Opioid receptor blockade in rat nucleus tractus solitarius alters amygdala dynorphin gene expression

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Received 9 August 2001; accepted in final form 7 March 2002

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Thus an opioidergic pathway appears to exist between the amygdala and the NTS, and we hypothesized that chronic opioid receptor blockade in the NTS would result in a lack of a downstream opioid stimulus to the amygdala, resulting in increased opioid gene expression in the amygdala. Also, because peripheral infusion of naltrexone increases NPY gene expression in the arcuate nucleus (ARC) and NPY peptide levels in the PVN, we predicted a similar change in NPY levels and gene expression after infusion of naltrexone in the mNTS. To test these predictions, amygdaloid proDynorphin (proDyn) mRNA, proEnkephalin (proEnk) mRNA, ARC NPY mRNA, and PVN NPY levels were measured in the following three groups of rats: 1) artificial cerebrospinal fluid (aCSF) infused in the NTS, 2) naltrexone (100 μg/day) infused for 13 days in the NTS, and 3) aCSF infused in the NTS of rats pair fed to the naltrexone-infused group. We also measured serum leptin, food intake, and body weight to evaluate whether mNTS-infused naltrexone would affect parameters influencing energy balance.

METHODS

**Animals.** Experimental protocols involving animals and their care were approved by the Animal Studies Committee at the Minneapolis Veterans Affairs Medical Center and conformed to National Institutes of Health (NIH) guidelines. Male Sprague-Dawley rats (Harlan, Madison, WI) weighing 280–305 g were individually housed in conventional hanging cages with a 12:12-h light-dark photoperiod (lights on at 0700) in a temperature-controlled room (21–22°C). Teklad Lab Chow and water were allowed ad libitum, except where noted.

**Cannulation.** Rats were anesthetized with Nembutal (40 mg/kg) and fitted with a 28-gauge stainless steel L-shaped cannula (ALZET Brain Infusion Kit; Alza, Palo Alto, CA) placed just above the mNTS. One end of this cannula was attached to tubing that was connected to an osmotic minipump (Alzet, model 1007D, 0.5 μl/h; Alza) prefilled with either aCSF or naltrexone (8.33 μg·μl⁻¹·h⁻¹). The pump was inserted subcutaneously. The dose of naltrexone (100 μg/day) was comparable to that given in a previous study in which 25 μg naltrexone were injected in the NTS every 6 h for 24 h (21). Stereotaxic coordinates for the guide cannula were determined from the rat brain atlas by Paxinos and Watson (28) and are as follows: 1.4 mm lateral and 3.7 mm posterior to the ear bars. At least 7 days elapsed after surgery before experimental trials.

**Verification of cannula placement.** After both experiments, brains were dissected out and stored in a 10% formaldehyde solution for later placement verification by histological examination. Cannula placement was deemed incorrect when the injection occurred outside a 0.25-mm radius from the targeted site. Data from animals with incorrectly placed cannulas (n = 2) were excluded from the final analysis.

**Drugs.** Naltrexone was purchased from RBI (Natick, MA) and was dissolved in aCSF just before use.

**Food intake measurements.** Food was allowed ad libitum throughout the experiment with the exception of rats in the pair-fed group. For pair-fed rats, the mean food intake of the naltrexone-treated rats was determined first, and then an amount ~1 g above that amount (to allow for spillage) was placed inside the rat’s cage 2 h before the dark cycle. These preweighed pellets of chow and collected spillage were weighed daily and subtracted from the initial weight to quantify the amount of food eaten.

**Experimental design.** Twenty-eight animals were randomly assigned to one of three groups as follows (3 rats died before completion of the study): 1) naltrexone-treated (100 μg/day infusion) with food allowed ad libitum (naltrexone, final n = 8), 2) aCSF-treated with food allowed ad libitum (aCSF, ad libidum; final n = 7), and 3) aCSF-treated with food intake matched to that of the naltrexone-treated animals (aCSF, pair fed; final n = 8). The third group was necessary to control for changes in gene expression because of alterations in food intake. Body weight and food intake measurements were made daily for 13 days after surgery. On day 8, the pumps were replaced with new pumps filled with the appropriate drug (naltrexone or aCSF). The animals then received either drug or control infusion for 5 days. After a total of 13 days of treatment, animals were rapidly decapitated, and tissues were dissected and stored at −110°C. ARC, PVN, and amygdala punches were removed by microdissection using the rat brain atlas of Paxinos and Watson (28), as previously described (22).

**Leptin RIA.** Blood samples were centrifuged for 20 min at 2,000 g, and sera were stored at −4°C until use. On the day of the RIA, samples were slowly thawed, and 100 μl sera were taken and added to the RIA tube for use in the rat leptin RIA kit (Linco Research, St. Louis, MO). Standard concentrations ranged between 0.5 and 50 ng/ml, which is within the limit of linearity. All samples were within this range. The assay sensitivity is 0.5 ng/ml (100 μl sample size). The cross-reactivity test, provided by Linco Research, indicated no cross-reaction with insulin, glucagon, or somatostatin release inhibitory factor.

**NPY RIA.** PVN were homogenized in 1 ml of 1 M CH₃COOH-95% ethanol (20:80 vol/vol) in propylene glycol. The homogenates were centrifuged at 13,000 g for 15 min. The pellets were reextracted with the same procedure, and the two supernatants were combined, lyophilized, and later used for rat NPY RIA (Peninsula Laboratories, Belmont, CA). The NPY RIA kit was validated before use with tissue extracts. Dose-response curves for PVN tissue extract and increasing concentrations of the NPY standard added to a rat PVN tissue extract were parallel (P > 0.05) to the standard curve. NPY (ranging from 4 to 32 pg) added to rat PVN tissue extract was consistently recovered from 100 μl of extract (90–100%). The assay sensitivity was 16 pg/tube. The cross-reactivity test, provided by Peninsula Laboratories, indicated a cross-reaction of <3% with human pancreatic polypeptide.

**NPY, proDyn, and proEnk mRNA determination.** Total RNA was extracted from the ARC and amygdala. Extraction was carried out using the guanidine thiocyanate-phenol-chloroform method (6). Tissue samples were collected in guanidine thiocyanate with added β-mercaptoethanol and homogenized in this buffer and water-saturated molecular biology-grade phenol. Sarcosyl, 2 M sodium acetate, and chloroform were then added. After centrifugation, the aqueous phase was precipitated with isopropanol, resuspended in guanidine thiocyanate buffer, and reprecipitated with isopropanol. The RNA pellet was washed with 75% ethanol and stored at −70°C in 100% ethanol. The RNA pellet was reconstituted in RNA storage buffer. The total amount of RNA was measured using a spectrophotometer.

Samples were analyzed by the slot-blot method using nylon membranes (Zeta-Probe, Hercules, CA). Aliquots of total
RNA were dissolved in 7.4% formaldehyde [6× saline-sodium citrate (SSC); 1× SSC = 0.15 M NaCl and 0.015 M sodium citrate] and denatured for 15 min at 65°C. Duplicates of 2 μg of each sample were slotted on 6× SSC-soaked nylon and then prehybridized for 24 h at 42°C in 50% formamide, 5× SSC, 10× Denhardt’s solution, 0.1% SDS, and denatured salmon sperm DNA in 50 mM sodium phosphate, pH 6.5. For NPY, the hybridization procedure was carried out using a radiolabeled cDNA pBLNPY-1 probe grown in our laboratory and generously provided by Dr. Steven L. Sabol (Laboratory of Biochemical Genetics, National Heart, Lung, and Blood Institute, NIH, Bethesda, MD). This cDNA contains a 511-bp sequence comprising most of the rat-prepro-NPY gene. For proEnk and proDyn, hybridization was performed using radiolabeled cDNA probe produced in our laboratory from transformed cells generously provided by Dr. J. O. Douglass (Vollum Institute for Advanced Biomedical Research, Oregon Health Sciences University, Portland, OR). The cDNA utilized were 1,070- and 1,700-bp nucleotide sequences coding for the genes of rat proEnk and proDyn, respectively. Specificity of the opioid probes was previously verified using Northern blot analysis (15). The hybridization medium was 50% formamide, 5× SSC, 2× Denhardt’s solution, 0.2% SDS, denatured salmon sperm DNA, and yeast tRNA in 50 mM sodium phosphate, pH 6.5, with the addition of 10⁶ counts·min⁻¹·ml⁻¹ [³²P]dCTP random primer-labeled probe. After hybridization for 45 h at 42°C, the nylon membrane was subjected to high- and low-salt washing and then placed in a cassette with a phosphor screen (Molecular Dynamics, Sunnyvale, CA) for 7 days exposure. The screen was then scanned using a PhosphorImager (Molecular Dynamics). Levels of mRNA are expressed in optical density units.

Statistical analysis. Data were analyzed by one-factor ANOVA, followed by multiple comparisons of means using the Least Significant Difference t-test. All data are expressed as means ± SE. It should be noted that, in several assays, some data were lost because of technical errors (indicated by fewer rats/group).

**RESULTS**

Food intake and body weight. There was a main effect of treatment on cumulative food intake [days 1–13; F(2,20) = 12.36, P = 0.0003; Fig. 1]. Post hoc analysis indicates that naltrexone in the mNTS resulted in a significant reduction in food intake (P < 0.05; Fig. 1). As expected, food intake of the aCSF-treated pair-fed rats was not significantly different from the naltrexone-treated rats (P > 0.05; Fig. 1). Body weight changes followed the pattern of food intake. There was a significant main effect of treatment on cumulative body weight change [days 1–13; F(2,20) = 21.23, P < 0.0001; Fig. 2]. Post hoc analysis indicates that naltrexone in the mNTS resulted in significant weight loss compared with the ad libitum control group (P < 0.05; Fig. 2). As expected, the aCSF-treated pair-fed rats lost a significant amount of weight compared with the ad libitum control group. There was no difference in body weight change between the naltrexone-treated and pair-fed rats (Fig. 2).

proDyn, proEnk, and NPY. There was a main effect of drug treatment on proDyn mRNA levels in the amygdala [F(2,18) = 5.16, P = 0.017; Fig. 3]. Post hoc analysis indicates that proDyn mRNA was significantly higher in naltrexone-treated rats compared with both aCSF-treated ad libitum- and aCSF-treated pair-fed rats (P < 0.05; Fig. 3). There was no difference in proDyn mRNA levels between aCSF-treated pair-fed and aCSF-treated ad libitum-fed rats (P > 0.05; Fig. 3). There were no main effects of treatment on amygdalar proEnk mRNA [F(2,14) = 0.67, P = 0.524; Fig. 3], ARC NPY mRNA [F(2,17) = 2.13, P = 0.149; Fig. 4], or NPY peptide levels in the PVN [F(2,20) = 68.25, P = 0.187; Fig. 4].

Leptin. There was a main effect of treatment on serum leptin [F(2,20) = 8.68, P = 0.002; Fig. 5]. Post hoc analysis indicates that the aCSF-treated pair-fed rats had significantly decreased serum leptin (P <
In the naltrexone-treated animals, in which food intake was similar to the aCSF-treated pair-fed rats, serum leptin was significantly elevated compared with the aCSF-treated pair-fed rats (\( P < 0.05 \)) but was not significantly different from the aCSF-treated ad libitum-fed rats (\( P > 0.05 \); Fig. 5).

**DISCUSSION**

Elevated levels of proDyn mRNA in the amygdala paralleled the decreased food intake accompanying mNTS naltrexone infusion. This latter finding is consistent with the results of previous studies showing that chronic intracerebroventricular administration of naltrexone is associated with increased proDyn mRNA expression in the rat hypothalamus, hippocampus, and striatum (34). The increased amygdala proDyn mRNA in the naltrexone-treated rats did not result from changes in food intake or body weight per se, since these parameters did not differ between the naltrexone-treated and aCSF-treated pair-fed rats. There was also no difference in proDyn mRNA in the aCSF-treated ad libitum-fed and pair-fed groups. Although it has been reported that chronic food restriction is associated with elevated proDyn mRNA in the central nucleus of the amygdala (2), there was no change in proDyn mRNA in the current study in which aCSF-treated pair-fed rats were mildly food restricted. Differences in restriction schedules, size of tissue samples, and molecular assays between these studies may account for this discrepancy.

Several potential anatomical mechanisms may account for the relationship between mNTS opioid receptor blockade and changes in amygdalar dynorphin gene expression. These include direct connections between the mNTS- and proDyn-expressing cell bodies in the amygdala, indirect connections through local interneu-
rons, which in turn contact proDyn-expressing cells, or some other multisynaptic pathway. NTS efferents project to a variety of brain stem and forebrain regions, including the amygdala (33). The amygdala, particularly the central nucleus is densely populated by peptidergic soma (4), and Dyn-immunoreactive neurons have been found within the amygdala, especially the lateral portion (8). Alternatively, the present findings may reflect actions along an indirect pathway from the NTS to the amygdala via the parabrachial nucleus (PBN; see Refs. 14 and 26), which is a major target of visceral/gustatory fibers from the NTS (27). The amygdala is a likely candidate for relevant opioidergic projections from the NTS, since injection of naltrexone in the rNTS inhibits food intake stimulated by DAMGO administered in the amygdala and injection of naltrexone in the amygdala inhibits food intake stimulated by DAMGO administered in the rNTS (9). Regardless of the signaling pathway, a possible explanation for the current findings is that naltrexone administration in the NTS interrupts a signal for opioid synthesis in the amygdala. Gene expression is presumed to reflect biosynthetic demand for peptide (37). Consequent to blockade of opioid receptors is the lack of opioid receptor signaling, and thus increases in proDyn mRNA may result from interoceptive cues, indicating a need for Dynorphin peptide.

In addition to measurement of amygdala opioid gene expression, levels of ARC NPY mRNA and PVN NPY peptide levels were also determined. There were no differences in levels of ARC NPY mRNA among any of the treatment groups. Although it has been previously demonstrated that ARC NPY gene expression increases with energy restriction (3, 16, 25, 29), the degree of the energy restriction in the current study was relatively mild. We previously noted that peripheral infusion of naltrexone results in an increase in ARC NPY gene expression with no change in NPY peptide levels in the PVN (22). The previous study had two major differences in design that may help explain the difference in NPY gene expression response between that study and the current one. The first difference was the feeding paradigm: in the previous study, we either gave rats ad libitum access to food or we completely restricted food; thus there was no pair fed group, and the food restriction in the previous study was much more intense (48 h deprivation). The second difference was the site of naltrexone infusion; in the previous study, the naltrexone was given peripherally and thus targeted not only peripheral opioid receptors but also opioid receptors throughout the brain. Thus the ARC likely received multiple signals, which potentially led to the more profound results. However, the current results may be a more reliable indicator of NTS/opioid and ARC/NPY signaling because the pair-feeding design better detects differences resulting from treatment (naltrexone) vs. energy levels, and the naltrexone was administered directly to the mNTS, which selectively targets opioid receptors that may directly signal the ARC.

We found that infusion of naltrexone in the mNTS decreased food intake and body weight. Previous studies show that chronic peripheral administration of naltrexone or naltrexone reduced food intake and body weight, accompanied by alterations in energy expenditure, as measured by the respiratory quotient (22–24). The present study is in agreement with these findings and suggests that the mNTS may be an important central locus for opioid involvement in energy balance. Other observations compatible with this hypothesis are that acute administration of naltrexone in the NTS blocks food intake and alterations in thermogenic capacity induced by NPY in the PVN (21). Additionally, food intake is stimulated by mu receptor agonist administration in the NTS (9, 19). Recent evidence from regional NTS microinjection feeding studies suggests that opioid receptors in the medial-intermediate subregion of the NTS are the most important in these effects (20). As expected with changes in energy-balance, the present results show that leptin levels decrease with a decrease in food intake. This finding is consistent with the hypothesis that food ingestion results in elevated leptin levels (18), presumably because of insulin-stimulated leptin release from adipose tissue (1, 17).

There was a significant decrease in leptin levels in the pair-fed group compared with the naltrexone-infused group. This may indicate that chronic opioid blockade decreases the impact of food restriction on leptin levels. However, it may also indicate a difference in energy state of the pair-fed and naltrexone-injected group. Pair-fed restricted rats were given their food 2 h before the dark cycle and most likely behaved as schedule-fed rats, eating most of their food over a brief period of time. On the other hand, the naltrexone-injected rats may have ingested their food over a longer time period, beginning at the onset of the dark cycle.
Nonetheless, both groups ingested the same amount of energy during the 13-day study period. The present results further support a relationship between opioid central nervous system pathways and energy metabolism. Blockade of opioid receptors in mNTS (unilateral) is associated with disruption of energy regulatory circuits, which results in alterations in energy balance. Associated with these changes is an increase in Dyn gene expression in the amygdala. We should note that such changes might have been more robust if naltrexone was injected bilaterally in the NTS. The precise pathway through which these alterations occur and the nature of accompanying behavioral effects need further elaboration.

This research was supported by General Research Funds of the Department of Veterans Affairs, National Institute of Drug Abuse Grant DA-03999, and National Institute of Diabetes and Kidney Diseases Grant P09-DK-30456.

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