introduction

It is well established that manipulations of the endogenous opioid system significantly alter feeding behavior in that opioid agonists typically stimulate intake, and opioid antagonists typically inhibit intake (see reviews: Bodnar, 2004; Cooper et al., 1988; Gosnell and Levine, 1996; Levine et al., 1985; Morley et al., 1983). In turn, behavioral states related to ingestive behavior alter levels of opioid peptides, receptors and genes. These behavioral states include food restriction, food deprivation, streptozotocin-induced diabetes and exposure to palatable diets. Although chronic food restriction increased [3H] naloxone binding in the midbrain, it decreased mu opioid binding in the basal amygdala, parabrachial nucleus (PBN) and habenula. Kappa opioid binding was respectively decreased (habenula) and increased (bed nucleus of the stria terminalis (BNST), ventral pallidum, medial preoptic area and PBN) following food restriction (Tsujii et al., 1986a; Wolinsky et al., 1994, 1996b). Dynorphin (DYN) A1-17 is increased by chronic food restriction in the dorsomedial, ventromedial and paraventricular hypothalamic nuclei, and decreased in the central amygdala. The shorter DYN A1-8 peptide is increased in the NAC, BNST, cortex, striatum, midbrain and LH (Berman et al., 1994, 1997; Tsujii et al., 1986b). Whereas chronic food restriction decreases hypothalamic arcuate beta-endorphin (BEND) and DYN (Brady et al., 1990; Kim et al., 1996), its combination with exercise increases hypothalamic BEND and DYN (Aravich et al., 1993). Food-restricted rats display increased c-Fos immunoreactivity in

the BNST, central nucleus of the amygdala and NAC following naltrexone pretreatment, in the BNST and amygdala following kappa antagonism, and in the NAC following mu antagonism (Carr et al., 1998, 1999). Pairing general opioid antagonists with food restriction also increases arcuate NPY mRNA, while decreasing brown fat uncoupling protein (Kotz et al., 1996). In addition to food restriction, streptozotocin-induced diabetes similarly increased DYN A1-17 in the dorsomedial and ventromedial hypothalamus, and DYN A1-8 in the LH (Berman et al., 1995, 1997). In contrast, diabetes decreased hypothalamic BEND (Kim et al., 1999; Locatelli et al., 1986). Both diabetes and food restriction increase kappa binding in the medial preoptic area and decrease mu binding in the lateral habenula (Wolinsky et al., 1996a). Moreover, glucoprivation selectively decreases mRNA levels of pro-dynorphin, but not pro-opiomelanocortin (POMC) or pro-enkephalin (Giraudo et al., 1998). Further, food deprivation lowers mRNA levels for the NOP receptor in the paraventricular (PVN) and LH as well as the central nucleus of the amygdala, and lowers pro-OFQ/N mRNA levels in the central nucleus of the amygdala (Rodi et al., 2002).

Long-term exposure to palatable solutions, but not the accompanying weight gain increases hypothalamic DYN protein and mRNA levels (Welch et al., 1996). Sucrose consumption significantly enhances the ability of naloxone to increase c-Fos activity in the lateral hypothalamus, ventral tegmental area, central nucleus of the amygdala and the medial preoptic area (Park and Carr, 1998; Pomonis et al., 2000). Antagonism of mu-1 opioid receptors blocks palatability-induced stimulation of dopamine release in the NAC (Tanda et al., 1998). Long-term exposure to a high fat diet increased mu opioid receptors in the hypothalamus (Barnes et al., 2003), but reduced enkephalin gene expression in

striatal, and particularly ventral striatal regions (Kelley et al., 2003). Although BEND levels were initially associated with overeating in genetically obese ob/ob mice and fa/fa rats (Margules et al., 1978), genetically-obese Zucker rats display reductions in POMC mRNA levels that correspond to reductions in alpha-melanocyte stimulating hormone, yet no changes in BEND (Kim et al., 2000). Further, DYN levels are increased and [3H]-naloxone binding is decreased in obese Zucker rats (Roane et al., 1988).

Following the initial observation that naloxone administration decreased intake in food-deprived rats (Holtzman, 1974), subsequent studies observed general opioid antagonist-induced decreases in both food and water intake in deprived and non-deprived rats and mice following systemic administration (Brown and Holtzman, 1979; Cooper, 1980; Frenk and Rogers, 1979; Holtzman, 1975; Levine et al., 1990a; Maickel et al., 1977) as well as direct injections into the ventromedial hypothalamus (VMH), LH, NAC, PVN and ventral tegmental area (VTA) (Bodnar et al., 1995; Kelley et al., 1996; Koch et al., 1995; Ragnauth et al., 1997; Thornhill and Saunders, 1984). Analysis of opioid receptor subtype antagonist effects upon deprivation-induced feeding reveal a strong effect for mu opioid antagonists, a moderate effect for kappa opioid antagonists, and relatively weak effects for delta and mu-1 opioid antagonists following ventricular administration (Arjune and Bodnar, 1990; Arjune et al., 1990, 1991; Koch and Bodnar, 1994; Levine et al., 1990b, 1991; Simone et al., 1985; Ukai and Holtzman, 1988). Mu opioid antagonists are also effective in reducing deprivation-induced intake following direct administration into the PVN and NAC, but not the VTA (Bodnar et al., 1995; Kelley et al., 1996; Koch et al., 1995; Ragnauth et al., 1997). Mu-selective opioid antagonists also potently decrease chow intake following glucoprivation and

lipoprivation as well as intake of palatable diets high in simple carbohydrates, complex carbohydrates and/or fat (Arjune and Bodnar, 1990; Arjune et al., 1990, 1991; Beczkowska et al., 1992, 1993; Cole et al., 1995; Islam and Bodnar, 1990; Koch and Bodnar, 1994; Stein et al., 2000) with the latter effects due to the orosensory actions of these antagonists given their effectiveness in sham-feeding animals (Leventhal and Bodnar, 1996; Leventhal et al., 1995).

The identification of the MOP or MOR-1 (mu), KOP (kappa), DOP (delta) and NOP (nociceptin) opioid peptide genes (see reviews: Pasternak, 2001; Uhl et al., 1994) allowed the study of the relationship of cloned opioid receptors to opioid-mediated actions in vivo, particularly through the use of antisense oligodeoxynucleotide (AS ODN) sequences complementary to specific regions of mRNA that can down-regulate receptor proteins (see review: Pasternak and Standifer, 1995). The use of highly selective AS ODN probes directed against individual exons of opioid receptor genes revealed unique exon-specific profiles of sensitivity to these probes for opioid agonists in analgesic studies (see review: Rossi et al. 1997) that was reproduced for opioid agonist-induced feeding responses. Thus, feeding responses elicited by the mu-selective opioid agonists, morphine and DAMGO, are blocked by MOP AS ODN probes directed against exons 1 and 4, but not exons 2 or 3, whereas feeding elicited by the active morphine metabolite of morphine M6G is blocked by MOP AS ODN probes directed against exons 2 and 3, but not 1 or 4 (Leventhal et al., 1997, 1998). Further, feeding elicited by the opioid peptide, BEND, is most potently blocked by mu, secondarily kappa and minimally by delta opioid antagonists, and is also most potently blocked by AS ODN probes directed against MOP (exons 1, 3 and 4), and minimally by DOP, KOP and NOP (Silva et al., 2001). Moreover,

feeding elicited by the opioid peptide, DYN, is most potently blocked by kappa, secondarily mu and minimally by delta opioid antagonists, and is also most potently blocked by AS ODN probes directed against KOP and NOP (exons 1 and 2) and minimally by DOP and MOP (Silva et al., 2002).

MOP AS ODN probes are very effective in reducing feeding and body weight under spontaneous intake conditions (exons 1, 2, 3 and 4: Leventhal et al., 1996) as well as markedly reducing intake following either glucoprivation (exons 1 and 2: Burdick et al., 1998) or lipoprivation (exons 1, 2 and 3: Stein et al., 2000). However, in contrast to the rank-order potency of mu > kappa > delta opioid antagonist effects upon deprivation-induced feeding, potent reductions in deprivation-induced feeding were only observed following administration of a KOP AS ODN probe (exon 2). Significant though modest reductions were noted for deprivation-induced feeding following MOP AS ODN probes (exons 2, 3 and 4: Hadjimarkou et al., 2003). The differential actions of MOP AS ODN probes upon agonist-induced and environmentally-induced ingestive responses in general, and upon deprivation-induced intake in particular suggests that the classic MOP gene may not be fully responsible for all mu-mediated effects, but rather these effects might be mediated by recently-identified MOP isoforms (Bare et al., 1994; Pan et al., 1999, 2000a, 2000b, 2001; Pasternak and Pan, 2000; Zimprich et al., 1995).

The anatomical localization of some of these MOP splice variants (MOR-1, MOR-1C, MOR-1D) has been identified, and demonstrated important site-specific differences in density and distribution (Abbadie and Pasternak, 2001; Abbadie et al., 2000a, 2000b; 2001; Ding et al., 1996). Importantly, the MOR-1 and particularly, the MOR-1C isoforms are differentially localized in sites intimately implicated in the opioid

mediation of ingestive behavior (see reviews: Bodnar, 2004; Glass et al., 1999; Gosnell and Levine, 1996), including the paraventricular, periventricular, ventromedial, and arcuate hypothalamic nuclei as well as such extra-hypothalamic areas as the amygdala, BNST, NAC, lateral septum, parabrachial nuclei and nucleus tractus solitarius.

Therefore, the goal of this study was to examine central adaptive changes and opioid receptor plasticity in MOR-1 and MOR-1C immunohistochemistry in rats exposed to different levels of food restriction (controls, 2 days, 7 days, 14 days or 14 days followed by a 7 day recovery period) or food deprivation (controls, 24 h, 48 h or 48 h followed by a 7 day recovery period).

Methods

Perfusion, Tissue Processing and Immunohistochemistry: Rats were anesthetized with euthasol (Delmarva, Henry Schein, NY) and perfused intracardially with saline (0.9%) in 0.1 M phosphate buffer (PB) (pH=7.4; 50 ml) followed by 4% formaldehyde in 0.1M PB, (pH 7.4, 300 ml). The brains will be removed and placed in 4% formaldehyde for 2 hrs, and then cryoprotected in 30% sucrose (in 0.1M PB). Sectioning was performed on a freezing stage microtome (Leica) at sections 40μm thick and collected into 0.1 M PB. Immunostaining was performed using the avidin-biotin peroxidase method (Hsu et al., 1981). Sections were incubated for 1 hr with a blocking solution of 0.1 M PB with 0.9% saline, 3% normal goat serum and 0.3% Triton-X. The sections were then incubated overnight at room temperature in the primary antiserum. The antibodies used for these experiments, which recognize an epitope in the carboxy terminus of MOR-1 and MOR-1C were previously characterized (Arvidsson et al., 1995; Abbadie et al., 2000). The sections were washed and then incubated in biotinylated goat anti-rabbit IgG (1:200;

Vector Labs, Burlingame, CA) and avidin-biotin-peroxidase complex (1:100; Vector Labs). To localize the HRP immunoreaction product, we used a nickel-intensified diaminobenzidine protocol with glucose oxidase adapted from Llewellyn-Smith and Minson (1992). Finally, the sections were washed in PB, mounted on gelatin-coated slides, dried, and coverslipped with DPX (Aldrich, Milwaukee, WI).

Quantification and Statistical Analysis

For each anatomical region, we measured the density of the immunoreactivity in 2 sections from each rat using a computer-assisted imaging analysis system (Spot, Mac, NIH Image). Using a 10X objective and a CCD camera we captured an image of the region of interest. In order to account for background staining, we took two measurements: the first was the area of interest and the second was a density value of the same size area outside of that region, which was subtracted. In order to reduce variability to the immunohistochemistry reaction, each reaction included rats from each condition. The investigator responsible for quantification was unaware of the treatment of the animals.

For statistical analysis, we used a one-way analysis of variance to test for effect of treatment. For multiple comparisons, we used the Tukey multiple comparison test.

Procedure

Animals participated in a 21-day paradigm. Control animals had water and food available at all times whereas food-deprived animals had no food available. Animals in the food-restriction paradigm had food available in restricted amounts (~15g/day). More specifically, food-deprived animals were fed *ad lib* for the whole time course except for the last 24 or 48 hours before sacrifice. An additional group of animals was exposed to

48 h of food deprivation but was allowed 7 days of *ad libitum* intake before sacrifice, for recovery. Throughout the time course of food deprivation the animals had water available at all times. Food-restricted animals were fed *ad lib* throughout the 21-day paradigm except for 2, 7 or 14 days prior to sacrifice. In addition, a group of animals was food-restricted for 14 days but before sacrifice it was allowed 7 days of *ad libitum* intake for recovery.

Results

Body Weight Changes Across Experimental Conditions: Animals in the eight groups displayed matched weights on the first experimental day ($F(7,48)= 0.019$, n.s.; Mean ~325 g). Significant differences were observed among groups ($F(7,70)= 41.13$, $P<0.0001$), between the pre-treatment and post-treatment conditions ($F(1,10)= 55.42$, $P<0.0001$) and for the interaction between groups and treatments ($F(7,70)= 85.83$, $P<0.0001$). Thus, whereas control *ad-libitum* fed rats gained weight from days 15 (390 g) to 21 (405 g), deprived rats displayed significant reductions in weight at 24 (pre: 370 g; post: 358 g) and 48 (pre: 385g; post: 360 g) h of deprivation. In contrast, rats deprived of food for 48 h (341 g) and sacrificed 7 days later (7days *ad lib*) recovered their body weight (411 g). Time-dependent and significant reductions in weight were noted for animals that were restricted for 2 (pre: 398 g; post: 376 g), 7 (pre: 375 g; post: 342 g) and 14 (pre: 408 g; post: 321 g) days. In contrast, rats that were food-restricted for 14 days (276 g) and sacrificed 7 days later recovered their body weight (365 g).

Comparison of Exon 4-LI and Exon 7/8/9-LI across sites in Control Animals: The anatomical distribution of immunoreactivity for MOR-1 (Exon 4-LI) and MOR-1C (Exon 7/8/9-LI) differed across the sites involved in feeding behavior (Table 4). Exon 7/8/9-LI

Table 4. Anatomical distribution of MOR-1- and MOR-1C-Like-Immunoreactivity

Site	MOR-1-LI	MOR-1C-LI
	Exon 4-LI	exons 7/8/9-LI
Lateral Septum	+	+++
Nucleus Accumbens, Core and Shell	++	-/+
Bed Nucleus of the Stria Terminalis	-/+	++
Amygdala, Central Nucleus	++	-/+
Paraventricular Hypothalamic Nucleus	-/+	++
Periventricular Hypothalamic Nucleus	-/+	++
Arcuate Nucleus and Median Eminence	+/++	+++/++++
Ventromedial Hypothalamic Nucleus	-/+	+
Parabrachial Nucleus, Lateral and Medial Divisions	++/+++	+/++
Nucleus of the Solitary Tract	+++	+

Intensity of immunoreactivity: -/+, absent to minimal; +, low; ++, moderate; +++, strong; ++++, intense.

was most intense in the hypothalamic arcuate nucleus/ median eminence and the lateral septum in control animals, and was greater than that observed for Exon 4-LI. Exon 7/8/9-LI was moderate in the periventricular hypothalamus and the BNST, and again correspondingly higher than that observed for Exon 4-LI. In contrast, Exon 4-LI was most intense in the PBN and NTS, and correspondingly higher than that observed for Exon 7/8/9-LI. Further, Exon 4-LI was moderate in the NAC and amygdala, and correspondingly higher than that observed for Exon 7/8/9-LI. This pattern of immunoreactivity is consistent with that reported previously for Exon 4-LI (Ding et al., 1996) and Exon 7/8/9-LI (Abbadie et al., 2000a).

Food Restriction, Food Deprivation and Exon 4-LI: Neither food restriction nor food deprivation significantly altered either the density or number of Exon 4-LI in any of the nuclei examined, suggesting that these manipulations fail to change MOP gene expression. In contrast, site-specific and condition-specific changes in Exon 7/8/9-LI were observed across the sites, and are subsequently reported.

Food Restriction and Exon 7/8/9-LI in the PVN: The PVN was examined in three different parts: the magnocellular division of the PVN and the dorsal and ventral parvocellular subdivisions of the PVN. First, whereas Exon 7/8/9-LI was present in the two parvocellular PVN subdivisions (see below), the magnocellular division of the PVN was almost devoid of Exon 7/8/9-LI activity either in terms of optical density or cell counts in control or restricted animals. Significant differences in the number of cells were observed among the control and restriction conditions in the dorsal ($F(4,32)= 2.58$, $p<0.05$) and ventral ($F= 2.76$, $p<0.45$) parvocellular PVN subdivisions. As the length of restriction increased, a corresponding increase in the number of Exon 7/8/9-LI cells was

observed for the dorsal (Figure 7A) and ventral (Figure 7B) parvocellular PVN subdivisions with 14 days of food restriction producing significant results. Interestingly, animals restricted for 14 days and then allowed ad libitum access to food for seven days (recovery) still showed strong increases in the number of Exon 7/8/9-LI cells in both parvocellular subdivisions. Correspondingly, significant differences in optical density were observed among the control and restriction conditions in the dorsal ($F= 4.14$, $p<0.008$) and ventral ($F= 5.54$, $p<0.002$) parvocellular PVN subdivisions. Again, as the length of restriction increased, a corresponding increase in Exon 7/8/9-LI optical density was observed for the dorsal (Figure 8A) and ventral (Figure 8B) parvocellular PVN subdivisions with the 14 days of food restriction with and without recovery producing significant results. As indicated, Exon7/8/9-LI was more intense in the dorsal and ventral parvocellular PVN subdivisions in a representative animal exposed to 14 days of food restriction (Figure 9, right panel) relative to a representative control animal under *ad libitum* feeding conditions (Figure 9, left panel).

Food Deprivation and Exon 7/8/9-LI in the PVN: The effects of food restriction in the parvocellular PVN upon Exon 7/8/9-LI appeared to be condition-specific. First, the magnocellular division of the PVN was almost devoid of Exon 7/8/9-LI activity either in terms of optical density or cell counts in control or deprived animals. Moreover, significant differences in the number of cells failed to be observed among the control and deprivation conditions in the dorsal ($F(3,22)= 1.12$, ns) and ventral ($F= 0.91$, ns) parvocellular PVN subdivisions (Figures 10A and 10B). Similarly, significant differences in optical density failed to be observed among the control and deprivation conditions in

Figure 7. Alterations (Mean, ±SEM) in the number of MOR 1C-like immunoreactive cells in the dorsal (Panel A) and ventral (Panel B) hypothalamic paraventricular nucleus in animals exposed to either *ad libitum* feeding (control), 2, 7 or 14 days of food restriction, or exposed to 14 days of food restriction followed by a 7-day *ad libitum* recovery period. The asterisks in this and the following anatomical summary figures indicate significant differences from *ad libitum* control values (Tukey comparison, $P<0.05$).

Figure 7.

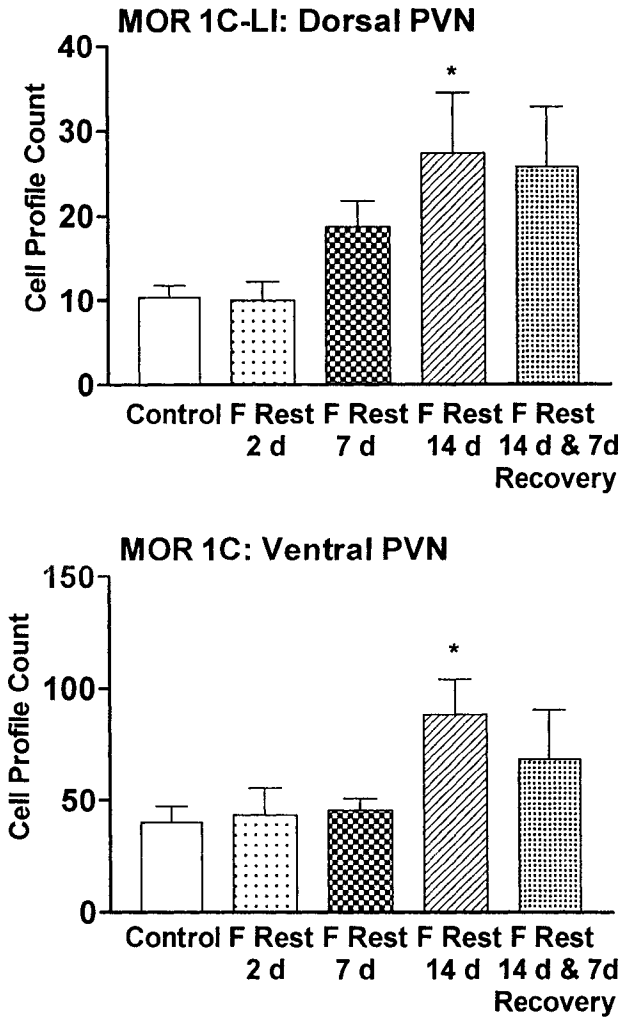


Figure 8. Alterations (Mean, ±SEM) in the optical density of MOR 1C-like immunoreactive cells in the dorsal (Panel A) and ventral (Panel B) hypothalamic paraventricular nucleus in animals exposed to either *ad libitum* feeding (control), 2, 7 or 14 days of food restriction, or exposed to 14 days of food restriction followed by a 7-day *ad libitum* recovery period.

Figure 8.

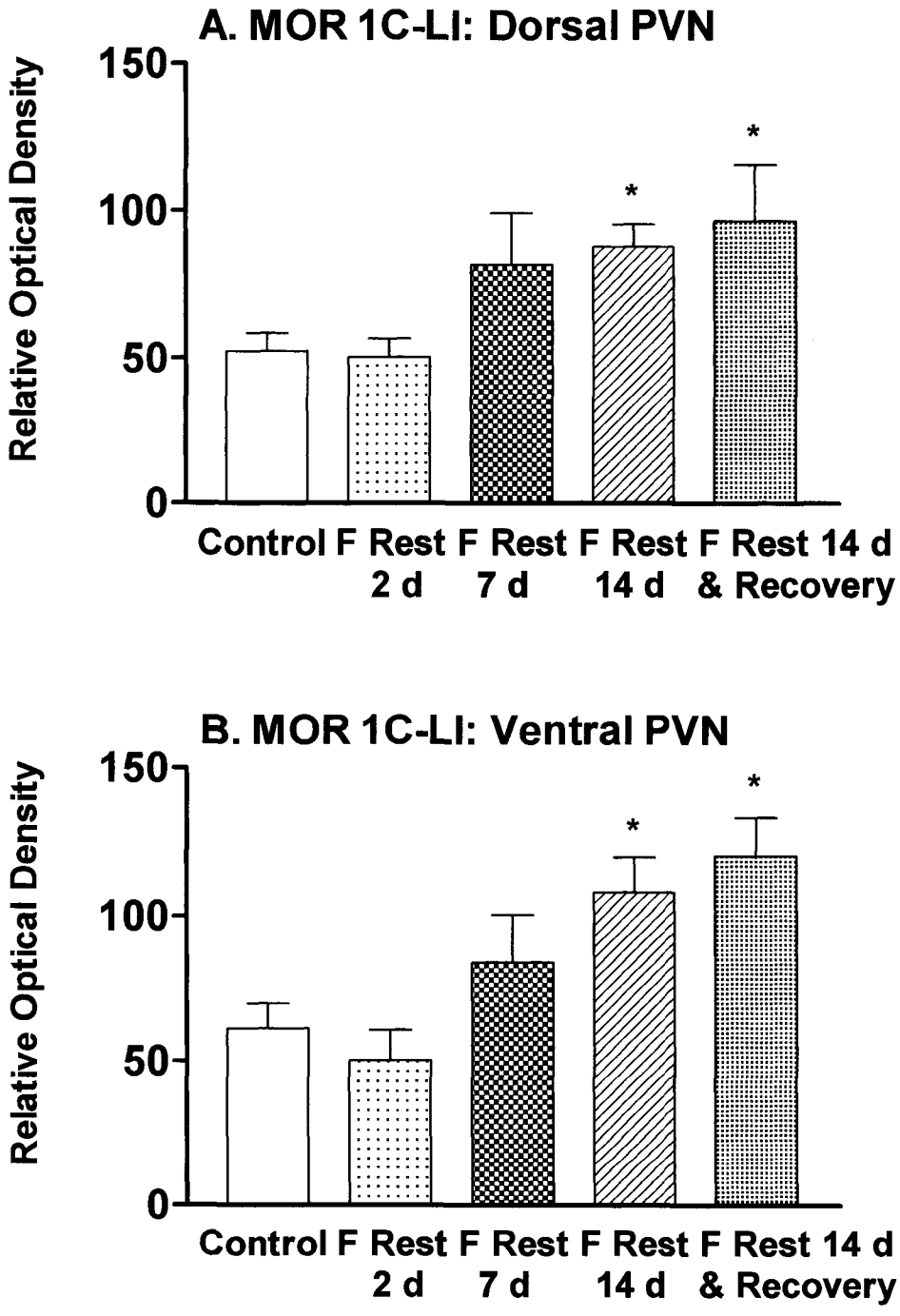


Figure 9. Photomicrographs of PVN MOR 1C-like immunoreactivity in *ad libitum* (control) and food restricted rats for 14 days.

Figure 9.

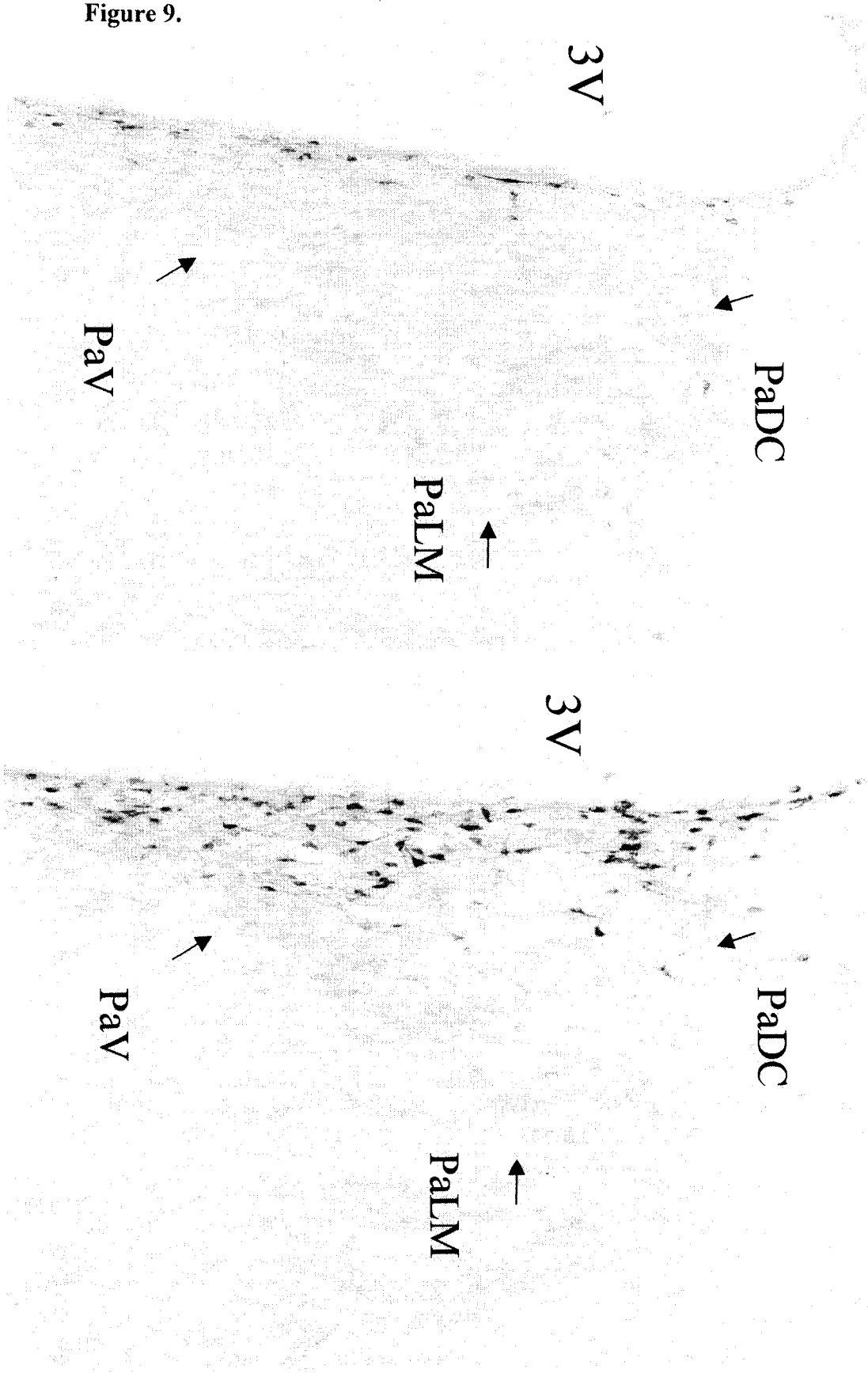
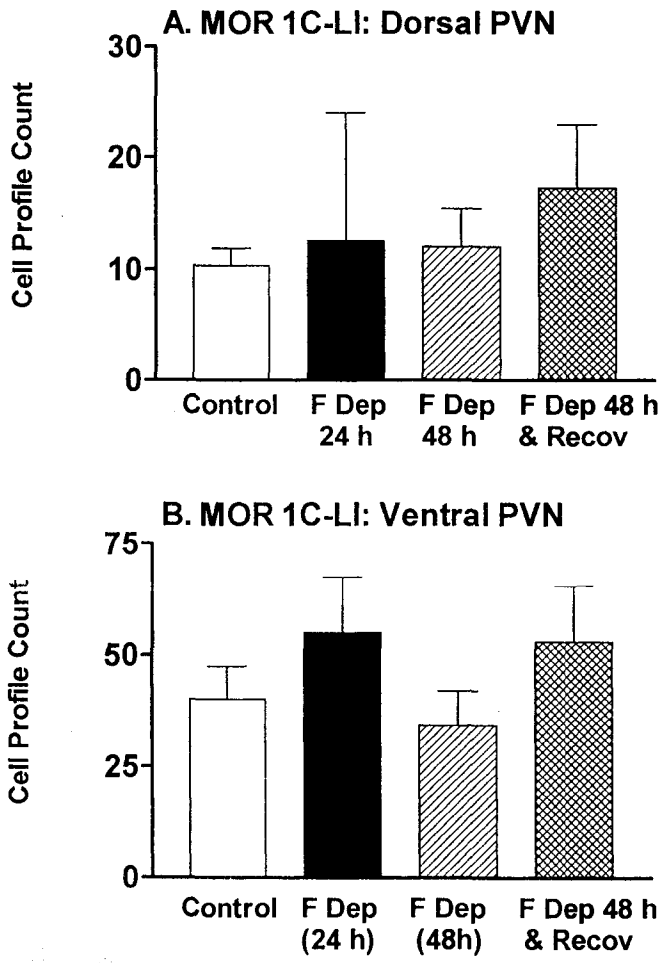


Figure 10. Alterations (Mean, ±SEM) in the number of MOR 1C-like immunoreactive cells in the dorsal (Panel A) and ventral (Panel B) hypothalamic paraventricular nucleus in animals exposed to either *ad libitum* feeding (control), 24 or 48 h of food deprivation, or exposed to 48 h of food deprivation followed by a 7-day *ad libitum* recovery period.

Figure 10.



the dorsal (F=0.59, ns) and ventral (F=1.07, ns) parvocellular PVN subdivisions (Figures 11 A &B).

Food Restriction, Food Deprivation and Exon 7/8/9-LI in Other Hypothalamic Sites: The effects of food restriction in the parvocellular PVN upon Exon 7/8/9-LI also appeared to be site-specific within the hypothalamus. Thus, in the adjacent periventricular hypothalamus, Exon 7/8/9-LI failed to differ for food-restricted animals relative to controls in either cell numbers (F= 0.24, ns) or optical density (F= 1.36, ns) (Figures 12A and 12B) or for food-deprived animals relative to controls in either cell numbers (F= 0.17, ns) or optical density (F= 0.24, ns) (Figures 13A and 13B). The optical density of Exon 7/8/9- LI in the VMH failed to differ for food-restricted animals (F= 1.07, ns: Figure 14A) or for food-deprived animals (F= 1.14, ns: Figure 14B) relative to controls. Although the optical density of Exon 7/8/9-LI in the arcuate nucleus significantly differed among food restriction conditions (F= 2.97, $p<0.034$), this was due to a reduced density observed only in animals restricted for 14 days and then allowed ad libitum access to food for seven days (recovery: Figure 15A). In contrast, the optical density of Exon 7/8/9-LI in the arcuate nucleus failed to differ for food-deprived animals relative to controls (F= 0.57, ns: Figure 15B).

Food Restriction, Food Deprivation and Exon 7/8/9-LI in Extra-hypothalamic Sites: Site-specific, but not condition-specific effects were observed for Exon 7/8/9-LI in extra-hypothalamic sites. Again, cell numbers of Exon 7/8/9-LI in the NTS, PBN, lateral septum, BNST and amygdala were relatively low and variable, and therefore not formally analyzed. Furthermore, optical density measures of Exon 7/8/9-LI in the BNST and amygdala were quite variable, and therefore not formally analyzed.

Figure 11. Alterations (Mean, ±SEM) in the optical density of MOR 1C-like immunoreactive cells in the dorsal (Panel A) and ventral (Panel B) hypothalamic paraventricular nucleus in animals exposed to either *ad libitum* feeding (control), 24 or 48 h of food deprivation, or exposed to 48 h of food deprivation followed by a 7-day *ad libitum* recovery period.

Figure 11.

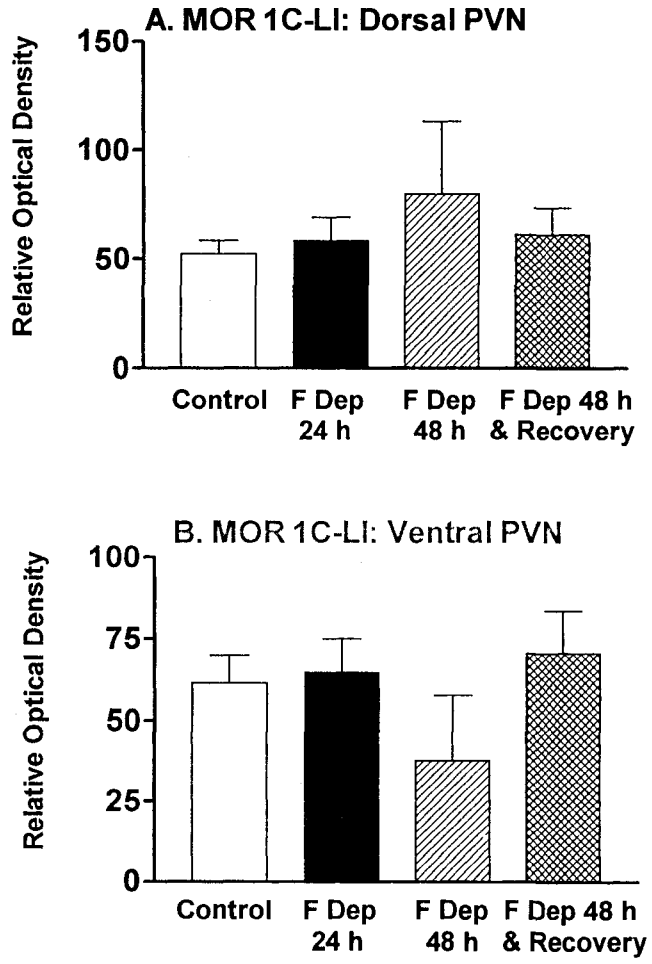


Figure 12. Alterations (Mean, ±SEM) in the number (Panel A) and optical density (Panel B) of MOR 1C-like immunoreactivity in the hypothalamic periventricular nucleus in animals exposed to either *ad libitum* feeding (control), food restriction or food restriction followed by recovery.

Figure 12.

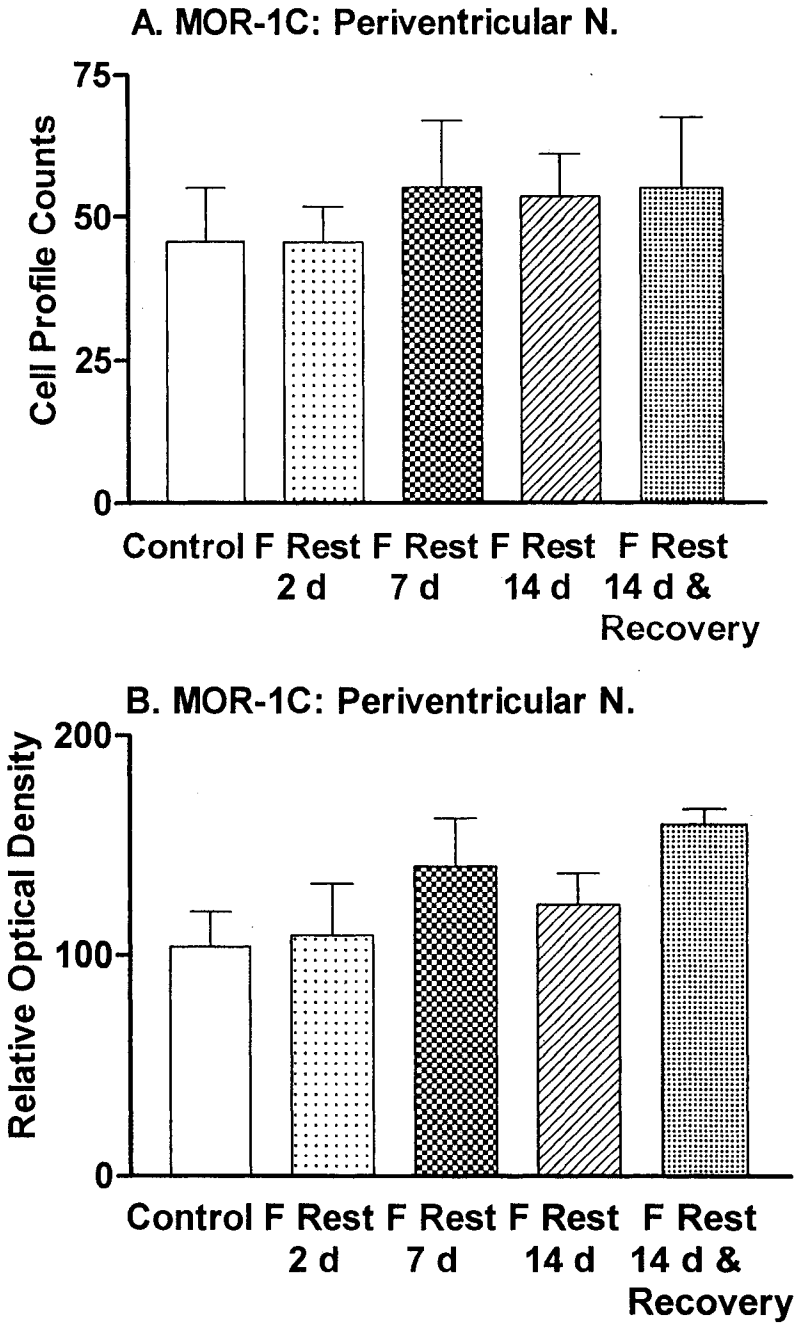


Figure 13. Alterations (Mean, ±SEM) in the number of cells (Panel A) and optical density (Panel B) of MOR 1C-like immunoreactivity in the hypothalamic periventricular nucleus in animals exposed to *ad libitum* feeding (control), food deprivation or food deprivation followed by recovery.

Figure 13.

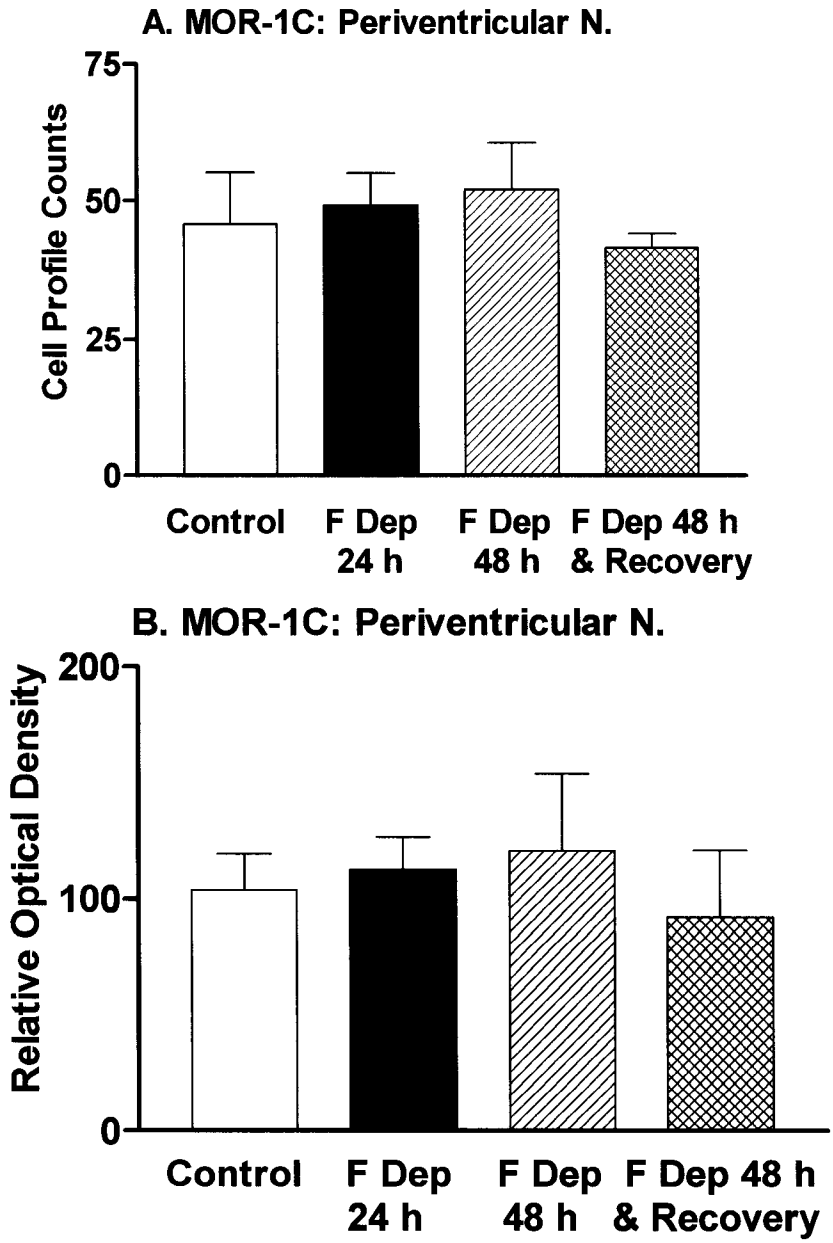


Figure 14. Alterations (Mean, ±SEM) in the optical density of MOR 1C-like immunoreactivity in the hypothalamic ventromedial nucleus in animals exposed to *ad libitum* feeding (control), food restriction (Panel A) or food deprivation (Panel B).

Figure 14.

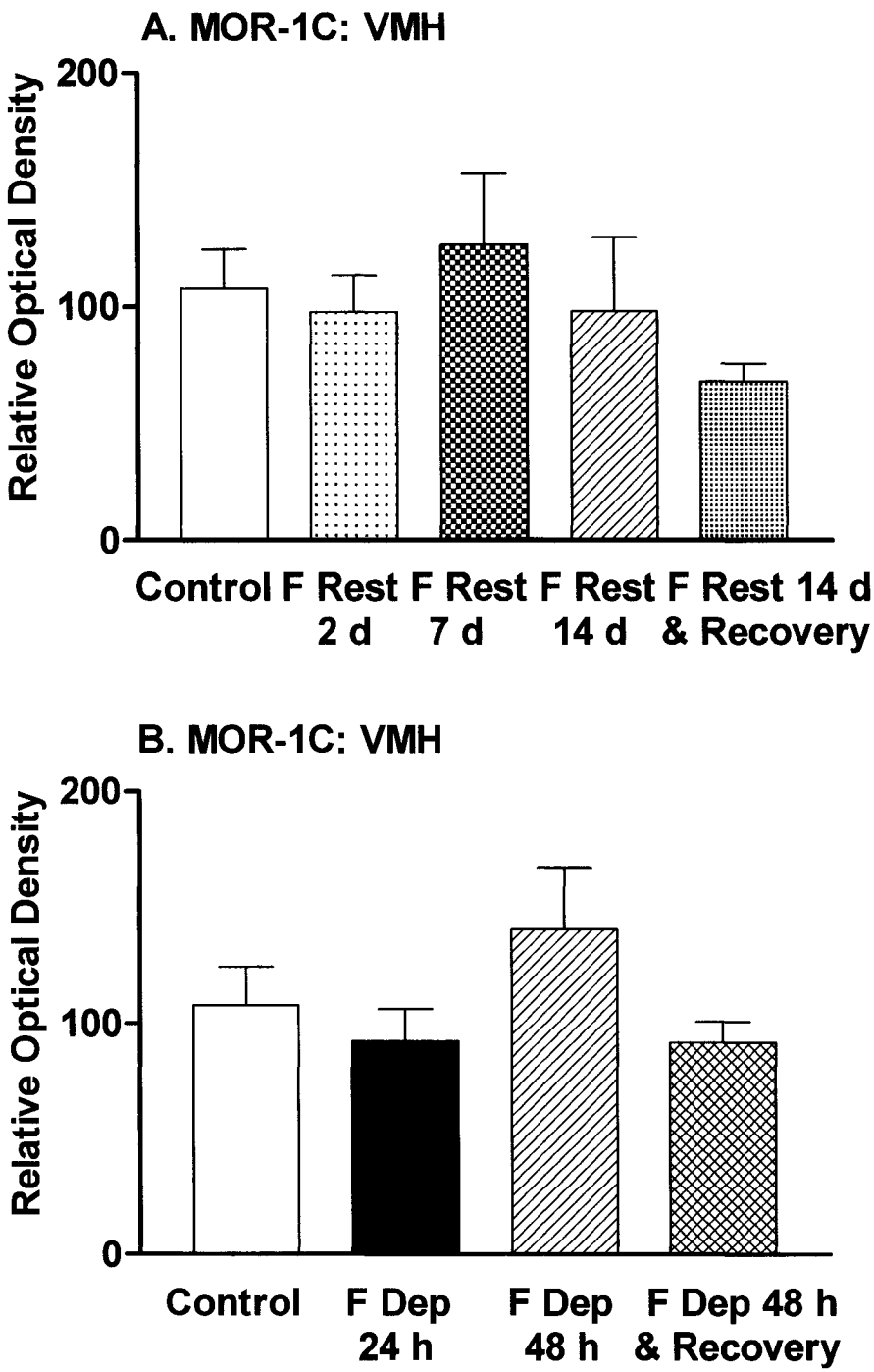
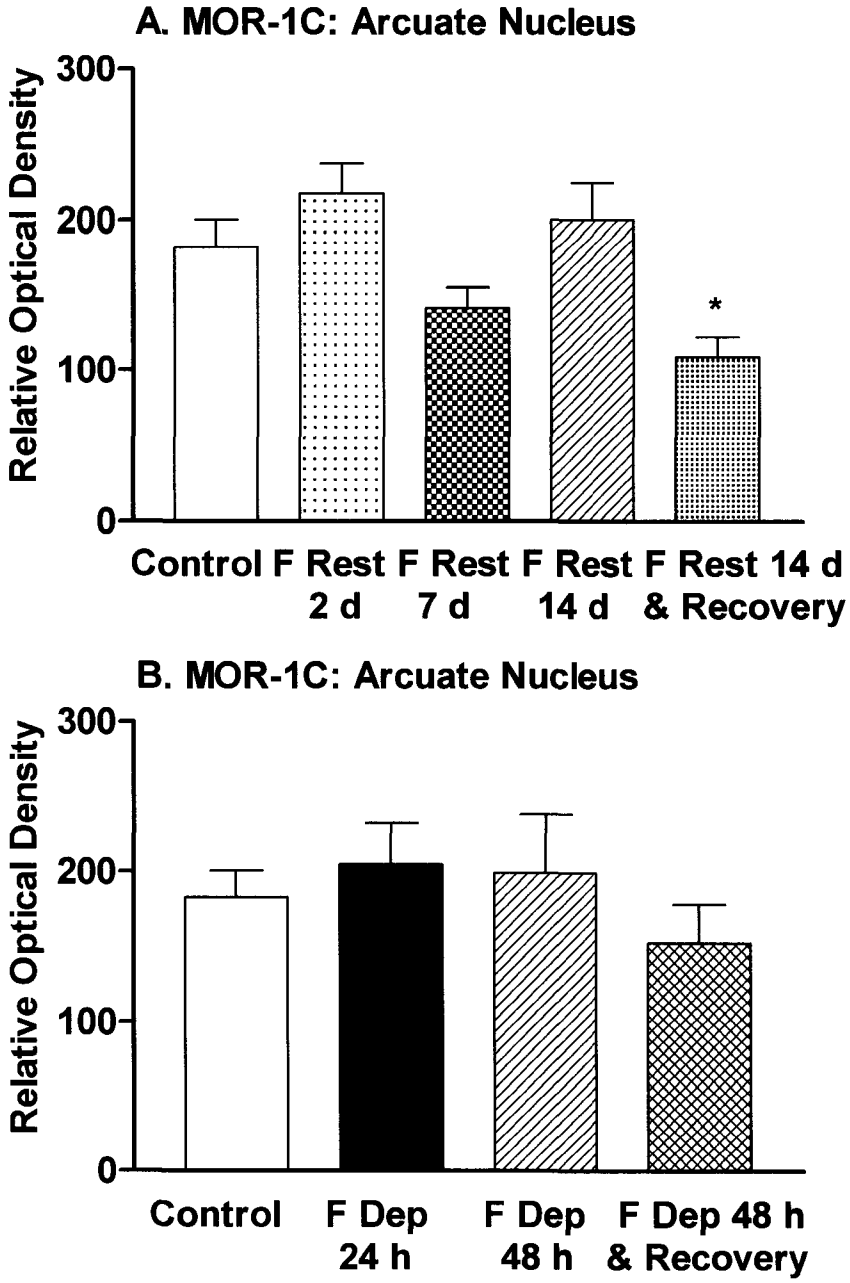


Figure 15. Alterations (Mean, ±SEM) in the optical density of MOR 1C-like immunoreactivity in the hypothalamic arcuate nucleus in animals exposed to either *ad libitum* feeding (control), food restriction (Panel A) or food deprivation (Panel B).

Figure 15.



In the NTS, significant differences in the optical density of Exon 7/8/9-LI were observed in food- restricted animals ($F= 3.04$, $p<0.03$) and in food-deprived animals ($F= 3.90$, $p<0.02$) relative to ad libitum fed controls. Interestingly, the direction and pattern of effects differed from that observed in the parvocellular PVN. Thus, animals restricted for either 2 or 7 days displayed significant reductions in the density of NTS Exon 7/8/9-LI relative to controls, whereas animals restricted for 14 days with or without recovery showed normal density measures (Figure 16A). Similarly, animals deprived of food for 24 or 48 h displayed significant reductions in the density of NTS Exon 7/8/9-LI relative to controls, whereas animals deprived for 48 h followed by a 7-day recovery showed normal density measures (Figure 16B). Thus as indicated, Exon7/8/9-LI was less intense in the NTS in representative animals exposed to either 2 or 7 days of food restriction (Figure 17 middle panel) and 24 or 48 h of food deprivation (Figure 17, right panel) relative to control animals under *ad libitum* feeding conditions (Figure 17, left panel). These decreases in immunoreactivity may be interpreted by the lack of food that the animals experienced for a period of time (24 or 48 h).

In contrast, the optical density of Exon 7/8/9-LI in the PBN failed to differ for food-restricted animals ($F= 1.44$, ns: Figure 18A) or for food-deprived animals ($F= 1.42$, ns: Figure 18B) relative to controls. Moreover, the optical density of Exon 7/8/9-LI in the lateral septum failed to differ for food-restricted animals ($F= 0.78$, ns: Figure 18C) or for food-deprived animals ($F= 0.72$, ns: Figure 18D) relative to controls.

Discussion

This study had the following major findings: a) there was a differential distribution of Exon 4-LI (MOR-1/MOP) and Exon 7/8/9-LI (MOR-1C) in sites

Figure 16. Alterations (Mean, ±SEM) in the optical density of MOR 1C-like immunoreactivity in the nucleus tractus solitarius in animals exposed to either *ad libitum* feeding (control), food restriction (Panel A) or food deprivation (Panel B).

Figure 16.

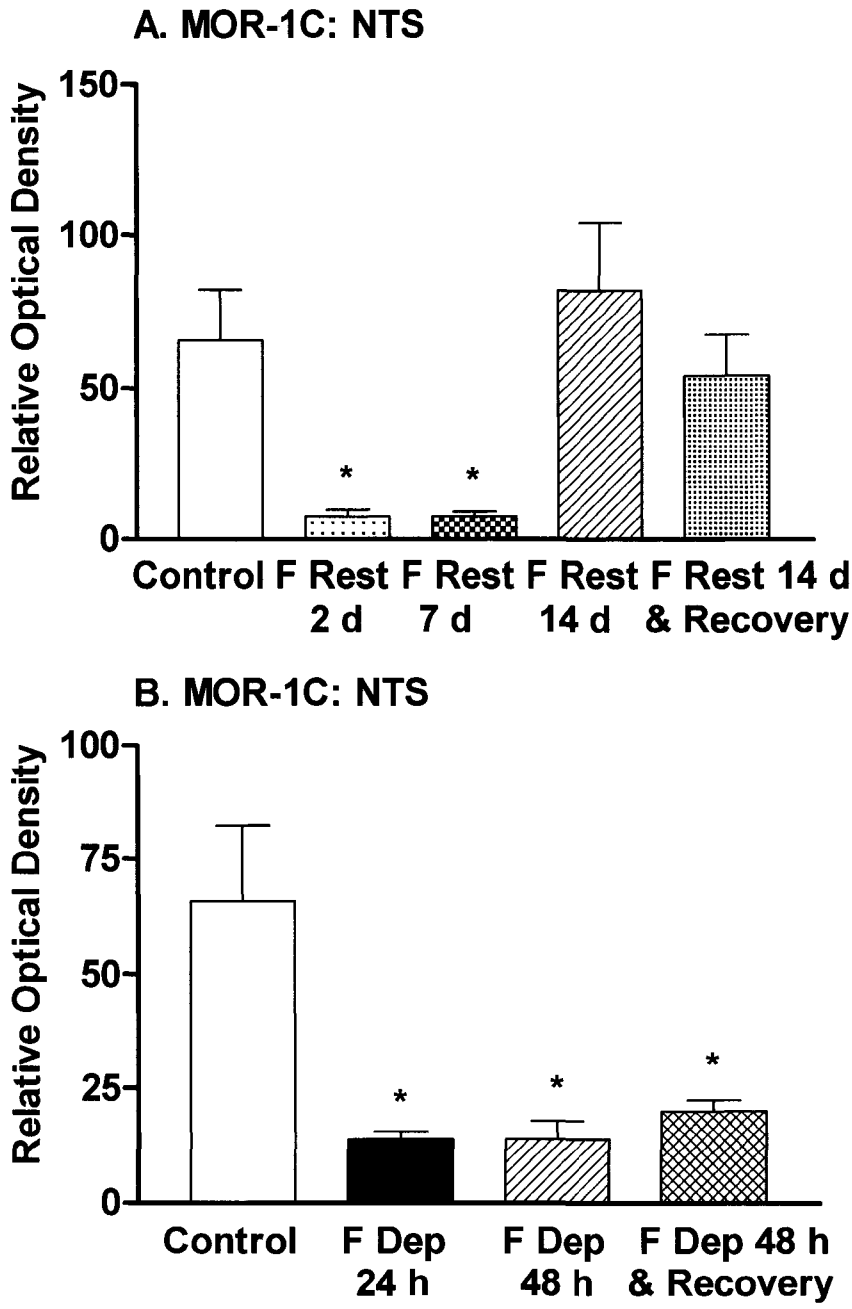


Figure 17. Photomicrographs of NTS MOR 1C-like-immunroeactivity in control, food restricted and food deprived animals

Figure 17.

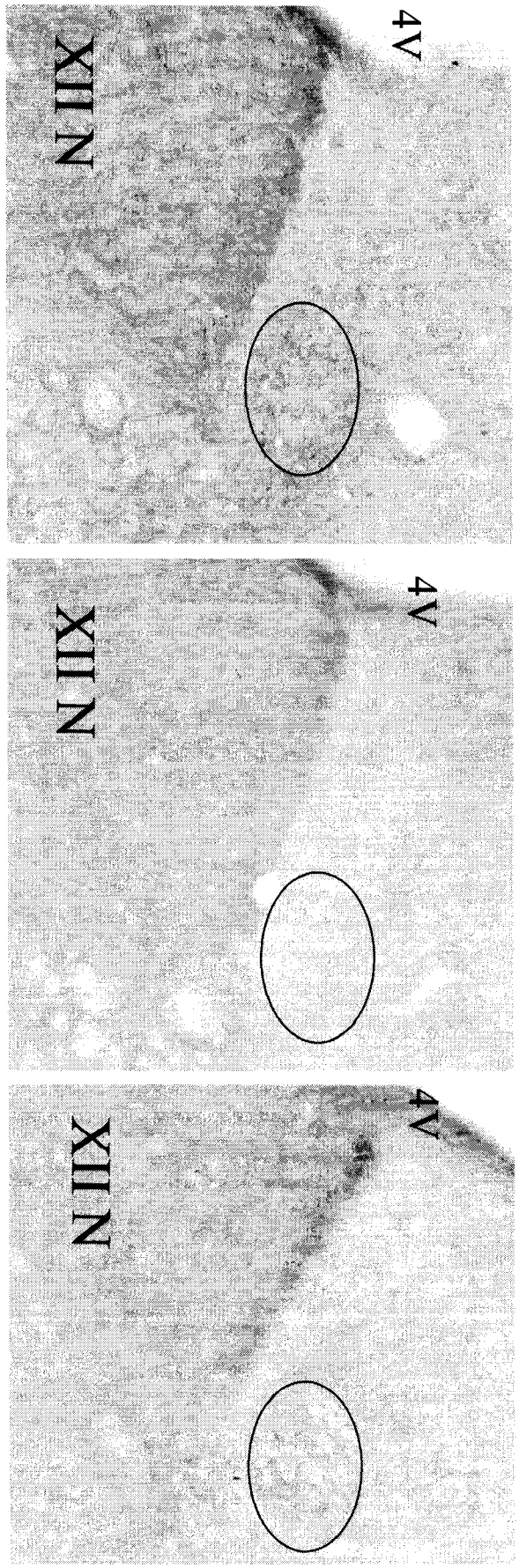
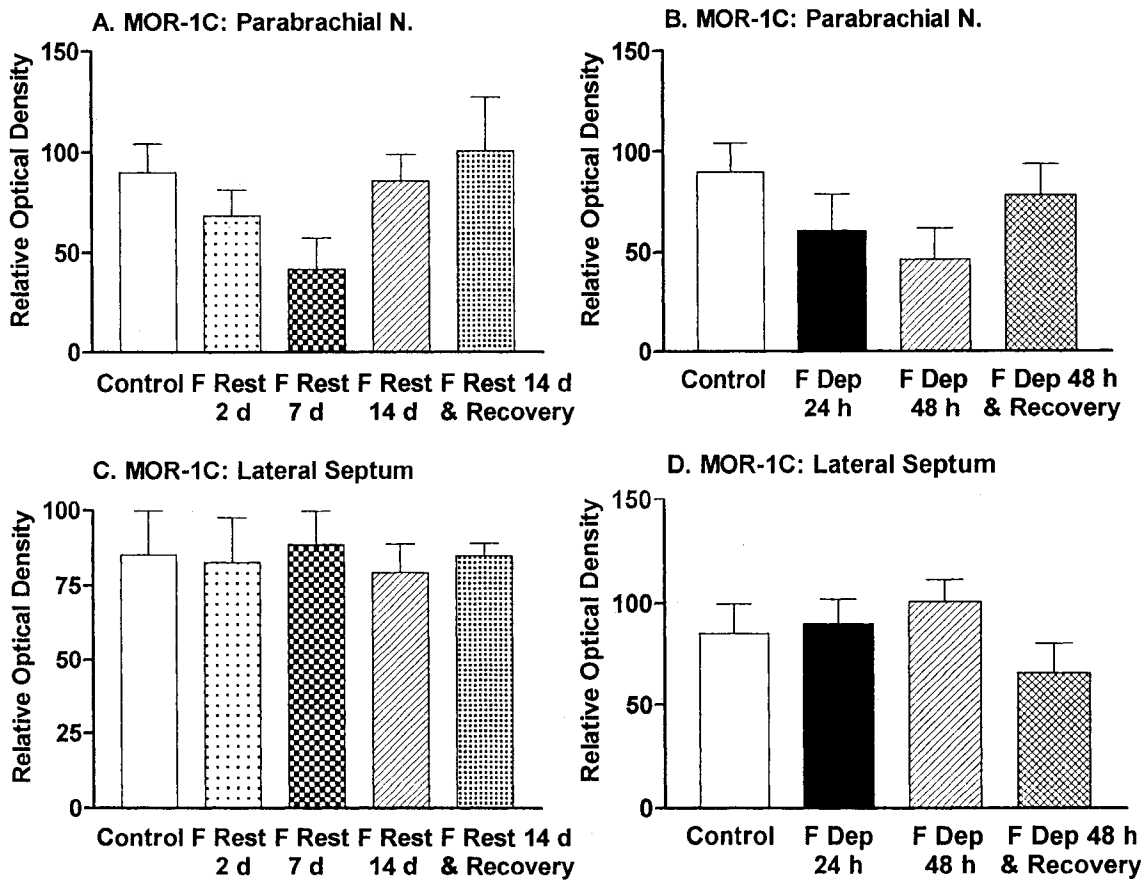


Figure 18. Alterations (Mean, ±SEM) in the optical density of MOR 1C-like immunoreactivity in the parabrachial (PBN: Panels A & B) and lateral septal nuclei (Panels C & D) in animals exposed to either *ad libitum* feeding (control), food restriction (Panels A & C) or food deprivation (Panels B & D).

Figure 18.



associated with the opioid mediation of feeding; b) neither food restriction nor food deprivation appreciably altered Exon 4-LI; c) the parvocellular subdivisions, but not the magnocellular subdivision of the PVN displayed significant time-dependent increases in the number and density of Exon 7/8/9-LI following extended food restriction, but not following extended food deprivation, an effect that persisted in animals subsequently allowed ad libitum access to food for seven days; d) hypothalamic changes in Exon 7/8/9-LI following food restriction were limited to the PVN, and not the periventricular, ventromedial or arcuate nuclei; e) the NTS displayed significant time-dependent reductions in the density of Exon 7/8/9-LI following brief exposure to either food restriction (2 and 7 days) or deprivation (24 and 48 h); and f) these effects were not observed in other extra-hypothalamic sites (PBN, lateral septum, BNST, amygdala) associated with opioid mediation of feeding.

Differential Exon 4-LI and Exon 7/8/9-LI Distribution in Feeding-Sensitive Sites: Previous anatomical studies have indicated differential distributions of the MOP and MOR-1C isoforms (Abbadie et al., 2000b; Ding et al., 1996). In examining sites either directly related to the intracerebral ingestive actions of opioid agonists and antagonists, and/or sites in which opioid peptides, receptors or mRNA is changed as a function of ingestive state, the present study indicated quite striking site-specific differences in immunoreactivity. Thus, the MOP gene showed greater immunoreactivity than the MOR-1C gene in the two brainstem sites, the NTS and PBN, and in two of the forebrain sites, the nucleus accumbens (core and shell) and the central nucleus of the amygdala. In contrast, the MOR-1C gene displayed greater immunoreactivity than the MOP gene in all four hypothalamic nuclei (paraventricular, periventricular, arcuate and ventromedial) as

well as the BNST and lateral septum. It is important to note that these differences are matters of degree, not a situation in which one isoform is present and the other is absent. That said, one can evaluate these differences in terms of three emerging ideas about potential opioid mechanisms of food intake control: a) sensory (e.g., taste, smell, texture of food stimuli and salient signals accompanying these stimuli) and metabolic (e.g., post-ingestive signals related to further food intake and regulation of body weight) integration; b) emotional (e.g., palatable, hedonic and incentive salient characteristics of food) integration; and c) issues related to energy needs (e.g., types of macronutrients; homeostatic state of the animal including deprivation, lipoprivation or glucoprivation; body adiposity and obesity models) (see review: Bodnar, 2004). Levine and co-workers (see review: Glass et al., 1999) have hypothesized site-specific actions for these three classes, suggesting that opioids in the hindbrain (NTS, PBN) are presumably involved in the sensory and metabolic integration regulating food intake, opioids in the amygdala (and other forebrain sites) may regulate "emotional" processing of food intake, and opioids in the hypothalamus are thought to regulate energy needs. Such a schema appears to fit some of the present results observed in food-deprived and food-restricted rats.

Minimal Sensitivity of Exon 4-LI to Food Restriction or Food Deprivation:

Neither food restriction nor food deprivation significantly altered either the density or number of Exon 4-LI in any of the nuclei examined, suggesting that these manipulations fail to change Opr*m* gene expression in these opioid-sensitive ingestion-related sites. Deprivation-induced feeding is reduced to the same degree by general and mu-selective opioid antagonists (Arjune et al., 1990; Bodnar et al., 1995; Brown and Holtzman, 1979; Cooper, 1980; Frenk and Rogers, 1979; Holtzman, 1974, 1975; Kelley et al., 1996; Koch

and Bodnar, 1994; Koch et al., 1995; Levine et al., 1990a, 1991; Maickel et al., 1977; Ragnauth et al., 1997; Simone et al., 1985; Thornhill and Saunders, 1984; Ukai and Holtzman, 1988). Yet only modest, but significant reductions in deprivation-induced feeding following administration of MOP AS ODN probes (exons 2, 3 and 4: Hadjimarkou et al., 2003). This stands in contrast to the ability of MOP AS ODN probes directed against each of its four exons to reduce spontaneous intake and body weight (Leventhal et al., 1996), and the ability of mu-selective antagonists and MOP AS ODN probes to produce comparable levels of inhibition of feeding elicited by glucoprivation (Arjune et al., 1990; Burdick et al., 1998; Koch and Bodnar, 1994), lipoprivation (Stein et al., 2000) and by mu-selective opioid agonists (morphine, DAMGO, morphine-6-glucuronide, BEND: Leventhal et al., 1997, 1998; Silva et al., 1998). The fact that Exon 4-LI is not appreciably altered by either food restriction or food deprivation thereby complements the modest actions of MOP AS ODN probes upon deprivation-induced feeding, and suggests that another isoform(s) of the MOP is (are) responsible for the mu-mediated actions upon deprivation-induced feeding. The results strongly suggest that the MOR-1C isoform is involved in these processes.

Exon 7/8/9-LI is Increased in the Parvocellular PVN Subdivisions following Food Restriction: The PVN is divided into magnocellular and parvocellular subdivisions with the former providing major axonal output to the neurohypophysis, and the latter providing output to the zona externa of the median eminence, to limbic and other forebrain areas, and to midbrain, hindbrain and spinal areas. The present study clearly demonstrated that Exon 7/8/9-LI was far more pronounced in the dorsal and ventral parvocellular subdivisions of the PVN relative to the magnocellular subdivision in

control *ad libitum*-fed animals, and that the low levels of Exon 7/8/9-LI in the magnocellular PVN subdivision were minimally affected by either food restriction or food deprivation. In contrast, both the number of cells and the optical density of Exon 7/8/9-LI were significantly increased in animals placed on a food restriction schedule for 14 days, and even for animals food restricted for 14 days followed by 7 days of *ad libitum* feeding. This effect was time-dependent in that 7 days of food restriction increased, albeit not significantly, Exon 7/8/9-LI. It should be noted that absolute food deprivation over the previous 24 or 48 h failed to alter Exon 7/8/9-LI, a lack of an effect that is not readily explained. Yet it should be noted that peptide levels of DYN A(1-17) in the PVN are also increased by either chronic food restriction (Berman et al., 1994, 1997; Tsujii et al., 1986b) or by palatability-induced hyperphagia in the absence of changes in met-enkephalin or BEND (Welch et al., 1996). Further, increased hypothalamic DYN levels are associated with corresponding increases in nocturnal intake (Przewlocki and Lason, 1982; Takahashi et al., 1986), and peripheral butorphanol, a mu/kappa opioid receptor agonist, stimulated PVN c-Fos activity (Kim et al., 2001). Finally, food deprivation lowers PVN NOP receptor mRNA (Rodi et al., 2002). Therefore, the parvocellular divisions of the PVN appear quite sensitive to several longer-term central adaptive changes in the opioid system by increasing the opioid peptide signals (e.g., DYN) or opioid receptor signals (Exon 7/8/9-LI) following food restriction and even following a short-term (7 days) recovery from the restriction regimen.

This finding is not surprising given the ability of opioid agonists and peptides to stimulate feeding following PVN microinjections (Gosnell et al., 1986; Kim et al., 2002; Leibowitz and Hor, 1982; McLean and Hoebel, 1982, 1983; Pomonis et al., 1996; Stanley

et al., 1989; Tepperman and Hirst, 1983; Woods and Leibowitz, 1985). Further, naltrexone pretreatment in the PVN blocked DAMGO-induced feeding elicited from either the central nucleus of the amygdala (Giraudo et al., 1998a) or the VTA (Quinn et al., 2003). Moreover, general opioid antagonist pretreatment in the PVN modestly reduces NPY-induced feeding from the same site without affecting NPY-induced reductions in brown fat thermogenesis (Kotz et al., 1995). Importantly, deprivation-induced feeding is markedly reduced by PVN pretreatment with mu and kappa, but not delta opioid antagonists, effects greater than that observed for glucoprivic and palatable intake (Koch et al., 1995).

Site-Specificity of Hypothalamic Changes in Exon 7/8/9-LI following Food Restriction: The ability of food restriction to increase cell numbers and optical density of Exon 7/8/9-LI was limited to the parvocellular PVN, as negligible effects of either food restriction or food deprivation were noted for Exon 7/8/9-LI in the neighboring periventricular, ventromedial or arcuate hypothalamic nuclei. These findings should not suggest that these sites are impervious to such central adaptive changes in the opioid system, since DYN A(1-17) is increased in the VMH following either chronic food restriction (Berman et al., 1994, 1997; Tsujii et al., 1986b) or streptozotocin-induced diabetes (Berman et al., 1995, 1997). In contrast, whereas chronic food restriction decreased hypothalamic arcuate beta-endorphin (BEND) and DYN (Brady et al., 1990; Kim et al., 1996), its combination with exercise increases hypothalamic BEND and DYN (Aravich et al., 1993). Therefore, it appears that separate hypothalamic components of the opioid system are differentially responding to these homeostatic challenges.

Exon 7/8/9-LI in Extra-hypothalamic Sites Respond Differentially to Food Restriction and Food Deprivation: In contrast to increased Exon 7/8/9-LI in the parvocellular PVN following long-term food restriction, the NTS displayed significant time-dependent reductions in the density of Exon 7/8/9-LI following brief exposure to either food restriction (2 and 7 days) or deprivation (24 and 48 h). Moreover, these effects were specific to the NTS, and not observed in the other extra-hypothalamic sites examined (PBN, lateral septum, BNST, amygdala) associated with opioid mediation of feeding. Microinjections of mu and delta opioid agonists into the NTS elicit feeding (Kotz et al., 1997), and there is a bidirectional opioid-opioid signaling pathway between the NTS and the central nucleus of the amygdala in that DAMGO-induced feeding elicited from one site is blocked by naltrexone pretreatment in the other site (Giraudo et al., 1998b). Moreover, DAMGO-induced feeding elicited from the NAC is blocked NTS inactivation with muscimol (Will et al., 2003). Whereas NTS c-Fos activity was stimulated by butorphanol (Kim et al., 2001) and OFQ/N (Olszewski et al., 2000), met-enkephalin suppresses neuronal activity induced by the sweet taste of sucrose in the NTS in a naltrexone-reversible manner (Li et al., 2003). This is consistent with the proposition (Glass et al., 1999) that opioids in the hindbrain including the NTS are presumably involved in the sensory (taste) and metabolic integration regulating food intake. Therefore, these factors would come into play following shorter periods of food restriction or brief food deprivation, and may provide a schema for the present findings that the optical density of Exon 7/8/9-LI is reduced in the NTS following short food restriction or deprivation.

The failure of the PBN, lateral septum, BNST and amygdala to display altered Exon 7/8/9-LI following restriction or deprivation does not mean that they are impervious to any central adaptive changes in the opioid system. Chronic food restriction decreased mu opioid binding in the amygdala and PBN, but increased kappa opioid binding in the BNST and PBN (Tsujii et al., 1986a; Wolinsky et al., 1994, 1996b). Dynorphin (DYN) A1-17 is decreased by chronic food restriction in the central amygdala, but the shorter DYN A1-8 peptide is increased in the NAC and BNST (Berman et al., 1994, 1997; Tsujii et al., 1986b). Food-restricted rats display increased c-Fos immunoreactivity in the BNST, central nucleus of the amygdala and NAC following naltrexone pretreatment, in the BNST and amygdala following kappa antagonism, and in the NAC following mu antagonism (Carr et al., 1998, 1999). Whereas food deprivation lowers both NOP receptor mRNA levels and pro-OFQ/N mRNA levels in the central nucleus of the amygdala (Rodi et al., 2002), sucrose consumption significantly enhances the ability of naloxone to increase c-Fos activity in the central nucleus of the amygdala (Park and Carr, 1998; Pomonis et al., 2000). Therefore, it appears that separate extra-hypothalamic components of the opioid system are differentially responding to these homeostatic challenges.

CHAPTER 7. SPECIFIC AIM FOUR.

Deprivation-Induced Feeding: Opioid Receptor Gene Role in Food-Deprivation Through Administration of Opioid Antisense Probes

Introduction

The endogenous opioid peptide and receptor system is intimately involved in the mediation of food intake as demonstrated by an initial study by Holtzman (1974) showing that systemic administration of the general opioid antagonist, naloxone, significantly reduced food intake elicited by food deprivation in rats. The ability of systemic naloxone and other general opioid antagonists like naltrexone to reduce deprivation-induced feeding was subsequently confirmed in both rats and mice (Brown and Holtzman, 1979; Cooper, 1980; Frenk and Rogers, 1979). General opioid antagonists also reduced deprivation-induced feeding in rats following intracerebral administration into the hypothalamic paraventricular nucleus, nucleus accumbens and to a lesser degree, the ventral tegmental area (Bodnar et al., 1995; Kelley et al. 1996; Koch et al., 1995; Ragnauth et al., 1997). The ability of selective μ, κ and δ opioid receptor subtype antagonists to alter deprivation-induced feeding in rats suggests differential opioid receptor subtype mediation.

Thus, ventricular pretreatment with either the μ-selective opioid antagonist, β-funaltrexamine (β-FNA), or the μ_1-selective opioid antagonist, naloxonazine, produces a 50-75% reduction in deprivation-induced intake in rats, effects equal in magnitude to general opioid antagonism (Arjune et al., 1990; Koch and Bodnar, 1994; Levine et al., 1991; Simone et al., 1985). In contrast, ventricular pretreatment with the κ-selective opioid antagonist nor-binaltorphamine (Nor-BNI) significantly, but moderately reduced

(~30%) deprivation-induced feeding in rats (Koch and Bodnar, 1994; Levine et al., 1990). Finally, ventricular pretreatment with the δ-opioid receptor antagonists, naltrindole or DALCE, failed to significantly alter deprivation-induced intake in rats (Arjune et al., 1991; Koch and Bodnar, 1994). Intracerebral microinjection studies indicate that deprivation-induced intake is markedly reduced by μ antagonists in the hypothalamic paraventricular nucleus and nucleus accumbens, and to a lesser degree in the ventral tegmental area in rats. Deprivation-induced feeding in rats is also modestly reduced by κ antagonists in each of the three sites, and modestly reduced by δ antagonists in only the ventral tegmental area (Bodnar et al., 1995; Kelley, et al. 1996; Koch et al., 1995; Ragnauth et al., 1997). Therefore, based on these data, it would appear that opioid receptor mediation of deprivation-induced feeding in rats occurs primarily through the μ receptor, secondarily through the κ receptor, and minimally through the δ receptor.

The AS ODN technique (see reviews: Pasternak and Standifer, 1995; Rossi and Pasternak, 1997) has been used in ingestive studies to correlate the molecular biology of the cloned opioid receptors (MOP [MOR-1: 4 exons], DOP [DOR-1: 3 exons], KOP [KOR-1: 3 exons], NOP [KOR-3/ORL-1: 3 exons]) (see reviews: Kieffer, 1995; Uhl et al., 1994) with their *in vivo* functional effects, and to provide converging and complementary lines of evidence to those supplied by opioid receptor subtype antagonist studies. Although there is only one identified MOP gene (Chen et al., 1993; Wang et al., 1993), the μ receptor antagonist-mediated actions upon deprivation-induced feeding may involve recently identified splice variants or isoforms of the MOP gene that have been identified in the mouse (Bare et al., 1994; Pan et al., 1999; Pan et al., 2000; Pan et al., 2001; Pasternak et al., 2001; Pasternak and Pan, 2000; Zimprich et al., 1995).

Thus, if one wanted to evaluate whether isoforms of the MOP (MOR-1) gene were responsible for μ opioid-mediated actions, one would use AS ODN probes directed against the additional identified exons 5, 6, 7, 8, 9, 10, 12 and 13. However, these exons and their AS ODN probes have only been identified in the mouse, and this animal would have to be employed in performing this analysis. Yet, although the mouse displays the same pattern of naloxone-induced inhibition of deprivation-induced feeding as the rat [e.g., Brown and Holtzman, 1979; Cooper, 1980; Frenk and Rogers, 1979; Holtzman, 1974) and indeed other species (e.g., wolves, tigers, woodchucks and deer, but not Chinese hamsters or raccoons: see review: Bodnar, 2004), studies using selective opioid receptor subtype antagonists have been exclusively carried out in the rat. Thus, a systematic comparison of selective opioid antagonist effects upon deprivation-induced feeding in the mouse and the rat is warranted. Indeed, given the different sizes and body weights of the two rodents, it is conceivable that 24 h of deprivation may constitute a greater homeostatic stressor upon the mouse than the rat, and therefore, the duration of deprivation becomes another issue for study of selective opioid antagonist effects in the mouse. Therefore, the first experiment will examine whether short-term (0.5-4 h) and long-term (24-48 h) intake and body weight (24-48 h) changes following 24 h of food deprivation in the mouse dose-dependently vary following ventricular and systemic administration of the general opioid antagonist, naltrexone, and following central administration of either μ (βFNA), κ (NBNI) or δ (NTI) opioid receptor subtype antagonists. The second experiment will examine whether short-term (0.5-4 h) and long-term (24-48 h) intake and body weight (24-48 h) changes following 12 h of food deprivation in the mouse dose-dependently vary following ventricular and systemic

administration of naltrexone, and following central administration of either μ (βFNA), κ (NBNI) or δ (NTI) opioid receptor subtype antagonists. The third experiment will examine whether short-term (0.5-4 h) and longer-term (24-48 h) intake and body weight (24-48 h) changes following 24 h of food deprivation in the rat dose-dependently vary following ventricular and systemic administration of naltrexone, and central administration of either μ (βFNA), κ (NBNI) or δ (NTI) opioid receptor subtype antagonists. The fourth experiment will examine whether AS ODN probes directed against each of the exons of the MOP (exons 1, 2, 3, 4, 5a, 6, 7, 8, 9, 10, 12, 13), DOP (exons 1, 2, 3), KOP (exons 1, 2, 3) and NOP (exons 1, 2) opioid receptor genes alter food intake and body weight changes following 24 h of food deprivation in the mouse.

Methods

AS ODN Probes: See General Methods: Chapter 3.

Opioid Antagonist testing procedures in mice: Experiment 1

Following acclimation of the mice in the cages equipped with metal grids at the floor of each cage, mice were weighed and assigned to a 4-day treatment paradigm. In the first control phase, food was removed from each animal's cage at 2 h into the light cycle (Day 1), and the animals were food-deprived for 24 h. Food was reintroduced on Day 2, and intake (±0.1 g) was measured by weighing standard chow pellets placed on the grid floor and adjusting for spillage collected by brown paper towels beneath the metal grid at 0.5, 1, 2, and 4 h thereafter. Food intake, adjusted for spillage, was also measured 24 (Day 3) and 48 (Day 4) h following food reintroduction. Body weight was monitored on all four days so that deprivation-induced weight loss and food reintroduction-induced weight recovery could be evaluated. The second experimental phase began two weeks

later after it was determined that all mice regained their original body weight, and demonstrated normal intake. Subgroups of mice, matched for 24 h deprivation-induced food intake, received either systemic naltrexone at doses of 0.1 (n=5), 1 (n=5) or 5 (n=10) mg/kg, ventricular naltrexone at doses of 10 (n=8) or 50 (n=10) μg, ventricular βFNA at doses of 5 (n=8) or 20 (n=9) μg, ventricular NBNI at doses of 5 (n=10) or 20 (n=9) μg, or ventricular NTI at doses of 5 (n=9) or 20 (n=9) μg. Antagonist treatments, reflecting peak time intervals, preceded reintroduction of food to deprived mice by 0.5 h for naltrexone, by 1 h for NBNI and NTI, and by 24 h for βFNA. Systemic naltrexone injections were administered subcutaneously in a 10 ml/kg volume. In performing ventricular injections in mice in this and subsequent protocols, animals were exposed to an isoflurane and oxygen combination for approximately 2-3 min until full anesthesia was observed. A midline incision along the sagittal suture exposed the sutures, and a freehand injection was administered in the lateral ventricle at 2 mm anterior to the lambda and 3-3.5 mm lateral to midline through an internal cannula (28-gauge) that extended 2mm below the surface of the skull. This procedure was periodically verified in control mice using luxol fast blue injections to visualize entry into the ventricles. Mice received one and only one experimental condition.

Opioid Antagonist testing procedures in mice: Experiment 2

New groups of mice were exposed to the same paradigm described in Experiment 1 with the following exception. Whereas animals in experiment 1 were food deprived at about 2h into the light cycle and remained deprived for 24 h subsequently, in this experiment, in both baseline and experimental phases animals were food-deprived at the onset of the dark cycle (Day 1), and remained as such for 12 h so that food was again

reintroduced at the onset of the light cycle on Day 2. Once baseline intake was assessed under these conditions of 12h of food deprivation, mice matched on their 12 h deprivation-induced food intake were assigned to the following opioid antagonists treatment conditions: systemic naltrexone (5 mg/kg, n=9), ventricular naltrexone (50 μg, n=10), ventricular βFNA (20 μg, n=8), ventricular NBNI (20 μg, n=10), or ventricular NTI (20 μg, n=9).

Opioid Antagonist testing procedures in rats: Experiment 3

Following recovery from surgery and approximately one month after arrival in the vivarium, food intake and body weight of 20 acclimated rats were assessed over 48 h to verify normal feeding responses. The rats underwent the following food deprivation paradigm four times over a ten-week period in which all rats were allowed two weeks between conditions to fully recover their normal body weight and normal food intake. In the first week, all 20 rats received a vehicle (5 μl normal saline) ventricular microinjection 30 min prior to food reintroduction. During the fourth, seventh and tenth weeks, subgroups of rats received a microinjection or a systemic (sc) injection of naltrexone, or a microinjection of one of three opioid selective antagonists. Specifically, the following treatment conditions were tested: systemic naltrexone at doses of either a) 0.1 (n=5) or b) 1 (n=5) or c) 5 (n=5) mg/kg; ventricular naltrexone at doses of either d) 10 (n=6) or e) 50 (n=5) μg; βFNA at doses of either f) 5 (n=5) or g) 20 (n=5) μg; NBNI at doses of either h) 5 (n=5) or i) 20 (n=5) μg, and NTI at doses of either j) 5 (n=5) or k) 20 (n=5) μg. Antagonist treatments, reflecting peak time intervals, preceded reintroduction of food to deprived rats by 0.5 h for naltrexone, by 1 h for NBNI and NTI, and by 24 h for βFNA. The order of the antagonist conditions and doses was counterbalanced across

the three tests, and the rats were matched for deprivation-induced intake values under vehicle control conditions.

On Day 1, the animals were weighed (pre-deprivation weight) and were food-deprived with water available for 24 h. On Day 2, the animals were re-weighed (post-deprivation weight) to assess body weight loss as a result of food deprivation, and Pre-weighed standard chow pellets were reintroduced on the floor of the cage with water available in a manner identical to that described in Experiment 1. Food intake ($\pm$0.1 g) was assessed by weighing food pellets adjusted for spillage collected by brown paper towels beneath the wire mesh cage at 0.5, 1, 2, and 4 h following food reintroduction. Next, a pre-weighed food bin was placed inside the cage with water available, and a brown paper towel was placed beneath the cage to collect intake and spillage for cumulative longer-term intake. On Days 3 and 4 cumulative intake was assessed after 24 and 48 h post food reintroduction, by measuring the food bin and adjusting for spillage. The rats were also re-weighed after 24 and 48 h (recovery weights) to assess food reintroduction-induced body weight gain.

AS ODN testing procedures in mice: Experiment 4

Individually housed mice acclimated to cages with metal grids at the bottom, were assigned to an 8-day testing paradigm. In this paradigm, animals were again food deprived for 24 h as described in Experiment 1. On days 1, 3 and 5 at 2-4 h into the light cycle, subgroups of 7-8 mice received a microinjection of one of the 12 MOR-1 AS ODN probes (directed against coding exons 1, 2, 3, 4, 5a, 6, 7, 8, 9, 10, 12 or 13), one of the DOP gene AS ODN probes (directed against coding exons 1, 2, 3), one of the KOP gene AS ODN probes (directed against coding exons 1, 2, 3), or one of the NOP gene AS

ODN probes (directed against coding exons 1 or 2) (Table 5). Following the third microinjection on day 5, the animals were food-deprived for 24 h with water available *ad libitum*. On day 6, food was reintroduced, and short-term cumulative intake was assessed after 0.5, 1, 2 and 4 h. Longer-term intake was assessed on days 7 and 8, 24 and 48 h following food reintroduction, respectively. Body weight was monitored throughout the paradigm as noted in Experiment 1.

Statistics

Randomized-block two-way analyses of variance were performed in each of the four experiments on short-term cumulative food intake data with the between-subject variable of vehicle and the different antagonist or AS ODN conditions, the within-subject variable of post-deprivation intake time (0.5, 1, 2, 4 h), and for the interaction between condition and time. Tukey comparisons ($P<0.05$) were used to assess significant antagonist or AS ODN effects relative to vehicle control treatment. Randomized-block two-way analyses of variance were also performed in each of the four experiments on longer-term cumulative food intake data with the between-subject variable of vehicle and the different antagonist or AS ODN conditions, the within-subject variable of post-deprivation intake time (24, 48 h), and for the interaction between condition and time. Deprivation-induced weight loss scores were assessed for each animal by subtracting the Post-Deprivation weight from its corresponding Pre-Deprivation weight, and recovery-induced weight gain scores were assessed for each animal by subtracting the corresponding Recovery weight from the Post-Deprivation weight. Separate one-way ANOVA were performed on deprivation-induced weight loss and two-way ANOVA for recovery-induced weight gain in each of the four experiments for each antagonist or AS

Table 5. Opioid antisense oligodeoxynucleotide probes targeted against the mouse genome

Probe	Sequence
MOP Gene:	
Exon 1 AS	CGC CCC AGC CTC TTC CTC T
Exon 2 AS	TTG GTG GCA GTC TTC ATT TTG G
Exon 3 AS	TGA GCA GGT TCT CCC AGT ACC A
Exon 4 AS	GGG CAA TGG AGC AGT TTC TG
Exon 5a AS	GGG GTT GGC ACC AGC ATT AGG TAC TC
Exon 6 AS	GGC TCA AAG ACA AGG GAC AGG TCA
Exon 7 AS	CCT GTA AAG ACT GTG GCA CCG C
Exon 8 AS	GGG CCA TCA TCA GGA AGA AGG
Exon 9 AS	GAA AGG CAT CTT CCC TCT CGC T
Exon 10 AS	CTT GCT GCC TTC GTA AGG ACC TGG
Exon 12 AS	GGA CAA AGT GAA CAT CAG AGC CAG
Exon 13 AS	CAG GAA AAG AAT GGA CAG AGG
DOP Gene:	
Exon 1 AS	TGT CCG TCT CCA CCG TGC
Exon 2 AS	ATC AAG TAC TTG GCG CTC TG
Exon 3 AS	AAC ACG CAG ATC TTG GTC AC
KOP Gene:	
Exon 1 AS	GCT GCT GAT CCT CTG AGC CCA
Exon 2 AS	CCA AAG CAT CTG CCA AAG CCA
NOP Gene:	
Exon 1 AS	GGG GCA GGA AAG AGG GAC TCC
Exon 2 AS	GAC GAG GCA GTT CCC CAG GA
Exon 3 AS	GGG CTG TGC AGA AGC CGA GA

ODN treatment relative to vehicle control values with Tukey comparisons ($P<0.05$) indicating individual significant effects relative to vehicle control treatment.

Results

Selective Opioid Receptor Antagonism and Deprivation (24 h)-Induced Feeding in the Mouse: Significant differences in short-term (0.5-4 h) intake were observed among drug conditions ($F(11,1023)= 266.45$, $P<0.0001$), across test times ($F(3,279)= 24978.91$, $P<0.0001$) and for the interaction between conditions and times ($F(33,3069)= 232.90$, $P<0.0001$). Systemic administration of the general opioid antagonist, naltrexone significantly and dose-dependently reduced short-term deprivation-induced feeding at the 0.1 (0.5-2 h), 1 (0.5-4 h) and 5 (0.5-4 h) mg/kg doses (Figure 19A). Correspondingly, short-term deprivation-induced feeding was significantly reduced across the 4 h time course following ventricular naltrexone doses of 10 and 50 μg (Figure 19B). Moreover, short-term deprivation-induced feeding was significantly reduced across the 4 h time course by both the 5 and 20 μg doses of the μ antagonist, BFNA (Figure 20A), the κ antagonist, NBNI (Figure 20B) and the δ antagonist, NTI (Figure 20C). Significant differences in longer-term (24-48 h) intake were observed among drug conditions ($F(11,1001)= 517.40$, $P<0.0001$), across test times ($F(1,91)= 1307.26$, $P<0.0001$) and for the interaction between conditions and times ($F(11,1001)= 298.35$, $P<0.0001$). Systemic naltrexone administration significantly (11-12%) and dose-dependently (1-5 mg/kg) reduced longer-term deprivation-induced feeding after 24, but not after 48 h; the lowest (0.1 mg/kg) actually increased intake after 48 h (Figure 19C). Correspondingly, longer-term deprivation-induced feeding was significantly reduced after 24 h following both ventricular naltrexone doses with the low and high doses respectively decreasing and

Figure 19. Alterations in short-term (0.5-4 h, Panels A & C) and longer-term (24-48 h, Panels B & D) cumulative intake in mice food-deprived for 24 h following systemic (Panels A & B) or ventricular (Panels C & D) administration of naltrexone. The asterisks in this and subsequent figures denote significant differences (Tukey comparisons, $P<0.05$) from corresponding vehicle control conditions. The dotted line indicates 24 h intake under normal (non-deprivation) conditions.

Figure 19.

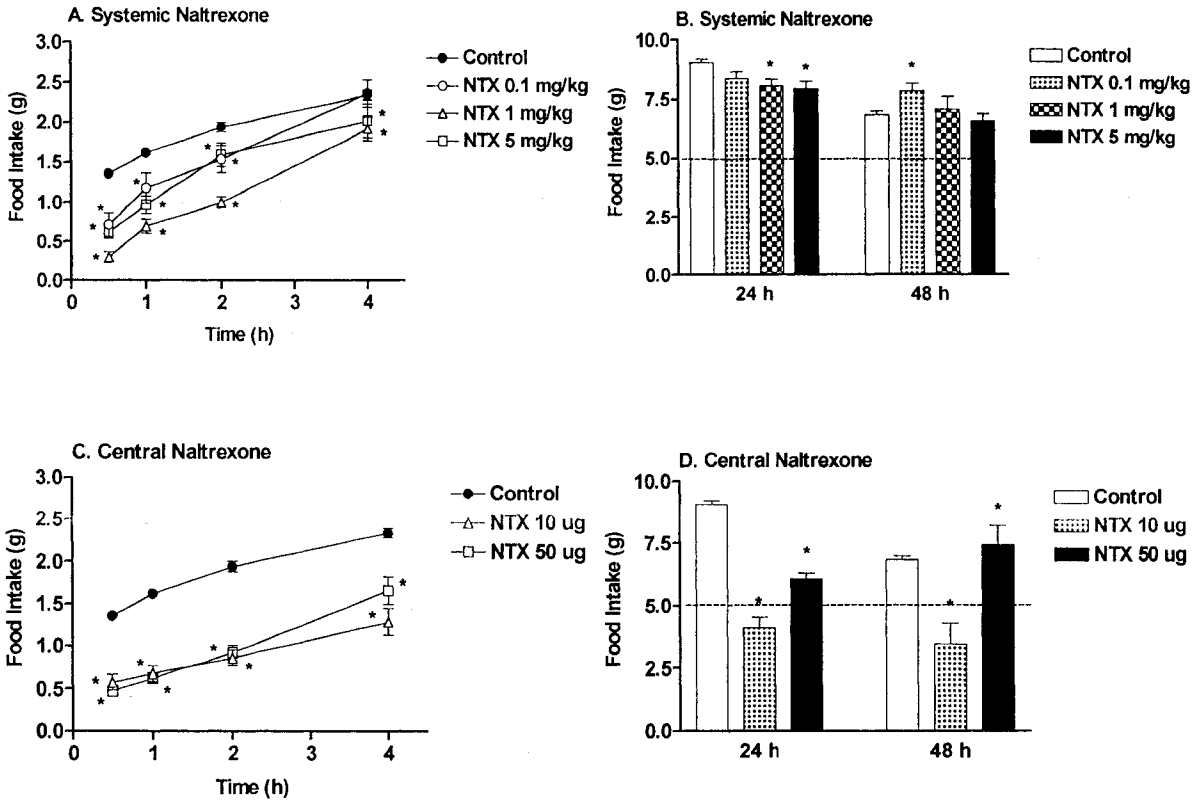
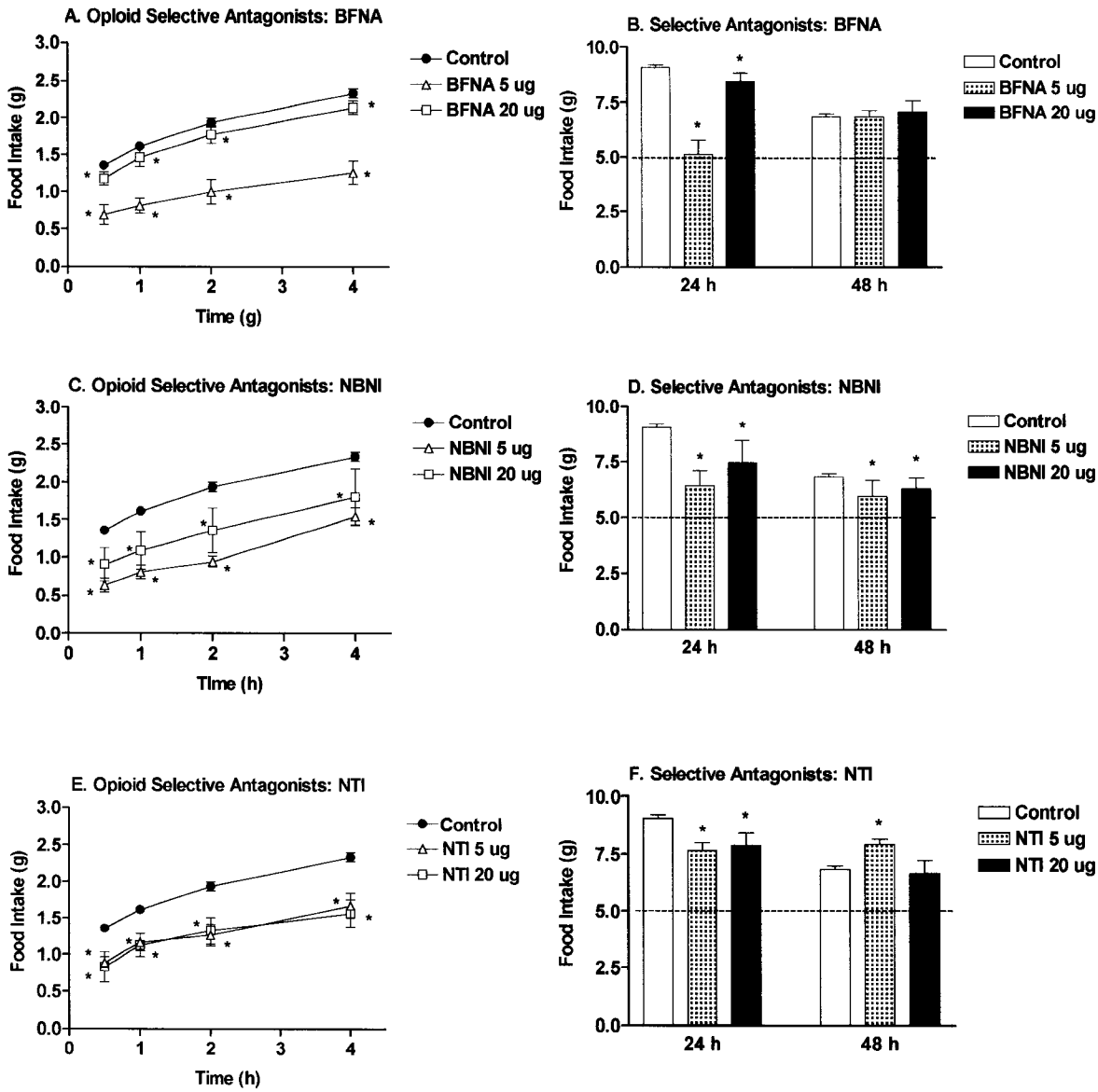


Figure 20. Alterations in short-term (0.5-4 h, Panels A, C & E) and longer-term (24-48 h, Panels B, D & F) cumulative intake in mice food-deprived for 24 h following ventricular administration of selective mu (β-FNA: Panels A & B), kappa (NBNI: Panels C & D) and delta (NTI: Panels E & F) opioid receptor subtype antagonists. The dotted line indicates 24 h intake under normal (non-deprivation) conditions.

Figure 20.



increasing intake after 48 h (Figure 19d). Moreover, longer-term deprivation-induced feeding was significantly reduced after 24 and 48 h following both NBNI doses (Figure 20E), and after 24 h by both doses of BFNA (Figure 20D) and NTI (Figure 20F). The low NTI dose significantly increased longer-term deprivation-induced intake after 48 h (Figure 20F). Although significant differences in deprivation-induced body weight loss occurred among drug groups ($F(11, 172)= 2.93$, $P<0.001$), all of the antagonists failed to differ from control treatment in the magnitude of body weight loss (Table 6). Significant differences in body weight recovery following deprivation were observed among drug conditions ($F(11,1001)= 699.16$, $P<0.0001$), across test times ($F(1,91)= 393.21$, $P<0.0001$) and for the interaction between conditions and times ($F(11,1001)= 155.90$, $P<0.0001$). Systemic naltrexone administration significantly reduced body weight recovery after 24 and 48 h following the 1 mg/kg dose, whereas ventricular naltrexone significantly reduced body weight recovery following the 10 (24-48 h) and 50 (24 h) μg doses (Table 6). Body weight recovery was significantly reduced following the 5 (24-48 h) and 20 (24 h) μg doses of BFNA, the 5 (24-48 h) and 20 (24-48 h) μg doses of NBNI, and the 5 (24 h) and 20 (24-48 h) μg doses of NTI (Table 6).

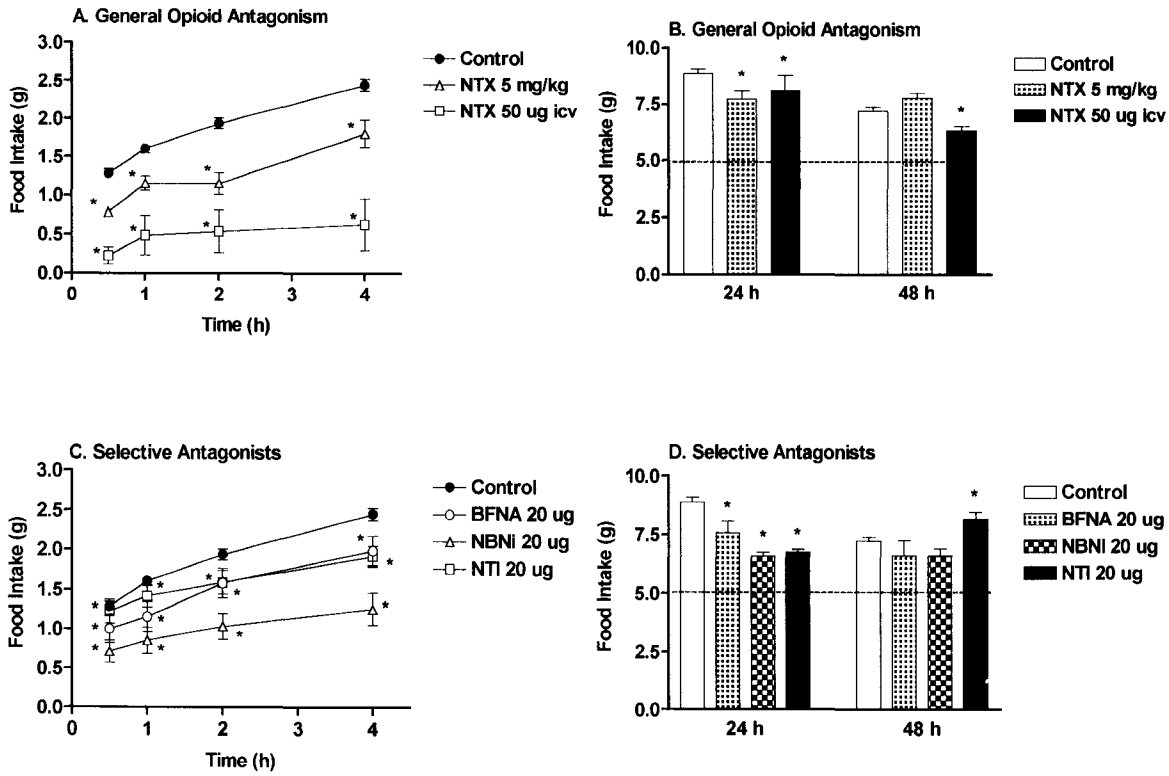
Selective Opioid Receptor Antagonism and Deprivation (12 h)-Induced Feeding in the Mouse: Significant differences in short-term (0.5-4 h) intake were observed among drug conditions ($F(5,220)= 169.53$, $P<0.0001$), across test times ($F(3,132)= 1213.94$, $P<0.0001$) and for the interaction between conditions and times ($F(15,660)= 36.58$, $P<0.0001$). Both systemic and ventricular naltrexone administration significantly reduced short-term deprivation-induced feeding across the time course (Figure 21A).

Table 6. Body weight loss and body weight recovery in 24 h-food-deprived mice treated with general and selective opioid receptor antagonists.

Treatment	BW (g) *M (±SEM)*	BW Recovery 24 h Post *M (±SEM)*	BW Recovery 48 h Post *M (±SEM)*
Control	-5.35 (0.13)	5.32 (0.16)	5.78 (0.19)
Systemic Naltrexone, sc			
0.1 mg/kg	-5.18 (0.24)	5.14 (0.53) *	5.62 (0.53) *
1.0 mg/kg	-4.28 (0.19)	3.82 (0.36)	2.24 (1.79)
5.0 mg/kg	-5.13 (0.53)	5.27 (0.54)	5.81 (0.48)
Central Naltrexone, icv			
10 μg	-4.67 (0.75)	-0.89 (0.62) *	-1.05 (1.14) *
50 μg	-5.77 (0.23)	4.66 (0.31) *	5.87 (0.29)
β-FNA			
5 μg	-5.58 (0.43)	2.89 (0.80) *	3.99 (0.47) *
20 μg	-6.57 (0.31)	6.06 (0.48) *	6.31 (0.62)
Nor-BNI			
5 μg	-4.57 (0.27)	2.53 (0.70) *	2.73 (1.20) *
20 μg	-4.84 (0.60)	3.52 (0.64) *	4.23 (0.88) *
NTI			
5 μg	-4.91 (0.28)	3.70 (0.41) *	5.28 (0.39)
20 μg	-3.73 (0.72)	4.00 (0.84) *	3.42 (3.33) *

Figure 21. Alterations in short-term (0.5-4 h, Panels A & C) and longer-term (24-48 h, Panels B & D) cumulative intake in mice food-deprived for 12 h following ventricular administration of selective mu (β-FNA), kappa (NBNI) and delta (NTI) opioid receptor subtype antagonists (Panels C & D). The dotted line indicates 24 h intake under normal (non-deprivation) conditions.

Figure 21.



Correspondingly, short-term deprivation-induced feeding was significantly reduced across the time course following BFNA, NBNI and NTI (Figure 21B). Significant differences in longer-term (24-48 h) intake were observed among drug conditions ($F(5,220)= 30.57$, $P<0.0001$), across test times ($F(1,44)= 242.92$, $P<0.0001$) and for the interaction between conditions and times ($F(5,220)= 128.50$, $P<0.0001$). Longer-term deprivation-induced intake was significantly reduced by systemic (24 h) and ventricular (24-48 h) naltrexone (Figure 21C), and by BFNA (24 h), NBNI (24 h) and NTI (24-48 h) pretreatment (Figure 21D). Although significant differences in deprivation-induced body weight loss failed to occur among drug groups ($F(5,85)= 0.86$, n.s., Table 7), significant differences in body weight recovery following deprivation were observed among drug conditions ($F(5,220)= 18.37$, $P<0.0001$), across test times ($F(1,44)= 552.94$, $P<0.0001$) and for the interaction between conditions and times ($F(5,220)= 56.08$, $P<0.0001$). Body weight recovery was significantly slowed by systemic (24 h), but not ventricular naltrexone (Table 7). Body weight recovery was significantly reduced following BFNA (24-48 h) and NBNI (24-48 h) and significantly increased by NTI (48 h) (Table 7).

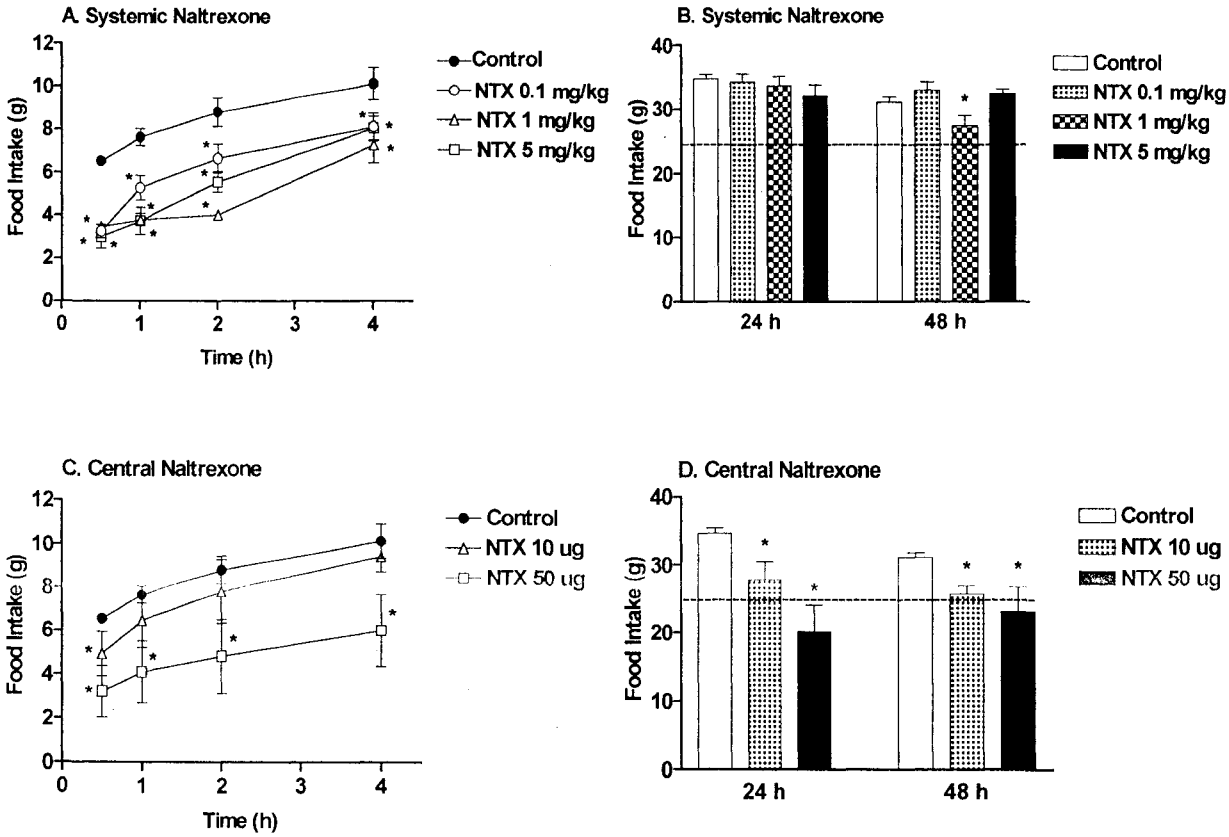
Selective Opioid Receptor Antagonism and Deprivation (24 h)-Induced Feeding in the Rat: Significant differences in short-term (0.5-4 h) intake were observed among drug conditions ($F(11,209)= 45.69$, $P<0.0001$), across test times ($F(3,57)= 1078.53$, $P<0.0001$) and for the interaction between conditions and times ($F(33,627)= 13.03$, $P<0.0001$). Whereas short-term deprivation-induced feeding was significantly reduced across the time course by all systemic naltrexone doses (Figure 22A), it was dose-dependently reduced by the low (0.5 h) and higher (0.5-4 h) ventricular naltrexone doses (Figure 22B). Whereas both BFNA doses significantly reduced short-term deprivation-

Table 7: Body weight loss and body weight recovery in 12 h-food-deprived mice treated with general and selective opioid receptor antagonists.

Treatment	BW Loss (g) *M (±SEM)*	BW Recovery 24 h Post *M (±SEM)*	BW Recovery 48 h Post *M (±SEM)*
Control	-4.38 (0.21)	4.06 (0.21)	4.39 (0.26)
Systemic Naltrexone, sc			
5.0 mg/kg	-4.41 (0.28)	3.27 (0.35) *	4.94 (0.40)
Central Naltrexone, icv			
50 μg	-3.86 (0.34)	3.62 (0.58)	3.77 (0.41)
β-FNA			
20 μg	-3.63 (0.44)	3.35 (0.46) *	3.68 (0.76) *
Nor-BNI			
20 μg	-3.85 (0.40)	2.17 (0.58) *	3.66 (0.48) *
NTI			
20 μg	-4.01 (0.40)	3.61 (0.96)	5.57 (1.02) *

Figure 22. Alterations in short-term (0.5-4 h, Panels A & C) and longer-term (24-48 h, Panels B & D) cumulative intake in rats food-deprived for 24 h following systemic (Panels A & B) or ventricular (Panels C & D) administration of naltrexone. The dotted line indicates 24 h intake under normal (non-deprivation) conditions.

Figure 22.



induced feeding across the time course (Figure 23A), it was dose-dependently reduced by the low (1 h) and higher (0.5-4 h) NBNI doses (Figure 23B). In contrast, NTI failed to alter short-term deprivation-induced intake in the rat (Figure 23C). Significant differences in longer-term (24-48 h) intake were observed among drug conditions ($F(11,209)= 50.37$, $P<0.001$) and for the interaction between conditions and times ($F(11,209)= 46.34$, $P<0.001$), but not for test times ($F(1,19)= 0.41$, n.s.). Whereas systemic naltrexone (1 mg/kg) only transiently (48 h) reduced longer-term deprivation-induced feeding (Figure 22C), both ventricular naltrexone doses decreased longer-term deprivation-induced feeding after 24 and 48 h (Figure 22D). Longer-term deprivation-induced intake was reduced was significantly reduced after 24 and 48 h following both BFNA doses (Figure 23D) and the low NBNI dose (Figure 23E), and after 24 h by the high dose of NBNI (Figure 23E) and NTI (Figure 23F). The significant differences in deprivation-induced body weight loss occurred among drug groups ($F(11,65)= 2.41$, $P<0.014$) indicated that weight loss was significantly greater following the low NTI dose (Table 8). Significant differences in body weight recovery following deprivation were observed among drug conditions ($F(11,209)= 67.22$, $P<0.0001$), across test times ($F(1,19)= 755.10$, $P<0.0001$) and for the interaction between conditions and times ($F(11,209)= 25.70$, $P<0.0001$). Both systemic and ventricular naltrexone administration significantly reduced body weight recovery after 24 and 48 h following all tested doses (Table 8). Moreover, both doses of the μ, κ, and δ antagonists also significantly reduced body weight recovery after 24 and 48 h (Table 8).

Opioid Receptor Antisense Probes and Deprivation (24 h)-Induced Feeding in the Mouse: Significant differences in short-term (0.5-4 h) intake were observed among

Figure 23. Alterations in short-term (0.5-4 h, Panels A, C & E) and longer-term (24-48 h, Panels B, D & F) cumulative intake in rats food-deprived for 24 h following ventricular administration of selective mu (β-FNA: Panels A & B), kappa (NBNI: Panels C & D) and delta (NTI: Panels E & F) opioid receptor subtype antagonists. The dotted line indicates 24 h intake under normal (non-deprivation) conditions.

Figure 23.

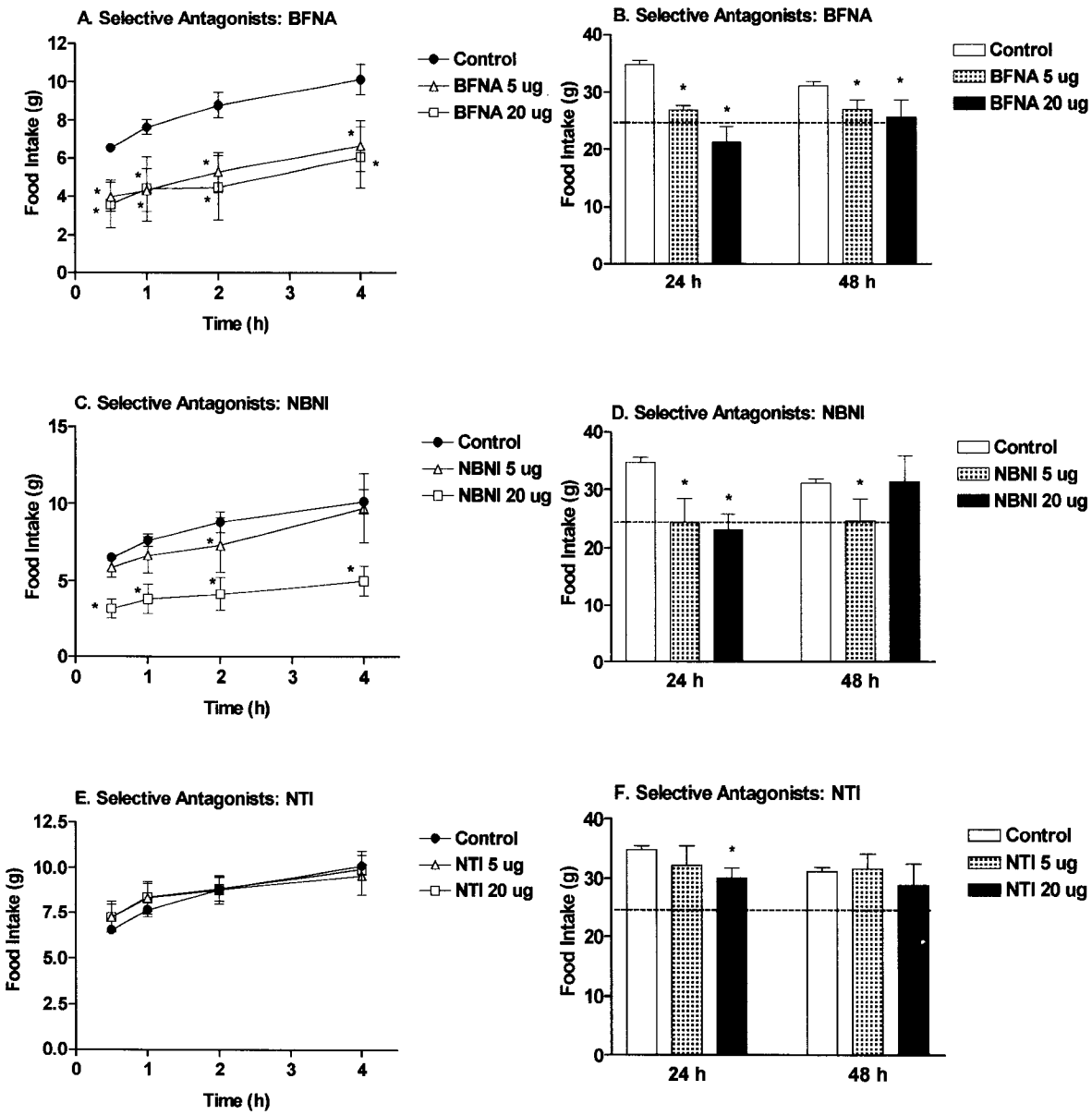


Table 8: Body weight loss and body weight recovery in 24 h-food-deprived rats treated with general and selective opioid receptor antagonists.

Treatment	BW Loss (g) *M (±SEM)*	BW Recovery 24 h Post *M (±SEM)*	BW Recovery 48 h Post *M (±SEM)*
Control	-40.70 (1.00)	31.60 (1.45)	40.70 (1.75)
Systemic Naltrexone, sc			
0.1 mg/kg	-41.17 (1.68)	26.17 (1.85) *	32.50 (2.16) *
1.0 mg/kg	-35.60 (2.73)	23.40 (4.00) *	26.20 (0.97) *
5.0 mg/kg	-36.40 (1.50)	26.60 (2.73) *	24.60 (2.74) *
Central Naltrexone, icv			
10 μg	-39.67 (3.00)	12.50 (4.60) *	17.33 (4.19) *
50 μg	-42.20 (4.65)	4.60 (9.20) *	6.00 (9.35) *
β-FNA			
5 μg	-38.80 (2.15)	25.60 (0.93) *	25.20 (2.31) *
20 μg	-46.00 (1.32)	16.60 (6.93) *	21.80 (6.10) *
Nor-BNI			
5 μg	-36.00 (4.37)	6.40 (6.95) *	13.20 (6.71) *
20 μg	-39.60 (1.43)	11.60 (2.69) *	17.80 (1.32) *
NTI			
5 μg	-49.60 (2.11) *	20.00 (5.09) *	28.20 (6.95) *
20 μg	-43.40 (2.17)	13.00 (2.45) *	22.20 (2.08) *

control and MOR-1 AS ODN conditions ($F(12,1032)= 124.16$, $P<0.0001$), across test times ($F(3,258)= 13321.76$, $P<0.0001$) and for the interaction between conditions and times ($F(36,3096)= 65.06$, $P<0.0001$). Short-term deprivation-induced intake was significantly and consistently reduced by AS ODN probes directed against coding exons 2 (2-4 h), 4 (0.5-4 h), 7 (1-4 h), 8 (0.5-4 h) and 13 (4 h) of the MOR-1 clone (Figure 24). Short-term deprivation-induced intake was significantly and consistently increased by AS ODN probes directed against coding exons 3 (0.5-4 h) (Figure 24A), 5a (0.5-1 h) (Figure 24B) and 9 (0.5-1, 4 h) (Figure 24C) of the MOR-1 clone. Significant biphasic effects upon short-term deprivation-induced intake were observed by AS ODN probes directed against coding exons 1 (0.5-1 h: increase; 4 h: decrease) and 10 (0.5 h: increase; 2-4 h: decrease) of the MOR-1 clone (Figure 24 A, D). AS ODN probes directed against coding exons 6 and 12 of the MOR-1 clone (Figure 24 C, D) failed to affect short-term deprivation-induced intake. Significant differences in longer-term (24-48 h) intake were observed among control and MOR-1 AS ODN conditions ($F(12,1032)= 179.19$, $P<0.0001$), across test times ($F(1,86)= 3051.82$, $P<0.0001$) and for the interaction between conditions and times ($F(12,1032)= 72.11$, $P<0.0001$). Longer-term deprivation-induced intake was significantly and consistently reduced by an AS ODN probe directed against coding exon 4 (24 h) of the MOR-1 clone (Figure 25A). Longer-term deprivation-induced intake was significantly and consistently increased by AS ODN probes directed against coding exons 3 (24 h), 5a (24-48 h), 6 (24-48 h), 9 (48 h), 12 (24-48 h) and 13 (24-48 h) of the MOR-1 clone (Figure 25). Longer-term deprivation-induced intake failed to be affected by AS ODN probes directed against coding exons 1, 2, 7, 8 and 10 of the MOR-1 clone (Figures 25).

Figure 24. Alterations in short-term cumulative intake in mice food-deprived for 24 h following ventricular administration of antisense probes directed against the four exons of the MOP gene (Panel A: exons 1, 2, 3, 4), two extended MOR-1 exons (Panel B: exons 5a, 6), three extended exons of the MOR-1C clone (Panel C: exons 7, 8, 9), and three other extended MOR-1 exons (Panel D: exons 10, 12, 13).

Figure 24.

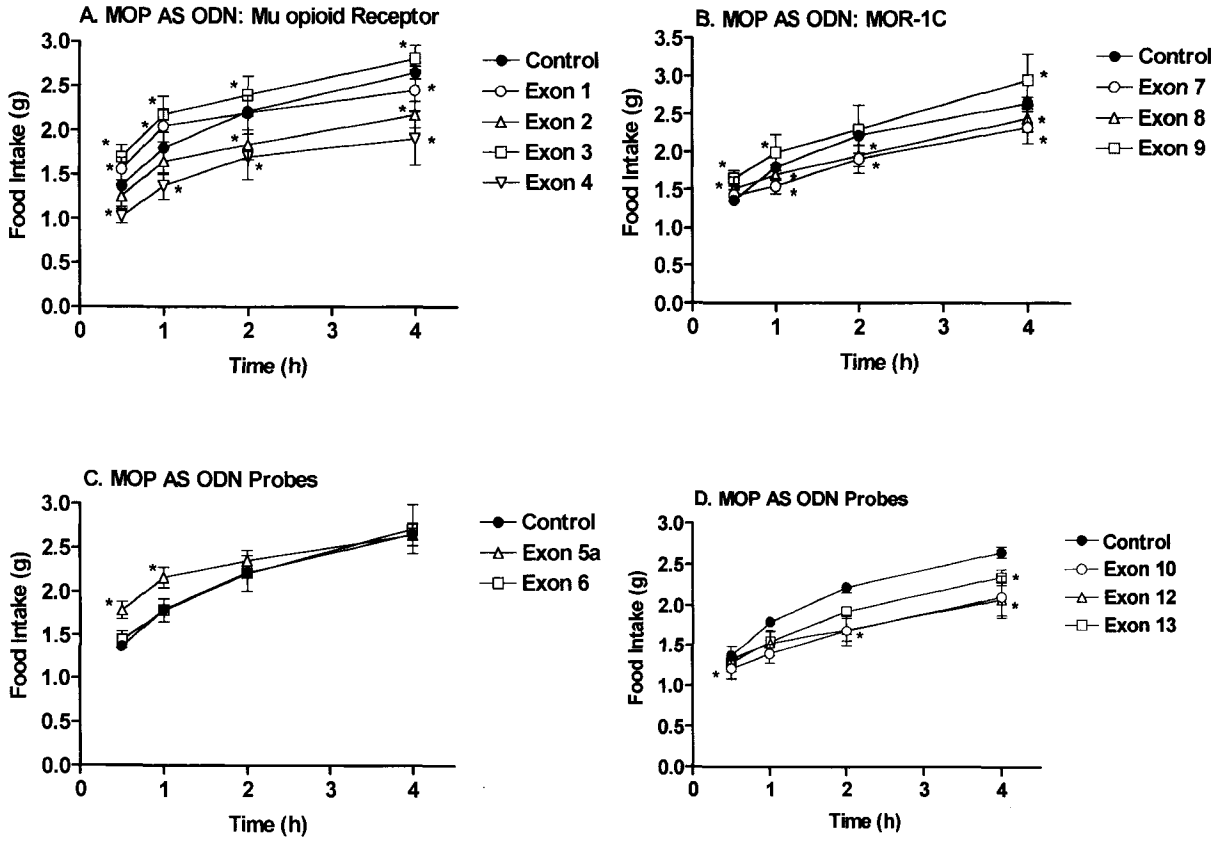
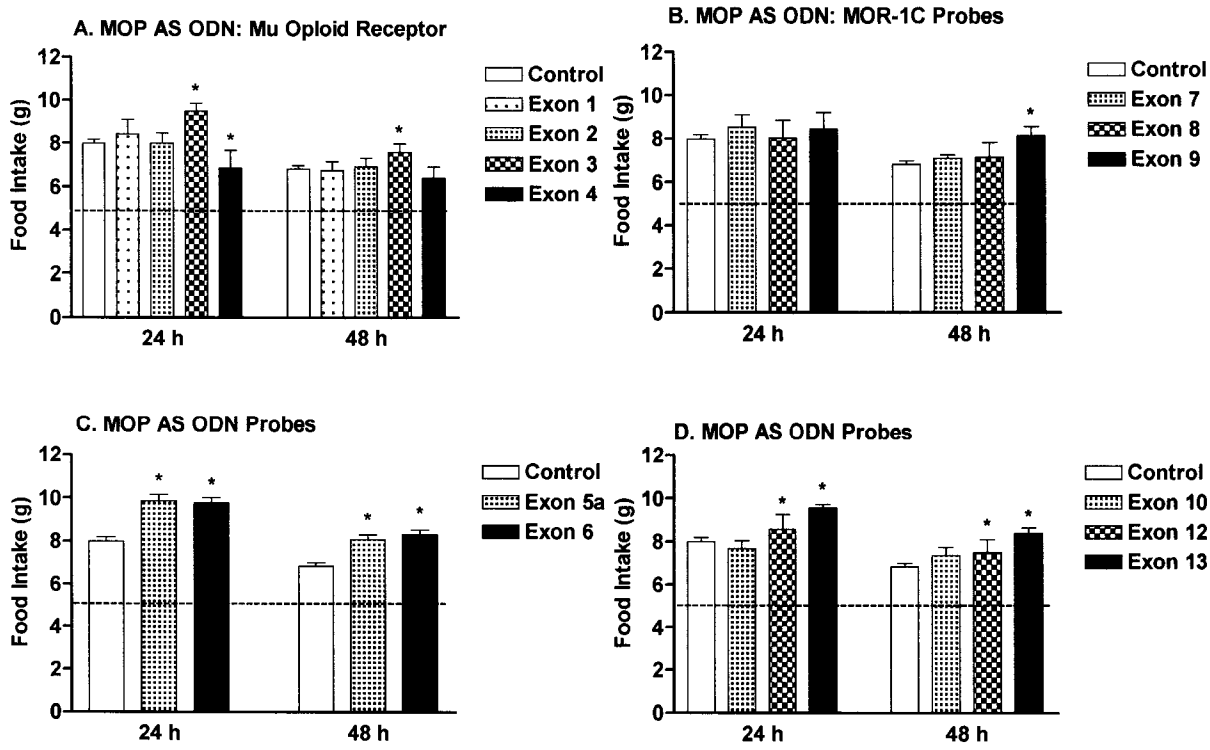


Figure 25. Alterations in longer-term cumulative intake in mice food-deprived for 24 h following ventricular administration of antisense probes directed against the four exons of the MOP gene (Panel A: exons 1, 2, 3, 4), two extended MOR-1 exons (Panel B: exons 5a, 6), three extended exons of the MOR-1C clone (Panel C: exons 7, 8, 9), and three other extended MOR-1 exons (Panel D: exons 10, 12, 13). The dotted line indicates 24 h intake under normal (non-deprivation) conditions.

Figure 25.

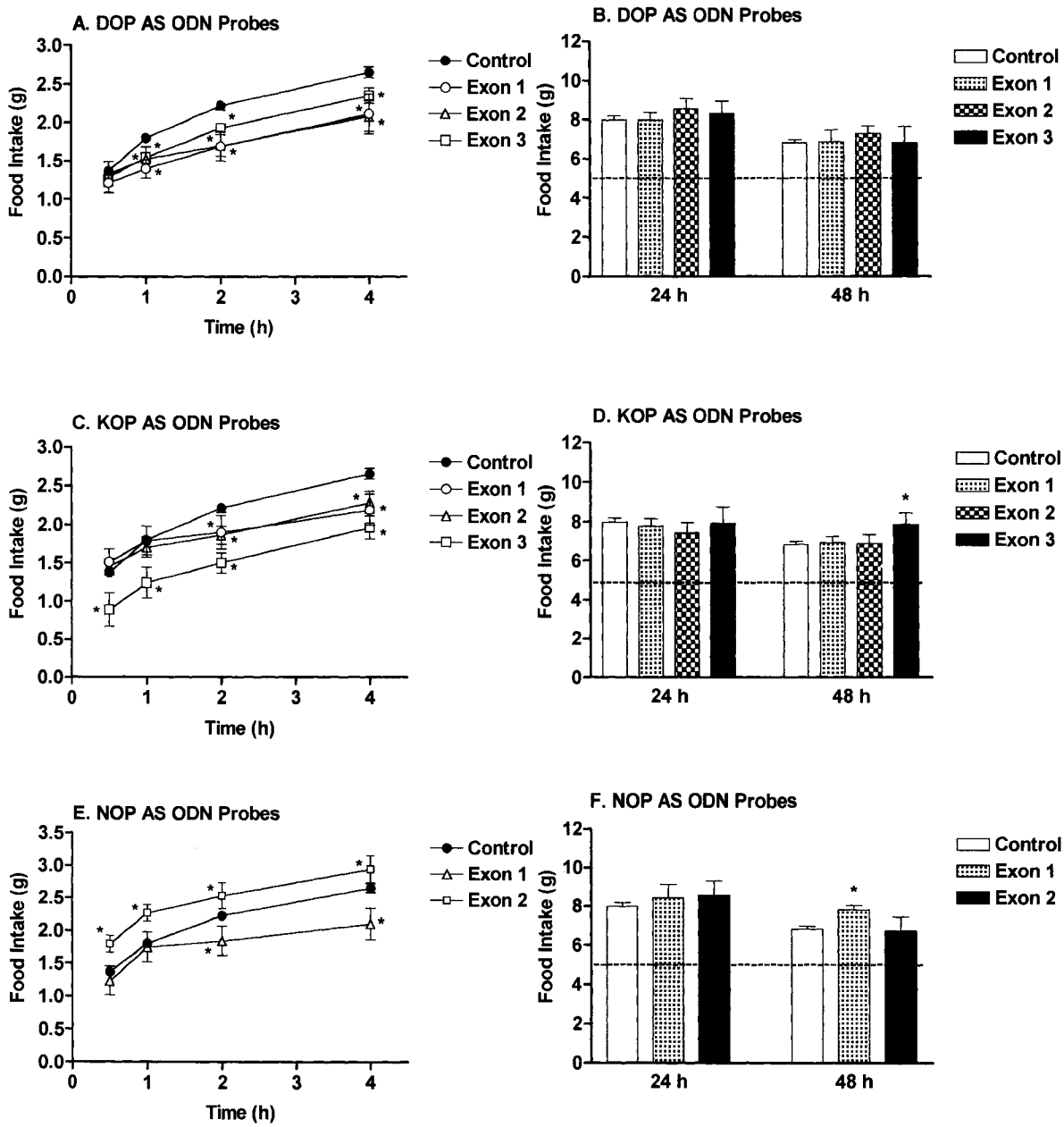


Significant differences in short-term (0.5-4 h) intake were observed among control and DOP, KOP and NOP AS ODN conditions ($F(9,774)= 190.47$, $P<0.0001$), across test times ($F(3,258)=7174.17$, $P<0.0001$) and for the interaction between conditions and times ($F(27,2322)=63.28$, $P<0.0001$). Short-term deprivation-induced intake was significantly and consistently reduced by AS ODN probes directed against coding exons 1 (1-4 h), 2 (1-4 h) and 3 (1-4 h) of the DOP gene, coding exons 1 (2-4 h), 2 (2-4 h) and 3 (0.5-4 h) of the KOP gene, and coding exon 1 (2-4 h) of the NOP gene (Figure 26). Short-term deprivation-induced intake was significantly and consistently increased by an AS ODN probe directed against coding exon 2 (0.5-4 h) of the NOP gene (Figure 26). Significant differences in longer-term (24-48 h) intake were observed among control and DOP, KOP and NOP AS ODN conditions ($F(9,774)= 44.84$, $P<0.0001$), across test times ($F(1,86)= 3168.20$, $P<0.0001$) and for the interaction between conditions and times ($F(9,774)= 68.36$, $P<0.0001$). Longer-term deprivation-induced intake was significantly and consistently increased by AS ODN probes directed against coding exon 3 (48 h) of the KOP gene and coding exon 1 (48 h) of the NOP gene. Longer-term deprivation-induced intake failed to be affected by AS ODN probes directed against coding exons 1, 2 and 3 of the times ($F(3,258)= 7174.17$, $P<0.0001$) and for the interaction between conditions and times ($F(27,2322)= 63.28$, $P<0.0001$).

DOP gene, coding exons 1 and 2 of the KOP gene and coding exon 2 of the NOP gene (Figure 26). Significant differences in deprivation-induced body weight loss occurred among control and MOR-1 AS ODN conditions ($F(12,211)= 2.08$, $P<0.019$), but not among control and DOP, KOP or NOP conditions ($F(8,182)= 1.45$, n.s.). Significant changes in weight loss were greater following AS ODN probes directed

Figure 26. Alterations in short-term (0.5-4 h; Panels A, C & E) and longer-term (24-48 h; Panels B, D, & F) cumulative intakes in mice food-deprived for 24 h following ventricular administration of antisense probes directed against the three exons of the DOP gene (Panels A & B), the three exons of the KOP gene (Panels C & D), and two exons of the NOP gene (Panels E & F). The dotted line indicates 24 h intake under normal (non-deprivation) conditions.

Figure 26.



against coding exons 9 and 10 of the MOR-1 gene, and were less following an AS ODN probe directed against coding exon 2 of the MOR-1 gene (Table 9).

Significant differences in body weight recovery following deprivation were observed among control and MOR-1 AS ODN conditions ($F(12,1524)= 212.68$, $P<0.0001$), across test times ($F(1,127)= 1604.21$, $P<0.0001$) and for the interaction between conditions and times ($F(12,1524)= 32.31$, $P<0.0001$). Significant differences in body weight recovery following deprivation were observed among control and DOP, KOP and NOP AS ODN conditions ($F(8,1016)= 160.53$, $P<0.0001$), across test times ($F(1,127)= 760.09$, $P<0.0001$) and for the interaction between conditions and times ($F(8,1016)= 96.05$, $P<0.0001$). Deprivation-induced weight recovery was significantly and consistently reduced by AS ODN probes directed against coding exons 1 (24-48 h), 4 (24 h) and 5a (24 h) of the MOR-1 clone, coding exons 1 (24-48 h) and 2 (48 h) of the DOP gene, coding exon 1 (48 h) of the KOP gene, and coding exons 1 (24-48 h) and 2 (24-48 h) of the NOP gene (Table 10). Deprivation-induced weight recovery was significantly and consistently increased by AS ODN probes directed against coding exons 6 (48 h) and 9 (24-48 h) of the MOR-1 clone (Table 9). Deprivation-induced weight recovery failed to be affected by AS ODN probes directed against coding exons 2, 3, 7, 8, 10, 12 and 13 of the MOR-1 clone, coding exon 3 of the DOP gene and coding exons 2 and 3 of the KOP gene (Table 10).

Discussion

The present study combined a pharmacological antagonist approach with an antisense approach to provide converging evidence to suggest the relative contributions of the different opioid receptors and genes in the regulation of the ultimate homeostatic

Table 9: Body weight loss and body weight recovery in 24 h-food-deprived mice treated with MOP antisense probes targeted against exons 1, 2, 3, 4, 5a, 6, 7, 8, 9, 10, 12, and 13.

Treatment	BW Loss (g) *M (±SEM)*	BW Recovery 24 h Post *M (±SEM)*	BW Recovery 48 h Post *M (±SEM)*
Control	-5.04 (0.11)	4.84 (0.15)	5.28 (0.17)
MOP Gene			
Exon 1	-4.58 (0.32)	3.64 (0.39) *	3.64 (0.48) *
Exon 2	-3.94 (0.83)	5.21 (0.39)	5.41 (0.65)
Exon 3	-4.90 (0.30)	4.45 (0.19)	5.00 (0.28)
Exon 4	-5.55 (0.50)	4.03 (0.83) *	4.65 (1.08) *
Exon 5a	-4.41 (0.43)	4.21 (0.26) *	5.18 (1.70)
Exon 6	-4.11 (0.59)	5.84 (0.64) *	6.78 (0.87) *
Exon 7	-4.40 (1.13)	3.98 (1.13) *	4.51 (1.10) *
Exon 8	-5.16 (0.36)	4.94 (0.41)	5.53 (0.77)
Exon 9	-6.08 (0.54)	6.09 (0.42) *	6.78 (0.41) *
Exon 10	-6.23 (0.59)	5.35 (0.54)	5.70 (0.94)
Exon 12	-5.56 (0.25)	4.69 (0.68)	4.94 (0.98)
Exon 13	-4.54 (0.64)	4.66 (0.32)	5.40 (0.43)

Table 10: Body weight loss and body weight recovery in 24 h-food-deprived mice treated with antisense probes targeted against the coding exons of the DOP (exons 1-3), KOP (exons 1-3) and NOP (exons 1-2) genes.

Treatment	BW Loss (g) *M (±SEM)*	BW Recovery 24 h Post *M (±SEM)*	BW Recovery 48 h Post *M (±SEM)*
Control	-5.04 (0.11)	4.84 (0.15)	5.28 (0.17)
DOP Gene			
Exon 1	-3.83 (1.12)	3.88 (0.59) *	4.25 (0.89) *
Exon 2	-4.94 (0.22)	4.31 (0.35)	4.54 (0.49) *
Exon 3	-4.91 (0.46)	4.98 (0.50)	4.69 (0.74)
KOP Gene			
Exon 1	-5.11 (0.19)	4.46 (0.28)	4.55 (0.85) *
Exon 2	-5.63 (0.21)	4.51 (0.47)	5.94 (1.27)
Exon 3	-4.26 (0.64)	4.28 (0.96)	5.76 (0.61)
NOP Gene			
Exon 1	-4.78 (0.28)	3.29 (0.51) *	3.93 (0.38) *
Exon 2	-4.44 (0.43)	3.23 (0.64) *	2.87 (1.19) *

challenge of food deprivation. As indicated throughout this dissertation, feeding behavior has traditionally been studied in the rat, the default animal subject for feeding, mostly because of their similar ingestive responses to other species and the measurable magnitude of their consumatory behavior. The ability of initially general and then selective opioid receptor subtype antagonists to differentially reduce feeding following food deprivation in rats revealed that mu opioid receptor antagonists (β-FNA) potently reduce deprivation-induced intake, kappa opioid antagonists (NBNI) moderately reduce deprivation-induced intake, whereas delta opioid antagonists (NTI) are generally ineffective. Previous studies revealed similar patterns of action between antagonists on the one hand and antisense probes on the other hand for feeding elicited by the mu opioid agonist, DAMGO, the active morphine metabolite, M6G, the opioid peptides, beta-endorphin and dynorphin, the glucoprivic challenge, 2DG, and the lipoprivic challenge, mercaptoacetate (Burdick et al., 1998; Leventhal et al., 1997, 1998; Silva et al., 2001, 2002; Stein et al., 2000). In contrast, although the first study and specific aim of the dissertation indicated congruence of moderate reductions in deprivation-induced intake following kappa antagonists and KOP antisense probes, and minimal reductions in deprivation-induced intake following delta antagonists and DOP antisense probes, the effects of mu antagonism and MOP antisense probes were divergent in the rat. Hence, mu antagonists potently reduced deprivation-induced intake in the rat, but antisense probes directed against exons of the MOP gene produced at best modest effects.

The incongruity between antagonist and antisense effects was addressed in part by the third study and third specific aim in which homeostatic challenges such as food restriction and food deprivation altered immunoreactivity of the MOR-1C isoform as

compared to the MOR-1 (MOP) gene. This was especially apparent in two sites intimately involved in the opioid control of feeding, the paraventricular nucleus and the nucleus tractus solitarius. Since little data have been collected regarding isoforms of the MOR-1 clone in the rat, it was of necessity to examine such possibilities in the mouse. However, to accomplish this, one had to conclusively demonstrate that the mouse and the rat display similar selective opioid antagonist actions. This initial examination follows.

General Opioid Antagonism and Deprivation-Induced Feeding in the Mouse: Comparisons with the Rat: As expected, both systemic and ventricular administration of the general opioid antagonist naltrexone dose-dependently (0.1-5 mg/kg) and potently (24-54 %) reduced deprivation induced intake in the rat. Peak antagonist effects were typically observed at 0.5-1 h following administration, but the magnitude (20%) of reductions declined 24 and 48 h following food reintroduction to about. Importantly, a similar pattern of responses was observed in mice food deprived for 24 h with the magnitude of reductions ranging from 40-78 % at 0.5-1 h following food reintroduction and subsequently declining after 24 and 48 h. It was important to examine whether mice, being smaller in size than the rat, would successfully cope with the stress of food deprivation for 24 h. Indeed, these animals appeared more affected than rats following this deprivation period displaying a less groomed and more "stressed" appearance. This experiment thus included the effects of a shorter (12 h) food-deprivation challenge to evaluate whether similar or different patterns of opioid antagonist effects emerged. Importantly, an almost identical pattern of naltrexone effects were observed in mice deprived for only 12 h (during the dark cycle) with reductions noted following systemic (20-39%) and central (70-82%) naltrexone.

Selective Opioid Antagonism and Deprivation-Induced Feeding in the Mouse: Comparisons with the Rat: The present study showed that the opioid selective antagonist β-FNA significantly decreased by 35-50% deprivation-induced intake in rats as expected. In the mouse, reductions in deprivation-induced intake after 24 h were of similar magnitudes (46-50 %), especially using the lower (5 μg) mu antagonist dose. Longer-term mu antagonist effects at 24 and 48 h were similar for both mice and rats deprived of food for 24 h. Despite naltrexone-induced marked reductions in deprivation-induced intake after a 12 h deprivation period in the mouse, mu antagonism produced milder magnitudes (~20%) of reductions. With this proviso, it is therefore clear that mu opioid antagonism works quite similarly upon deprivation-induced intake in the mouse and rat.

A somewhat similar conclusion can be drawn for kappa-mediated effects. Previous studies (Koch and Bodnar, 1994; Levine et al., 1990) demonstrated that ventricular administration of the kappa opioid antagonist NBNI, reduced deprivation-induced intake in the rat by about 30%, effects consistent with dose-dependent reductions of 16-53% observed in the present experiment in rats. A less consistent dose-dependent effect was observed in mice deprived of food for either 12 or 24 h with the lower dose reducing deprivation-induced intake by 44-53% after 0.5-4 h, and the higher dose reducing deprivation-induced intake by 20-33% after 0.5-4 h. Longer-term intakes (24 and 48 h) in mice were significantly reduced by NBNI by 20-34% in the rat, but to a lesser degree (8-29%) in the mouse.

A more striking species-specific pattern emerged for delta-mediated effects upon deprivation-induced intake. Previous studies (e.g., Arjune et al., 1991; Koch and Bodnar, 1994) failed to observe effects of the delta opioid antagonist NTI upon deprivation-

induced feeding in rats; this finding was essentially replicated in the present experiment. Based on these studies, we expected the same lack of NTI-induced effects when administered in food-deprived mice. Despite our expectations, the same doses of the delta opioid receptor antagonist, NTI significantly reduced intake induced by 24 h of food deprivation in the mouse by 28-39 % after 0.5-4 h, but by only 13-15% after 24-48 h. Milder (~20%), but similar degrees of delta antagonist inhibition were noted in mice deprived of food for 12 h. These data thereby suggests that delta opioid receptors play a more important role in mediating deprivation-induced intake in the mouse than in the rat.

NOP-selective Antisense Probes and Deprivation-Induced Feeding in the Mouse: Comparisons with the Rat: Antisense probes directed against each of the exons of the NOP gene are effective in reducing feeding elicited by its putative endogenous peptide, OFQ/N (Leventhal et al., 1998). Further, antisense probes directed against exons of the NOP gene reduced intake elicited by dynorphin (Silva et al., 2002). In contrast, the same antisense probes exerted relatively minor effects upon intake elicited by M6G, beta-endorphin, 2DG and mercaptoacetate (Burdick et al., 1998; Leventhal et al., 1998; Silva et al., 2001; Stein et al., 2000). A similar modest role for the NOP gene can be observed for deprivation-induced feeding. Like the effect observed in the rat in the first series of studies, antisense probes directed against exon 1 of the NOP gene modestly reduced deprivation-induced feeding in the mouse. Notably, the antisense probe directed against exon 2 of the NOP gene significantly though modestly increased deprivation-induced feeding specifically in the mouse.

KOP-selective Antisense Probes and Deprivation-Induced Feeding in the Mouse: Comparisons with the Rat: Antisense probes directed against exons of the KOP

gene effectively reduce feeding elicited by dynorphin and beta-endorphin (Silva et al., 2001, 2002) and reduce intake elicited by 2DG and mercaptoacetate (Burdick et al., 1998; Stein et al., 2000). A surprising effect in the first series of studies was the relatively potent (43%) reductions in deprivation-induced feeding in the rat by an antisense probe directed against exon 2 of the KOP gene. Although antisense probes directed against each of the three exons of the KOP gene reduced deprivation-induced feeding in the mouse, the antisense probe directed against exon 3 of the KOP gene produced the most pronounced (26-35%) effects. The pattern of KOP antisense and NBNI antagonist actions upon deprivation-induced feeding in both the mouse and the rat provide strong and convincing convergence of kappa-mediated actions for this important homeostatic challenge.

DOP-selective Antisense Probes and Deprivation-Induced Feeding in the Mouse: Comparisons with the Rat: Antisense probes directed against exons of the DOP gene only effectively reduces feeding elicited by the delta agonist, deltorphin (Leventhal et al., 1998) with more modest actions observed for dynorphin, beta-endorphin, 2DG and mercaptoacetate (Burdick et al., 1998; Silva et al., 2001, 2002; Stein et al., 2000). In agreement with the ineffectiveness of NTI to reduce deprivation-induced intake in the rat in the present and prior (Arjune et al., 1991; Koch and Bodnar, 1994), the first series of studies indicated only modest reductions in deprivation-induced feeding in the rat by an antisense probe directed against exon 1 of the DOP gene. As indicated in an earlier part of this discussion, a strong species-specific effect emerged in that delta antagonism modestly though significantly reduced deprivation-induced intake in the mouse. The antisense approach completely validated the antagonist actions of NTI in that antisense

probes directed against exon 1 (20-24%), 2 (15-23%) or 3 (11-23%) of the DOP gene modestly though significantly reduced deprivation-induced intake after 0.5-4 h in the mouse. Thus, again, convergence between the two antagonist and antisense approaches not only indicated the existence of delta-mediated effects in the mouse, but also indicated their absence in the rat.

MOP-selective and MOR-1-selective Antisense Probes and Deprivation-Induced Feeding in the Mouse: Comparisons with the Rat: Antisense probes directed against the MOP gene have typically produced the most pronounced effects upon feeding responses including spontaneous intake and body weight (Leventhal et al., 1996), 2DG-induced glucoprivation (Burdick et al., 1998), mercaptoacetate-induced lipoprivation (Stein et al., 2000), and intake induced by the opioid agonists, morphine, M6G, DAMGO, beta-endorphin and dynorphin (Leventhal et al., 1997, 1998; Silva et al., 2001, 2002). Indeed, it was the ability of antisense probes directed against different exons of the MOP gene to differentiate between the mu-mediated actions of feeding responses elicited by morphine and DAMGO on the one hand (exons 1 and 4) and M6G on the other (exons 2 and 3) (Leventhal et al., 1997, 1998). These data, together with similar effects on analgesic responses spoke directly to the functional significance of identified MOR-1 isoforms (Bare et al., 1994; Pan et al., 1999; Pan et al., 2000; Pan et al., 2001; Pasternak et al., 2001; Pasternak and Pan, 2000; Zimprich et al., 1995) that are summarized in Table 5. In these isoforms, exons 1, 2 and 3 are conserved, and thus, it is the exon(s) that follow that differentiate them from one another. Thus, exon 5a is the unique aspect of the coding for the MOR-1B isoform, and the combination of exons 7, 8 and 9 are the unique aspect of the coding for the MOR-1C splice variant. Moreover, the combination of exons 7 and 8,

but not 9 are coding for the MOR-1D isoform. The extended exons 10, 12 and 13 are participating in other MOR-1 isoforms (Pan et al., 2000; Pan et al., 2001; Pasternak et al., 2001; Pasternak and Pan, 2000).

Our ability to observe potent reductions in deprivation-induced feeding in the rat by mu-selective antagonists was followed by meager reductions in the same response by MOP-specific antisense probes. As indicated earlier, mice like rats, respond similarly to mu antagonists, and since the extended isoforms were largely identified in the mouse, it became necessary to test these antisense probes in this species. As in the rat (exons 2, 3, 4: ~30%), traditional MOP antisense probes produced modest effects. Thus, deprivation-induced intake in the mouse was reduced by antisense probes directed against exons 1 (18%), 2 (18%) or 4 (23-28%), but was increased by an antisense probe directed against exon 3 (15%). Our findings in Specific Aim 3 strongly suggested that the MOR-1C splice variant would be important in this deprivation response since restriction and deprivation altered MOR-1C immunoreactivity. Thus, antisense probes directed against exons 7 or 8 significantly reduced deprivation-induced intake by 14% and 12% respectively, where the antisense probe directed against exon 9 actually increased (10-17%) this response. None of the other probes produced any more marked effects, leaving us with an inability to clearly correlate pronounced mu antagonist effects with weaker antisense effects. One possible explanation may be the fact that changes in expression of opioid receptor genes take around 72 h to take place. Thus, the 24 and 12 h of food deprivation paradigms may be insufficient to produce these kinds of alterations.

Another possible explanation of the small antisense probe effects may be the fact that both the oligos as well as the antagonists were infused in the lateral ventricle and

thus their effects may have been limited by diffusion. It is likely that structures critical in feeding behavior remained unaffected by the treatments. The weak effects following the antisense probe treatments compared to the more substantial antagonist treatments, especially in mice, can be explained by this inability to access "key" targets in the mediation of feeding behavior.

Given that food deprivation represents an admixture of many ingestive actions that could be differentially regulated by separate mu-mediated mechanisms, the effects of antisense probe combinations would determine whether these isoforms are implicated in deprivation-induced feeding when knocked out simultaneously. One potential reason as to why antisense probes produce relatively mild reductions in deprivation-induced intake in the mouse is the potential activation of other compensatory mechanisms. Thus, the absence of food in addition to the limited constitutive (e.g., fat stores) resources on the part of the mouse render food deprivation as even more challenging of an experience than that in the rat, who may have more resources stored.

CHAPTER 8. GENERAL DISCUSSION

The present dissertation aimed to examine the role of the opioid receptor system in the regulation of feeding behavior following the homeostatic challenge of food deprivation. The studies reviewed used five different and somewhat complementary approaches: a) generalization of effects across rodent species in the rat and the mouse; b) validation of general and selective antagonist effects across species and with relevance to prior studies; c) evaluation of traditional MOP, DOP, KOP and NOP antisense probes in both the mouse and rat to examine effects on deprivation-induced feeding as well as isoforms of the mouse MOR-1 clone to examine their relevance; d) evaluation of G-protein alpha subunits in the mediation of deprivation-induced feeding; and e) the reciprocity of pharmacological and molecular manipulations in altering deprivation-induced feeding with the ability of homeostatic challenges to alter molecular receptor processes as indicated by immunohistochemistry.

The mu opioid receptor is thought to be the major receptor involved in the regulation of feeding behavior induced by homeostatic challenges, such as glucoprivation, lipoprivation and food deprivation (Burdick et al., 1998; Stein et al., 2000; Ragnauth et al., 1997). This premise has been supported by studies using mu opioid antagonists and antisense probes targeting the four coding exons of this receptor gene in the glucoprivic and lipoprivic paradigms, but not in the food deprivation paradigm. Two of the studies in this dissertation aimed to demonstrate the involvement of the opioid receptor genes in the regulation of feeding induced by food deprivation (24 h). The data suggest that the effects of the mu opioid receptor antagonists in blocking

deprivation-induced feeding may in fact be due to their actions on one or more splice variants of the Oprm gene.

The second study investigated the role of the G-protein alpha subunits in the regulation of deprivation-induced feeding since the opioid receptors are coupled to these proteins. The results suggest that the challenge of food deprivation is in fact not relying on the opioid system but is rather recruiting resources from other neurotransmitter systems, particularly dopamine receptors. This was supported by the fact that the most potent reductions of deprivation-induced feeding followed probes targeting the Gs and Gq alpha subunits.

Moreover, the use of multiple species has proven to be enlightening in terms of the opioid receptor system and its role in feeding behavior. The fourth study of this dissertation has shown that the delta opioid receptor in mice is playing an important role in the regulation of feeding induced by food deprivation, whereas in the rat this receptor has been termed ineffective. Thus, there appear to be different ways by which different species use opioid receptor systems to regulate this most important homeostatic challenge.

Food restriction but not food deprivation seems to activate the parvocellular divisions of the PVN since immunoreactivity levels of MOR-1C increase in a time-dependent fashion. Interestingly, the NTS is sensitive to both food restriction and food deprivation and these challenges result in decreases in MOR-1C immunoreactivity. The PVN and NTS are both sites associated with opioid-induced feeding following microinjections of mu and delta opioid agonists with reciprocal connections (Kotz et al., 1997).

Future experiments could examine the effects of individual exons of the MOP or other genes in mice, using the knockout approach. Given the fact that food restriction caused significant increases in MOR-1C immunoreactivity, a possible experiment would be one that studied feeding behavior in mice which lack exons 7, 8 and 9 (unique to the MOR-1C variant) in comparison to mice which lack exon 4 (unique to the traditional MOP gene), in addition to wild type animals. Other types of knockout mice could be created to allow study of these or more isoforms and examine the possibility of providing converging evidence from both the knockout and knockdown approaches, in addition to the pharmacological approach. Moreover, since the mu opioid receptor and its gene have been implicated in the regulation of homeostatic challenges (food restriction) through changes in protein expression, it would be of interest to conduct experiments in which glucoprivic or lipoprivic animals, or animals exposed to different types of diets (i.e. high fat) were assessed in terms of immunoreactivity levels of several Opr*m* isoforms in relevant neuroanatomical sites. It is possible, that the small effects observed by the antisense probes relative to the antagonist treatments are due to diffusion which follows intraventricular administration. Thus, once sites of interest are identified, one could administer antisense probes directly in these sites, which may allow more sensitivity in detecting the effects of regulatory challenges. A means of more effective delivery of the probes could be through the use of viral vectors in order to minimize degradation, which is not a major concern with intraventricular administration. Yet another way to approach these questions could be through the use of different inbred mouse strains (CXBK, SWR, etc), which recently have shown different sensitivities towards opiate mediated effects.

In conclusion, this dissertation has contributed new information in terms of the role of the opioid receptor subtypes and genes in the regulation of the ultimate homeostatic challenge of food deprivation. Our choice to use multiple approaches to study this system made it possible for us to gain insight as to how the opioid system may work under this state of need. In addition, the inter-species comparison has been fruitful in discovering information that distinguishes one species from the other and the specific role that opioid receptors and genes play in each. This information may be essential in terms of each animal's success or failure to cope in times of privation and ultimately survive.

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