Dysregulation of Nociceptin/Orphanin FQ Activity in the Amygdala Is Linked to Excessive Alcohol Drinking in the Rat

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Background: Alcoholism is a complex behavioral disorder in which interactions between stressful life events and heritable susceptibility factors contribute to the initiation and progression of disease. Neural substrates of these interactions remain largely unknown. Here, we examined the role of the nociceptin/orphanin FQ (N/OFQ) system, with an animal model in which genetic selection for high alcohol preference has led to co-segregation of elevated behavioral sensitivity to stress (Marchigian Sardinian alcohol-preferring [msP]).

Methods: The msP and Wistar rats trained to self-administer alcohol received central injections of N/OFQ. In situ hybridization and receptor binding assays were also performed to evaluate N/OFQ receptor (NOP) function in naïve msP and Wistar rats.

Results: Intracerebroventricular (ICV) injection of N/OFQ significantly inhibited alcohol self-administration in msP but not in nonselected Wistar rats. The NOP receptor messenger RNA expression and binding was upregulated across most brain regions in msP compared with Wistar rats. However, in msP rats [^{35}S]GTPγS binding revealed a selective impairment of NOP receptor signaling in the central amygdala (CeA). Ethanol self-administration in msP rats was suppressed after N/OFQ microinjection into the CeA but not into the bed nucleus of the stria terminalis or the basolateral amygdala.

Conclusions: These findings indicate that dysregulation of N/OFQ-NOP receptor signaling in the CeA contributes to excessive alcohol intake in msP rats and that this phenotype can be rescued by local administration of pharmacological doses of exogenous N/OFQ. Data are interpreted on the basis of the anti–corticotropin releasing factor (CRF) actions of N/OFQ and the significance of the CRF system in promoting excessive alcohol drinking in msP rats.

Key Words: Addiction, alcohol preferring rats, alcoholism, central amygdala, nociceptin/orphanin FQ, NOP receptors

Nociceptin/Orphanin FQ (N/OFQ) is the endogenous ligand of the opioid receptor-like 1 receptor, recently included in the opioid receptor family and renamed NOP (1,2). Neuroanatomical studies (3,4) have shown a wide distribution of N/OFQ and its receptor in structures involved in motivational effects of addictive drugs (5–7), including the amygdala, the bed nucleus of the stria terminalis (BNST), the nucleus accumbens, and various fronto-cortical areas. Evidence for a role of the N/OFQ system in behavior motivated by drugs of abuse is particularly robust in the case of alcohol (8–11). Studies in genetically selected Marchigian Sardinian alcohol-preferring (msP) rats demonstrated that intracerebroventricular (ICV) treatment with N/OFQ inhibits ethanol-induced conditioned place preference, home cage ethanol drinking, operant ethanol self-administration, and relapse (8,9,12,13).

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Recent data suggest that preclinical findings with N/OFQ might have a good potential for clinical translation. In fact, the opioid agonist/partial agonist buprenorphine, which has long been in clinical use for the treatment of pain (14–16) and management of heroin dependence (17–19), reduces alcohol-drinking in msP rats via activation of NOP receptors (20). There are, consistent with this finding, clinical data indicating that buprenorphine given to heroin addicts lowers not only opiate but also alcohol consumption (17,19). These data point to the possibility that buprenorphine effects on alcohol-drinking are mediated by activation of NOP receptors.

In the interest of future clinical application of novel and selective NOP agonists, here we examined the effects of N/OFQ in msP rats, in which genetic selection for high ethanol preference has co-segregated with hypersensitivity to stress and anxiety (21,22). We first evaluated the effect of subchronic ICV application of N/OFQ on ethanol self-administration in msP rats and compared it with effects obtained in nonselected Wistar rats. The results revealed that N/OFQ significantly inhibits alcohol drinking in msP rats but not Wistar rats. An extensive brain in situ hybridization and NOP receptor autoradiography study showed that NOP receptors are upregulated in msP, compared with Wistar rats. Surprisingly, however, N/OFQ-stimulated [^{35}S]GTPγS binding in the central amygdala (CeA) was lower in msP rats, indicating dysregulated NOP receptor/G protein coupling mechanisms in this brain area that renders the system hypofunctional. Subsequent brain microinjection studies demonstrated that administration of N/OFQ into the CeA but not into the BNST or the basolateral amygdala (BLA) selectively reduces ethanol self-administration in msP rats. Together, these findings suggest that dysregulation of the NOP receptor system in the CeA contributes

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to the elevated alcohol-drinking of msP rats and that exogenous administration of NOP receptor agonists might act by restoring N/OFQ neurotransmission in this brain area.

Methods and Materials

Subjects

Genetically selected male Marchigian Sardinian alcohol-preferring (msP) and nonselected Wistar rats (350–450 g) were studied. The msP rats were bred at the Department of Experimental Medicine and Public Health of the University of Camerino (Italy). Wistar rats, purchased from Charles River (Germany) were habituated to the same housing condition as msP rats for 1 month before the beginning of the experiments. Experiments were conducted during the dark phase of a 12-hour light/dark cycle (lights on 8:00 PM). All procedures were conducted in adherence with the European Community Council Directive for Care and Use of Laboratory Animals.

Drugs

Nociceptin/Orphanin FQ was a generous gift of Dr. R. Guerrini (University of Ferrara, Italy); it was dissolved in sterile isotonic saline and injected in a volume of 1.0 μL/rat or .5 μL/site/rat into the lateral cerebroventricle or into the specific brain areas, respectively.

Intracranial Surgery

Anesthetized rats were stereotaxically implanted (for coordinates see Table 1) with guide cannula aimed at the respective brain structure. Experiments began 1 week after surgery. After completion of the experiments, rats were killed and cannula placements were verified by histological analysis.

Alcohol Self-Administration Training

Rats were trained to self-administer 10% (v/v) ethanol in 30-min daily sessions under a fixed-ratio 1 (FR1) schedule of reinforcement where each response resulted in delivery of .1 mL of fluid. A saccharin fading procedure was used to train the rats to 10% ethanol self-administration (9,23). Briefly, animals were first trained to lever-press for .2% (w/v) saccharin solution. After successful acquisition of saccharin-reinforced responding for 2 days, rats were trained to self-administer .2% saccharin solution containing 5.0% ethanol. Beginning on day 3, the concentration of ethanol was gradually increased from 5.0% to 8.0% and finally 10%, while the concentration of saccharin was correspondingly decreased to 0%. From the first day, rats began to press for 10% ethanol, and the house light located on the front panel was turned on for 5.0 sec.

Table 1. Brain Stereotaxic Coordinates for Cannula Placement

Region	Antero-Posterior	Lateral	Ventral
Lateral Cerebroventricle (ICV)	−1.0	1.8	2.0
BNST	−.1	±1.3	6.0
CeA	−1.5	±4.3	7.0
BLA	−1.5	±5.1	7.0

Rats were anesthetized by intramuscular injection of 100–150 μL of a solution containing tiletamine chloridrate (58.17 mg/mL) and zolazepam chloridrate (57.5 mg/mL). Guide cannulas (.65 mm outside diameter) were stereotaxically implanted and cemented to the skull. Nociceptin/orphanin FQ (N/OFQ) was injected through a stainless-steel injector (.25 mm outside diameter) protruding beyond the cannula tip: 2.5 mm for the lateral ventricle; 1.2 mm for the bed nucleus of the stria terminalis (BNST); and 1.5 mm for the central amygdala (CeA) and the basolateral amygdala (BLA). ICV, intracerebroventricular.

During the experiments responses at the active and the inactive levers were always recorded.

Experiment 1: Effect of Subchronic ICV Injections of N/OFQ on Alcohol Self-Administration in msP and Wistar Rats

After acquisition of a stable 10% ethanol self-administration baseline (10 days), msP rats (n = 21) and Wistar rats (n = 27) were divided into three and four groups, respectively. During the last 4 days of training (pre-treatment), immediately before the self-administration sessions, rats were given 1 μL of ICV saline to familiarize them with the injection procedure. Then, for 6 consecutive days msP (n = 7/group) and Wistar rats (n = 6–7/group) received .0, .5, and 1.0 μg/rat or .0, 1.0, 2.0, and 4.0 μg/rat of N/OFQ or its vehicle. Rats were tested for 10% ethanol self-administration immediately after drug administration. At completion of drug testing, ethanol self-administration was monitored for an additional 4 days (post-treatment) during which all rats received only ICV saline.

Experiment 2: N/OFQ and NOP Receptor In Situ Hybridization

For in situ hybridization, receptor binding, and receptor activity (see also Experiments 3 and 4), rats were killed in their inactive phase by decapitation and brains were quickly removed, snap frozen in −40°C isopentane, and stored at −70°C. Coronal brain sections (10 μm) were taken at Bregma levels as illustrated in Figure 1 according to the atlas of Paxinos and Watson (24) and stored at −70°C until use (24).

In situ hybridization for rat-specific riboprobes for N/OFO (gene reference sequence in PubMed database [http://www.ncbi.nlm.nih.gov/Entrez/]: NM_013007, position 275–535 base pair [bp]) and NOP receptor (gene reference sequence in PubMed database [http://www.ncbi.nlm.nih.gov/Entrez/]: NM_031569, position 879–1142 bp) have been recently shown in Hansson *et al.* (22). Procedures for RNA probe synthesis in both antisense and sense direction and all the hybridization steps have been described in detail in Hansson *et al.* (25).

For data visualization phosphor imaging plates (Fuji-film for BAS-5000, Fujifilm, Tokyo, Japan) were exposed for 48 hours to hybridized sections. Phosphor imager-generated (Fujifilm Bio-Imaging Analyzer Systems, BAS-5000, Fujifilm) digital images were analyzed with MCID Image Analysis Software (Imaging Research, St-Catharine, Ontario, Canada). Regions of interest were defined by anatomical landmarks as described in the atlas Paxinos and Watson (24) and illustrated in Figure 1. Signal density was measured as photostimulable luminescence/mm^2 and converted into integrated optical density values, expressed in calibration standards units (nCi/g) with a C^{14} standard curve (Microscale C^{14}, Amersham GE Healthcare, Chalfont St. Giles, United Kingdom). For detailed visualization, films (Kodak BioMax MR, Eastman Kodak Company, Rochester, New York) were subsequently exposed for 1 month to hybridized sections.

Experiment 3: NOP Receptor Autoradiography

For autoradiography, sections were brought up to room temperature, incubated for 15 min at room temperature in 50 mmol/L Tris-hydrochloric acid (HCl) buffer (pH 7.5) containing 5 mmol/L magnesium chloride ($MgCl_2$) and 1 mmol/L ethylenediaminetetraacetic acid (EDTA). This incubation step was repeated with a fresh buffer. Sections were then transferred into humidified chambers, 800 μL of reaction mix was applied to each slide, and sections were incubated for 2 hours at room temperature. Reaction mix contained 1 nmol/L [^{3}H]-nociceptin (specific activity 90 Ci/mmol, New England Nu-

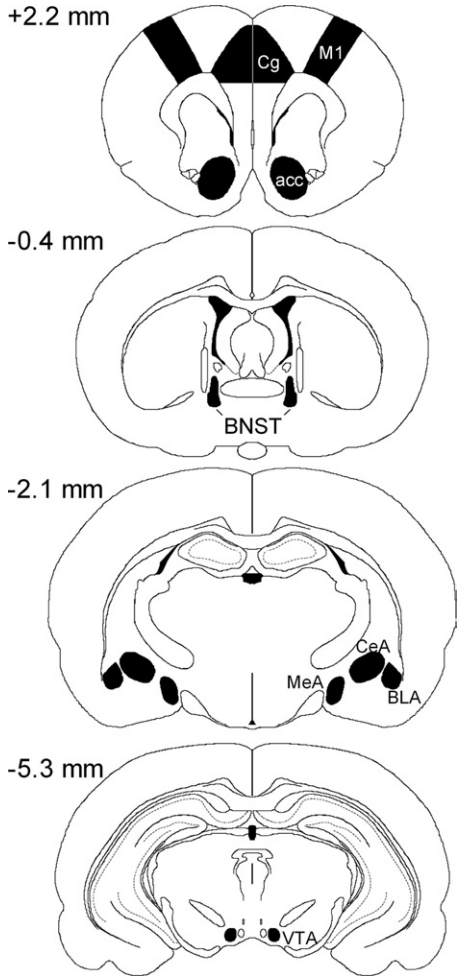

Figure 1. Schematic representation of the sampled areas for the densitometric evaluation of messenger RNAs in a coronal section through the rat forebrain at Bregma levels +2 to −5 mm. CG, cingulate cortex; M1, primary motor cortex; acc, nucleus accumbens; BNST, bed nucleus of stria terminalis; CeA, central amygdaloid nucleus; MeA, medial amygdaloid nucleus; BLA, basolateral amygdaloid nucleus; VTA, ventral tegmental area.

clear, Boston, Massachusetts) prepared in a buffer containing 50 mmol/L Tris-HCl (pH 7.5), 5 mmol/L $MgCl_2$, 1 mmol/L EDTA, .1 mmol/L bacitracin, and .1% bovine serum albumine. Nonspecific binding was measured on adjacent sections by adding 1 μmol/L UFP-101 (N/OFQ receptor antagonist, Tocris Bioscience, Ellisville, Missouri). Incubation was stopped by washing the sections twice in ice-cold buffer followed by a dip in ice-cold deionized water. Sections were dried under a stream of cold air and were used to expose Fuji BAS-5000 Phosphorimager tritium plates for 10 days. Density values were measured as described previously and compared against standard curves generated with [^{3}H]-microscales (Amersham), and data (nCi/mg) were converted to fmol receptor/mg protein tissue equivalence. Specific binding was defined as a difference between total and nonspecific binding.

Experiment 4: NOP Receptor Signaling

Sections were brought up to room temperature and incubated for 15 min at room temperature in a 50-mmol/L Tris-HCl (pH 7.5) buffer containing 5 mmol/L $MgCl_2$ and 1 mmol/L EDTA. Incubation was repeated once, and sections were transferred into humidified chambers. Incubation buffer (500 μL: 50 mmol/L Tris-HCl pH 7.5, 5 mmol/L $MgCl_2$, 1 mmol/L EDTA, 100 mmol/L sodium chloride, 1 mmol/L (R,R)-Dithiothreitol (DTT), .1% N,O-Bis(trimethylsilyl)acetamide [BSA]) containing 1 mmol/L 5′-guanylate diphosphate (GDP) was applied to each slide and sections were incubated for 15 min at room temperature. Solution was discarded, and a mix of 50 pmol/L [^{35}S]GTPγS, 1 mmol/L GDP, and 1 μmol/L N/OFQ or vehicle in incubation buffer was applied. Sections were incubated for 60 min at 30°C, and incubation was stopped by washing the slides for 2 min in ice-cold buffer followed by a dip in ice-cold deionized water. Sections were dried under a stream of cold air and used to expose Fuji BAS-5000 Phosphorimager plates. Agonist stimulated and baseline [^{35}S]GTPγS binding was measured on adjacent sections as described earlier with [^{14}C]-microscales (Amersham) standard curve. Percent stimulation was calculated as the difference between agonist-stimulated and baseline bindings and expressed as percent of baseline value in the same region and animal.

Experiment 5: Effect of N/OFQ Microinjections into the CeA, BLA, or BNST on Alcohol Self-Administration in msP Rats

The msP rats were trained, with the same procedure described in Experiment 1, to operant ethanol self-administration until stable baseline of responding was reached. At this point rats were subjected to intracranial surgery, and then ethanol self-administration baseline was re-established. One group of msP rats (n = 30) with brain cannulae implanted bilaterally into the CeA was subdivided into three groups that were treated with N/OFQ .125 or .25 μg site/rat or drug vehicle, respectively. A second group of msP rats (n = 30) with brain cannulae implanted bilaterally into the BLA was subdivided into three groups to receive N/OFQ .25, .5 μg site/rat, or N/OFQ vehicle, respectively. A third group of rats (n = 24) implanted bilaterally with cannulae aimed at the BNST was subdivided into three groups to receive N/OFQ .25, .5 μg site/rat, or N/OFQ vehicle, respectively.

On experimental day 1 (pre-treatment) all rats were subjected to a mock bilateral injection of vehicle to familiarize them with the treatment procedure. On day 2 (test day), rats were tested for the effect of N/OFQ, whereas on day 3 (post-treatment), all rats received .5 μL site/rat of vehicle again. Immediately after brain microinjections, rats were placed in the self-administration chambers and tested for 10% ethanol self-administration.

At the end of the experiments, cannula placement was verified by histological analysis. Correct cannulae placement was confirmed in 21 of 30 rats in the CeA (Figure 2), 22 of 30 rats in the BLA, and 19 of 24 rats in the BNST. Only data obtained in rats with correct cannulae placement were subjected to statistical evaluation.

Statistical Analysis

Data from experiments with subchronic N/OFQ treatment (Experiment 1) were analyzed by means of two-way analysis of variance (ANOVA) with one within-subjects factor (test session) and one between subjects factor (N/OFQ dose). The effect of N/OFQ given into the CeA, BNST, and BLA (Experiment 5) was analyzed by one-way ANOVA with one between subject factor (N/OFQ dose). For behavioral experiments, post hoc comparisons were performed by Newman-Keuls tests. Gene expression (Experiment 2), receptor binding (Experiment 3), and receptor activity (Experiment 4) data had homogeneous variances within respective regions and were therefore compared by region-wise

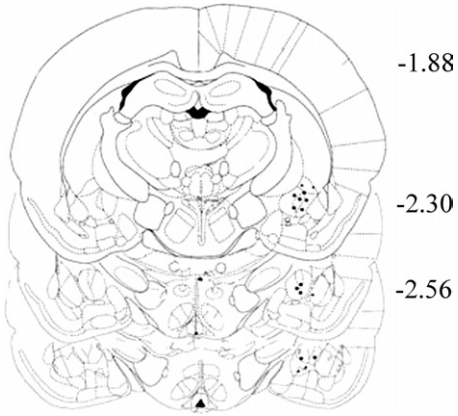

Figure 2. Representation of the location of the tips of the injectors (filled circles) within the central amygdaloid nucleus (CeA). Numbers indicate the distances from bregma in millimeters. Data from Paxinos and Watson [24].

one-way parametric ANOVAs, followed by Holm-Bonferroni correction.

Results

Experiment 1: Effect of Subchronic ICV Injections of N/OFQ on Alcohol Self-Administration in msP and Wistar Rats

As shown in Figure 3A, subchronic (6 days) ICV treatment with N/OFQ (.5 and 1.0 μg/rat) markedly reduced responding on the active lever in msP rats [$F(2,18) = 6.7$, $p < .01$]. Newman-Keuls post hoc test confirmed a significant difference between control subjects and rats treated with .5 and 1.0 μg/rat of N/OFQ.

Conversely, N/OFQ, did not significantly modify [$F(3,23) = .44$, p = ns] ethanol-maintained responding in Wistar rats (Figure 3C). In these rats the peptide was given at doses up to 8 times higher than that effective in reducing alcohol self-administration in msP rats. Responses on the inactive lever were negligible in all treatment conditions for both msP [$F(2,18) = 2.731$, p = ns] (Figure 3B) and Wistar rats [$F(3,23) = 2.02$, p = ns] (Figure 3D).

Experiment 2: N/OFQ and NOP Receptor In Situ Hybridization

The N/OFQ and NOP receptor messenger RNAs (mRNAs) showed, consistent with existing literature, an overlapping pattern of distribution. The highest expression of N/OFQ mRNA was found in the CeA, BNST, and medial amygdala. Moderate expression was identified in various hippocampal subregions, whereas in the BLA expression of the peptide precursor was undetectable. The expression of NOP transcript was highest in the paraventricular nucleus of the hypothalamus; moderate levels were measured in the BNST and in various amygdaloid and hippocampal subregions. Comparison between msP and Wistar rats revealed that N/OFQ and NOP receptor mRNA levels were significantly higher in the alcohol-preferring rats. As shown in Supplement 1 and Figure 4, the largest differences between the lines were identified in the motor cortex (M1), BNST, and CeA for the N/OFQ precursor and in the BNST, CeA, and M1 for the NOP precursor, respectively.

Experiment 3: NOP Receptor Autoradiography

To evaluate whether differences in NOP mRNA expression translates into changes in binding capacity, a binding study was carried out. Confirming existing data (26,27), we found high levels of NOP receptor binding in various cortical and amygdaloid subregions, including M1, cingulate cortex, CeA, medial amygdaloid nucleus (MeA), and BLA. Moderate NOP receptor density was found in the BNST. As shown in Supplement 1 and in Figure 5, comparison between msP and Wistar rats revealed that NOP receptor density was significantly higher in the alcohol-preferring line. The largest differences were found in the CeA, MeA, BNST, nucleus accumbens, and cingulate cortex. In the BLA, the difference between rat strains was not significant.

Experiment 4: NOP Receptor Signaling

An autoradiographic [35S]GTPγS binding assay was used to measure G-protein signaling after activation of the NOP receptor. Consistent with the receptor autoradiographic results, agonist stimulated NOP receptor function was generally

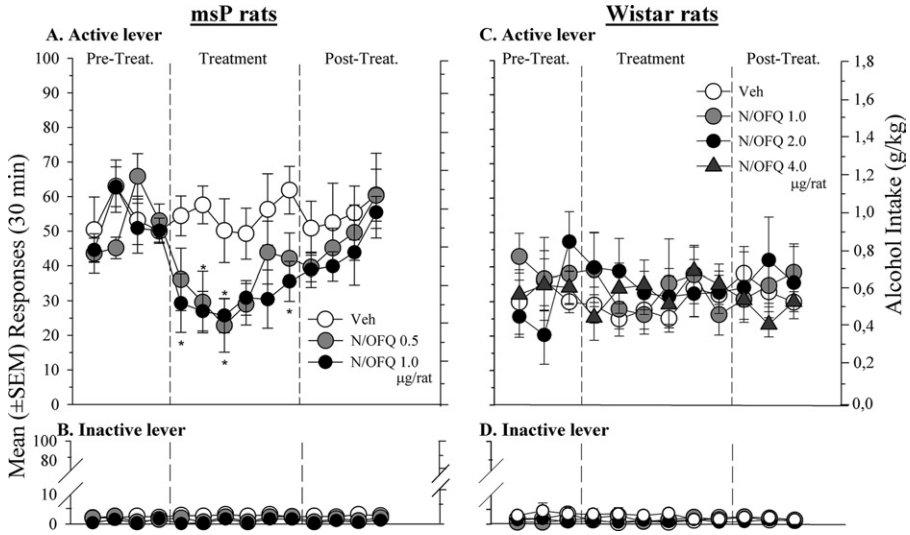

Figure 3. Self-administration for 10% ethanol under a fixed-ratio (FR)-1 schedule of reinforcement in: (Left Panel) Marchigian Sardinian alcohol-preferring (msP) rats treated intracerebroventricular (ICV) for 6 consecutive days with .5 or 1.0 μg/rat of nociceptin/orphanin FQ (N/OFQ) or its vehicle (Veh); and (Right Panel) Wistar rats treated ICV for 6 consecutive days with 1.0, 2.0, and 4.0 μg/rat of N/OFQ or Veh. **(A)** and **(C)** correspond to the number of reinforced active lever presses, whereas **(B)** and **(D)** correspond to the number of inactive lever presses. Values represent the mean (± SEM) of 6–7 subjects/group. Difference from vehicle *$p < .05$.

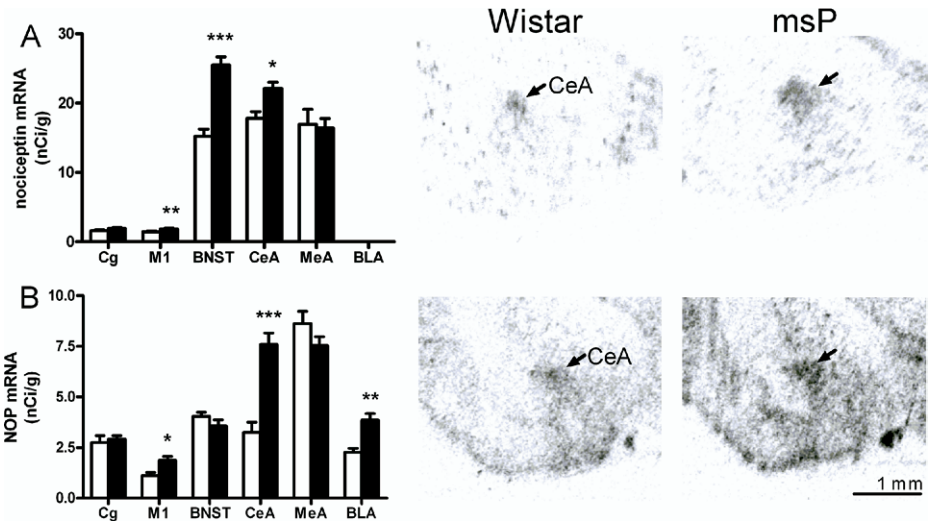

Figure 4. (A) Left panel: upregulated N/OFQ messenger RNA (mRNA) expression in msP rats compared with Wistar rats (nCi/g ± SEM, $n = 7–8$; ***$p < .001$; **$p < .01$; *$p < .05$). Right panel: illustrative autoradiogram showing increased N/OFQ expression in msP (scale bars, 1 mm). **(B)** Left panel: upregulated N/OFQ receptor (NOP) receptor mRNA expression in msP rats (black bars) compared with Wistar (white bars) rats (nCi/g ± SEM, $n = 7–8$; ***$p < .001$; **$p < .01$; *$p < .05$). Right panel: illustrative autoradiogram showing increased NOP expression in msP (scale bars, 1 mm). For details on treatment, see Methods and Materials. Other abbreviations as in Figures 1 and 3.

higher in msP rats, presumably reflecting the increased receptor density (Supplement 2). Surprisingly, however, after N/OFQ stimulation, a significantly lower response was detected in the CeA of msP rats despite higher NOP receptor density in this region (Figure 6). This unexpected finding prompted us to further investigate NOP receptor functionality by analyzing the effect of N/OFQ microinjection into the CEA on ethanol self-administration in msP rats.

Experiment 5: Effects of N/OFQ Microinjections into the CeA, BLA, or BNST on Alcohol Self-administration in msP Rats

All rats acquired responding reinforced by 10% ethanol and developed stable ethanol self-administration. Analysis of variance revealed a main effect of treatment [$F(2,18) = .61$, $p < .05$] in rats injected into the CeA. As shown in Figure 7A, Newman-Keuls post hoc tests showed that microinjection of N/OFQ (.25 μg/site/rat) into this brain area markedly reduced ethanol self-administration in msP rats ($p < .05$). After treatment with a lower dose of N/OFQ (.125 μg/site/rat), a trend toward reduced ethanol self-administration was observed, although this effect was not statistically significant (Figure 7A).

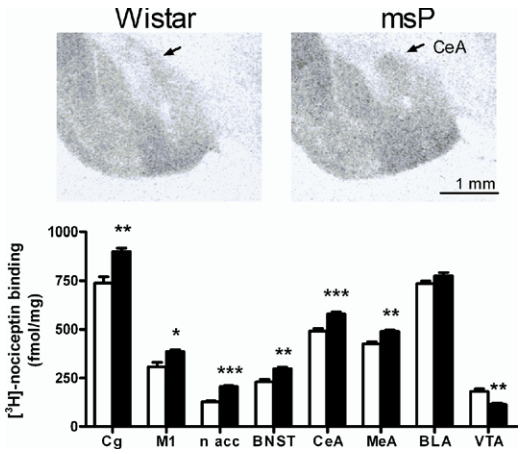

Figure 5. Lower panel: elevated NOP mRNA expression in msP rats was accompanied by increased density of NOP binding sites in numerous brain regions, shown with [^{3}H]-nociceptin using UFP-101 (N/OFQ receptor antagonist) to measure nonspecific binding (rats; $n = 8$/group; mean ± SEM; ***$p < .001$; **$p < .01$; *$p < .05$). Upper panel: illustrative autoradiogram showing increased total [^{3}H]-nociceptin binding in msP rats. For details on treatment, see Methods and Materials. Other abbreviations as in Figures 1 and 4.

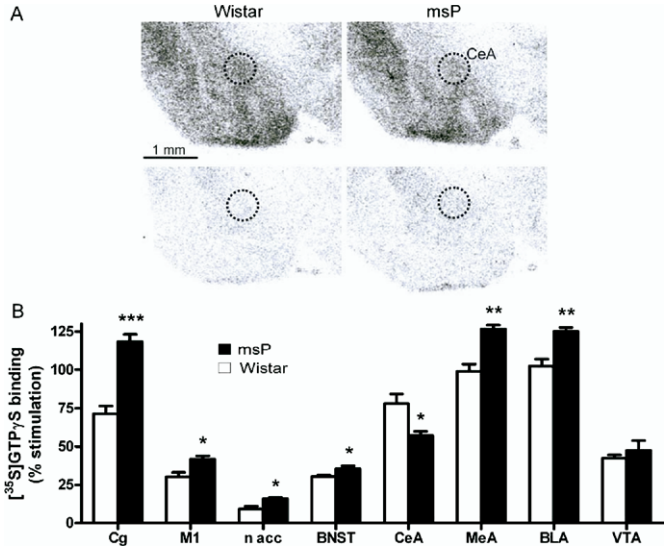

Figure 6. (A) Illustrative autoradiogram showing [^{35}S]GTPγS binding in stimulated (upper panel) and baseline condition (lower panel). The MsP rats show lower nociceptin-stimulated [^{35}S]GTPγS binding in CeA (right upper panel) as compared with Wistar rats (left upper panel). **(B)** Nociceptin-stimulated [^{35}S]GTPγS binding. Percent stimulation was calculated as a difference between agonist-stimulated and baseline bindings and expressed as percent of baseline value in the same region and animal (rats; $n = 8$/group; mean ± SEM; ***$p < .001$; **$p < .01$; *$p < .05$). Results depicted a general increase of NOP receptor functionality in msP rats except in the central amygdala where it was significantly lower. For details on treatment, see Methods and Materials. Other abbreviations as in Figures 1 and 4.

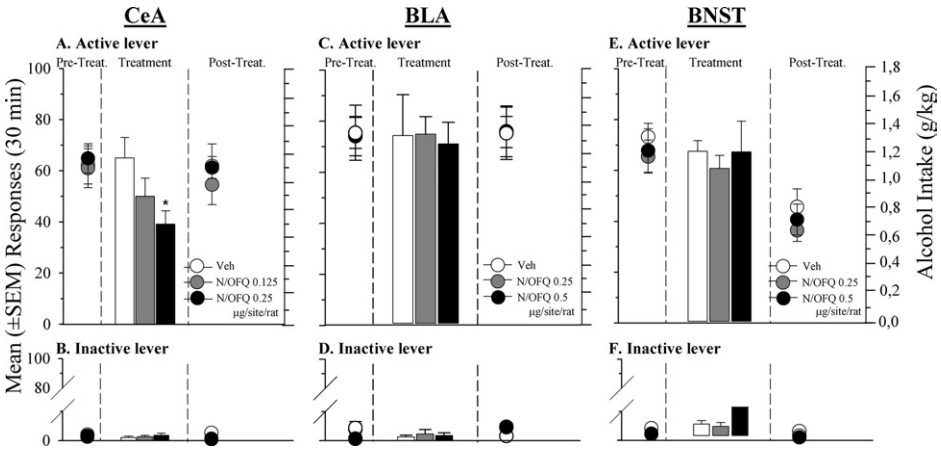

Figure 7. Self-administration for 10% ethanol under an FR-1 reinforcement schedule in msP rats treated into: (left panel) the CeA with .125 and .25 μg/site/rat of N/OFQ or Veh; (central panel) the BLA with .25 and .5 μg/site/rat of N/OFQ or Veh; (right panel) BNST with .25 and .5 μg/site/rat of N/OFQ or Veh at: **(A, B, and C)** number of reinforced active lever presses; **(D, E, and F)** number of inactive lever presses. Values represent the mean (± SEM) of 6–8 subjects/group. Difference from control subjects (vehicle) *$p < .05$. Abbreviations as in Figures 1 and 3.

In the BLA (Figure 7C) and in the BNST (Figure 7E) microinjection of N/OFQ, at doses two times higher than those used in the CeA, did not significantly modify ethanol self-administration [$F(2,19) = .02$, p = ns and $F(2,16) = .21$, p = ns, respectively]. During the post-treatment phase of the BNST study, we observed a trend-level [$F(4,32) = .07$, p = ns] reduction of lever presses after both N/OFQ and vehicle microinfusion. This effect seems to be nonspecific and is presumably related to the microinjection procedure rather than to a drug effect.

Responses at the inactive lever (Figures 7B, 7D, and 7F) were almost absent in all treatment conditions and were not affected by intracerebral injections of the peptide ([$F(2,18) = .63$, p = ns], [$F(2,19) = .09$, p = ns], and [$F(2,16) = .83$, p = ns] in the CeA, BLA, and BNST, respectively).

Discussion

In agreement with previous reports, we show here that subchronic ICV administration of N/OFQ significantly reduces ethanol self-administration in msP rats (9). In contrast, in nonselected Wistar rats tested under the same experimental conditions, N/OFQ did not alter ethanol consumption. These findings parallel recently published data showing that administration of corticotropin-releasing factor (CRF) receptor-1 antagonists reduces ethanol-drinking in msP but not in unselected nondependent Wistar rats (22,28). Several previous studies have demonstrated that N/OFQ acts as a functional antagonist at the extrahypothalamic CRF_1 receptor system (29). For example, central administration of the peptide prevents several behavioral effects of stress, including anorexia elicited by intracranial injection of CRF or footshock-induced reinstatement of alcohol-seeking (29–31).

The msP rats are highly sensitive to stress, show an anxious phenotype, and exhibit depression-like behavioral signs that recover after ethanol-drinking (21,32). Elevated expression and function of the CRF_1 receptor in numerous brain areas has recently been identified as a molecular substrate of both the anxious, stress-sensitive phenotype and the high alcohol preference of msP rats (22). We therefore hypothesize that exogenous administration of N/OFQ, through its ability to reduce CRF_1-mediated activity, might help to reduce the negative affective state of msP rats and therefore lower their excessive ethanol intake by reducing the ethanol negative reinforcement. In contrast, in nonselected Wistar rats, ethanol-drinking is predominantly positively reinforced via activation of the brain reward systems, an action that occurs independently from the brain CRF_1 receptors system.

The in situ hybridization results showed higher expression of N/OFQ and NOP receptor mRNAs in numerous brain areas of msP rats compared with Wistar rats. In particular, we found significantly higher levels of N/OFQ mRNA in the M1, BNST, and CeA of msP rats, whereas NOP receptor transcript was higher in the M1, CeA, and BLA of msP rats. Autoradiographic analysis showed that these gene expression changes were accompanied by significant increases in NOP receptor binding capacity in several brain areas, including the CeA, BNST, ventral tegmental area, and various cortical structures. Furthermore, the results of the [^{35}S]GTPγS assay showed that this upregulation of NOP binding sites was also accompanied by a substantial increase in agonist-induced NOP receptor signaling in almost all the brain areas investigated. On the basis of these findings we propose that upregulation of the N/OFQ/NOP system occurs in msP rats in response to and in an attempt to compensate the up-regulation of CRF_1 receptors in this line of rats (22). This adaptive response might be one of several compensatory functional adaptations that occur in msP rats, such as upregulation of neuropeptide Y and its receptors (22).

Whereas consistent upregulation of NOP receptor expression, binding, and signaling were observed in msP versus Wistar rats in the majority of brain regions examined, a distinct and unexpected pattern of differences was found in the CeA. In this brain region N/OFQ-stimulated [^{35}S]GTPγS binding was significantly lower in msP rats than in Wistar rats despite elevated NOP receptor expression and binding in the msP line. This finding suggests uncoupling of the NOP receptor from its G proteins, leading to lower responsiveness to NOP receptor stimulation in CeA cells of msP rats. This in turn suggests that although the N/OFQ system is generally recruited in msP rats to counteract upregulated CRF activity, uncoupling of the NOP receptor in CeA leads to selective breakdown of this adaptation in CeA. This is of particular interest, because recent data obtained in post-dependent rats indicate that CRF activity in this structure is critical for the regulation of excessive ethanol self-administration (33,34).

This hypothesis predicts that stimulation of NOP receptor

activity in the CeA might help restore CeA functional equilibrium. We found, in agreement with this prediction, that agonist administration into this brain area suppressed ethanol self-administration, at doses lower than those needed to reduce alcohol consumption after ICV administration. Alcohol self-administration was not affected by microinjection of N/OFQ into the BNST or BLA (two areas adjacent and neuroanatomically connected to the CeA), supporting the notion that the CeA is the brain site of action for this effect of N/OFQ.

Ethanol mediates its reinforcing properties through interactions with several neuronal systems (5). Among these, the CeA has recently been identified as a key region (35,36), and γ-aminobutyric acid (GABA)ergic transmission in this brain area seems to play an important role. In fact, microinjection of $GABA_A$ receptor antagonists into the CeA significantly reduces ethanol self-administration in the rat (37,38). In addition, electrophysiological studies show an increased GABAergic transmission in the CeA after ethanol superfusion (39), an effect blocked by N/OFQ pretreatment (10).

Interestingly, within CeA, CRF is expressed in GABAergic neurons, where it is co-released with GABA (40,41) and ethanol-induced increase in GABAergic transmission within the CeA depends on CRF_1 receptor activation (42). To our knowledge no studies have until now examined the interaction among GABA, N/OFQ, and the CRF systems in the CeA. However, on the basis of these indirect data, one might speculate that reduction of ethanol intake after CeA injection of N/OFQ depends on the ability of this neuropeptide to inhibit ethanol-induced GABA release via functional CRF antagonism.

Activation of NOP receptors also results in marked functional anti-opioid activity (43) that might contribute to the effect of N/OFQ on ethanol self-administration. It is known, in fact, that treatment with anti-opioidergic agents significantly reduces alcohol-drinking in rats, and the CeA has been found to be an important site for these effects (44). However, opioid antagonists reduce alcohol consumption not only in msP rats (21,45) but also in nonselected Wistar rats (44,46,47). Given our finding that N/OFQ selectively reduces alcohol self-administration in msP rats, it is unlikely that N/OFQ-induced reduction of ethanol-drinking depends on its anti-opioid activity.

In conclusion, the present findings together with previously published reports suggest that msP rats have an upregulated CRF_1 receptor-mediated activity in the CeA and that the compensatory increase in NOP receptor neurotransmission found in other brain regions is absent or attenuated in this structure, because of uncoupling of receptors from G proteins. We speculate that the imbalance between these "stress" (CRF) and "anti-stress" (N/OFQ) systems might play a causal role in sustaining excessive ethanol-drinking in msP rats. Restoration of the stress response set point, either by administration of CRF_1 receptor antagonists or NOP receptor agonist, might re-establish equilibrium in CeA function, thereby reducing ethanol-drinking in msP rats.

Lastly, the results of the present investigation support that agonism at NOP receptors or antagonism at CRF_1 receptors might both be attractive pharmacotherapeutic strategies for alcohol addiction treatment. The combined anti-CRF and anti-opioid actions of N/OFQ might be synergistic and thus particularly attractive. These findings provide a rationale for evaluating N/OFQ agonism as a mechanism to treat alcohol dependence.

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Supplementary material cited in this article is available online.

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