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Orphanin FQ acts as an anxiolytic to attenuate behavioral responses to stress

(anxiety/benzodiazepine/neuropeptide/plus maze/operant conflict)

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ABSTRACT **Orphanin FQ (OFQ, Nociceptin) is a recently discovered 17-amino acid neuropeptide that is structurally related to the opioid peptides but does not bind opioid receptors. OFQ has been proposed to act as an anti-opioid peptide, but its widespread sites of action in the brain suggest that it may have more general functions. Here we show that OFQ plays an important role in higher brain functions because it can act as an anxiolytic to attenuate the behavioral inhibition of animals acutely exposed to stressful/anxiogenic environmental conditions. OFQ anxiolytic-like effects were consistent across several behavioral paradigms generating different types of anxiety states in animals (light-dark preference, elevated plus-maze, exploratory behavior of an unfamiliar environment, pharmacological anxiogenesis, operant conflict) and were observed at low nonsedating doses (0.1–3 nmol, intracerebroventricular). Like conventional anxiolytics, OFQ interfered with regular sensorimotor function at high doses (>3 nmol). Our results show that an important role of OFQ is to act as an endogenous regulator of acute anxiety responses. OFQ, probably in concert with other major neuropeptides, exerts a modulatory role on the central integration of stressful stimuli and, thereby, may modulate anxiety states generated by acute stress.**

Orphanin FQ (OFQ, Nociceptin) is a 17-amino acid neuropeptide that is structurally related to the opioid peptides but does not act on μ, δ, or κ opioid receptor subtypes (1, 2). OFQ selectively binds its own receptor (OFQR), which is also sequentially related to the opioid receptors, yet does not bind opioid ligands (3–8). The OFQR couples to G proteins to modulate second messenger systems and cell excitability (9–11). When delivered intracerebroventricularly (i.c.v.) in a large dose range (0.1–10 nmol), OFQ was found to block stress and opioid-mediated antinociception (12, 13), to stimulate feeding in satiated rats (14), and to increase or decrease, depending on dosage, locomotion (1, 15) or nociception (16, 17) in rodents. OFQ, its precursor, and OFQR are present in several brain regions involved in integration of the emotional components of fear and stress such as the amygdaloid complex, thalamic and hypothalamic regions, or central gray regions (1–8, 18, 19). This has led us to investigate whether OFQ might also have a role in higher brain functions and would control behavioral responses to stress that relate to anxiety states. To investigate this hypothesis, a battery of behavioral models of anxiety and fear (light-dark aversion, elevated plus-maze, exploratory behavior of an unfamiliar environment, pharmacological anxiogenesis, operant conflict) were used on mice and rats. In these assays, fear-like responses of a composite nature are generated by exposure to various stressful environmental conditions (20–27). These paradigms have been established for their sensitivity to conventional anxiolytic tranquilizers and anxiogenic compounds of various structural classes and mechanisms of action. They were pharmacologically validated with prototypical anxiolytic compounds (i.e., diazepam) before OFQ testing and were controlled for effects on sensorimotor function.

MATERIALS & METHODS

Animals. The experimental procedures used in this investigation received approval from a local committee based on adherence to Swiss federal regulations and guidelines on animal experimentation. Animals were purchased from Biological Research Laboratories (Fullinsdorf, Switzerland) and were acclimatized to the laboratory conditions for at least 1 week before the start of the experiments. They were group-housed under controlled laboratory conditions (temperature, 20 ± 2°C; relative humidity, 50–60%; 12-h normal light/dark cycle). Tests were performed between 9 and 12 a.m. Male MORO mice (30–50 g) were used in the light-dark, horizontal wire, and conflict tests, and male BALB/c mice (20 g) were used in the urocortin-induced phobic hypolocomotion test. Free-hand transcranial injection were made for local i.c.v. delivery in mice that were directly euthanized at the end of the experiment. Male Wistar RoRo rats (250 g) were used in the elevated plus-maze, horizontal wire, and general locomotion tests and were stereotaxically implanted, under ketamine/xylazine (90/10 mg/kg i.p., respectively) anesthesia with stainless steel guide cannulae (model C313G, Plastics One, Roanoke, VA) in the lateral ventricles (0.3 mm posterior to bregma, 1.4 mm lateral, and 3.6 mm ventral to the skull). Guides were fastened to the skull by means of dental cement and stainless steel microscrews and dummy cannulae (model C313DC, Plastics One) were inserted. Animals received postoperative buprenorphine (0.05 mg/kg s.c., twice daily) for 1 day and a 3-day postoperative recovery period. Injection cannulae (model C313I, Plastics One) were used for i.c.v. infusions. Ventricular localization was controlled by measuring the dipsogenic effects of angiotensin II.

Drugs. Synthetic OFQ was purchased from Research Genetics (Huntsville, AL) and dissolved in artificial cerebrospinal fluid (CSF) for local i.c.v. delivery (in 5 μl volume). Diazepam was synthesized at Hoffmann–La Roche; it was freshly pre-

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Abbreviations: OFQ, orphanin FQ; OFQR, orphanin FQ receptor: CRF, corticotropin releasing factor; i.c.v., intracerebroventricular; p.o., per os; CSF, cerebrospinal fluid.
A commentary on this article begins on page 14217.
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pared as a light suspension in saline containing 0.3% of Tween 80 and administered orally or i.p. (5 ml/kg injection volume) with a 30-min pretreatment time to allow for absorption and distribution.

Light-Dark Aversion and Forced Motor Performance in Mice. The light-dark aversion apparatus consisted of two Plexiglass boxes (27 × 21 × 14 cm, black, and covered on one side; translucent and illuminated with a 30-W lamp placed 30 cm above the other side) with an interconnecting dark tunnel (7 × 10 cm). Five min following i.c.v. infusion of OFQ or 30 min following oral administration of diazepam, each mouse was placed in front of the tunnel and the total time spent in the lit area and the total number of transitions from dark to lit area were recorded during 5 min (20, 21). Forced motor performance was subsequently evaluated in a standard horizontal wire test (traction test) that consisted in allowing mice to grasp a horizontally strung wire (20 cm above the bench level, 1 mm in diameter, 15 cm long) with their forepaws. The ability to actively grasp and climb up to the wire with at least one hindpaw was scored individually; groups scores were expressed as the percentage of animals performing to this task. Animals were used once (n = 10–20/dose group).

Exploration. In a test of free exploratory behavior, BALB/c mice were placed in a large unfamiliar environment (23–25). Exploration was measured in activity monitors (40 × 40 × 30 cm, Omnitech Electronics, Columbus, OH) placed in a sound-proof room with no light. Locomotion was monitored via a grid of invisible infrared light beams. Horizontal and vertical activities were used in this study to describe the dynamic picture of mice treated with the test drugs. A vertical sensor monitoring rearing and jumping activity was attached 4 cm above the cage floor. An analyzer constantly collected the beam status information from the activity monitor, and activity detected by the horizontal sensors was expressed as total distance run during the 30-min test. When compared with artificial CSF control tests, both corticotrophin releasing factor (CRF) and urocortin, a recently described endogenous ligand for CRF receptors (28, 29), induced comparable, dose-dependent decreases in behavioral performance of BALB/c mice exposed to a novel environment. Horizontal and vertical activities were reduced by 0.1 or 0.3 μg CRF or by 0.1, 0.3, or 1 μg urocortin (26) and were reversed by diazepam. A value of 0.3 μg (0.06 nmol) urocortin was selected as the challenge dose (the lowest dose to reach maximal "anxiogenic-like" efficacy). OFQ and urocortin were administered i.c.v. as a single mixture infusion 10 min before testing. Animals were used once (for OFQ, n = 16/dose group; for diazepam n = 8/dose group).

Elevated Plus-Maze and Forced Motor Performance in Rats. A standard elevated plus-maze (50 × 50 × 50 cm) made of gray Plexiglas and placed in a sound-proof observation room with controlled light (200 ± 10 Lux on the central platform of the maze) was used in rats. This test is based on the natural aversion of rodents for open spaces and uses a maze with two open and two enclosed arms (22). The maze was carefully cleaned between each rat exposure. It was positioned in a closed, white environment, and rats were observed and scored via a closed-circuit TV camera fixed at the ceiling and an observation monitor located in an adjacent room. Ten minutes following i.c.v. infusion of OFQ or 30 min following oral administration of diazepam, rats were exposed to the maze, and time spent in the open arms in seconds, number of transitions from closed to open arms, and number of transitions from closed to closed arms were recorded and expressed as absolute values (total test duration was 5 min). Forced motor performance was subsequently evaluated in a traction test that consisted of forcing rats to grasp a horizontally strung wire (20 cm above the bench level, 2 mm in diameter, 20 cm long) with their forepaws. Various neurobehavioral items were scored: the grasp reflex (score 1), body weight support (score 2), climb reflex (score 3), and escape (score 4); groups scores were calculated by averaging individual scores. The maximum of two permissible readings was recorded. Forelimb grip strength was then quantitatively assessed by using a digital strain gauge. Animals held by the tail grasped a triangular bar (2 mm diameter, 5 cm wide) and were gently pulled away from the bar with a smooth steady pull until they released the triangle. The strain gauge remains fixed at its maximum deflection, and three readings were taken for each animal and the maximum of three permissible readings was recorded as forelimb grip strength (in g). Individual strengths were averaged over each dose group. Total duration of these tests was 1 min. Spontaneous locomotor behavior was then recorded for an additional 5 min in animal activity monitors (40 × 40 × 30 cm, Omnitech Electronics), identical to those used and described above for mice, except that the vertical sensors were attached 8 cm above the cage floor. Horizontal and vertical activity were also used in this study to describe the dynamic picture of rats treated with the test drugs. The OFQ study was made of two experimental groups (n = 16 in each group) in which two doses and a vehicle control were administered i.c.v. and counterbalanced in a cross-over design so that each animal received each treatment once, and a 1-week delay separated two consecutive i.c.v. infusions.

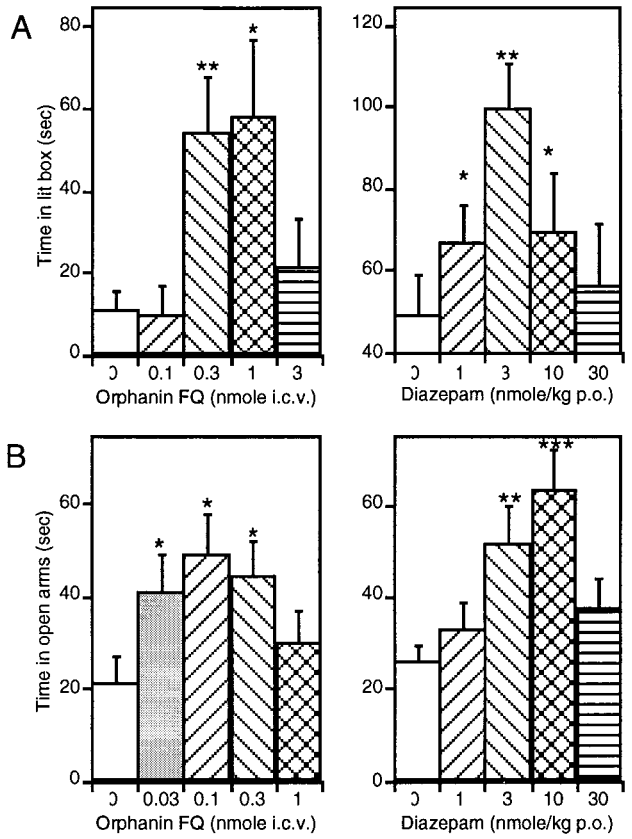

FIG. 1. (*A*) Increase in open area exposure time induced by i.c.v. OFQ (*Left*) and oral diazepam (*Right*) in the light-dark preference test in mice. Note that baseline was significantly lower after transcranial injection than after oral administration. Motor performance subsequently evaluated in a horizontal wire test is described in Table 1. (*B*) Increase in open arm activity induced by i.c.v. OFQ (*Left*) and oral diazepam (*Right*) in the elevated plus-maze test in rats. Locomotion in the closed parts of the maze was not modified; performance was evaluated in tests of forced locomotion and spontaneous exploratory activity (Table 2). Data are means ± SEM (n = 10–20 in *A* and n = 16 in *B*, except in OFQ's control group where n = 32), and statistical significance was determined by a single factor analysis of variance followed by post-hoc Bonferroni test for multiple comparisons (*, $P < 0.05$; **, $P < 0.01$ vs. vehicle).

Table 1. Effects of OFQ on forced motor performance in mice

	Mice with motor impairment	
	Proportion	Percent
OFQ (i.c.v.)		
Vehicle	0/10	0
0.1 nmol	0/20	0 NS
0.3 nmol	0/20	0 NS
1 nmol	5/20	25 NS
3 nmol	5/10	50 NS
Diazepam (p.o.)		
Vehicle	0/10	0
1 nmol/kg	0/10	0 NS
3 nmol/kg	0/10	0 NS
10 nmol/kg	3/10	30 NS
30 nmol/kg	8/10	80†

Motor performance was measured in a traction test as described. NS, not significant.
†$P < 0.05$, Fisher exact probability test.

Conflict. In the conflict procedure (27), food-deprived mice (80–85% of free feeding body weight) were given the opportunity to press a lever for 20-mg food pellets delivered on a fixed ratio schedule. Following training, lever pressing was either associated (punished) or nonassociated (unpunished) with a mild electric foot-shock. Scrambled shock (0.2 mA, 300 msec) was delivered through the grid floor of a sound-attenuated 18 × 18 × 21 cm cage equipped with the lever connected to a food dispenser. Animals were repeatedly exposed to the conflict situation (5-min period of unpunished responding followed by a 15-min period of punished responding), and at least 4 days separated two consecutive drug exposures. Animals received unpunished food delivery on intervening days without drug testing. Diazepam [30 nmol/kg per os (p.o.), 30 min pretreatment] vs. vehicle were initially tested; mice were subsequently subdivided in a cross-over design into two groups ($n = 9$ in each group) receiving either artificial CSF or 3 nmol OFQ as a final injection via the transcranial i.c.v. route. The punished session started 5 min following infusion and lasted for 15 min. All animals were euthanized at the end of the experiment and for practical reasons—the conflict procedure involves several weeks of training, and a single dose (3 nmol) only was tested to investigate the effects of OFQ. This dose was selected based on efficacy in other tests for anxiety and for sensorimotor deficit in mice, knowing that operant procedures such as Geller–Seifter conflict tests are generally insensitive to even gross motor deficits because of the high motivation of hungry mice to press the lever.

Affinity for CRF Receptors. OFQ was tested for its ability to compete for [^{3}H]urocortin (0.2 nM) binding to membranes from HEK 293 cells transfected with the human CRF1 receptor, the rat CRF2α receptor, or the rat CRF2β receptor. Bound and free ligands were separated by filtration through glass fiber filters. OFQ was also examined for its ability to compete for [^{3}H]urocortin (0.6 nM) binding to the medium harvested from HEK 293 cells transfected with the human CRF binding protein. Free ligand was separated from bound by precipitation with activated charcoal.

RESULTS

OFQ was found to be active in the two-compartment light-dark preference test in mice. This test records the natural aversion of mice for brightly lit areas (neophobia) as compared with their preference for dark and protected spaces. OFQ (0.3 and 1 nmol) significantly increased the exploratory behavior of mice in the lit aversive section of the light-dark box when compared with control injections of artificial CSF (Fig. 1*A*). Reduced aversion for open spaces is characteristic of anxiolytic agents such as diazepam (Fig. 1*A*). Nonsignificant increases were also detected in the number of transitions from dark to light following 0.3 and 1 nmol of OFQ (1.4 ± 0.5 and 1.0 ± 0.4, respectively, vs. 0.5 ± 0.5, 0.5 ± 0.2, and 0.3 ± 0.2 for vehicle, for 0.1 and 3 nmol OFQ, respectively). Diazepam induced significant increases in transitions at 1 and 3 nmol/kg (9.0 ± 0.8 and 11.5 ± 1.4 vs. 6.5 ± 0.9, respectively) but not at 10 and 30 nmol/kg (6.9 ± 1.3 and 3.3 ± 0.9, respectively). A dose of 0.1 nmol of OFQ was below threshold for drug activity whereas 3 nmol did not increase the time spent in the lit area above baseline levels, presumably caused by deficits in motor function. Motor performance subsequently evaluated in a horizontal wire test was intact following vehicle and low doses of OFQ, but mild to moderate deficits were observed at higher doses (Table 1).

In rats, OFQ also attenuated the fear response to open spaces as measured by using the elevated plus-maze. The time spent and the number of entries into open arms are indices of open-space anxiety in animals, as supported by the fact that anxiolytics such as diazepam increase these parameters (Fig. 1*B*). OFQ (0.03, 0.1, and 0.3 nmol) induced an increase in the number of entries and time spent in the open arms (Fig. 1*B*). Transitions from closed to open arms were also recorded. When compared with their respective baselines, they increased by 22%, 27%, 125%, and 44% following 0.03, 0.1, 0.3, and 1 nmol of OFQ, respectively, and by 20%, 88%, 152%, and 76% following 1, 3, 10, and 30 nmol/kg of diazepam, respectively. Locomotion in other parts of the maze was not modified, and performance was essentially unaffected at high doses (0.3 and 1 nmol OFQ) when animals were subsequently evaluated in tests of forced locomotion and spontaneous exploratory activity (Table 2). Consistently with the reduced effects seen with 1 nmol OFQ in the plus-maze, trends for decrease in locomotion were detected at this dose in the test for spontaneous exploratory activity.

The behavioral properties of OFQ were further investigated in a test of free exploratory behavior in mice placed in a large unfamiliar environment. In this test, urocortin (a CRF analog) decreased exploratory horizontal activity and rearing in BALB/c mice, presumably as the result of increased fear of exposure. Benzodiazepines, such as diazepam, are active in reversing urocortin-induced decreases in exploration (Fig. 2*A*)

Table 2. Effects of OFQ on forced motor performance and spontaneous locomotion in rats

	Artificial CSF	0.3 nmol OFQ	1 nmol OFQ
Forced motor performance			
Traction test, scores	2.71 ± 0.16	2.75 ± 0.17 NS	2.50 ± 0.17 NS
Grip strength, grams	784.0 ± 58.9	706.2 ± 105.8 NS	760.7 ± 50.9 NS
Spontaneous locomotion			
Total distance, cm	1581 ± 171	1624 ± 161 NS	1457 ± 162 NS
Vertical time, s	57.6 ± 6.8	55.6 ± 6.2 NS	39.4 ± 4.1 NS

Motor performance and locomotion were measured in horizontal wire traction and forelimb grip strength procedures followed by a measure of spontaneous exploratory behavior recorded in automated activity monitors as described. Data are given as mean ± SEM. Same drug treatment and statistics as in Fig. 1*B*. NS, not significant.

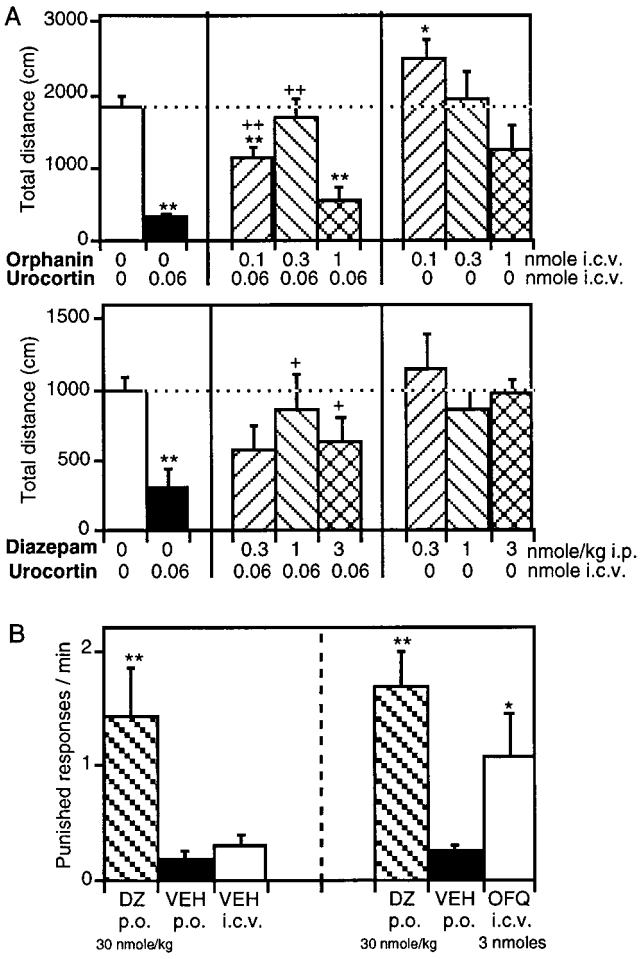

FIG. 2. (*A*) Attenuation by i.c.v. OFQ (*Upper*) and i.p. diazepam (*Lower*) of urocortin-induced behavioral inhibition (*Middle*) and effects on spontaneous exploration (*Right*) in an unfamiliar environment in mice. Note that baseline is lower with multiple injections (i.c.v. combined with i.p. injections). Combinations of OFQ, diazepam, urocortin, or artificial CSF were administered as indicated at the bottom of the bar graphs. Data are means ± SEM (n = 8–16), and statistical significance was determined by a single factor analysis of variance followed by post-hoc Bonferroni tests for multiple comparisons (∗ $P < 0.05$; ∗∗, $P < 0.01$ vs. vehicle/vehicle; ++, $P < 0.01$ vs. vehicle/urocortin 0.06 nmol). (*B*) Increase in punished responding induced by i.c.v. OFQ in a conflict procedure in mice. Mice were initially tested orally with diazepam (10 mg/kg p.o., 30 min pretreatment) vs, vehicle and were subsequently subdivided into two groups (n = 9/group) receiving either artificial CSF (*Left*) or 3 nmol OFQ (*Right*). Data are means ± SEM and statistical significance was determined by a Wilcoxon test for diazepam and a Mann–Whitney test for OFQ (∗, $P < 0.05$; ∗∗, $P < 0.01$ vs. the respective vehicle).

at doses that do not interfere with motor function (0.3, 1 and 3 nmol/kg). We found that OFQ (0.1 and 0.3 nmol, but not 1 nmol) also reverses these urocortin-induced inhibitory effects on a measure of horizontal activity (Fig. 2*A*). Very similar effects were observed on measures of vertical activity (rearings). This was not caused by direct interaction with CRF receptors because, in competition binding assays, OFQ was found not to interact with [^{3}H]urocortin binding to CRF1, CRF2α, CRF2β receptors or to the CRF binding protein (no significant inhibition of binding was observed in any of the assays at concentrations of OFQ up to 1,000 nM). When administered by itself, 0.1 nmol OFQ significantly stimulated exploration, a result that is consistent with its antianxiety properties; at 1 nmol of OFQ, however, locomotor activity was reduced, presumably as a consequence of the motor deficits that emerge at this dose (see above). OFQ also does not interact with [^{3}H]flumazenil binding to the benzodiazepine site of the $GABA_A$ receptor (no inhibition recorded at OFQ concentration up to 10 μM).

Finally, OFQ was also tested in an operant conflict procedure in which animals are not exposed to a novel environment or to an endogenous anxiogenic agent, but to hunger and aversive electric shocks to the feet. In this assay, food-deprived mice press a lever for food and concomitantly receive mild footshocks; behavioral suppression generated by the conflict between food-seeking and pain is measured. When compared with i.c.v. vehicle, OFQ (3 nmol, see *Materials and Methods* for dosing rationale) significantly increased the rate of punished lever responding, a characteristic effect of clinically effective anxiolytics such as diazepam (Fig. 2*B*). Unpunished responding, which serves as a measure of performance, was not significantly affected by OFQ as compared with its vehicle (1.6 ± 0.1 vs 2.4 ± 0.2 responses/min, respectively).

DISCUSSION

OFQ plays an important role in higher brain functions because it can act as an anxiolytic to attenuate the behavioral inhibition of animals acutely exposed to stressful/anxiogenic environmental conditions. OFQ's anxiolytic-like effects were observed at low nonsedating doses (0.1–3 nmol dose range, i.c.v.) and were consistent across several behavioral paradigms generating different types of anxiety states in animals. At these doses, stimulation of spontaneous locomotion and exploration have been reported (15), but no conditioned place preference or aversion reminiscent of the motivational effects of psychostimulants was detected (30). Our data suggest that these increases in locomotion might be related to OFQ's anxiolytic-like activity, because increased exploration is an intrinsic manifestation of reduced anxiety in animals and because classical antianxiety drugs exert similar behavioral effects in rodents. Conversely, high doses of OFQ (>1–3 nmol) have opposite effects as these doses interfere with normal sensorimotor function and decrease locomotion (1, 30, 31). Such inverted U-shaped functions are typical of conventional anxiolytics (i.e., benzodiazepines, buspirone) that, in addition to reducing anxiety, also impair motor performance at high doses. Biphasic effects are generally suggestive of an heterogeneity in sites of action of the drug; in the case of OFQ, the existence of subtypes of OFQR with distinct regional localization is not yet elucidated.

The attenuating effects of OFQ on stress-related behavioral responses were highly consistent across a range of tests involving different sets of environmental stressors. They were detected at low doses of OFQ and in two rodent species. Behavioral paradigms that use exploration of novel environments, drug-induced fear, and conflict procedures are widely used as animal models of anxiety. These assays may well reflect different subtypes of anxiety; hence, we hypothesize that OFQ might be a general modulator of acute behavioral responses to stress and may contribute in a general way to the regulation of anxiety states generated by stress. These anxiolytic-like properties could be related to the presence of OFQ receptors on neurons in the locus coeruleus, central gray, raphe nucleus, hypothalamus, or amygdala, regions associated with the integration and transduction of stressful stimuli (32, 33). Other neuropeptides such as CRF, neuropeptide Y (NPY), or cholecystokinin (CCK) exert an important role in the processing of stressful stimuli in these same brain regions (33–35). CRF and CCK have been reported with anxiogenic-like properties, whereas OFQ and NPY seem to exert anxiolytic-like effects. Thus, neuropeptides seem to exert a reciprocal regulation of behavioral responsiveness to stressful stimuli, and the OFQ system may play a major role in these elaborate interactions.

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