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Antisense Mapping of the MOR-1 Opioid Receptor Clone: Modulation of Hyperphagia Induced by DAMGO[1]

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ABSTRACT

The *mu* opioid receptor mediates ingestive behavior: *mu*-selective agonists stimulate food intake and antagonists reduce intake in many ingestive situations. Antisense oligodeoxynucleotides directed against each of the four exons of the MOR-1 clone were equally effective in reducing spontaneous food intake and body weight in rats. However, antisense probes directed against only exon 1 or 4 of the MOR-1 clone reduced *mu*-mediated analgesia. The present study examined whether central administration of antisense probes directed against each of the four exons of the MOR-1 clone or a missense control altered hyperphagia elicited by the *mu* agonist DAMGO across a range of doses. Antisense probes directed against only exon 1 or 4 blocked hyperphagia at agonist doses of 0.5 and 1.0 μg; this pattern was identical to that observed for *mu*-mediated analgesia. A missense control failed to exert significant effects, which suggests specificity of antisense actions. The effective antisense probes failed to reduce hyperphagia at a higher (5 μg) agonist dose, a result consistent with limitations in down-regulation of receptor proteins by antisense. The *mu* antagonist β-funaltrexamine produced a similar pattern of effects on *mu*-mediated hyperphagia. The selective actions of antisense probes directed against different exons of the MOR-1 clone in reducing hyperphagia induced by DAMGO suggest that multiple splice variants of the MOR-1 clone exist and raise the possibility of further opioid receptor subclassifications.

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

The initial cloning of DOR-1 (Evans *et al.*, 1992; Kieffer *et al.*, 1992) quickly led to the identification of other structurally related G protein-mediated receptors (MOR-1, KOR-1, KOR-3, ORL-1; see review: Uhl *et al.*, 1994) and has opened new areas of research, including investigation of the relationship of the cloned receptors to opioid actions *in vivo*. The cloning of the specific opioid receptors made possible the identification of short (15–25 bases) AS ODN sequences that are complementary to specific regions of mRNA that can down-regulate receptor proteins. The efficacy and specificity of AS ODNs directed against opioid receptor clones have been confirmed functionally and biochemically (see review: Pasternak and Standifer, 1995), particularly in analgesic assays. AS ODNs directed against the 5′-untranslated regions of DOR-1, MOR-1 or KOR-1 selectively and respectively reduced *delta*-mediated (Bilsky *et al.*, 1994; Lai *et al.*, 1994; Standifer *et al.*, 1994; Tseng *et al.*, 1994), *mu*-mediated (Chen *et al.*, 1995; Rossi *et al.*, 1994, 1995a, 1995b) and $kappa_1$-mediated (Adams *et al.*, 1994; Chien *et al.*, 1994) forms of analgesia.

Although MOR-1 encodes a *mu* opioid receptor, its relationship to the pharmacologically defined *mu* receptor subtypes has been unclear. Using the AS ODN technique to map individual exons within MOR-1, we were able to determine which individual exons modulate *mu*-mediated analgesia (Rossi *et al.*, 1995a, 1995b, 1996, 1997). AS ODN probes directed against exons 1 and 4 of MOR-1 blocked morphine and *mu* agonist-mediated analgesia, whereas probes targeted against exon 2 or 3 of MOR-1 were ineffective. In contrast, AS ODN probes directed against exon 2 or 3 of MOR-1 blocked analgesia induced by the morphine metabo-

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ABBREVIATIONS: AS ODN: antisense oligodeoxynucleotide; βFNA: β-funaltrexamine; KOR-1: *kappa* opioid receptor clone; KOR-3: $kappa_3$ opioid receptor clone; MS ODN: mismatch oligodeoxynucleotide; M6G: morphine-6β-glucuronide; MOR-1: *mu* opioid receptor clone; ORL-1: orphanin opioid-like receptor clone; VEH: vehicle; DOR-1 = *delta* opioid receptor clone.

lite M6G, whereas AS ODNs directed against exon 1 or 4 of MOR-1 were ineffective. Analgesic responses induced by heroin, fentanyl and etonitazine are reduced by AS ODNs directed against either exon 1 or 2 of MOR-1 (Rossi *et al.*, 1996). Thus these studies raised the possibility that various *mu* receptor subtypes could result from alternative splice variants of MOR-1 (Pasternak and Standifer, 1995).

The AS ODN strategy has recently been applied to opioid modulation of ingestive behavior (Leventhal *et al.*, 1996). Body weight and food intake were significantly reduced by AS ODNs directed against each of the four exons of MOR-1. In contrast, a MS ODN control was ineffective. The sensitivity of spontaneous intake and body weight to all four exons suggests that the receptor responsible for this action is encoded by MOR-1. *mu*-selective opioid agonists such as morphine and DAMGO stimulate food intake after systemic and central administration (Bakshi and Kelley, 1993; Gosnell *et al.*, 1986a, 1986b; Sanger and McCarthy, 1980), and this effect is blocked by *mu*-selective opioid antagonists (Levine *et al.*, 1991). The first goal of the present study was to determine whether AS ODNs directed against MOR-1 would block hyperphagia elicited by the *mu* agonist DAMGO. If so, the second goal was to determine whether the profile of MOR-1 AS ODN effects mirrored those effects observed for *mu* agonist analgesia (only exons 1 and 4 AS ODNs effective) or those effects observed for spontaneous food intake and body weight (all four exons effective). Thus the present study examined whether i.c.v. administration of AS ODNs directed against exon 1, 2, 3 or 4 of MOR-1 or administration of a MS ODN altered hyperphagia elicited by the selective *mu* agonist DAMGO. Although AS ODNs produce quite dramatic behavioral and physiological effects, they are accompanied by rather modest (30%–40%) reductions in receptor protein levels (see review: Pasternak and Standifer, 1995). Therefore, it is possible that the presence or absence of AS ODN effects on agonist-induced hyperphagia depends on the dose of the agonist employed as well as on the efficacy of the AS ODN. Thus our third goal was to determine whether effective AS ODNs would block DAMGO-induced hyperphagia across a range of effective DAMGO doses. Finally, the present study confirmed that DAMGO-induced hyperphagia was a *mu*-mediated effect by blocking this response with the *mu* antagonist βFNA (Levine *et al.*, 1991).

Materials and Methods

Subjects. Male albino Sprague-Dawley rats (90–120 days of age, Charles River Laboratories, Kingston, NY) were housed individually in wire mesh cages and maintained on a 12-h light: 12-h dark cycle with water and rat chow available *ad libitum*. Each rat was pretreated with chlorpromazine (3 mg/kg i.p.) and anesthetized with Ketamine HCl (120 mg/kg i.m.). A stainless steel guide cannula (22-gauge, Plastics One, Roanoke, VA) was implanted stereotaxically (Kopf Instruments, Tujunga, CA) into the left lateral ventricle using the following coordinates: incisor bar (+5 mm), 0.5 mm anterior to the bregma suture, 1.3 mm lateral to the sagittal suture and 3.6 mm from the top of the skull. Cannulas 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 began. Rats weighed between 275 and 300 g before surgery and weighed 400 to 550 g after completion of testing. After completion of behavioral testing, all animals were killed with an overdose of anesthetic, and cannula placements were verified visually.

AS ODNs and opioid agonists and antagonists. All phosphodiester oligodeoxynucleotides (Midland Certified Reagent Company, Midland, TX) were dissolved (5 μg/μl) in 0.9% normal saline and purified in our (G.W.P., G.C.R.) laboratory. AS ODNs (19–22 bases long) were directed against four regions of MOR-1 (table 1). An AS ODN control consisted of a MS ODN in which four bases from the AS1 sequence were switched without altering the remaining sequence. All sequences are specific to MOR-1 and are not present in other opioid receptor cDNAs. Three infusions were administered i.c.v. at 48-h intervals over 15 s through a stainless steel internal cannula (28-gauge, Plastics One, Roanoke, VA). DAMGO (0.5–5 μg, Peninsula Laboratories, Belmont, CA) and βFNA (0.2–20 μg, Research Biochemicals Intl., Natick, MA) were dissolved in 0.9% normal saline and administered i.c.v. in 5-μl volumes over 30 s through an internal cannula. βFNA was administered 24 h before agonist administration to allow for full development of irreversible *mu* antagonist effects (Portoghese *et al.*, 1980).

Protocols. All rats were tested over 4 to 10 days at 3 to 9 h into the light cycle to ensure the stability of base-line spontaneous food intake. Preweighed pellets were placed directly on the floor of the wire mesh cages to optimize accessibility, because this factor can interfere with DAMGO-induced feeding (Badiani *et al.*, 1995). Cumulative intakes were assessed 2 and 4 h before and after each condition, adjusting for spillage that was collected on paper placed under the cage. After intake stabilization, all rats received a VEH condition (PRE-VEH, 5 μl of 0.9% normal saline i.c.v.). Because DAMGO is known to produce sedative and hypoactive effects, we treated each animal twice with DAMGO (1 μg i.c.v.) without measuring intake. In the assessment of DAMGO-induced hyperphagia, rats received one of three (0.5, 1 or 5 μg) doses (PRE-DAMGO), and intake was assessed after 2 and 4 h. Intake elicited by each DAMGO dose was matched across the subgroups of rats receiving AS ODNs and the MS ODN control. During the test phase of the experiment, rats received one of four AS ODN sequences (AS1, AS2, AS3 or AS4: 10 μg, 2 μl, i.c.v.) or a mismatch sequence (MS1) (table 1) on days 1, 3 and 5 as previously described (Leventhal *et al.*, 1996). This AS ODN dose was most efficacious in analgesic dose-response assays (Rossi *et al.*, 1997). This time course of treatment both down-regulates the synthesis of new receptors and permits the turnover of existing receptors (see review: Pasternak and Standifer, 1995). Twenty-four hours after the last AS ODN or MS ODN treatment (day 6), rats were retested with their respective DAMGO dose (0.5–5 μg), and food intake was assessed after 2 and 4 h.

In order to confirm the *mu*-selective actions of DAMGO-induced hyperphagia, separate but identically screened groups of rats screened for DAMGO-induced hyperphagia received the *mu*-selective antagonist βFNA (0.2–20 μg: Arjune *et al.*, 1990; Levine *et al.*, 1991; Ukai and Holtzman, 1988) 24 h before DAMGO treatment. Cumulative intakes were again assessed after 2 and 4 h.

Statistics. Separate split-plot analyses of variance were performed on cumulative food intake data at 2 and 4 h for different doses of DAMGO (0.5, 1 and 5 μg) as a function of either AS ODN treatment (AS1, AS2, AS3, AS4 or MS1) or βFNA treatment. Significant differences in intake measures were determined for each subgroup relative to both corresponding VEH control values and corresponding DAMGO doses before AS ODN or antagonist treatment (Tukey comparisons, $P < .05$).

TABLE 1
Sequence of AS ODNs and the MS ODN

Probe	Sequence	Location
MS1	CGC CCC GAC CTC TTC CCT T	Exon 1 195–213
AS1	CGC CCC AGC CTC TTC CTC T	Exon 1 195–213
AS2	TTG GTG GCA GTC TTC ATT TTG G	Exon 2 572–593
AS3	TGA GCA GGT TCT CCC AGT ACC A	Exon 3 959–979
AS4	GGG CAA TGG AGC AGT TTC TG	Exon 4 1457–1476

The identified locations are based on the MOR-1 sequence (GenBank accession number U26915).

Results

DAMGO-induced hyperphagia. Significant dose-dependent differences in DAMGO-induced hyperphagia were observed after 2 [$F(3,86) = 217.89$, $P < .0001$] and 4 [$F = 291.43$, $P < .0001$] h. DAMGO significantly increased food intake relative to VEH after 4 h (fig. 1). These effects were dose-dependent in that the effect of the 5-μg dose was significantly greater than those of the 0.5- and 1-μg doses, and the effect of the 1-μg dose was significantly greater than that of the 0.5-μg dose (fig. 1). The patterns of effects after 2 h in this and the other conditions were identical.

MOR-1 AS ODNs and DAMGO (0.5 μg)-induced hyperphagia. Significant differences in food intake were observed among conditions after 2 [$F(5,23) = 67.92$, $P < .0001$] and 4[$F = 69.87$, $p < .0001$] h. The 0.5-μg dose of DAMGO significantly increased intake after 2 and 4 h, and this effect was significantly reduced by pretreatment with either AS1 (76%) or AS4 (70%) (fig. 2A). In contrast, neither AS2 (17% increase) nor AS3 (10% increase) significantly altered DAMGO hyperphagia (fig. 2A).

MOR-1 AS ODNs and DAMGO (1.0 μg)-induced hyperphagia. Significant differences in food intake were observed among conditions after 2 [$F(6,37) = 66.42$, $P < .0001$] and 4 [$F = 73.38$, $P < .0001$] h. The 1.0-μg dose of DAMGO significantly increased intake after 2 and 4 h, and this effect was significantly reduced by pretreatment with either AS1 (100%) or AS4 (53%) (fig. 2B). In contrast, neither AS2 (10% increase) nor AS3 (7% increase) significantly altered DAMGO hyperphagia (fig. 2B). Further, MS1 failed to alter DAMGO-induced hyperphagia significantly at this dose (fig. 2B).

MOR-1 AS ODNs and DAMGO (5.0 μg)-induced hyperphagia. Significant differences in food intake were observed among conditions after 2 [$F(4,17) = 15.93$, $P < .0001$] and 4 [$F = 21.74$, $P < .0001$] h. The 5.0-μg dose of DAMGO significantly increased intake after 2 and 4 h. In contrast to the differential effectiveness of AS ODNs on DAMGO-induced hyperphagia at lower doses, neither AS1 nor AS4 significantly altered hyperphagia induced by a 5-μg dose of DAMGO (fig. 2C). Again, MS1 also failed to affect DAMGO-induced hyperphagia at this dose (data not shown).

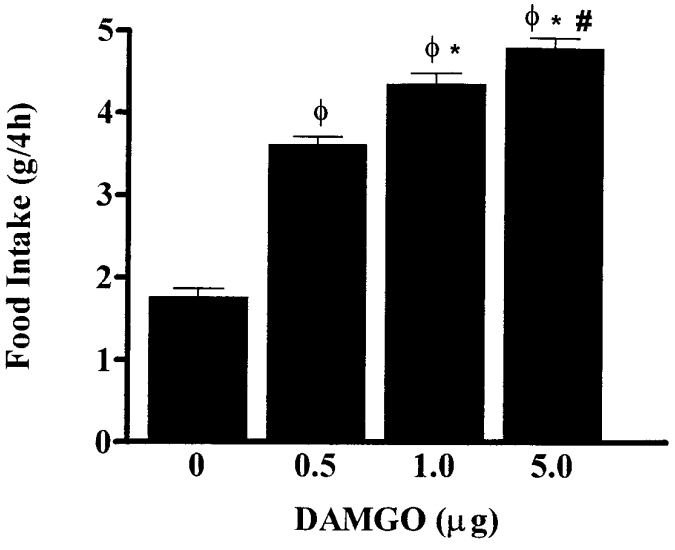

Fig. 1. Alterations (mean ± S.E.M.) in food intake after i.c.v. administration of 0 ($n = 87$), 0.5 ($n = 24$), 1.0 ($n = 39$) or 5.0 ($n = 24$) μg of DAMGO. Significant differences are denoted relative to vehicle (ϕ), 0.5 μg (*) or 1.0 μg (#) DAMGO doses (Tukey comparisons, $P < .01$).

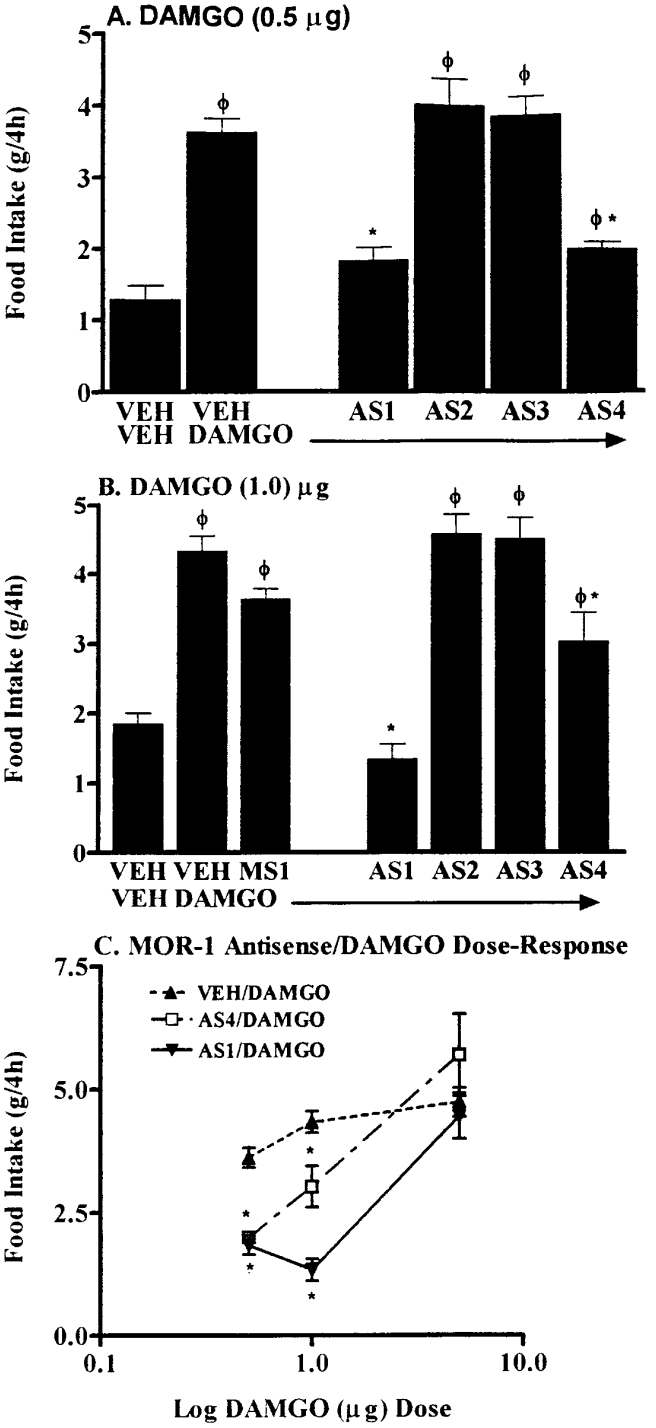

Fig. 2. Alterations (mean ± S.E.M.) in DAMGO-induced hyperphagia at doses of 0.5 (panel A, $n = 24$) or 1.0 (panel B, $n = 38$) μg after i.c.v. administration of AS ODNs directed against exons 1 (AS1: panel A, $n = 7$; B, $n = 8$), 2 (AS2: A, $n = 6$; B; $n = 8$), 3 (AS3: A, $n = 5$; B, $n = 8$) or 4 (AS4: A, $n = 6$; B, $n = 8$) of MOR-1 or a missense control (MS1: B, $n = 6$). Panel C depicts alterations in food intake as a function of the DAMGO dose (0.5–5.0 μg i.c.v.) after the administration of effective (AS1 and AS4) AS ODNs of MOR-1. Significant differences are denoted relative to either VEH-VEH (ϕ) or VEH-DAMGO (*) values (Tukey comparisons, $P < .01$).

βFNA and DAMGO-induced hyperphagia. Significant differences were observed for DAMGO-induced hyperphagia among conditions for doses of 0.5 [$F(2,23) = 91.24$, $P < .0001$], 1.0 [$F(4,37) = 112.28$, $P < .0001$] and 5.0 [$F(2,17) = 31.53$, $P < .0001$] μg. A fixed 20-μg dose of βFNA significantly and dose-dependently reduced DAMGO-induced hyperphagia at doses of 0.5 (88%), 1.0 (100%) and 5.0 (59%) μg (fig. 3A). Further, hyperphagia induced by a fixed 1.0-μg dose of DAMGO was significantly reduced by a dose range (0.2–20 μg) of βFNA (80%–100%; fig. 3B).

Discussion

The present findings confirmed the major goals of the study. First, specific AS ODNs directed against MOR-1 blocked hyperphagia elicited by the *mu* agonist DAMGO. In contrast, a MS ODN control failed to alter the magnitude of DAMGO-induced hyperphagia. Second, the observed effects of AS ODNs directed against MOR-1 were specific to the targeted regions of the clone in that AS ODNs directed against exon 1 or 4 significantly reduced DAMGO-induced hyperphagia. In contrast, AS ODNs directed against exon 2 or 3 of MOR-1 failed to exert significant effects. This pattern of AS ODN effects for *mu* agonist-induced hyperphagia mirrored the pattern of AS ODN effectiveness observed for *mu* agonist-induced analgesia (Rossi *et al.*, 1995a, 1995b, 1996, 1997). The present pattern of results for DAMGO-induced hyperphagia stands in marked contrast to reductions in spontaneous food intake and body weight after the administration of AS ODNs directed against each of the four exons of MOR-1 (Leventhal *et al.*, 1996). Third, there are dose-dependent limitations to the AS ODN approach, such that effective AS ODNs reduced hyperphagia at DAMGO doses of 0.5 and 1 μg but not at a higher, 5-μg dose. Hence the presence or absence of AS ODN effects on agonist-induced hyperphagia appears to depend on the dose of the agonist employed as well as on the efficacy of the AS ODN. This pattern can be explained by the modest (40%) reductions in receptor protein levels resulting from AS ODN administration (see review: Pasternak and Standifer, 1995). Finally, the ability of the *mu*-selective antagonist βFNA to reduce DAMGO-induced hyperphagia confirmed previous findings (Levine *et al.*, 1991). However, the magnitude of *mu* antagonist effects on dose-response relationships for DAMGO-induced hyperphagia paralleled AS ODN effects such that the potency of a fixed (20 μg) βFNA dose to reduce DAMGO-induced hyperphagia declined as a function of increased DAMGO doses.

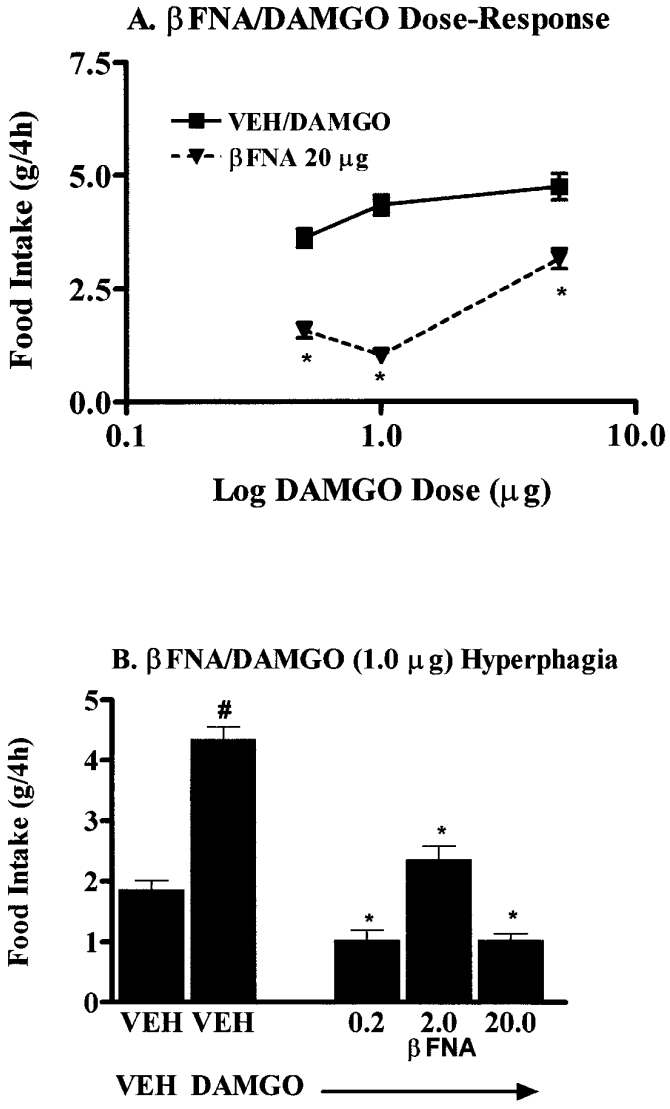

Fig. 3. A) Alterations (mean ± S.E.M.) in DAMGO-induced hyperphagia after pretreatment with a fixed (20-μg) dose of the *mu* opioid antagonist βFNA as a function of the DAMGO dose. B) Alterations (mean ± S.E.M.) in DAMGO (1.0 μg)-induced hyperphagia after pretreatment with different doses (0.2–20 μg) of βFNA. Significant differences induced by βFNA ($n = 5$ or 6/dose) are denoted relative to either VEH-VEH (ϕ) or VEH-DAMGO (*) values (Tukey comparisons, $P < .01$).

Our previous study (Leventhal *et al.*, 1996) failed to observe any significant AS ODN effects on hyperphagia elicited by 2-deoxy-D-glucose or hyperdipsia elicited by angiotensin II, a surprising finding given the effectiveness of *mu* antagonists to block both responses (Arjune *et al.*, 1990; Koch and Bodnar, 1994; Ruegg *et al.*, 1994). This apparent discrepancy might be explained by the present findings of differential AS ODN effectiveness as a function of the DAMGO dose. The 2-deoxy-D-glucose (500 mg/kg i.p.) and angiotensin II (20 ng i.c.v.) doses utilized in the previous (Leventhal *et al.*, 1996) study were at the high end of their respective dose-response curves for hyperphagia and hyperdipsia. It will be important to determine whether lower effective doses of the two physiological compounds are sensitive to AS ODNs against MOR-1.

The activity of AS ODNs that target various regions of mRNA that encode opioid receptors suggests that alternative splicing of transcripts can be explored by mapping exons with AS ODN probes (see review: Pasternak and Standifer, 1995). This approach was especially fruitful in distinguishing the analgesic responses of morphine and its metabolite M6G (Pasternak *et al.*, 1987; Paul *et al.*, 1989). Supraspinal and spinal mediation of morphine analgesia have been defined pharmacologically by the respective actions of mu_1 and mu_2 receptors (see review: Pasternak, 1993). M6G labels the traditional *mu* receptors in binding assays with an affinity slightly less than that of morphine. Yet when it is administered centrally in mice, its analgesic potency is 100-fold greater. The efficacy differences between the two drugs raised the possibility that M6G might act through a subtype of the *mu* receptor. Supraspinal morphine analgesia was significantly and selectively reduced by AS ODNs directed

against only exon 1 or 4 of the MOR-1 clone, whereas supraspinal M6G analgesia and heroin analgesia were potently and selectively reduced by AS ODNs directed against only exon 2 or 3 (Rossi *et al.*, 1995a, 1995b, 1996, 1997). Further, spinal morphine analgesia is blocked only by AS ODNs directed against exon 4. Thus the selectivity profiles of the AS ODN probes are not consistent with a single receptor, which implies that these subtypes might represent isoforms of MOR-1 generated by alternative splicing (Rossi *et al.*, 1995a).

The reductions in DAMGO-induced hyperphagia by AS ODNs directed against only exon 1 or 4 parallel effects observed for *mu* analgesic actions, which suggests that a common splice variant of the MOR-1 clone may be mediating both analgesic and hyperphagic actions. It should be noted that inactivity of a particular AS ODN might result from a variety of technical factors, including unanticipated mRNA structures. This concern is alleviated by the fact that the inactive probes in the DAMGO-induced hyperphagia paradigm are active in reducing both M6G analgesia (Rossi *et al.*, 1995a, 1995b, 1997) and spontaneous food intake and body weight (Leventhal *et al.*, 1996). Further, although the same mRNA receptor substrate mediates both responses, the substrate is probably located in different supraspinal loci. Thus *mu*-mediated analgesic responses are most potently elicited from the periaqueductal gray, the rostroventromedial medulla and the locus ceruleus (Bodnar *et al.*, 1988, 1991; Fang *et al.*, 1986; Smith *et al.*, 1988). In contrast, *mu*-mediated hyperphagic responses are most potently elicited from the hypothalamic paraventricular nucleus (Koch *et al.*, 1995), the nucleus accumbens (Bakshi and Kelley, 1993; Bodnar *et al.*, 1995; Cador *et al.*, 1986; Majeed *et al.*, 1986) and the ventral tegmental area (Mucha and Iversen, 1986; Noel and Wise, 1993, 1995). The differential actions of multiple MOR-1 splice variants may explain some of the different *mu*-mediated ingestive effects obtained using selective opioid antagonists. Thus *mu* antagonism with βFNA significantly reduces food intake under spontaneous, deprivation, glucoprivic and palatable conditions (see review: Bodnar, 1996). In contrast, mu_1 antagonism with naloxonazine significantly reduces food intake only under spontaneous and deprivation conditions.

Two possible profiles of AS ODN effects on DAMGO-induced hyperphagia were hypothesized: the observed differential pattern described in the previous sections and consistent with *mu* agonist-induced analgesia, and equal effectiveness of AS ODNs directed against each exon of the MOR-1 clone as observed in spontaneous intake and weight studies (Leventhal *et al.*, 1996). That the former, but not the latter, hypothesis was confirmed strongly suggests that the *mu* mediation of *mu* agonist-induced hyperphagia and the *mu* mediation of spontaneous intake and weight are different. Spontaneous food intake is an outcome of multiple factors acting on organisms, and the experimental alteration of any of them may increase feeding. For instance, *mu* opioid agonists increase feeding by altering the palatability of certain constituents of food, including either the macronutrient itself, such as fat (Marks-Kaufman, 1982; Marks-Kaufman and Kanarek, 1980), or the preference for a preferred macronutrient (Doyle *et al.*, 1993; Gosnell *et al.*, 1990). It is conceivable that DAMGO elicits feeding by acting on such specific mechanisms and that AS ODNs directed against exon 1 or 4 block this effect. In contrast, body weight and spontaneous intake may be influenced by additional factors mediated by all four exons of MOR-1. Such a distinction could not be made in traditional opioid antagonist studies. The *mu* antagonist βFNA both significantly reduced DAMGO-induced hyperphagia (Levine *et al.*, 1991) and significantly reduced spontaneous intake and weight under both acute (Arjune *et al.*, 1990; Ukai and Holtzman, 1988) and chronic (Cole *et al.*, 1995) microinjection conditions. Therefore, the AS ODN approach appears to be a more precise tool in dissecting differences in the functional roles of specific receptors. The selective actions of AS ODNs directed against different exons of MOR-1 in reducing DAMGO-induced hyperphagia support the hypothesis that multiple splice variants of MOR-1 exist and raise the possibility of further opioid receptor subclassifications.

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