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Nociceptin/Orphanin FQ Increases Anxiety-Related Behavior and Circulating Levels of Corticosterone During Neophobic Tests of Anxiety

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Intracranial administration of nociceptin/orphanin FQ (N/OFQ) increases circulating concentrations of adrenocorticotrophic hormone and corticosterone in unstressed rats, and elevates the responsiveness of these hormones during mild stress. Furthermore, N/OFQ and its cognate receptor are both abundant in a variety of limbic nuclei, and stress exposure decreases neuronal N/OFQ content in forebrain neurons. In light of these and other findings, we examined the potential involvement of N/OFQ in regulation of anxiety-related behaviors in rats. In the open field, elevated plus maze, and dark-light neophobic tests, intracerebroventricular N/OFQ (1.0 pmole–1.0 nmole) increased the expression of anxiety-related behaviors. Specifically, N/OFQ increased the latency to enter, decreased the number of entries into, and decreased the time spent in the exposed or brightly lit environments of all three tests. N/OFQ also enhanced thigmotactic responses in the open field test. The effects of diazepam and of the benzodiazepine inverse agonist FG 7142 were also assessed in independent groups of rats. In all three tests, the behavioral effects of N/OFQ resembled the anxiogenic actions of FG 7142, and contrasted with the anxiolytic actions of diazepam. N/OFQ administration also increased circulating concentrations of corticosterone during anxiety testing, in comparison with the concentrations in vehicle-treated controls. We conclude that N/OFQ administration is anxiogenic, and elevates responsiveness of the hypothalamic pituitary-adrenal axis during neophobic tests of anxiety. This supports the possibility that N/OFQ neurotransmission participates in processing of emotionally-salient and stressful stimuli, and suggests that normal functioning of the N/OFQ system may be important in physiological and psychological well-being.
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INTRODUCTION

Nociceptin/orphanin FQ (N/OFQ) is an opioid-related 17-amino acid peptide (Meunier *et al*, 1995; Reinscheid *et al*, 1995) that binds saturably and with high affinity to the NOP receptor (formerly known as ORL1 or LC132), but does not bind to the mu-, delta-, and kappa-opioid receptor types. The NOP receptor is a member of the super-family of seven transmembrane, Gi protein-coupled receptors. It is negatively linked to adenylate cyclase, inhibits N-type Ca^{2+} channels, and activates inward rectifying K^{+} channels (Bunzow *et al*, 1994; Chen *et al*, 1994; Fukuda *et al*, 1994; Mollereau *et al*, 1994; Wick *et al*, 1994; Lachowicz *et al*, 1995; Connor *et al*, 1996a, b; Vaughan and Christie, 1996). NOP exhibits a high degree of amino acid sequence homology with the cloned opioid receptor types, but it does not selectively bind prototypical opioid agonists or antagonists (Bunzow *et al*, 1994; Chen *et al*, 1994; Fukuda *et al*, 1994; Mollereau *et al*, 1994; Wick *et al*, 1994; Lachowicz *et al*, 1995). Accordingly, N/OFQ and NOP represent an opioid-related neurotransmitter system that has the potential to mediate physiological and behavioral actions that are distinct from those that are mediated by the opioid system.

N/OFQ and NOP each exhibit widespread distribution in the central nervous system (Bunzow *et al*, 1994; Mollereau *et al*, 1994; Lachowicz *et al*, 1995; Neal *et al*, 1999a, b), and the distribution reflects the functional diversity of this neuropeptide system. N/OFQ administration generally inhibits locomotor activity (Reinscheid *et al*, 1995; Devine *et al*, 1996b; Rizzi *et al*, 2001a) after intracerebroventricular (i.c.v.) administration of higher doses (1–10 nmoles), and it has been reported to increase locomotion after i.c.v. administration of very low doses (0.005–0.05 nmoles; Florin *et al*, 1996). N/OFQ alters responses to nociceptive stimuli (Meunier *et al*, 1995; Reinscheid *et al*, 1995; Grisel *et al*,

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1996; Erb *et al*, 1997; Hao *et al*, 1997; Helyes *et al*, 1997; King *et al*, 1997; Morgan *et al*, 1997; Tian *et al*, 1997; Yamamoto *et al*, 1997; Zhu *et al*, 1997). It appears not to exert any specific motivational actions in tests of place conditioning, when administered alone (Devine *et al*, 1996a; Ciccocioppo *et al*, 2000; Le Pen *et al*, 2002), but it stimulates feeding behavior (Pomonis *et al*, 1996; Stratford *et al*, 1997; Polidori *et al*, 2000; Ciccocioppo *et al*, 2002a), blocks morphine place conditioning (Murphy *et al*, 1999; Ciccocioppo *et al*, 2000), and interferes with the behaviorally-reinforcing effects of ethanol (Ciccocioppo *et al*, 1999, 2002b; Martin-Fardon *et al*, 2000). Furthermore, N/OFQ administration appears to interfere with learning and memory functions (Sandin *et al*, 1997; Hiramatsu and Inoue, 1999; Higgins *et al*, 2002), and it may alter sensory processing (Wang *et al*, 1996).

N/OFQ and NOP are abundantly expressed in a variety of limbic and limbic-associated brain structures (Bunzow *et al*, 1994; Fukuda *et al*, 1994; Mollereau *et al*, 1994; Wick *et al*, 1994; Lachowicz *et al*, 1995; Florin *et al*, 1997; Foddi and Mennini, 1997; Neal *et al*, 1999a, b) that are involved in the processing of emotionally-relevant stimuli. This raised the possibility that N/OFQ might contribute to regulation of the hypothalamic-pituitary-adrenal (HPA) axis, and to regulation of emotional states. In fact, i.c.v. administration of N/OFQ increases the circulating concentrations of adrenocorticotrophic hormone (ACTH) and corticosterone (CORT) in unstressed rats, and augments these hormonal responses in mildly stressed rats (Devine *et al*, 2001). Furthermore, acute stress exposure decreases N/OFQ content in forebrain neurons, implicating endogenous N/OFQ neurotransmission in physiological stress responses (Devine *et al*, 2002). In addition, a preproN/OFQ knockout mouse expresses high levels of anxiety-related behavior in neophobic tests (Koster *et al*, 1999), suggesting that an intact N/OFQ system is necessary for normal anxiety responses.

In our investigations of the interactions between N/OFQ and anxiety, we have focused on three neophobic tests of anxiety (the open field, elevated plus maze, and dark-light tests). These tests take advantage of two innate characteristics of rodents. Rodents are foraging animals that tend to explore novel environments, but they also tend to avoid exposed and brightly-lit areas in which they may be vulnerable to predation. Rats and mice exhibit thigmotaxis (exploration that is largely restricted to the periphery) when placed in an open field (Hughes, 1972). They preferentially explore the enclosed arms of an elevated maze (Pellow *et al*, 1985) when presented with a choice between enclosed and open arms, and they will spend more time in the dark side of a shuttle box in which one side is dark, and one side is brightly illuminated (Crawley and Goodwin, 1980). These behaviors are altered by administration of drugs that humans report are anxiolytic (eg diazepam; Barrett and DiMascio, 1966; McDowall *et al*, 1966; Zbinden and Randall, 1967) or anxiogenic (eg FG 7142; Dorow, 1987), and so these behaviors have been widely used in assays to evaluate the anxiety-modulating effects of drugs and other manipulations. Anxiolytic drugs increase, and anxiogenic drugs decrease exploration of the open arms of the elevated plus maze (Handley and Mithani, 1984; Pellow and File, 1986), the central region of an open field (Hughes, 1972; Stefanski *et al*, 1992; Plaznik *et al*, 1994; Simon *et al*, 1994), and the lighted compartment of a dark-light shuttle box (Crawley and Goodwin, 1980; Costall *et al*, 1989; Onaivi and Martin, 1989; Chaouloff *et al*, 1997). We now report that i.c.v. administration of N/OFQ (0.001–1.0 nmole) increases the expression of anxiety-related behaviors and plasma concentrations of CORT in rats during exposure to the three neophobic tests of anxiety. The behavioral findings conflict with previous reports that N/OFQ and an N/OFQ analogue (Ro64-6198) are anxiolytic in a variety of assays (Jenck *et al*, 1997, 2000; Griebel *et al*, 1999; Wichmann *et al*, 2000; Dautzenberg *et al*, 2001; Gavioli *et al*, 2002). The reasons for these contradictory results are discussed.

EXPERIMENTAL PROCEDURES

Animals and Surgery

Two hundred and twelve male Long-Evans rats (Charles River, Raleigh NC), weighing 250–300 g, were housed in pairs in 43 cm × 21.5 cm × 25.5 cm plexiglas cages and kept on a 12 h/12 h light-dark cycle (lights on 0700 h). Food and water were available *ad libitum*. The temperature ($22 \pm 2^{\circ}C$) and relative humidity (55–60%) were controlled. The rats were acclimated to these controlled housing conditions for at least one week.

After acclimation to the housing room, 112 of the rats were implanted under ketamine: xylazine anesthesia (83.3 mg ketamine/ml; 16.7 mg xylazine/ml; 0.75 ml/kg) with chronic stainless steel guide cannula (22 gauge) fastened to the skull by dental cement and microscrews, and terminating 0.5 mm above the lateral ventricle in the right hemisphere (0.8 mm posterior to bregma, 1.4 mm lateral to the midsagittal suture, 2.7 mm ventral to dura). Stainless steel obturators (28-gauge) extending 1.1 mm beyond the guide cannula tip were put in place at the time of surgery and removed at the time of drug injection. All guide cannulae, obturators, and injectors were purchased from Plastics One (Roanoke, VA). The rats were housed individually after surgery and allowed 7–10 days recovery before anxiety testing.

All experimental procedures were pre-approved by the University of Florida Institutional Animal Care and Use Committee (IACUC) and were conducted in compliance with the NIH Guide for the Care and Use of Laboratory Animals.

Drugs and Injections

Diazepam was purchased from Elkins Sinn Inc. (Cherry Hill, NJ), dissolved in 40% propylene glycol (PG; v/v) and administered by intramuscular (i.m.) injection (0 or 2.0 mg/kg) in a volume of 1.0 ml/kg. The benzodiazepine inverse agonist FG 7142 (Dorow *et al*, 1983; Petersen and Jensen, 1984; Stephens *et al*, 1984) was purchased from Sigma-Aldrich Inc. (St. Louis, MO), dissolved in 1.0% carboxymethylcellulose (CMC; w/v) and administered by intraperitoneal (i.p.) injection (0 or 10 mg/kg) in a volume of 1.0 ml/kg.

N/OFQ was purchased from Sigma-Aldrich Inc. The peptide was dissolved in artificial cerebrospinal fluid (aCSF), composed of: 2.0 mM Sorensen's phosphate buffer at pH 7.4, containing Na^+ 145 mM; K^+ 2.7 mM; Mg^{2+}

1.0 mM; Ca^{2+} 1.2 mM; Cl^{-} 150 mM; ascorbate 0.2 mM (Moghaddam and Bunney, 1989). N/OFQ microinjections (0, 0.001, 0.01, 0.10, or 1.00 nmole in 1.0 μl aCSF) were delivered by i.c.v. microinjection over 2.0 min, using a Hamilton microsyringe driven by a syringe pump. The injectors (that extended 1.0 mm beyond the guide cannula) were kept in place an additional 3.0 min to allow for drug diffusion. The rats were freely moving during drug administration.

Experiment 1: The Open Field

The open field consisted of a 90 cm × 90 cm square black acrylic arena with an adjoining start box (20 cm × 30 cm) that was separated from the open arena by a guillotine door. The walls of the arena and the start box were 60 cm high. Both the open field and the start box had open tops. The door (20 cm × 20 cm) was opened from outside the testing room by a rope and pulley system. Illumination of the open field arena and the start box were approximately equal (30–50 lux).

Open field behavior was recorded using a video camera mounted to the ceiling. The videotapes were scored by trained observers who were blind to the treatment conditions. The video image of the open field arena was partitioned (ie grid placed over television screen) into 25 equal-size squares—16 peripheral or outer zone squares and 9 inner zone squares. Latency to enter the open field, latency to enter the inner zone, time spent in the open field and inner zone, and entries to both areas were scored. An entry into the open field or inner zone was scored when the rat placed all four paws in the respective area. Time spent in the open field or inner zone began at this point and ended when the rat had completely left the region. Inter-observer reliability was 95.8–100% for the various behavioral measures.

After at least 7 days acclimation to the housing rooms, 32 of the rats were prepared for testing in the open field. These rats did not undergo surgery, and they were tested with i.m. injections of diazepam (2.0 mg/kg) or i.p. injections of FG 7142 (10 mg/kg), or the respective vehicle solutions ($n = 8$ rats/group). The rats were handled on each of three consecutive days, and allowed one day free of handling before testing in the open field. All open field tests were conducted in the morning, between 9:00 and 12:00. Each rat was injected by an experimenter who was blind to drug treatment, and immediately placed into a holding cage that was identical to its home cage, where it remained for the next 30 min to allow time for drug absorption. Then, each rat was individually placed into the open field start box. The door separating the start box from the open field arena was opened after a 60 s acclimation period, and the rat then had free access to the entire apparatus for 5 min, before being returned to the holding cage. Each rat was sacrificed by decapitation 60 min after the drug injection.

Twenty eight of the surgically-cannulated rats were tested with i.c.v. injections of N/OFQ (0.001, 0.01, 0.1, or 1.0 nmole) or aCSF vehicle ($n = 5$–6 rats/group). After the 7–10 days recovery from surgery, each rat was handled in the same manner as the diazepam- and FG 7142-treated rats were. Once again, the open field tests were all conducted between 9:00 and 12:00. Five minutes after the start of the N/OFQ or aCSF injection, each rat was placed in the start box, acclimated for 60 s, and then allowed 5 min free access to the apparatus, before being returned to its home cage. Each rat was rapidly decapitated 30 min after the drug microinjection.

When the rats in each treatment group (diazepam, FG 7142, N/OFQ, and all vehicles) were decapitated, trunk blood (6 ml) was collected in polyethylene tubes on ice, containing 600 μl Na_2EDTA at 20 μg/μl. All blood samples were centrifuged at 1000 × gravity for 5 min. Plasma was aliquotted and stored at −80°C for later quantification of CORT concentrations, using radioimmunoassay (RIA). The CORT RIAs were performed with kits from Diagnostic Products Corp. (Los Angeles, CA). The brains were removed and frozen in 2-methyl butane at −40°C. They were stored at −80°C, and later sectioned at 30 μm in the coronal plane for verification of the cannula placements. The adrenal and thymus glands were removed, stored at −80°C, and weighed.

Experiment 2: The Elevated Plus Maze

The elevated plus maze was constructed of black acrylic, and consisted of two opposed open and two closed arms (45 cm × 12 cm each) adjoined by a common central platform (12 cm × 12 cm). The maze was elevated 90 cm above the floor and illuminated by a light bulb above the central platform. The closed arms were enclosed by 45 cm high walls, and the open arms were surrounded by 0.6 cm ledges. Illumination of the open and closed arms, and the central platform was approximately equal (35–70 lux).

Elevated plus maze trials were recorded with a video camera mounted to the ceiling, and the videotapes were scored by trained observers who were blind to the treatment conditions. Latency to first enter the open arms, time spent in the open arms, and entries made to the open arms were scored. Entries to the closed arms were also scored (as a measure of locomotor activity; Pellow *et al*, 1985). An arm entry was scored when the rat placed four paws onto the arm. Open arm time began when the rat placed all four paws onto an open arm, and concluded when the rat placed all four paws onto a closed arm. Inter-observer reliability was 97.4–100% for the various behavioral measures.

The rats were acclimated to the housing room, recovered from surgery (if applicable), and were handled three times prior to the test day in the same manner as in the open field experiment. The elevated plus maze tests were also conducted between 9:00 and 12:00, at which time each rat was injected with diazepam (0 or 2.0 mg/kg i.m.; $n = 10$ rats/group), FG 7142 (0 or 10 mg/kg i.p.; $n = 8$ rats/group) or N/OFQ (0, 0.01, 0.10, or 1.00 nmole, i.c.v.; $n = 9$–10 rats/group). Once again, the experimenter was blind to the treatment conditions. Thirty minutes after the systemic injections of diazepam, FG 7142, or vehicle, or five minutes after the start of the i.c.v. injections of N/OFQ or aCSF vehicle, each rat was placed in the center of the elevated plus maze facing a closed arm, and videotaped for 5 min. Each rat was then returned to its home cage. The rats were subsequently sacrificed. The brains were collected, frozen, and sectioned at 30 μm to verify the cannula placements.

Experiment 3: The Dark-Light Test

The dark-light test was conducted in automated shuttle boxes (Gemini II, San Diego Instruments, San Diego, CA) that were contained within sound-attenuating enclosures. The shuttle box compartments (25 cm × 20 cm × 17 cm) were distinguished only by wall color and illumination. The dark compartment had black walls, whereas the lit compartment had white walls. An automated guillotine door (9 cm × 6 cm) separated the two compartments. The dark compartment (4 lux) was not illuminated. The lit compartment (1000 lux) was illuminated by a light in the ceiling of the compartment.

The location of each rat was monitored with photocells and scored by software using a computer that was interfaced with the shuttle boxes. The photocells were located every 3 cm along the walls of the shuttle box, starting 6 cm from the door. Crossing from one compartment to the other was scored whenever the rat emerged far enough from one side to interrupt the first photocell beam while no longer interrupting the photocell beams in the original compartment. Latency to first enter the lit compartment, time spent in the lit compartment, and entries made to the lit compartment were recorded.

The rats were acclimated to the housing room, recovered from surgery (if applicable), and were handled in the same manner as in the open field and plus maze experiments. All testing was completed between 9:00 and 12:00. Each rat was injected with diazepam (0 or 2.0 mg/kg i.m.; $n = 8$ rats/group), FG 7142 (0 or 10 mg/kg i.p.; $n = 8$ rats/group) or N/OFQ (0, 0.01, 0.10, or 1.00 nmole, i.c.v.; $n = 6$ rats/group) by an experimenter who was blind to the treatment conditions. Thirty minutes after the systemic injections of diazepam, FG 7142, or vehicle, or five minutes after the start of the i.c.v. injections of N/OFQ or aCSF vehicle, each rat was placed in the dark compartment of the shuttle box apparatus facing the wall opposite the automatic guillotine door. The door separating the shuttle box compartments opened after a 60 s acclimation period. Each rat's location was then monitored for 5 min by the photocell apparatus. At the end of the test, each rat was returned to its home cage. The rats were subsequently sacrificed. The brains were collected, frozen, and sectioned at 30 μm to verify the cannula placements.

Experiment 4: The 'Dark-Dark' Test

An additional experiment was conducted to evaluate the potential impact that the locomotor-inhibiting actions of N/OFQ (Reinscheid *et al*, 1995; Devine *et al*, 1996b; Rizzi *et al*, 2001a) could have had on anxiety-related behavior. In this experiment, independent groups of rats were tested in the standard dark-light test, or in a modified 'dark-dark' shuttle box. In this modified shuttle box, the white walls were removed so that both compartments had identical black walls, and the lights were left off in both compartments. The rats were handled and injected with N/OFQ (0 or 0.10 nmole i.c.v.) as in the previous experiment, and placed into the left compartment (which was dark in both the dark-light, and dark-dark tests). Crossings from the left compartment to the right compartment were scored in the same manner as crossings from dark to light were previously scored. These rats were sacrificed and the brains were sectioned to verify the cannula placements.

Statistics

In the open field experiment, differences between the diazepam-treated and PG vehicle-treated rats were evaluated using *T*-tests for each behavioral measure (latencies, times, entries), and for the plasma CORT concentrations. Similarly, group differences were evaluated with *T*-tests for the FG 7142- and CMC vehicle-treated groups. Between-groups differences for the rats treated with aCSF and the four doses of N/OFQ were evaluated for each behavioral measure and for the CORT concentrations using one-way analyses of variance (ANOVAs). Significant effects ($p < 0.05$) were further evaluated with Student Newman Keuls post-tests comparing scores for each of the N/OFQ-treated groups with corresponding scores for the aCSF vehicle-treated group. Adrenal and thymus weights were each compared between all groups with one-way ANOVAs to evaluate whether the stress of surgical cannulation and recovery produced any significant changes in morphological indices of HPA axis function.

In the elevated plus maze and dark-light experiments, the effects of diazepam and of FG 7142 on behavioral measures were each evaluated with *T*-tests. In the elevated plus maze and dark-light experiments, between-groups differences among the aCSF- and N/OFQ-treated groups were evaluated with one-way ANOVAs for each of the behavioral measures. Significant effects were further evaluated with Student Newman Keuls post-tests.

In the 'dark-dark' experiment, the behaviors of the vehicle- and N/OFQ-treated rats that were exposed to the dark-light test, and the behaviors of the vehicle- and N/OFQ-treated rats that were exposed to the dark-dark test were each evaluated with *T*-tests.

RESULTS

Experiment 1: The Open Field

The diazepam-treated rats exhibited significantly shorter latencies to enter the open field from the start box ($T_{(14)} = 1.977$, $p < 0.05$; Figure 1a), and significantly greater time in the open field ($T_{(14)} = 2.684$, $p < 0.01$; Figure 1b), than did the PG-treated controls. The number of entries into the open field did not differ between these diazepam- and vehicle-treated groups ($T_{(14)} = 0.3247$, $p > 0.05$; Figure 1c). The diazepam-treated rats also exhibited significantly shorter latencies to enter the inner zone of the open field ($T_{(14)} = 3.814$, $p < 0.001$; Figure 1d), and significantly more time in the inner zone ($T_{(14)} = 1.812$, $p < 0.05$; Figure 1e) than did the vehicle-treated controls. The diazepam-treated rats seemed to exhibit more entries into the inner zone, but this did not reach statistical significance ($T_{(14)} = 1.682$, $p = 0.0574$; Figure 1f).

The FG 7142-treated rats exhibited significantly longer latencies to enter the open field from the start box ($T_{(14)} = 6.760$, $p < 0.0001$; Figure 1a), and significantly less time in the open field ($T_{(14)} = 6.574$, $p < 0.0001$; Figure 1b), than did the CMC-treated controls. The FG 7142-treated rats also exhibited significantly fewer entries into the open field

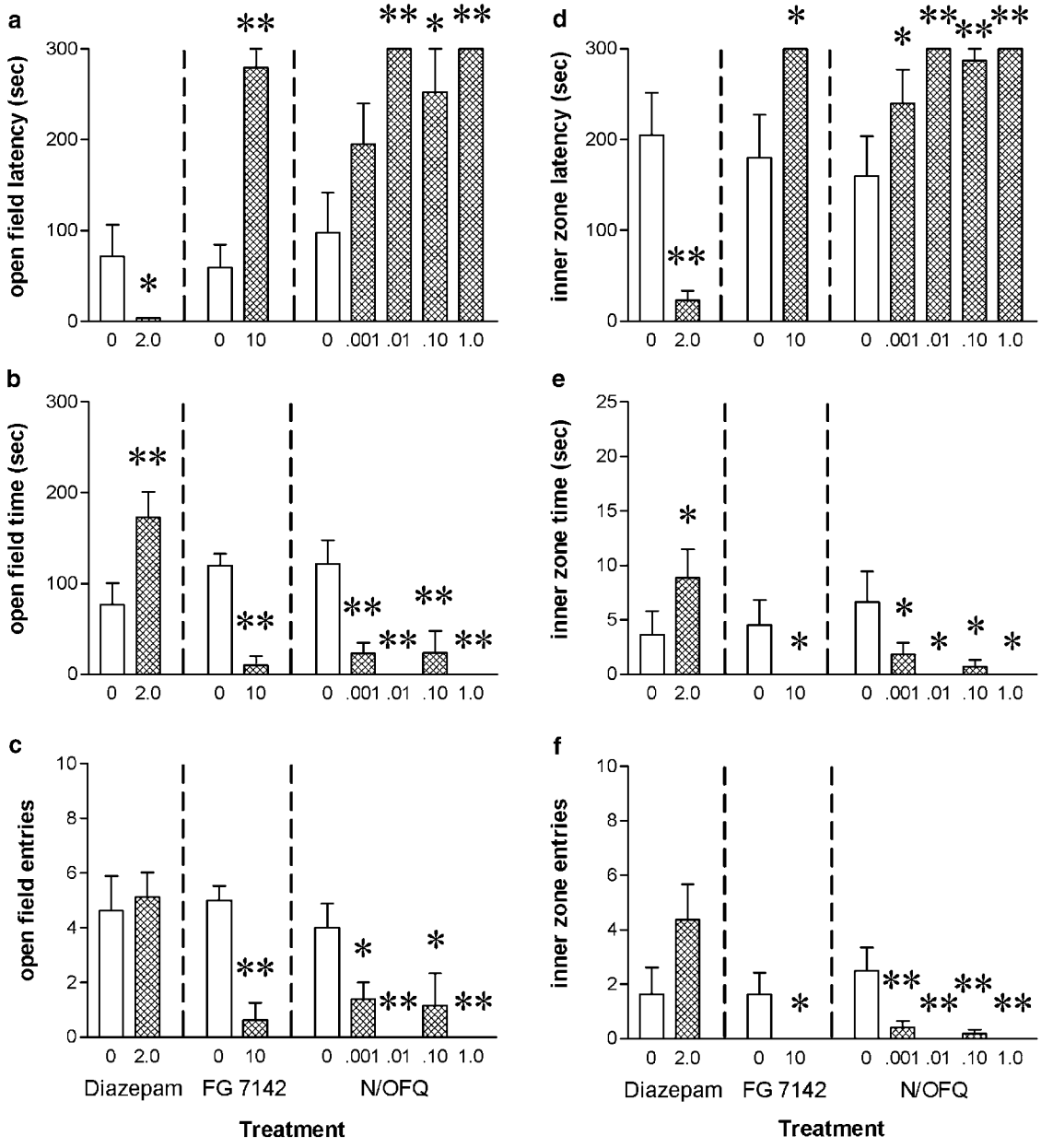


Figure 1 Experiment 1, The Open Field Test: Diazepam administration decreased the expression of anxiety-related behaviors, whereas FG 7142 increased the expression of these behaviors in the open field test. Specifically, diazepam decreased the latency to enter (a) the open field, and (d) the inner zone of the open field. Diazepam increased the time spent in (b) the open field, and (e) the inner zone of the open field. FG 7142 increased the latency to enter (a) the open field, and (d) the inner zone. FG 7142 decreased the time spent in (b) the open field and (e) the inner zone, and FG 7142 decreased the total entries into (c) the open field, and (f) the inner zone. N/OFQ administration increased the expression of anxiety-related behaviors in the same manner as FG 7142 did. The N/OFQ treated rats exhibited significantly longer latencies to enter (a) the open field and (d) the inner zone of the open field than did the aCSF-treated controls. These N/OFQ-treated rats spent significantly less time in (b) the open field, and (e) the inner zone, and they made fewer entries to (c) the open field and (f) the inner zone, than did the aCSF-treated rats. In fact, at some doses of FG 7142 and N/OFQ, all the rats failed to enter the open field or the inner zone, and so the latencies are 300 s and the standard errors are zero at these doses. Values expressed are group means ± the standard error of the mean (SEM) ($n = 8$ rats per group for diazepam and FG 7142, $n = 5$-6 rats per group for N/OFQ). Significant differences between drug-treated rats and the appropriate vehicle-treated (propylene glycol, carboxymethylcellulose, or artificial CSF) controls (*T*-tests for diazepam and FG 7142; Newman-Keuls tests for N/OFQ) are depicted as follows: $*p < 0.05$, $**p < 0.01$.

than did the vehicle-treated controls ($T_{(14)} = 5.320$, $p > 0.0001$; Figure 1c). The FG 7142-treated rats exhibited significantly longer latencies to enter the inner zone of the open field ($T_{(14)} = 2.547$, $p < 0.05$; Figure 1d), and significantly less time in the inner zone ($T_{(14)} = 1.900$, $p < 0.05$; Figure 1e) than did the vehicle-treated controls. The FG 7142-treated rats also exhibited fewer entries into the inner zone, than did the vehicle-treated controls ($T_{(14)} = 2.030$, $p < 0.05$; Figure 1f).

The rats that were treated with N/OFQ (0.01–1.00 nmole) exhibited significantly longer latencies to enter the open field than did the aCSF vehicle-injected controls ($F_{(4,23)} = 5.738$, $p < 0.01$; Figure 1a), and these rats exhibited significantly less time in the open field at all doses tested

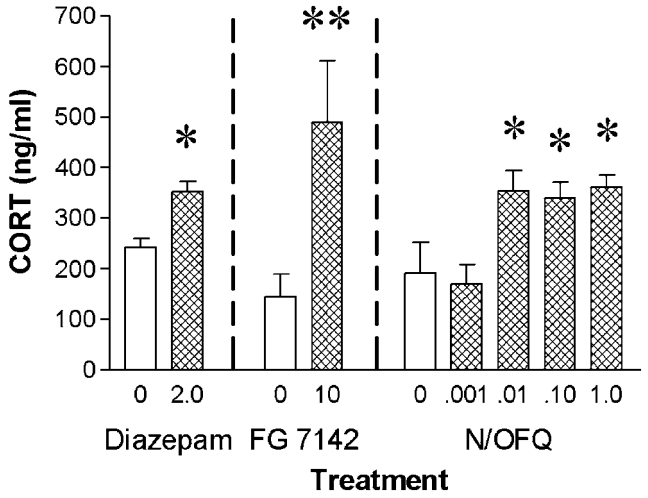


Figure 2 Experiment 1, The Open Field Test: Diazepam and FG 7142 administration both increased the open field-induced elevations in plasma CORT concentrations. Administration of N/OFQ augmented the open field-induced elevations in plasma CORT, when these concentrations were assayed 30 min after the i.c.v. injection. Values expressed are group means ± the standard error of the mean (SEM) ($n = 8$ rats per group for diazepam and FG 7142, $n = 5$–6 rats per group for N/OFQ). Significant differences between drug-treated rats and the appropriate vehicle-treated controls (*T*-tests for diazepam and FG 7142; Newman-Keuls tests for N/OFQ) are depicted as follows: $^{*}p < 0.05$, $^{**}p < 0.01$.

(0.001–1.00 nmole) when compared with the aCSF-treated controls ($F_{(4,23)} = 8.558$, $p < 0.001$; Figure 1b). All the rats that were treated with N/OFQ also exhibited significantly fewer entries to the open field than did the vehicle-treated rats ($F_{(4,23)} = 4.981$, $p < 0.01$; Figure 1c).

The N/OFQ-treated rats also exhibited significantly longer latencies to enter the inner zone of the open field ($F_{(4,23)} = 5.323$, $p < 0.001$; Figure 1d), and spent less time in the inner zone ($F_{(4,23)} = 3.952$, $p < 0.051$; Figure 1e) than did the aCSF-treated controls. The N/OFQ-treated rats also exhibited fewer entries to the inner zone ($F_{(4,23)} = 6.571$, $p < 0.01$; Figure 1f) than did the aCSF vehicle-treated controls at all doses tested.

The adrenal ($F_{(8,51)} = 1.991$, $p > 0.05$) and thymus ($F_{(8,51)} = 1.735$, $p > 0.05$) gland weights did not differ between any of the groups in the open field tests (data not shown), indicating that the surgical preparation of the rats did not produce substantial alterations in HPA axis functioning. Plasma CORT concentrations were significantly elevated in the diazepam-treated ($T_{(14)} = 2.047$, $p < 0.05$), FG 7142-treated ($T_{(14)} = 3.226$, $p > 0.01$), and N/OFQ-treated ($F_{(4,23)} = 5.158$, $p < 0.01$) groups when these concentrations were compared with the concentrations in the appropriate vehicle-treated controls (Figure 2).

Experiment 2: The Elevated Plus Maze

The diazepam-treated rats exhibited significantly shorter latencies to enter the open arms of the elevated plus maze ($T_{(18)} = 1.832$, $p < 0.05$; Figure 3a), and significantly more time in the open arms ($T_{(18)} = 2.487$, $p < 0.05$; Figure 3b), than did the PG-treated controls. The diazepam-treated rats also exhibited significantly more entries into the open arms than did the controls ($T_{(18)} = 3.585$, $p < 0.01$; Figure 3c). The number of entries into the closed arms did not differ between these diazepam- and vehicle-treated groups ($T_{(18)} = 0.1451$, $p > 0.05$; Figure 1d).

The FG 7142-treated rats exhibited significantly longer latencies to enter the open arms of the elevated plus maze ($T_{(14)} = 2.739$, $p < 0.01$; Figure 3a), and significantly less time on the open arms ($T_{(14)} = 4.605$, $p < 0.001$; Figure 3b), than did the CMC-treated controls. The FG 7142-treated rats also exhibited significantly fewer entries into the open arms than did the vehicle-treated controls ($T_{(14)} = 4.209$, $p > 0.001$; Figure 3c), and the number of entries into the closed arms did not differ between the FG 7142- and vehicle-treated groups ($T_{(18)} = 1.151$, $p > 0.05$; Figure 3d).

The rats that were treated with N/OFQ exhibited significantly longer latencies to enter the open arms of the elevated plus maze than did the aCSF vehicle-injected controls ($F_{(3,32)} = 4.211$, $p < 0.05$; Figure 3a), and these rats exhibited significantly less time in the open arms than did the aCSF-treated controls ($F_{(3,32)} = 3.010$, $p < 0.05$; Figure 3b). The rats that were treated with the highest dose of N/OFQ (1.0 nmole) also exhibited significantly fewer entries to the open arms ($F_{(3,32)} = 3.346$, $p < 0.05$; Figure 3c) and to the closed arms ($F_{(3,32)} = 7.497$, $p < 0.001$; Figure 3d) than did the vehicle-treated rats.

Experiment 3: The Dark-Light Test

The diazepam-treated rats exhibited significantly shorter latencies to enter the lit side of the dark-light test ($T_{(14)} = 2.414$, $p < 0.05$; Figure 4a), and significantly more time in the lit side ($T_{(14)} = 2.646$, $p < 0.01$; Figure 4b), than did the PG vehicle-treated controls. The number of entries into the lit compartment did not differ between the diazepam- and vehicle-treated groups ($T_{(14)} = 1.722$, $p > 0.05$; Figure 4c).

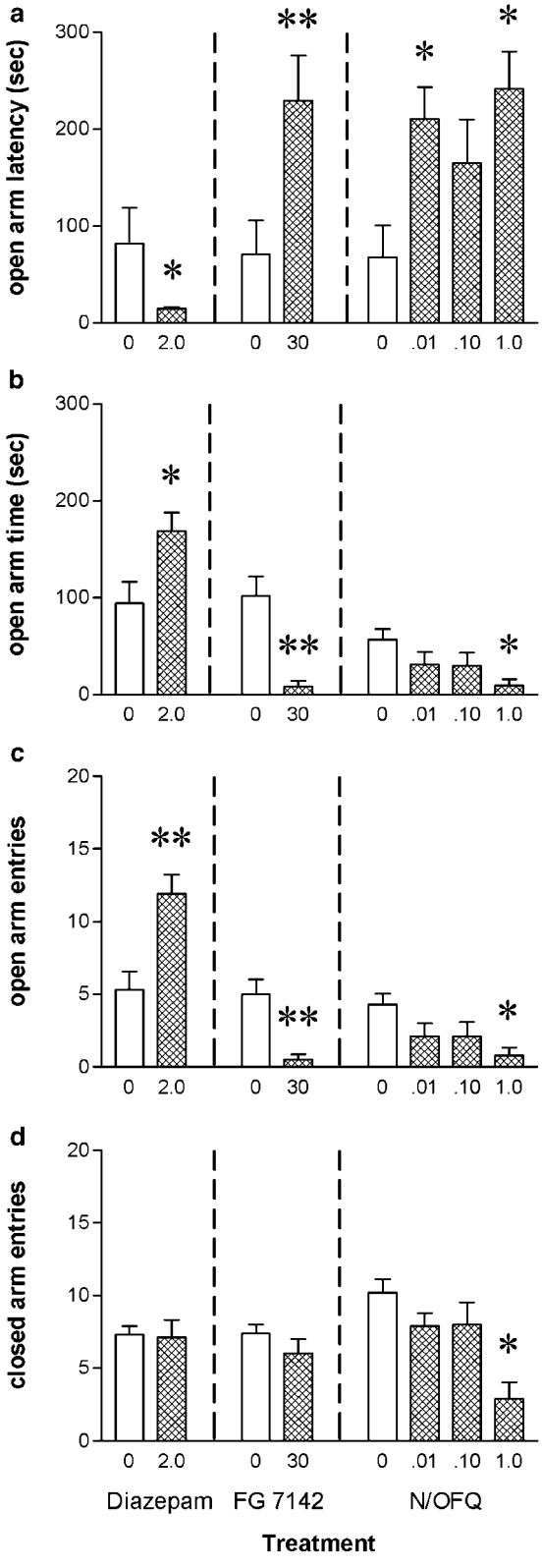
The FG 7142-treated rats exhibited significantly longer latencies to enter the lit side of the dark-light test ($T_{(14)} = 2.819$, $p < 0.01$; Figure 4a), and significantly less time on the lit side ($T_{(14)} = 1.945$, $p < 0.05$; Figure 4b), than did the CMC-treated controls. The number of entries into the lit compartment did not differ between the FG 7142- and CMC-treated groups ($T_{(14)} = 0.5407$, $p > 0.05$; Figure 4c).

The rats that were treated with N/OFQ exhibited significantly longer latencies to enter the lit side of the dark-light test than did the aCSF vehicle-injected controls ($F_{(3,20)} = 6.097$, $p < 0.01$; Figure 4a), and these rats exhibited significantly less time in the lit side than did the aCSF-treated controls ($F_{(3,20)} = 9.092$, $p < 0.001$; Figure 4b). The rats that were treated with N/OFQ also exhibited significantly fewer entries to the lit side ($F_{(3,20)} = 16.487$, $p < 0.0001$; Figure 4c) than did the vehicle-treated rats.

Experiment 4: The Dark-Dark Test

When rats were tested in the dark-dark apparatus (initially placed in the left side of the shuttle-box), there were no significant differences between the aCSF- and N/OFQ-treated rats in latencies to enter the right (dark) side ($T_{(10)} = 1.090$, $p > 0.05$; Figure 5a), time spent in the right side ($T_{(10)} = 1.180$, $p > 0.05$; Figure 5b), or entries to the right side ($T_{(10)} = 1.010$, $p > 0.05$; Figure 5c).

The rats that were treated with N/OFQ and tested in the dark-light test exhibited significantly longer latencies to enter the right (lit) side of the shuttle box than did the aCSF vehicle-injected controls ($T_{(10)} = 2.482$, $p < 0.05$; Figure 5a). These N/OFQ-treated rats also exhibited significantly less time in the right side ($T_{(10)} = 5.053$, $p < 0.001$; Figure 5b), and significantly fewer entries to the right side ($T_{(10)} = 2.894$, $p < 0.01$; Figure 5c) than did the aCSF vehicle-treated rats.



DISCUSSION

Our results with diazepam and FG 7142 concur with previous reports (Crawley and Goodwin, 1980; Pellow and File, 1986; Costall *et al*, 1989; Onaivi and Martin, 1989; Stefanski *et al*, 1992; Simon *et al*, 1994; Chaouloff *et al*, 1997), indicating that diazepam is anxiolytic and FG 7142 is anxiogenic in the open field, elevated plus maze, and dark-light neophobic tests of anxiety. Furthermore, the data from the diazepam and FG 7142 experiments reveal that our experimental conditions are appropriate to characterize both anxiogenic and anxiolytic actions of pharmacological agents under a single standard set of conditions (ie housing, handling, lighting, etc.). These conditions were established and validated in each test so that the vehicle-treated rats spend approximately 20–30% of the session in the open field, or on the open arms, or in the lit compartment of the test. Under these conditions, anxiolytic drugs like diazepam reliably increase the time spent in the anxiety-provoking environments (up to a ceiling of approximately 50%, a value at which there is no anxiety-based preference for either environment), and anxiogenic drugs like FG 7142 reliably decrease time spent in these anxiety-provoking environments. We believe it is important to conduct these tests in this manner so that anxiety-increasing or anxiety-decreasing actions of uncharacterized molecules can be identified without bias from the testing situation (Fernandez *et al*, 2002).

The rats that received i.c.v. injections of N/OFQ expressed more anxiety-related behaviors than did the vehicle-treated controls in each of the three tests of anxiety. Specifically, the N/OFQ-treated rats exhibited pronounced thigmotaxis in the open field (ie avoidance of the inner zone). These rats exhibited longer latencies to enter the open field and its inner zone, the open arms of the elevated plus maze, and the lit compartment of the dark-light test. They spent less time in these exposed and brightly-illuminated areas, and they

Figure 3 Experiment 2, The Elevated Plus Maze Test: Diazepam administration decreased the expression of anxiety-related behaviors, whereas FG 7142 increased the expression of these behaviors in the elevated plus maze. Diazepam (a) decreased the latency to enter the open arms, (b) increased the time spent in the open arms, and (c) increased the number of entries to the open arms, but (d) did not affect the number of entries to the closed arms. FG 7142 (a) increased the latency to enter the open arms, (b) decreased the time spent in the open arms, and (c) decreased the number of entries to the open arms, but (d) did not affect the number of entries to the closed arms. N/OFQ-treated rats exhibited significantly more anxiety-related behaviors than did the aCSF vehicle-treated controls. N/OFQ-treated rats (a) exhibited longer latency to enter the open arms, (b) shorter time on the open arms, and (c) fewer open arm entries. The rats that were treated with the highest dose of N/OFQ also exhibited fewer entries into the closed arms of the elevated plus maze. Values expressed are group means ± the standard error of the mean (SEM) ($n = 10$ rats per group for diazepam and 8 rats per group for FG 7142, $n = 9$–10 rats per group for N/OFQ). Significant differences between drug-treated rats and the appropriate vehicle-treated controls (*T*-tests for diazepam and FG 7142; Newman–Keuls tests for N/OFQ) are depicted as follows: $^{*}p < 0.05$, $^{**}p < 0.01$.

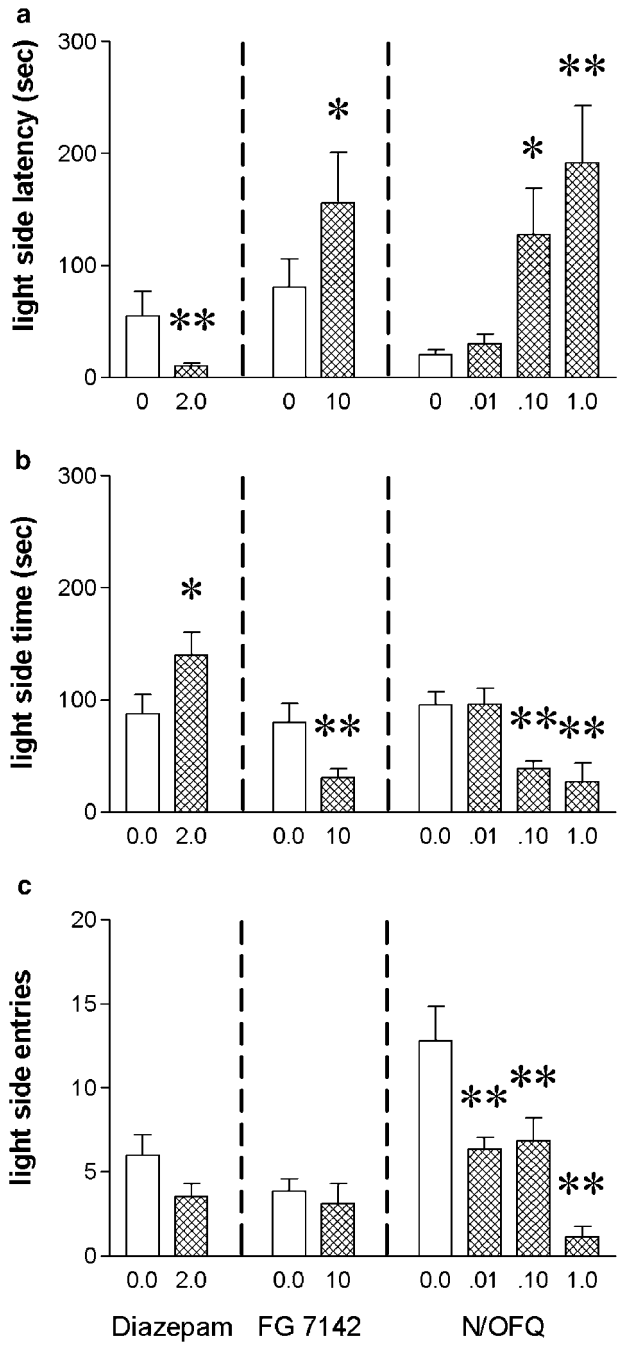


Figure 4 Experiment 3, The Dark-Light Test: Diazepam administration decreased the expression of anxiety-related behaviors, and FG 7142 increased the expression of these behaviors in the dark-light test. Specifically, diazepam (a) decreased the latency to enter the light side, and (b) increased the time spent in the light side. FG 7142 (a) increased the latency to enter the light side, and (b) decreased the time spent in the light side. N/OFQ administration increased the expression of anxiety-related behaviors in the same manner as FG 7142 did. The N/OFQ treated rats exhibited (a) significantly longer latencies to enter the light side, and (b) significantly shorter time spent in the light side. The N/OFQ-treated rats also made (c) significantly fewer entries to the light side than did the aCSF vehicle-treated rats. Values expressed are group means ± the standard error of the mean (SEM) ($n = 8$ rats per group for diazepam and FG 7142, $n = 6$ rats per group for N/OFQ). Significant differences between drug-treated rats and the appropriate vehicle-treated controls (*T*-tests for diazepam and FG 7142; Newman–Keuls tests for N/OFQ) are depicted as follows: $^{*}p < 0.05$, $^{**}p < 0.01$.

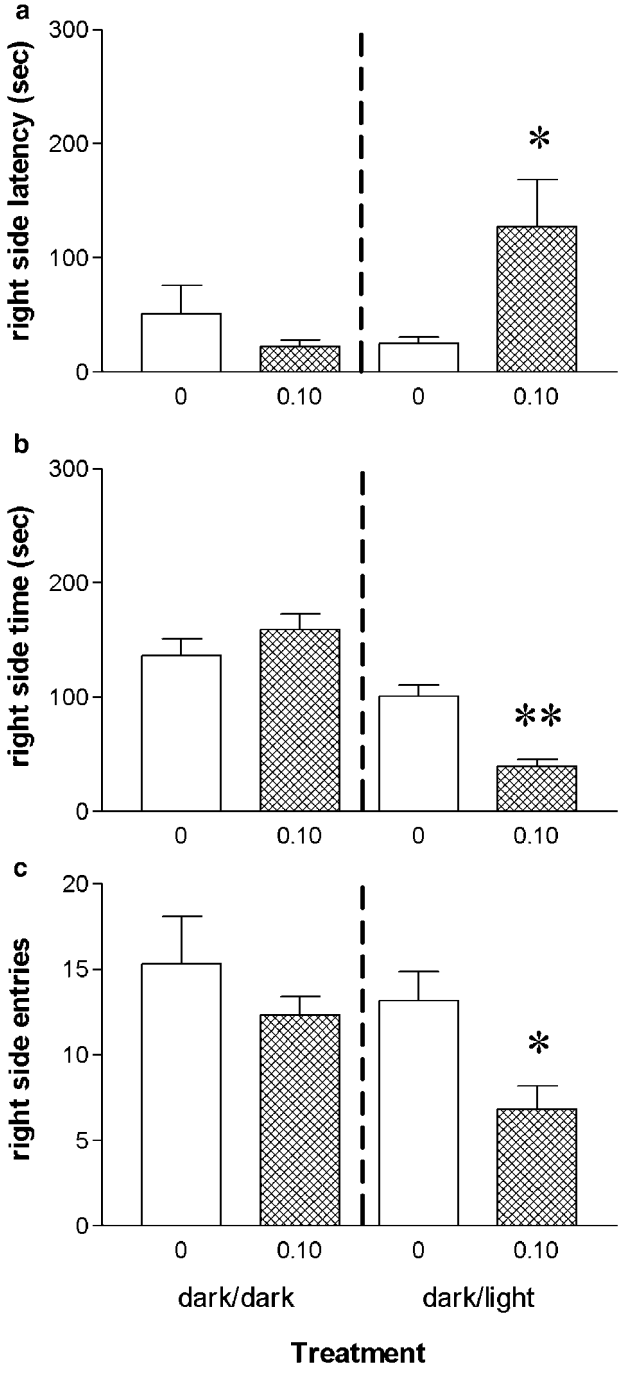


Figure 5 Experiment 4, The Dark-Dark Test: N/OFQ administration increased the expression of anxiety-related behaviors in the dark-light test, and had no effect in the dark-dark test. In the dark light test the N/OFQ treated rats exhibited (a) significantly longer latencies to enter the light side, (b) significantly shorter time in the light side, and (c) significantly fewer entries to the light side than did the aCSF vehicle-treated rats. In the dark-dark test, the aCSF- and N/OFQ-treated rats did not differ in any of the measures. Values expressed are group means ± the standard error of the mean (SEM) ($n = 6$ rats per group). Significant differences between drug-treated rats and the aCSF vehicle-treated controls (*T*-tests) are depicted as follows: $^{*}p < 0.05$, $^{**}p < 0.01$.

made fewer entries to these areas than did the aCSF vehicle-treated rats. These behaviors resembled the behaviors of the rats that were treated with FG 7142 in all three tests, and contrasted with the behaviors that were exhibited by the

rats that were treated with diazepam. Accordingly, N/OFQ appears to exert anxiogenic actions after i.c.v. administration. In fact, N/OFQ and FG 7142 both produced total avoidance of the open field and/or its inner zone by all the rats at some doses, demonstrating the severe expression of anxiety-related behavior after treatment with these drugs (Figure 1).

The fact that N/OFQ produced significant expression of anxiety-related behavior across a broad range of doses is interesting in light of the known effects of N/OFQ on locomotion. The effective dose range (1.0 pmole to 1.0 nmole, where the open field test was the most sensitive assay) spans the range of doses at which N/OFQ has been reported to produce locomotor activation (5.0 to 50 pmoles; Florin *et al*, 1996) or suppression (1.0–10 nmoles; Reinscheid *et al*, 1995; Devine *et al*, 1996b; Rizzi *et al*, 2001a). However, it should be noted that some of these studies (in particular the studies reporting locomotor activation) were performed in mice, and dose-equivalence cannot be assumed across these rodent species. Accordingly, we conducted an additional experiment in the shuttle box (Experiment 4), in order to verify that the observed effects of N/OFQ were actually a result of the specific anxiety-related manipulations in our tests, rather than a locomotor artifact. In this case, half the rats were tested in the standard 'dark-light' conditions (black walls and no light *vs* white walls and light). The remaining rats were tested in the 'dark-dark' conditions (black walls and low light in both compartments). When the rats were tested in the standard dark-light conditions, N/OFQ once again increased the expression of anxiety-related behavior (ie avoidance of the lit side). However, when the left and right sides were both dark with black walls, there were no differences in behaviors between the N/OFQ- and vehicle-treated rats, and all the rats spent equivalent amounts of time in the left and right compartments. Accordingly, the rats' avoidance of the lit compartment after N/OFQ treatment is specifically related to the lighting conditions, and cannot be explained in terms of sedative or locomotor suppressant actions of N/OFQ.

Exposure of the rats to the open field produced moderate elevations in the circulating concentrations of CORT in the vehicle-treated rats, and these circulating CORT responses were significantly enhanced by both the diazepam and FG 7142 treatments. These enhancements of the stress-induced elevations in circulating CORT concur with previous reports that circulating ACTH and CORT concentrations are elevated by acute administration of these drugs (Marc and Morselli, 1969; Pellow and File, 1985; Matheson *et al*, 1988). Accordingly, the association between the emotional (anxiolytic *vs* anxiogenic) and hormonal effects of these drugs is not straightforward, and the HPA axis-activating properties do not specifically predict the emotional effects of these drugs after acute administration.

N/OFQ microinjections also enhanced the CORT responses to the mild stress of the open field, when these hormonal responses were measured 30 min after the N/OFQ administration (approximately 20 min. after the open field test). This agrees with our previous report that N/OFQ activates the HPA axis in unstressed rats, and increases hormonal responses in the presence of a mild (novel environment) stressor (Devine *et al*, 2001). Accordingly, the anxiogenic actions of N/OFQ administration are accompanied by enhancement of HPA axis responses to the open field. However, it should be noted that the relationship between N/OFQ-induced HPA axis activity and N/OFQ-induced anxiety may also not be straightforward—for example, the 1.0 pmole dose of N/OFQ did not significantly alter the CORT response to the open field, but it did produce significant anxiety-related behavior in this assay. This demonstrates that the CORT response and the anxiety responses are dissociable. Therefore, the anxiogenic actions of N/OFQ likely arise from central actions of N/OFQ after i.c.v. administration, rather than some indirect response involving circulating CORT. It should also be noted that Le Cudennec *et al* (2002) reported that N/OFQ administration attenuated severe stress-induced elevations in plasma CORT concentrations in mice, when the mice were acutely injected into the lateral ventricle during manual restraint (ie without prior surgical cannulation). This procedure is considered to be highly stressful, and very high circulating concentrations of CORT were found in the saline-injected control mice. These methods contrast with our method for i.c.v. injections in chronically cannulated rats under unstressed and freely-moving conditions or under the conditions of mild stress during unrestricted exposure to a novel environment (Devine *et al*, 2001; and present results). In fact, we found that i.c.v. N/OFQ did not affect the highly elevated circulating CORT concentrations when cannulated rats were exposed to the severe stress of restraint (Devine *et al*, 2001).

In previous reports, it has been described that administration of N/OFQ (Jenck *et al*, 1997; Griebel *et al*, 1999; Gavioli *et al*, 2002) or the synthetic N/OFQ analogue Ro 64-6198 (Jenck *et al*, 2000; Wichmann *et al*, 2000; Dautzenberg *et al*, 2001) decreased fear- and anxiety-related behaviors in a variety of assays. In fact, when we initially reported that N/OFQ increases circulating ACTH and CORT concentrations in unstressed and mildly stressed rats (Devine *et al*, 2001), we reconciled our hormonal findings with the published behavioral data (Jenck *et al*, 1997; Griebel *et al*, 1999) on the basis that a variety of drugs that are anxiolytic can also increase circulating CORT concentrations in unstressed or mildly stressed rats (Ellis, 1966; Kakihana *et al*, 1968; Marc and Morselli, 1969; Tabakoff *et al*, 1978; Rivier *et al*, 1984; Matheson *et al*, 1988, 1997a, b; de Boer *et al*, 1991). However, the current findings indicate that the behavioral and hormonal effects of N/OFQ resemble the effects of drugs like FG 7142 (Pellow and File, 1985, 1986), yohimbine (Smythe *et al*, 1983; Pellow *et al*, 1985; Suemaru *et al*, 1989), amphetamine (Knych and Eisenberg, 1979; Pellow *et al*, 1985), and caffeine (Spindel *et al*, 1983; Pellow *et al*, 1985)—all of which are anxiogenic in neophobic tests of anxiety, and all of which elevate circulating ACTH and/or CORT concentrations after acute administration.

In general, we believe that the contradiction between our behavioral findings and those of the previous studies result from substantial differences in the methods employed to test and measure fear and anxiety responses. In the current studies, we report that N/OFQ administration produced anxiogenesis during neophobic tests that were specifically designed to test the rats under conditions in which they were exposed to a minimal amount of psychological stress (ie the novel environment of the test apparatus). One previous study reports behavioral observations during the

'mouse defense test battery' (MDTB; Griebel *et al*, 1999). Another study reports observations during a modified 'Geller-Seifter conflict test' (Jenck *et al*, 1997). These tests examine the animals' behaviors under conditions where there is specific and intense fear of imminent predation or footshock. Accordingly, the current and previous tests differ greatly in the amount of psychological stress the animals were exposed to at the time of N/OFQ administration. Furthermore, it should be noted that N/OFQ only altered measures of rearing and biting in the MDTB, and did not alter 5 other fear-related behaviors in this test (Griebel *et al*, 1999). In fact, the authors of the MDTB study concluded that N/OFQ administration only affected responses to extreme and unavoidable stressors in their test, and they were unable to observe any impact of N/OFQ on cognitive aspects of the stress exposure (ie risk assessment). In addition, it should be noted that the Geller-Seifter conflict test was conducted with food-deprived mice that were rewarded for lever presses with food accompanied by electric footshock (Jenck *et al*, 1997). N/OFQ is a potent orexigenic agent (Pomonis *et al*, 1996; Stratford *et al*, 1997), and so one additional possibility is that N/OFQ-induced stimulation of feeding behavior could have influenced the outcome of this experiment.

The apparently differing outcomes of these tests could also be attributed to the use of different species in these experiments, since rats were used in the present experiments, and mice were used in the MDTB and Geller-Seifter conflict tests. However, species differences do not really provide a satisfactory explanation, because rats have also been used in studies that report fear- and anxiety-reducing actions of N/OFQ (Jenck *et al*, 1997, 2000; Wichmann *et al*, 2000; Dautzenberg *et al*, 2001).

The contradiction between the anxiogenic behavioral actions of N/OFQ in the present neophobic tests and the anxiolytic actions in similar previous neophobic tests (Jenck *et al*, 1997; Gavioli *et al*, 2002) is particularly puzzling. There were no obvious differences in effective doses across the various neophobic tests in these studies. In one study, the doses were in the range of 10 pmoles–0.1 nmole i.c.v. in 2.0 μl phosphate buffered saline in mice (Gavioli *et al*, 2002). In another, the doses were 30 pmoles–3.0 nmoles i.c.v. in 5.0 μl aCSF in mice and rats (Jenck *et al*, 1997). In the present study, the doses were 1.0 pmole–1.0 nmole i.c.v. in 1.0 μl aCSF in rats. The effective doses overlap, and so the contradictory data cannot be accounted for by the doses that were tested.

On the other hand, there were substantial differences in the conditions under which anxiety-related behavior was evaluated, and in the dependent measures that were assayed across the three studies. In one of the previous studies (Gavioli *et al*, 2002), the N/OFQ-treated rats spent approximately 75% of the test session on the open arms of the elevated plus maze. This preference for the open arms suggests that something other than anxiety state affected the behavior of the mice. If rodents are tested under neutral conditions (ie test environment with no extraneous influences), and anxiety is completely eliminated from the test situation, the animals should explore all arms equally. Under these conditions, the maximum amount of time on the open arms should not be significantly more than 50% of the session. A clear preference for the open arms suggests that something attracted the mice to the open arms, or drove them from the closed arms of the test, and these behaviors do not appear to be related to anxiety. In the other study (Jenck *et al*, 1997), the vehicle-treated animals (mice and rats) spent only 4–8% of the session in the anxiety-provoking environments of the dark-light and elevated plus maze tests (ie lit compartment and open arms respectively). Under these conditions, where the control animals are exhibiting extremely high levels of anxiety-related behaviors, further N/OFQ-induced avoidance of the anxiety-provoking environments might be obscured by floor effects. However, this explanation is unsatisfactory, as it does not account for the fact that the N/OFQ-treated animals actually exhibited more time (approximately 15–20% of the session) in the anxiety-provoking environments than the vehicle-treated animals did. Another possibility is that the differences in the anxiogenic effects in the present study (where the vehicle-treated rats spent 20–30% of the session in the anxiety-provoking environments) and the anxiolytic effects in the previous study could be accounted for by the possibility that N/OFQ exerts differential effects on anxiety, depending upon the basal anxiety status of the animal at the time of N/OFQ administration. In this case, N/OFQ would increase anxiety in mildly stressed animals (current results), whereas it would attenuate anxiety in animals that are undergoing severe emotional stress (Jenck *et al*, 1997). The neurobiological mechanisms of such an effect are difficult to imagine, and this possibility will require further investigation.

In addition to the methodological differences between the current and previous tests of the elevated plus maze and dark-lights, there were also pronounced differences in the methodologies of the open field tests in the current and previous studies. In the current experiment, one of the important measures of anxiety was thigmotactic behavior, and this avoidance of the inner zone of the open field was increased by administration of N/OFQ—demonstrating an anxiogenic action. In the previous open field experiment, N/OFQ was found to reverse urocortin-induced inhibition of locomotion, and this was interpreted as an anxiolytic action (Jenck *et al*, 1997). Unfortunately, thigmotactic responses were not assessed in that experiment. Since thigmotactic behaviors are thought to reflect anxiety states more reliably than locomotor counts do (Sheldon, 1968; Archer, 1973; File, 1985), it would be interesting to evaluate the potential effect of N/OFQ on urocortin- or FG 7142-induced alterations in thigmotaxis.

The reasons for the contradiction between our behavioral evidence that N/OFQ is anxiogenic and reports that the NOP receptor agonist Ro64-6198 is anxiolytic (Jenck *et al*, 2000; Wichmann *et al*, 2000; Dautzenberg *et al*, 2001) are also unclear. Minor differences have been reported between the pharmacological actions of N/OFQ and those of Ro64-6198—for example, Ro64-6198 induces rapid internalization of NOP receptors, an effect that is not produced by N/OFQ, and Ro64-6198 is a very weak partial agonist at mu opioid receptors (Dautzenberg *et al*, 2001). However, it seems unlikely that these minor pharmacological differences could account for the pronounced behavioral differences between our study and the previous reports.

Another important concern in studies with Ro64-6198 comes from evidence for differences in the specificity of its

effects across rodent species. Specifically, Ro64-6198 inhibited electrically-stimulated contractions of isolated guinea pig ileum and mouse vas deferens, and these effects were not antagonized by $[Nphe^1]NC_{(1-13)}NH_2$ and J-113397. Ro-64-6198 effects were fully antagonized in the rat vas deferens, and N/OFQ effects were fully antagonized in all three tissue preparations (Rizzi *et al*, 2001b). Accordingly, N/OFQ effects were produced through actions on the NOP receptor in all three tissue preparations, whereas Ro64-6198 actions appeared to be specifically mediated by the NOP receptor only in the rat tissue. In relation to the behavioral studies, fear- and anxiety-reducing actions were found only in rats, and these effects were not apparent in mice tested with Ro64-6198 (Jenck *et al*, 2000). Accordingly, the *in vivo* behavioral profile matched with the *in vitro* pharmacological profile across species, and the effects of Ro64-6198 appear to have been mediated through actions on NOP receptors in rats.

So, since pharmacological differences between N/OFQ and Ro64-6198 do not appear to account for the differences between our finding with N/OFQ and previous findings with Ro64-6198, we must again look specifically at the nature of the anxiety tests to resolve the contradiction. In one study with Ro64-6198, (Wichmann *et al*, 2000) the behavioral data (open field exploration and time on open arms of plus maze) are not shown, so it is not possible to evaluate the behavioral effects of Ro64-6198 in this study. In another study (Jenck *et al*, 2000), three of the four reported anxiety tests involve substantial inherent psychological stress (fear-potentiated startle, conditioned conflict, and panic attack induced by stimulation of the dorsal periaqueductal gray). The fourth test was an elevated plus maze test in which the vehicle-injected control rats spent less than 12% of the session exploring the open arms of the maze. In the third study (Dautzenberg *et al*, 2001), the behavior of the vehicle-treated rats appeared similar to the behavior of the vehicle-treated rats in the study by Jenck *et al* (2000), with the rats spending only about 12% of the session on the open arms of the plus maze. Accordingly, it appears once again that differences in the amount of stress during the tests may account for the contradictory behavioral data.

One remaining possibility is that the differences in behavioral actions between the present and previous studies could reflect differences in the strains of rats that were used in some of the neophobic tests of anxiety. Long-Evans rats were used in the present studies, whereas Sprague-Dawley (Jenck *et al*, 2000; Wichmann *et al*, 2000; Dautzenberg *et al*, 2001) and/or Wistar rats (Jenck *et al*, 1997, 2000) were used in the previous studies. Although we consider rat strain differences to be an unlikely source of the contradictory behavioral results, we do not have data to address this issue, and it cannot be ruled out at the present time.

In summary, N/OFQ administration produced increases in specific anxiety-related behaviors in three neophobic tests of anxiety in the rat, when the tests were conducted under a standard set of mildly stressful conditions that will reveal both anxiolytic and anxiogenic actions of pharmacological treatments. This anxiogenic action of N/OFQ was accompanied by elevations in circulating CORT concentrations, replicating our previous finding (Devine *et al*, 2001) that N/OFQ administration activates the HPA axis and augments the responses of the axis when rats are exposed to mild stress. These findings, coupled with evidence that N/OFQ neurotransmission is increased by stress exposure (Devine *et al*, 2002) suggest that N/OFQ neurotransmission may participate in normal processing of emotionally-salient and stressful stimuli. Accordingly, disregulation of the N/OFQ-NOP system may be implicated in psychiatric dysfunction involving altered activity of the HPA axis and pathological anxiety states. Further studies with metabolically-stable agonists and antagonists for the NOP receptor will be required to confirm the involvement of endogenous N/OFQ neurotransmission in affective regulation.

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