EFFECTS OF NOCICEPTIN/ORPHANIN FQ MICROINJECTIONS INTO THE AMYGDALA ON ANXIETY-RELATED BEHAVIORS AND HYPOTHALAMIC-PITUITARY-ADRENAL AXIS ACTIVATION

By

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A THESIS PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE

UNIVERSITY OF FLORIDA

2005

This document is dedicated to all who believe in the scientific pursuit of knowledge and truth.

ACKNOWLEDGMENTS

I thank my committee members Dr. Henrietta Logan, Dr. John Petitto, and Dr. Margaret Bradley for their assistance in the preparation of this thesis. I also, and especially, thank my advisor, Dr. Darragh Devine, for his incredibly dedicated guidance and support of my graduate studies.

I would also like to thank my mother, Lisa, my stepfather, Michael, and my brother, Damion, for guiding and supporting me on my journey, in life and in academics. I thank Terry and Karen for their support during my first 2 years of graduate training. I thank "my" Michael for motivating me during the writing of this thesis. And I thank Simon Poindexter, the brilliant Siamese, for keeping my lap warm and occasionally editing this manuscript while I wrote.

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December 2005

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Major Department: Psychology

Intracerebroventricular (ICV) microinjections of the opioid-like neurotransmitter nociceptin/orphanin FQ (N/OFQ) produce elevations in hypothalamic-pituitary-adrenal axis (HPA axis) activity and anxiety-related behaviors in rats. Furthermore, these increases in HPA axis activity can be produced by N/OFQ injections into a number of limbic structures. We examined the potential role of one limbic structure, the amygdala, in the N/OFQ-induced anxiogenic effects. Male Long Evans rats were each implanted with a guide cannula into the right lateral ventricle or amygdala. Each rat received an injection of N/OFQ (0, 0.01, 0.1, or 1.0 nmole) prior to behavioral testing in a neophobic test of anxiety. In the anxiety test, each rat was placed in a start box connected to an open field, where the rats had free access to the field for 5 minutes. Latency to enter the open field, number of entries into the open field, total time spent in the open field, and thigmotactic behaviors were used as measures of anxiety. Following testing, the rats

were euthanized and plasma samples were obtained for analysis of HPA axis activity. Additionally, each rat brain was removed for cannula verification, and thymus glands, adrenal glands, and spleens were dissected out for analysis of overall health of the rats. N/OFQ injections produced dose-orderly increases in anxiety-related behaviors, and these effects were greater in the ICV-implanted rats than they were in the amygdala-implanted rats. The ICV N/OFQ-treated rats displayed longer latencies to enter the open field, fewer numbers of entries into the open field, and less total time spent in the open field than the ICV vehicle-treated rats did. The intra-amygdaloid N/OFQ-treated rats also exhibited longer latencies to enter the open field, but did not differ in the number of entries into the open field or the total time spent in the open field when compared with the behavior of the intra-amygdaloid vehicle-treated rats. N/OFQ injections into the lateral ventricle also produced elevations in circulating corticosterone, indicating that the HPA axis activity was greater in these rats. However, amygdaloid injections did not affect corticosterone levels. In conclusion, the amygdala appears to be involved in the anxiogenic behavioral effects of N/OFQ. However, the differences in potency of effects between ICV N/OFQ injections and intra-amygdaloid injections on anxiety-related behaviors and circulating CORT in rats indicate that the amygdala is not the primary site of drug action and that extra-amygdaloid sites are involved.

CHAPTER 1
INTRODUCTION

Nociceptin/Orphanin FQ (N/OFQ) and its cognate receptor NOP constitute a highly conserved (Danielson and Dores, 1999) peptide neurotransmitter system that affects an interesting range of very important behavioral and physiological activities. N/OFQ is a 17 amino acid peptide that is structurally similar to the endogenous opioids, particularly dynorphin A (Meunier et al., 1995; Reinscheid et al., 1995). However, N/OFQ does not bind to the μ, δ, or κ opioid receptors with high affinity (Shimohigashi et al., 1996), but does bind with high affinity to the NOP receptor (Butour et al., 1997; Reinscheid et al., 1995; Shimohigashi et al., 1996). The NOP receptor is a 7-transmembrane, G-protein coupled receptor (Bunzow et al., 1994; Chen et al., 1994; Lachowicz et al., 1995; Reinscheid et al., 1996; Wang et al., 1994; Wick et al., 1994) that is negatively linked to adenylate cyclase (Lachowicz et al., 1995; Mollereau et al., 1994; Reinscheid et al., 1995; Reinscheid et al., 1996), increases inward rectifying K^+ channel conductance (Connor et al., 1996a; Vaughan and Christie, 1996; Vaughan et al., 1997), and inhibits Ca^{2+} conductance (Connor et al, 1996b). The NOP receptor shows high structural homology with the opioid receptors (Bunzow et al., 1994; Chen et al., 1994; Lachowicz et al., 1995; Mollereau et al., 1994; Wang et al., 1994; Wick et al., 1994), although it does not bind any of the opioids with high affinity (Bunzow et al., 1994; Butour et al., 1997; Lachowicz et al., 1995; Wang et al., 1994). This low affinity between N/OFQ and opioid receptors and between NOP and opioid peptides suggests that the N/OFQ-NOP system is functionally distinct from the opioid system.

N/OFQ, its precursor protein, and the NOP receptor are all found widely distributed throughout the brain, spinal cord, and periphery (Bunzow et al., 1994; Chen et al., 1994; Devine et al., 2003; Lachowicz et al., 1995; Neal et al., 1999a&b; Nothacker et al., 1996; Mollereau et al., 1994; Wang et al., 1994; Wick et al., 1994), consistent with a wide range of functions including pain modulation (Meunier et al., 1995; Reinscheid et al., 1995; Tian et al., 1997), motor performance (Devine et al., 1996; Reinscheid et al., 1995), spatial learning (Sandin et al., 1997; Sandin et al., 2004), and feeding (Nicholson et al., 2002; Pomonis et al., 1996). However, localization is particularly high in limbic regions including the hypothalamus, septum, bed nucleus of stria terminalis (BNST), and amygdala (Bunzow et al., 1994; Devine et al., 2003; Lachowicz et al., 1995; Neal et al., 1999a&b; Nothacker et al., 1996; Wang et al, 1994). This limbic localization is consistent with the known role of N/OFQ in stress responses (physiological, homeostatic responses to stimuli that represent a change or potential change to the organism's environment; e.g., Herman and Cullinan, 1997) and anxiety responses (behavioral responses to non-specific, potentially threatening stimuli; e.g., Walker et al., 2003). For example, N/OFQ is released from forebrain neurons in rats following exposure to a mild stressor (Devine et al., 2003). Additionally, intracranial injections of N/OFQ alter hypothalamic-pituitary-adrenal axis (HPA axis) activity and anxiety-related behaviors. Unfortunately, some studies have described increased HPA-axis activity and anxiogenesis (Devine et al., 2001; Fernandez et al., 2004; Misilmeri and Devine, 2000; Misilmeri et al., 2002), while other studies have reported decreased HPA-axis activity and anxiolysis (Gavioli et al., 2002; Griebel et al., 1999; Jenck et al., 1997; Le Cudennec et al., 2002).

To examine anxiety-related behaviors, standard neophobic tests are generally used, such as the open field test, elevated plus maze, and light-dark test. These tests are based on the natural behaviors of rats, including the tendency to explore during foraging activities and, on the other hand, the tendency to avoid open spaces due to their vulnerability to predation. For example, rats show thigmotaxis in the open field (for example see Simon et al., 1994), and they show a preference for the enclosed arms of the elevated plus maze (Handley and Mithani, 1984; Pellow et al., 1985) and for the dark box of the light-dark test (Chaouloff et al., 1997; Costall et al., 1989; Crawley and Goodwin, 1980; Onaivi and Martin, 1989). The balance between exploration and avoidance can be manipulated in a highly reproducible manner by anxiolytic drugs (i.e., drugs that humans report to be anxiety-reducing, such as diazepam) and by anxiogenic drugs (i.e., drugs that humans report to be anxiety-inducing, such as FG 7142). Rats increase their exploration of open or lit spaces following administration of anxiolytic compounds and decrease their exploration following administration of anxiogenic compounds (Chaouloff et al., 1997; Costall et al., 1989; Crawley, 1981; Crawley and Goodwin, 1980; Fernandez et al., 2004; Handley and Mithani, 1984; Hughes, 1972; Onaivi and Martin, 1989; Pellow and File, 1986; Pellow et al., 1985; Simon et al., 1994; Stefanski et al., 1992).

In the present experiment, we use a modified version of the open field test, in which a start box was attached to one wall of the open field. This addition allows us to use latency to enter the open field and time spent in the open field as measures of exploratory behavior (in addition to thigmotactic behavior that has been reported in previous versions of the open field). Also, we divide the open field into 3 zones, a proximal peripheral zone, a distal peripheral zone, and an inner zone (see Methods

section for a full description). The separate zones allow us to measure exploration near the "safety" of the start box versus exploration farther away from this "safe" zone, as well as the traditional measure of inner zone exploration. Furthermore, we have calibrated the test so that our vehicle-treated rats spend approximately 25% of the test time in the open field, allowing us to easily observe both increases and decreases in anxiety-related behaviors under one set of conditions (lighting, handling, etc.). Under these conditions, rats that have been treated with diazepam generally show shorter latencies to enter the open field, more total time spent in the open field, and more exploration away from the start box and in the middle of the open field, as compared to vehicle-treated rats. On the other hand, rats treated with FG 7142 generally show longer latencies to enter the open field, less total time spent in the open field, and less exploration away from the start box and in the center of the open field. These data provide evidence that the modified open field is a valid and sensitive tool for measuring changes in expression of anxiety-related behaviors (Fernandez et al., 2002).

Intracerebroventricular (ICV) injections of N/OFQ have been shown to increase anxiety-related behaviors in the modified open field test, the elevated plus maze, and the light-dark test (Devine et al., 2004; Fernandez et al., 2004). N/OFQ-treated rats, as compared to vehicle-treated rats, display longer latencies to enter the open arms of the elevated plus maze, the light box of the light-dark test, and the open field of the open field test. Additionally, N/OFQ-treated rats spend less total time in the open arms, light box, and open field. These behaviors resemble the effects following injections of other anxiogenic drugs, such as FG 7142. Furthermore, injections of anxiolytic drugs, such as diazepam, produce behavioral effects that are opposite to those produced by the N/OFQ

injections. Specifically, injections of diazepam produce shorter latencies to enter open/lit areas and greater total time spent in these areas. These results suggest that N/OFQ has an anxiogenic action after ICV administration.

In addition to these behavioral effects, ICV injections of N/OFQ increase HPA axis activity in rats. When rats are injected into the lateral ventricle under unstressed conditions (i.e., the rats are allowed to recover from the stress of handling and cannula implantation prior to the delivery of the drug), they exhibit substantial elevations in circulating adrenocorticotropic hormone (ACTH) and corticosterone (CORT; Devine et al., 2001). These elevations of stress-related hormones are also observed when ICV N/OFQ injections are administered without allowing rats to recover from the stress of handling (Nicholson et al., 2002). Additionally, elevations of ACTH and CORT are observed when rats are injected and then exposed to the mild stress of a novel environment or following testing in the open field (Devine et al., 2001; Fernandez et al., 2004). These N/OFQ-induced elevations in circulating hormone concentrations are mediated by limbic inputs, including the septum, BNST, and amygdala (Misilmeri and Devine, 2000; Misilmeri et al., 2002). Specifically, unstressed injections into these limbic structures produce elevations in circulating ACTH and CORT. These data suggest that N/OFQ activates the HPA axis in unstressed conditions and enhances its activity during exposure to mild stress.

The behavioral and hormonal results described above conflict with other reports that N/OFQ is anxiolytic and attenuates stress-induced CORT elevations (Gavioli et al., 2002; Griebel et al., 1999; Jenck et al., 1997; Le Cudennec et al., 2002). The reasons for these discrepancies are currently unclear, but we have consistently observed dose-orderly

anxiogenic and HPA axis-activating effects under a variety of conditions and with experimenters who are blind to the treatment conditions. Anxiogenic actions have also been reported by Vitale and colleagues (2003), using an elevated plus maze.

In light of these observations that N/OFQ produces anxiogenic behavioral effects and activation of the HPA axis, and that the hormonal alterations produced by N/OFQ are mediated by limbic structures, we were interested in whether the anxiogenic behavioral effects are also mediated by such limbic regions. In particular, we examined the potential role of the amygdala, a limbic structure that is known to participate in the regulation of behavioral and hormonal responses to fear-inducing stimuli (for examples see Goldstein, 1965; Rogan et al., 1997; Walker and Davis, 1997).

CHAPTER 2
METHODS

Animals

Male Long Evans rats (n = 85, Harlan, Indianapolis, IN) were housed in polycarbonate cages (43 x 21.5 x 25.5 cm) on a 12hr-12hr light-dark cycle (lights on at 7:00 am). The rats were pair-housed until surgery, then singly-housed in a climate-controlled vivarium (temperature 21-23^{o} C, humidity 55-60%). Standard laboratory chow and tap water were available *ad libitum*. All procedures were pre-approved by the University of Florida's Institutional Animal Care and Use Committee, and the experiments were conducted in compliance with the NIH Guide for the Care and Use of Laboratory Animals.

Drugs

Ketamine and xylazine were both obtained from Henry Schein (Melville, NY) at concentrations of 100 mg/ml. Ketamine-xylazine was mixed by adding 2 ml of xylazine to 10ml of ketamine yielding a 12ml solution of 83.3 mg/ml ketamine and 16.7 mg/ml xylazine. Ketorolac tromethamine (30 mg/ml) and AErrane (99.9 % isoflurane) were also purchased from Henry Schein.

N/OFQ was obtained from Sigma-Aldrich (St. Louis, MO) and was dissolved in artificial extracellular fluid (aECF) composed of 2.0 mM Sorenson's phosphate buffer (pH 7.4) containing 145 mM Na^{+}, 2.7 mM K^{+}, 1.0 mM Mg^{2+}, 1.2 mM Ca^{2+}, 150 mM Cl^{-}, and 0.2 mM ascorbate. These ion concentrations replicate the concentrations found in extracellular fluid in the brain (Moghaddam and Bunney, 1989). N/OFQ was prepared at

concentrations of 0.01, 0.1, and 1.0 nmole per 1.0 µl for ICV injections or per 0.5 µl for amygdaloid injections.

Surgery

Each rat (260-355g) was implanted with a guide cannula under ketamine-xylazine anesthesia (62.5 mg/kg ketamine + 12.5 mg/kg xylazine, i.p. in a volume of 0.75 ml/kg). Ketorolac tromethamine (2 mg/kg, s.c.) was injected for analgesia at the time of surgery. During surgery, AErrane was administered as supplemental anesthesia as necessary. Once the rat was anesthetized, a stainless steel guide cannula (11mm, 22 gauge) was vertically implanted into the right lateral ventricle (ICV; 0.8 mm posterior to bregma, 1.4 mm lateral from the midline, and 2.7 mm ventral from dura; $n = 35$) or into the right amygdala (1.8 mm posterior to bregma, 3.9 mm lateral, and 6.2 mm ventral; $n = 50$). The cannula was secured with dental cement anchored to stainless steel screws (0.80 x 3/32", Plastics One Inc.). An obturator that extended 1.2 mm beyond the guide cannula tip was inserted at the time of surgery and removed on the day of the experiment at the time that an intracranial injection was administered. Following surgery, each rat was injected with 1.0 ml warm 0.9% NaCl and placed in a warm, clean cage to recover from anesthesia. The rats were then returned to the vivarium where they were given 7-10 days to fully recover from surgery.

Equipment

Anxiety-related behavior was measured in the modified open field test (see Fig. 2-1). The open field was composed of a 90 x 90 x 60 cm field with a 20 x 30 x 60 cm start box attached to the outside of the open field, at the midpoint of one side. The bottom and sides were constructed of black acrylic. Separating the start box and the open field was a black acrylic guillotine door attached to a rope and pulley system, which

allowed the door to be opened from outside the testing room. The tops of the start box and open field were open and a camera was mounted on the ceiling above the testing apparatus to record the rats' behaviors. Illumination of the start box was approximately 14 lux, and illumination throughout the open field was even at approximately 30 lux.

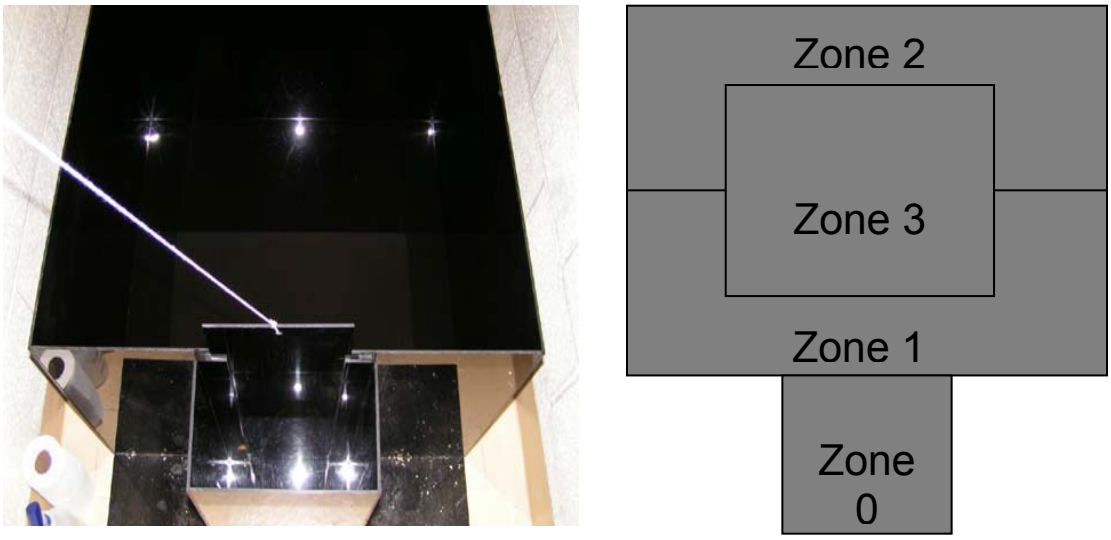


Figure 2-1. Photograph of the open field and a diagram of the zones used for scoring. Zone 0 represents the start box where the rat is placed for the first minute of the test. After the door opens and the rat enters the open field, he may move between zone 0 and the open field freely. Zone 1 represents the proximal periphery of the open field. Zone 2 represents the distal periphery, and zone 3 represents the central region of the open field.

An observer who was blind to the treatment conditions scored the exploratory behavior of the rats from the videotapes, using a grid that was superimposed on the video monitor. This grid divided the open field into 25 equal squares. The outer 16 squares defined an outer zone that was further subdivided such that the half of the outer zone proximal to the start box defined zone 1 and the half of the outer zone distal from the start box defined zone 2. Zone 3 was defined by the inner 9 squares. Additionally, for consistency, the start box was defined as zone 0. Latency to enter the open field (i.e. entry into zone 1), total time spent in the open field (sum of zones 1-3), latency to enter

and time spent in each of the 3 zones, and the number of entries into the open field and the inner zone 3 were used as measures of anxiety. An entry into the open field or movement from one zone to another was counted when all 4 paws of the rat left one zone and entered a new zone.

Anxiety-Testing Procedure

Beginning 7-10 days after surgery, each rat was handled for 5 minutes on each of 3 consecutive days, and then given one day with no disturbance. On the 5th day, each rat was fitted with a 28-gauge stainless steel injector connected with polyethylene (PE20) tubing to a Hamilton syringe (5 µl syringe for ICV injections and 1 µl syringe for amygdaloid injections) mounted in a syringe pump. Each rat then received a 1.0 µl (ICV) or 0.5 µl (amygdala) injection of aECF or aECF containing 0.01, 0.1, or 1.0 nmole N/OFQ by an experimenter blind to the dose. The injections were administered over a 2-minute period and the injector was left in place for 3 additional minutes for diffusion. Each rat was freely moving in its home cage during the injection procedure.

These injections and the subsequent behavioral tests were completed 90-210 minutes after the vivarium lights were turned on, the time during which the HPA axis is at its daily nadir (Ixart et al., 1977; Kwak et al., 1993). Five minutes after the injection, each rat was individually placed in the start box (zone 0) of the open field test, and the door to the testing room was closed, isolating the rat from the experimenter and any other disruptive influences. The rat was then given 1 minute to acclimate to the novel environment of the start box. After 1 minute the guillotine door was opened remotely, and it remained open throughout the test period. The rat was given 5 minutes to explore the start box and open field. Each rat was then returned to its home cage until sacrifice by rapid decapitation 30 minutes after the start of the injection.

Immediately after decapitation, 6 ml of trunk blood was collected into chilled polypropylene tubes containing 600 µl of Na_2EDTA at 20 µg/µl. The tubes were centrifuged at 1000x gravity. The plasma fraction was collected, aliquotted, and frozen at -80ºC until use. Each brain was removed, frozen in 2-methylbutane at -40°C, and stored at -80°C until use. Later each brain was sectioned at 30 µm and stained with cresyl violet for cannula placement verification. Thymus glands, adrenal glands, and spleens were dissected out and weighed for verification of the health status of the rats.

Radioimmunoassay (RIA) was performed for quantification of plasma concentrations of CORT using a kit by Diagnostic Products Corp. (Los Angeles, CA). The interassay variability for this kit increased with sample CORT concentrations, ranging from less than 5% for lower plasma CORT concentrations to less than 15% for higher plasma CORT concentrations.

Statistical Analyses

All between groups differences (0, 0.01, 0.1, and 1.0 nmole N/OFQ) for latency to enter the open field and the individual zones, total open field time and individual zone times, number of entries into the open field and inner zone 3, plasma CORT concentrations, and organ masses were analyzed by separate one-way ANOVAs for ICV and for amygdala placements. All significant effects were further analyzed by Newman-Keuls post-tests.

Standard deviations were calculated for circulating CORT concentrations for the ICV-injected rats and the amygdala-injected rats, separately. Outliers (values beyond 2 standard deviations from the mean) were removed from further analysis. Two outlying CORT values from the ICV-implanted groups and 2 values from the amygdala-implanted groups were eliminated prior to further analysis.

Anatomical misplacements occurred in 2 ICV-implanted rats and necrotic lesions at the site of injection were observed in 2 additional ICV-implanted rats. These 4 rats were removed from the study. Anatomical misplacements also occurred in 9 amygdala-implanted rats. Five of the rats with extra-amygdaloid placements were treated with N/OFQ (1 rat at 1.0 nmole, 2 rats at 0.1 nmole, and 2 rats at 0.01 nmole) and are reported as anatomical controls. The remaining 4 rats with misplacements were treated with aECF and were removed from the study. Additionally, 2 amygdala-implanted rats had necrotic lesions at the site of the injection and were also removed from the study.

Differences between anatomical controls and intra-amygdaloid vehicle controls in latency to enter the open field and the individual zones, total open field time and individual zone times, number of entries into the open field and inner zone 3, plasma CORT concentrations, and organ masses were analyzed by individual T-tests.

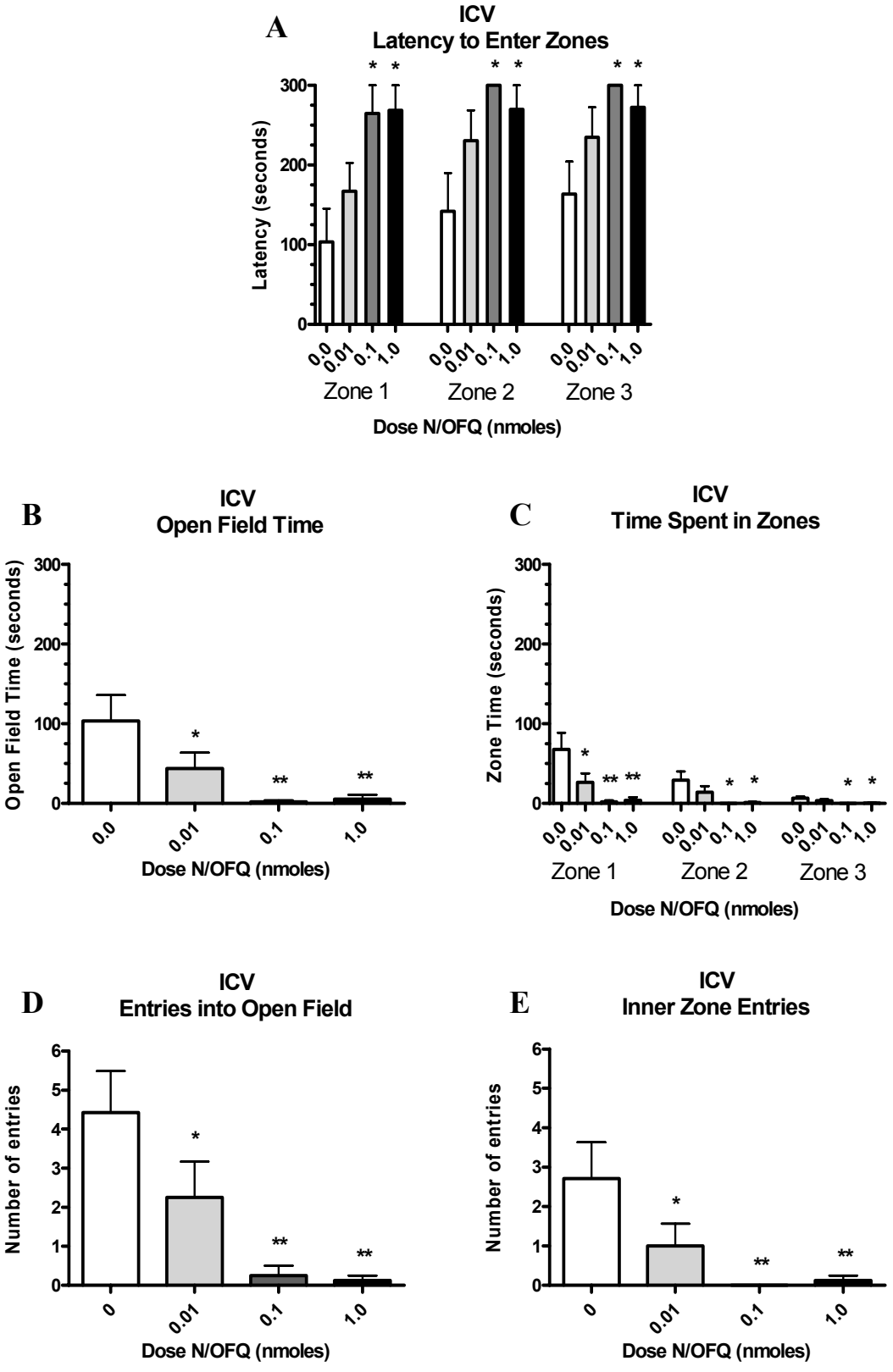
CHAPTER 3
RESULTS

Intracerebroventricular Injections

The N/OFQ-treated rats showed greater expression of anxiety-related behaviors than the aECF vehicle-treated rats did after ICV injections. These N/OFQ-treated rats displayed significantly longer latencies to enter the open field (i.e. zone 1; $F_{(3,27)} = 4.83$, $p < 0.01$) than did the aECF-treated rats. The N/OFQ-treated rats also exhibited significantly longer latencies to enter zone 2 ($F_{(3,27)} = 4.06$, $p < 0.05$) and zone 3 ($F_{(3,27)} = 3.62$, $p < 0.05$) (Fig. 3-1A). Additionally, the N/OFQ-treated rats spent significantly less time in the open field, at all N/OFQ doses (Fig 3-1B; $F_{(3,27)} = 6.44$, $p < 0.01$), in comparison with the behaviors of the aECF-treated rats. This decrease in open field time was also significant for zone 1 ($F_{(3,27)} = 6.90$, $p < 0.01$), zone 2 ($F_{(3,27)} = 4.44$, $p < 0.05$), and zone 3 ($F_{(3,27)} = 4.18$, $p < 0.05$) independently (Fig. 3-1C). The N/OFQ-treated rats also displayed significantly fewer entries into the open field from the start box (Fig. 3-1D; $F_{(3,27)} = 8.24$, $p < 0.01$) and significantly fewer entries into the inner zone 3 (Fig. 3-1E ; $F_{(3,27)} = 5.7$, $p < 0.01$).

Injections of N/OFQ into the right lateral ventricle produced significant elevations in circulating CORT at every dose administered as compared to the CORT concentrations in the aECF-treated rats (Fig. 3-2; $F_{(3,25)} = 5.6$, $p < 0.01$).

Figure 3-1. Anxiety-related behaviors following ICV injections of N/OFQ. N/OFQ-treated rats exhibited: (A) longer latencies to enter all 3 zones of the open field, (B) decreased total time in the open field, (C) decreased total time in each of the 3 zones, (D) fewer entries into the open field from the start box , and (E) fewer entries into zone 3 (the inner zone). All of these effects occurred in a manner that was generally dose-orderly. Values expressed are group means ± SEM (n = 7-8 rats per group). At some doses, all rats in the group failed to enter zones 2 or 3. These groups show a mean latency of 300s, a mean zone time of 0s, a mean numer of entries equal to 0, and a SEM of 0 for each of these measures. Significant differences between the N/OFQ-treated rats and the aECF-treated controls (0.0 nmoles) are expressed as * $p < 0.05$ and ** $p < 0.01$


A
ICV
Latency to Enter Zones
Latency (seconds)
300
200
100
0
0.0
0.01
0.1
1.0
Zone 1
Zone 2
Zone 3
Dose N/OFQ (nmoles)
B
ICV
Open Field Time
Open Field Time (seconds)
Dose N/OFQ (nmoles)
C
ICV
Time Spent in Zones
Zone Time (seconds)
Zone 1
Zone 2
Zone 3
Dose N/OFQ (nmoles)
D
ICV
Entries into Open Field
Number of entries
0
0.01
0.1
1.0
Dose N/OFQ (nmoles)
E
ICV
Inner Zone Entries
Number of entries
Dose N/OFQ (nmoles)

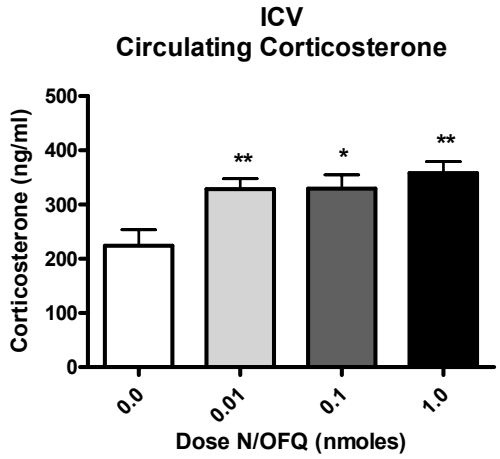


Figure 3-2. Concentrations of circulating corticosterone following ICV injections of N/OFQ. Injections of N/OFQ into the lateral ventricle produced dose-orderly elevations in circulating CORT at all doses administered. Values expressed are group means ± SEM (n = 7-8 rats per group). Significant differences between the N/OFQ-treated rats and the aECF-treated controls are expressed as * $p < 0.05$ and ** $p < 0.01$

Intra-Amygdaloid Injections

The N/OFQ-treated rats with amygdaloid implants exhibited elevations in anxiety-related behavior as indicated by significantly longer latencies to enter the open field (Fig. 3-3A; $F_{(3,35)} = 3.34$, $p < 0.05$) and significantly longer latencies to enter zone 2 ($F_{(3,35)} = 2.93$, $p < 0.05$). However, the latencies to enter zone 3 were not significantly different from the latencies of the aECF-treated rats ($F_{(3,35)} = 1.81$, $p > 0.05$). The N/OFQ-treated rats did not exhibit significant differences from aECF-treated rats in total open field time (Fig 3-3B.; $F_{(3,35)} = 1.5$, $p > 0.05$) or time spent in any of the 3 zones (Fig 3-3C.; $F_{(3,35)} = 0.98$, $p > 0.05$ for zone 1; $F_{(3,35)} = 2.63$, $p > 0.05$ for zone 2; $F_{(3,35)} = 0.3$, $p > 0.05$ for zone 3). These rats also did not display significant differences in the number of entries into the open field (Fig. 3-3D; $F_{(3,35)} = 0.45$, $p > 0.05$) or into the inner zone 3 (Fig. 3-3E; $F_{(3,35)} = 0.93$, $p > 0.05$).

Most of the amygdaloid placements were located in or around the basomedial (BMA) and the central (CeA) nuclei of the amygdala (see figures 3-4 and 3-5). The

effects of aECF injections into these amygdaloid sites were compared against the effects of injections into extra-amygdaloid sites (defined as anatomical controls). Comparisons between the vehicle-treated rats and the N/OFQ-treated anatomical controls showed no significant differences on any measure of latency (Fig. 3-3, A; $t_{(12)} = 0.87$, $p > 0.05$ for open field latency, $t_{(12)} = 0.72$, $p > 0.05$ for zone 2 latency, $t_{(12)} = 2.1$, $p > 0.05$ for zone 3 latency). Similarly, the anatomical controls did not differ from the vehicle-treated rats on any measure of time (Fig. 3-3, B-C; $t_{(12)} = 0.46$, $p > 0.05$ for open field time, $t_{(12)} = 0.29$, $p > 0.05$ for zone 1 time, $t_{(12)} = 0.62$, $p > 0.05$ for zone 2 time, $t_{(12)} = 0.49$, $p > 0.05$ for zone 3 time), or on any measure of zone entries (Fig. 3-3, D-E; $t_{(12)} = 1.92$, $p > 0.05$ for open field entries, and $t_{(12)} = 0.82$, $p > 0.05$ for inner zone 3 entries).

Injections of N/OFQ into the right amygdala did not produce significant changes in circulating CORT (Fig. 3-6; $F_{(3,34)} = 1.78$; $p > 0.05$) as compared with the CORT concentrations in the aECF-treated rats. Concentrations of circulating CORT were not significantly different between N/OFQ-treated anatomical controls and vehicle-treated rats (Fig. 3-6, $t_{(12)} = 2.10$, $p > 0.05$).

Figure 3-3. Anxiety-related behaviors following intra-amygdaloid injections of N/OFQ. N/OFQ-treated rats exhibited (A) longer latencies to enter zones 1 and 2 of the open field. However, injections of N/OFQ into the right amygdala did not significantly alter (B) total open field time, (C) time spent in any of the 3 zones, (D) the number of entries into the open field, (E) or the number of entries into the inner zone 3. Values expressed are group means ± SEM (n = 9-10 rats per group). Significant differences between the N/OFQ-treated rats and the aECF-treated controls (0.0 nmoles) are expressed as * $p < 0.05$. AC = anatomical controls

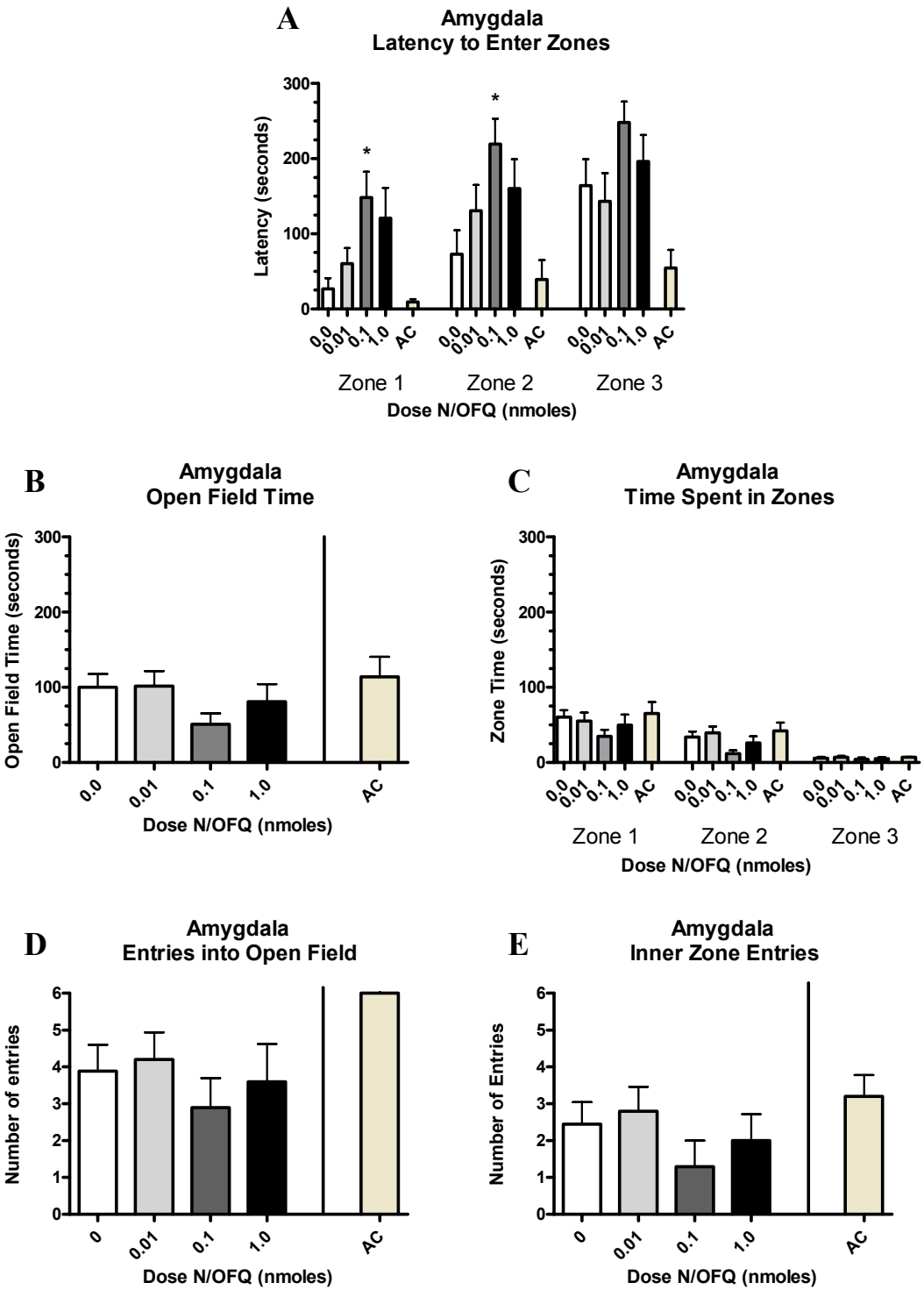

A
Amygdala
Latency to Enter Zones
Latency (seconds)
300
200
100
0
*
*
0.0
0.01
0.1
1.0
AC
Zone 1
Zone 2
Zone 3
Dose N/OFQ (nmoles)
B
Amygdala
Open Field Time
Open Field Time (seconds)
Dose N/OFQ (nmoles)
C
Amygdala
Time Spent in Zones
Zone Time (seconds)
Zone 1
Zone 2
Zone 3
Dose N/OFQ (nmoles)
D
Amygdala
Entries into Open Field
Number of entries
0
0.01
0.1
1.0
AC
Dose N/OFQ (nmoles)
E
Amygdala
Inner Zone Entries
Number of Entries
Dose N/OFQ (nmoles)

Figure 3-4. Anatomical map of amygdaloid placements showing latency to enter open field. Shown on the map are the placements for each rat, including anatomical controls. The shapes and colors of the symbols indicate the doses of N/OFQ and the latency to enter the open field for individual rats. The 1.0 nmole doses are marked by an upward facing triangle, 0.1 nmole doses are marked by a diamond, 0.01 nmole doses are marked by a downward facing triangle, and aECF controls are marked by a circle. Additionally, anatomical controls are identified by a black dot placed within the marker. Latency to enter the open field is identified by color with red ≥ 131 seconds, orange = 45-130 seconds, yellow = 10-44 seconds, and green ≤ 9 seconds. (Atlas diagrams taken from Paxinos and Watson, 1998.)

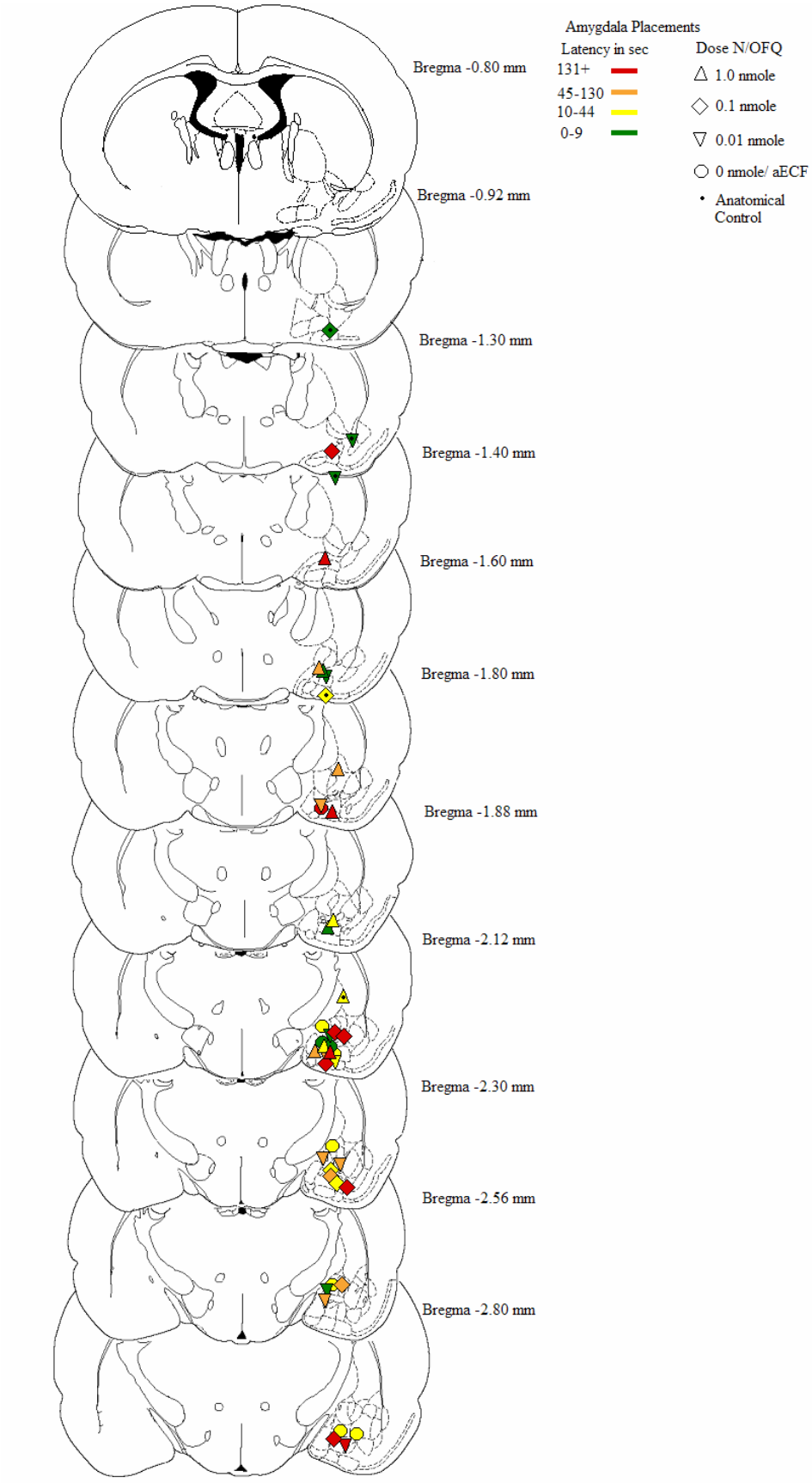
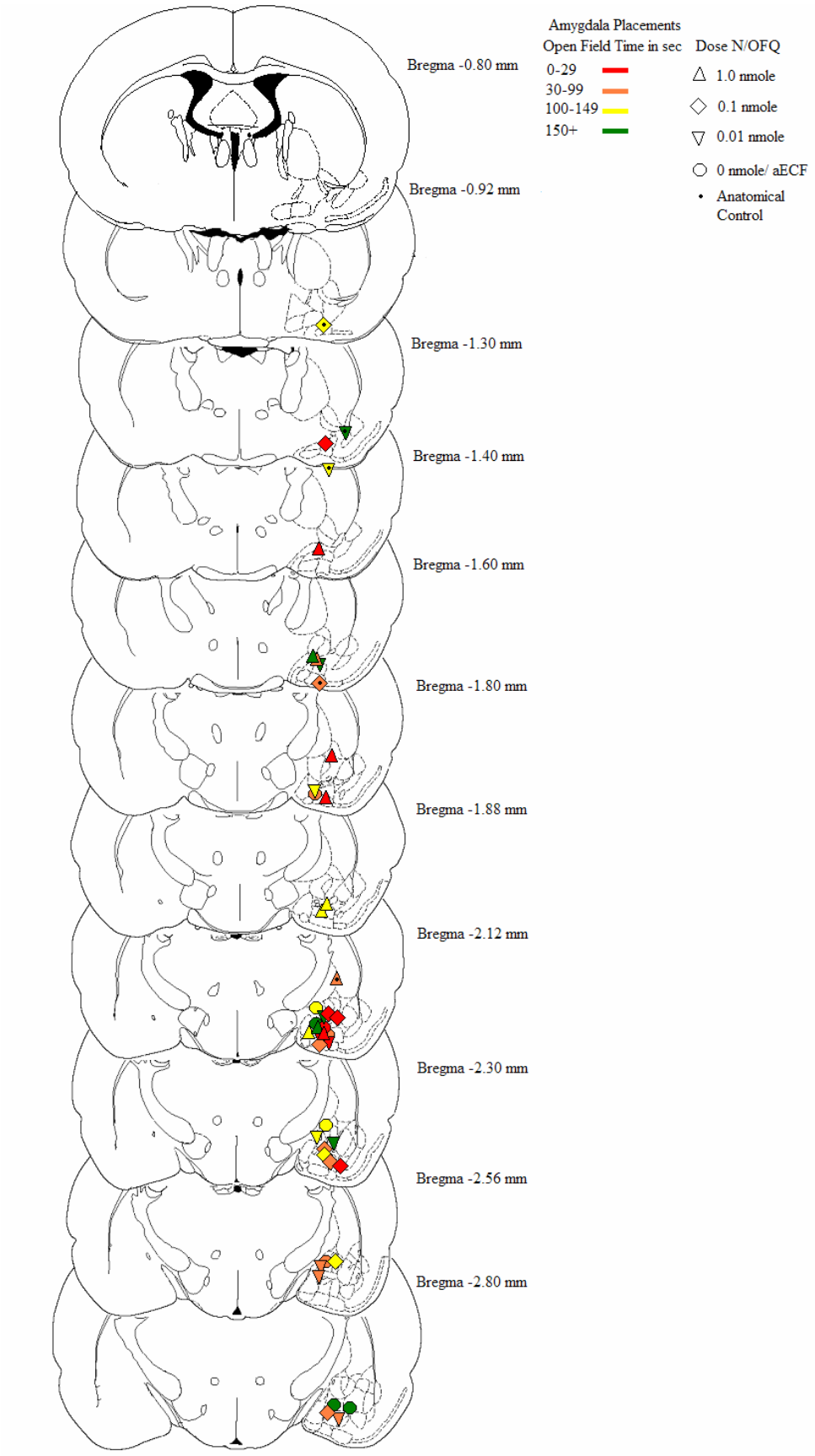

Amygdala Placements
Latency in sec
131+
45-130
10-44
0-9
Dose N/OFQ
1.0 nmole
0.1 nmole
0.01 nmole
0 nmole/ aECF
Anatomical Control
Bregma -0.80 mm
Bregma -0.92 mm
Bregma -1.30 mm
Bregma -1.40 mm
Bregma -1.60 mm
Bregma -1.80 mm
Bregma -1.88 mm
Bregma -2.12 mm
Bregma -2.30 mm
Bregma -2.56 mm
Bregma -2.80 mm

Figure 3-5. Anatomical map of amygdaloid placements showing time spent in the open field. Shown on the map are the placements for each rat, including anatomical controls. The shapes and colors of the symbols indicate the doses of N/OFQ and the time spent in the open field for individual rats. 1.0 nmole doses are marked by an upward facing triangle, 0.1 nmole doses are marked by a diamond, 0.01 nmole doses are marked by a downward facing triangle, and aECF controls are marked by a circle. Additionally, anatomical controls are identified by a black dot placed within the marker. Amount of time spent in the open field is identified by color with red ≤ 29 seconds, orange = 30-99 seconds, yellow = 100-149 seconds, and green ≥ 150seconds. (Atlas diagrams taken from Paxinos and Watson, 1998.)


Amygdala Placements
Open Field Time in sec
0-29
30-99
100-149
150+
Dose N/OFQ
1.0 nmole
0.1 nmole
0.01 nmole
0 nmole/ aECF
Anatomical Control
Bregma -0.80 mm
Bregma -0.92 mm
Bregma -1.30 mm
Bregma -1.40 mm
Bregma -1.60 mm
Bregma -1.80 mm
Bregma -1.88 mm
Bregma -2.12 mm
Bregma -2.30 mm
Bregma -2.56 mm
Bregma -2.80 mm

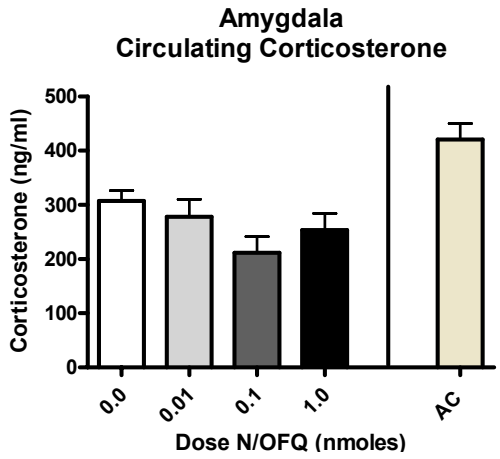


Figure 3-6. Concentrations of circulating corticosterone following intra-amygdaloid injections of N/OFQ. N/OFQ administration did not significantly alter the levels of circulating corticosterone. Values expressed are group means ± SEM (n = 9-10 rats per group). AC = anatomical controls

Thymus, Adrenal, and Spleen Masses

There were no significant differences in adrenal masses (Fig. 3-7A.; ICV: $F_{(3,26)} = 0.23$, $p > 0.05$; Amygdala: $F_{(3,30)} = 0.46$, $p > 0.05$), thymus gland masses(Fig. 3-7B; ICV: $F_{(3,27)} = 0.30$, $p > 0.05$; Amygdala: $F_{(3,35)} = 0.73$, $p > 0.05$), or spleen masses (Fig. 3-7C; ICV: $F_{(3,27)} = 0.13$, $p > 0.05$; Amygdala: $F_{(3,35)} = 0.49$, $p > 0.05$) between the aECF and N/OFQ-treated rats for ICV- or amygdala-implanted groups. Additionally, these organ masses were comparable to the masses that were found in previous experiments involving groups of rats that did or did not undergo surgical procedures (data not shown; Fernandez et al., 2004).

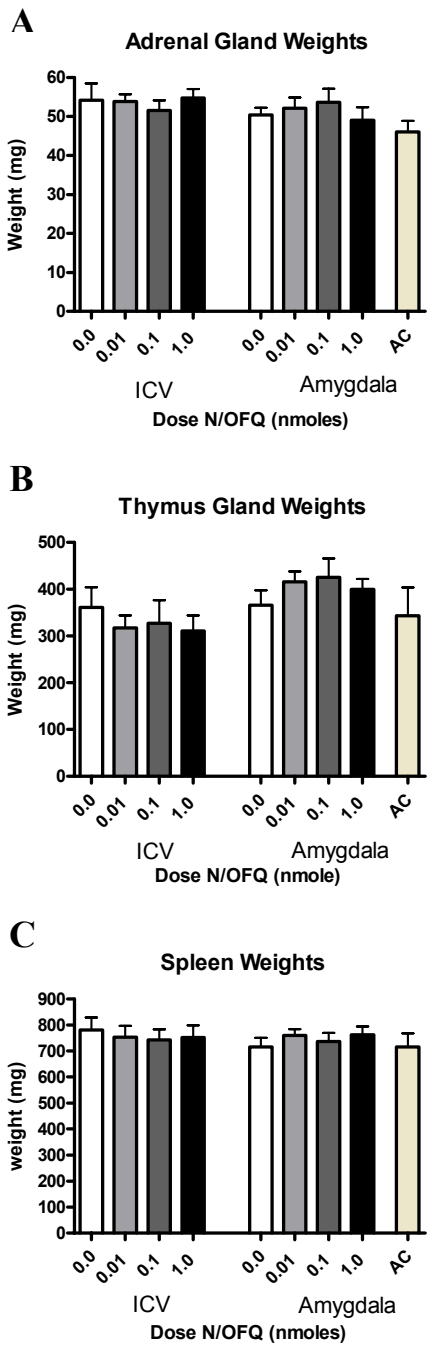


Figure 3-7. Analysis of glandular masses. (A) Adrenal gland masses, (B) thymus gland masses and (C) spleen masses showed no significant differences between groups, regardless of the injection site or N/OFQ treatment. Values expressed are group means ± the standard error of the mean (SEM) (n = 7-9 rats per group for adrenal glands; n = 7-10 for thymus glands and spleens).

CHAPTER 4
DISCUSSION

Anxiety-Related Behavior

Injections of N/OFQ into the right lateral ventricle were very effective at producing dose orderly elevations in all measured anxiety-related behaviors. These results replicate previous findings from our lab (Fernandez et al., 2004). Injections into the right amygdala also elevated anxiety-related behaviors as seen in measures of latency to enter the open field and zone 2. However, amygdaloid injections were less effective at altering the expression of these behaviors than were the ICV injections, when equimolar amounts of N/OFQ were injected.

There are multiple reasons why we might observe differences in potency between ICV and amygdaloid injections of N/OFQ. First, the amygdala may not be the primary site of drug action. Davis and colleagues (Lee and Davis, 1997; Walker et al., 2003) proposed that one condition for a structure to be considered a primary site of drug action is that the effects of direct injections into that structure mimic the effects of injections into the ventricle. In fact, in their studies of acoustic startle responses, Lee and Davis (1997) found that injections of corticotropin releasing hormone (CRH) into the BNST, like injections of CRH into the lateral ventricle, produced an enhancement of rats' acoustic startle reflexes. These increases in startle following intra-BNST injections occurred more rapidly than the enhancement produced by ICV injections. Additionally, the increases in startle occurred at much lower concentrations than were required with ICV injections (40 and 80 ng intra-BNST versus 1 μg ICV), although the ultimate

behavioral change was not as great with these low concentration intra-BNST injections (70% enhancement following intra-BNST injections versus approximately 200% enhancement following ICV injections). This, in addition to lesion and CRH antagonist data, demonstrates that the BNST is a primary site of action for the startle-enhancing effects of CRH. These results provide a model for localizing the effects of neurotransmitter action. In contrast, our results that equimolar injections of N/OFQ into the amygdala produced less potent effects than injections into the ventricle suggest that the amygdala may not be the primary site of action for the anxiogenic effects of NOFQ.

It could be argued that the effects of our amygdaloid injections were less potent because of the fact that they were done unilaterally. However, we injected into the right amygdala, which is generally more dominantly involved in emotionally-relevant behavioral responses (Adamec et al., 2001; Andersen and Teicher, 1999; Coleman-Mesches and McGaugh, 1995a&b; Peper et al., 2001; Scicli et al., 2004), or is at least no less involved than the left amygdala (Good and Westbrook, 1995; Izquierdo and Murray, 2004; LaBar and LeDoux, 1996). In fact, bilateral injections may add little in terms of changes in emotionally-relevant behaviors when compared to the effects of unilateral injections into the right amygdala. For example, in one study, Coleman-Mesches and McGaugh (1995b) found impairments in the retention of inhibitory avoidance learning following intra-amygdaloid injections of the GABA-A agonist, muscimol. Interestingly, unilateral right amygdaloid injections and bilateral amygdaloid injections produced equivalent degrees of learning impairment (and the impairment was minimal following unilateral injections into the left amygdala). These data suggest that the right amygdala is dominant in inhibitory avoidance learning tasks

and the left amygdala plays little role, which is consistent with other studies showing right amygdaloid dominance in emotionally-relevant behaviors. Accordingly, the fact that we did not get more potent effects with injections into the right amygdala versus the ventricle further supports the contention that the amygdala is not a primary site for the anxiogenic actions of N/OFQ.

Importantly, our ICV injections were also done unilaterally. Previous work in our lab with ^{125}I-N/OFQ demonstrated that N/OFQ injected unilaterally into the lateral ventricle primarily reaches ipsilateral structures, with little radioactive label detection in the contralateral hemisphere (Devine, D.P., unpublished). This provides further evidence that the potent effects seen following ICV injections in the present experiment were not likely due to bilateral actions at the amygdalae. ICV injections are, however, expected to diffuse more widely than intra-amygdaloid injections, and so, these injections are likely to have effects at multiple sites. Moreover, if there are multiple sites all contributing in an additive or synergistic manner to emotionally-relevant behavioral effects, then it is possible that no single site will meet the criteria of a primary site of drug action. ICV injections, then, may actually produce a stronger result by affecting multiple primary sites concurrently.

Another factor that could contribute to the apparently weak behavioral effects of intra-amygdaloid injections of N/OFQ is that the amygdala is a very complex structure that consists of multiple interconnected nuclei. A number of these nuclei are involved in fear-related behaviors including the central amygdala (CeA), the basolateral nucleus (BLA), the basomedial nucleus (BMA), the lateral nucleus (LA), the medial nucleus (MeA), and the intercalated neurons (for examples see Bhatnagar and Dallman, 1998;

Good and Westbrook, 1995; Goosen and Maren, 2001; Parè et al., 2003; Walker and Davis, 1997). However, among these nuclei, only the BLA and the MeA show high levels of NOP receptor mRNA expression and radiolabelled N/OFQ binding (Neal et al., 1999b). Additionally, the BMA and LA show some NOP mRNA expression and N/OFQ binding. However, the CeA and intercalated neurons show little to no NOP mRNA expression or N/OFQ binding. It is possible, then, that injections of N/OFQ into these different nuclei could have differing effects, introducing greater variability in the results. While there was some scatter in placement in the present study, most placements were in the CeA or the BMA. Although we did not statistically analyze differences between injections into these 2 sets of nuclei, there was no apparent difference in effect. This may be due, largely, to diffusion of N/OFQ following the injections, such that our injections affected multiple subnuclei. However, it is unclear how far our injections diffused within the amygdala.

An alternative possibility is that another limbic site, such as the septum or BNST, is the primary site of drug action, mediating the behavioral effects of N/OFQ after ICV administration. In fact, the amygdala and BNST can be differentiated in terms of their roles in fear and anxiety (Walker and Davis, 1997; for review see Walker et al., 2003). Although the distinctions are not entirely clear, the amygdala appears to play a larger role in fear-related behaviors (such as startle responses to a specific, usually conditioned, stimulus), and the BNST appears to be more important in generalized anxiety (for example increases in startle reflexes that are not produced by a specific and immediate stimulus). The modified open field test used in the present experiment more resembles tests of generalized anxiety, as there is no specific, conditioned fear stimulus. In this

respect, the BNST may be more involved in the behavioral responses during the open field test.

In conclusion, it appears that the amygdala plays a role in N/OFQ-induced increases in anxiety-related behaviors. However, because the behavioral effects observed in amygdala-implanted rats were not as great as those seen in ICV-implanted rats; it is evident that other structures must also be involved. The specific brain regions involved and the manner in which these regions interact to yield the anxiogenic effect of N/OFQ remain to be determined.

Corticosterone

ICV injections of N/OFQ enhanced HPA axis-activity following exposure of the rats to mild stress (i.e., handling and exposure to the novel environment of the anxiety test). These data for ICV injections are consistent with previous research (Fernandez et al., 2004). However, in this and the Fernandez experiment, the CORT concentrations appear to be higher after injection of N/OFQ at all the doses that were tested, than they were following ICV injections of equimolar N/OFQ doses in unstressed rats (Devine et al., 2001). This supports the assertion that the handling and injection procedures, as well as the exposure to a novel environment (the open field), are mildly to moderately stressful for the rats. Nevertheless, the rats that were injected with N/OFQ still displayed higher levels of circulating CORT in response to these stressors than did the vehicle-treated rats. These data provide evidence that N/OFQ actions produce further enhancement of HPA axis activity beyond that produced by stressor exposure. In fact, Devine and colleagues (2001) demonstrated that injections of N/OFQ into the lateral ventricle prolonged the CORT elevation produced by exposure to a mild stressor such that at 30 minutes following injection, the CORT levels of vehicle-treated rats were

returning to baseline while the CORT levels of N/OFQ-treated rats remained highly elevated. This suggests that N/OFQ has an enduring pharmacological effect or that it is interfering with negative feedback mechanisms of the HPA axis (Devine et al., 2001). While we did not conduct a time course examination of CORT levels in the present experiment, the elevation in CORT levels observed at 30 minutes post-injection in the ICV N/OFQ-treated rats is compatible with the idea that ICV N/OFQ administration prolongs CORT elevation through this time point.

Injections of N/OFQ into the amygdala did not enhance HPA axis activity following exposure to mild stress. This may seem inconsistent with the behavioral data; however, hormonal and behavioral responses to pharmacological manipulations can be dissociated. For example, diazepam, an anxiolytic, does produce elevations in circulating corticosterone (Chabot et al., 1982; Fernandez et al., 2004; Marc and Morselli, 1969; Massoco and Palermo-Neto, 1999). Nevertheless, previous research in our lab found that intra-amygdaloid injections of N/OFQ in unstressed rats did produce elevations of circulating CORT, although the elevations were relatively small (Misilmeri and Devine, 2000). It is possible, then, that the experiences of handling, injection, and exposure to the open field produce stress effects in rats that are great enough to obscure the modest effects of intra-amygdaloid N/OFQ injections on CORT concentrations. Additionally, if the hypothesis of Devine and colleagues (2001), that ICV injections of N/OFQ are reaching structures involved in negative feedback regulation, is correct, then it would be expected that intra-amygdaloid injections of N/OFQ would not produce the same enhancement of HPA axis activity.

It is important to note that the CORT data from 4 rats (2 ICV-implanted rats and 2 amygdala-implanted rats) were removed prior to analysis, as they were outlying values. Of major concern were the very low CORT concentrations (under 100 ng/ml) of the 2 ICV-implanted rats, considering the level of stressor exposure these rats experienced. This may have been due to high amounts of coagulation in the plasma tested, although this was not systematically recorded. For consistency, standard deviations were also calculated for the amygdala-implanted groups and outlying values were subsequently removed. The removal of the 2 outlying values from the 1.0 nmole ICV group did affect the final statistical analysis; however, removal of the 2 values from the 0.01 nmole and 1.0 nmole intra-amygdaloid groups did not affect further analysis.

Organ Masses

In the present study, we measured the thymus, adrenal, and spleen masses to establish that there were no systematic differences in health status or stress exposure between the various groups of rats (especially since the rats underwent differing types of intracranial cannulation surgery). Thymus glands and spleens tend to decrease in mass and adrenal glands tend to increase in mass following exposure to physiological stressors (such as physical insults, poor diet, or exposure to toxic chemicals) and psychological stressors (such as restraint, crowding, or predator exposure), particularly after chronic exposure to these stressors (for examples see Blanchard et al., 1998; Bryant et al., 1991; Dominguez-Gerpe and Rey-Mendez, 2001; Hasegawa and Saiki, 2002; Selye, 1936; Watzl et al., 1993). While we did not include any rats that did not undergo the stress of surgery and handling, the gland masses in this experiment were similar to those measured in other experiments where non-surgical unstressed controls were included (Fernandez et al., 2004). Additionally, there were no significant differences in thymus gland masses,

spleen masses, or adrenal gland masses between any of the groups tested. Therefore, we can conclude that there were no apparent differences in the health of the rats that may have affected the ultimate results of the experiment.

Summary

These data show that N/OFQ injections affect anxiety and HPA axis activity through actions in the amygdala (among other potential sites), suggesting the possibility that amygdaloid N/OFQ neurotransmission may be involved in regulation of affect. However, because ICV injections of N/OFQ produced greater, more potent effects than injections into the right amygdala did, additional structures must be involved. This may include actions in the contralateral amygdala or other limbic structures such as the BNST or, potentially, in multiple limbic regions involving synergistic actions at these sites. To examine other structures involved, we are currently conducting a number of studies. For example, we are examining the effects of injecting N/OFQ into the BNST to determine if the BNST contributes to, or is the primary site of action for, the anxiogenic behavioral effects of N/OFQ. Additionally, we are using other tools, such as in situ hybridization, to examine changes in N/OFQ and NOP expression throughout the brain following social stress. These studies will help us to better understand the role of N/OFQ in the neurocircuitry of stress, anxiety, and HPA axis functioning.

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BIOGRAPHICAL SKETCH

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