ROLE OF THE NOCICEPTIN/ORPHANIN FQ-NOP RECEPTOR SYSTEM IN STRESS RESPONSES

By

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To Susan L. Menet.
She left this world on November 5, 2007, but she will never leave our hearts.

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Intracerebroventricular (ICV) microinjections of the neuropeptide nociceptin/orphanin FQ (N/OFQ) produce elevations in hypothalamic-pituitary-adrenal (HPA) axis activity and anxiety-related behaviors. Injections into the amygdala also elevate anxiety-related behaviors, although to a lesser extent than do ICV injections. Therefore, the effects of N/OFQ were examined following injections into another limbic structure, the bed nucleus of stria terminalis (BNST). Injections into the BNST produced elevated anxiety-related behaviors and circulating corticosterone. These results suggest that N/OFQ is involved in regulation of affective behaviors and the HPA axis through limbic actions. The potent effects of N/OFQ following ICV injections may involve additive or synergistic activity at multiple limbic sites.

To verify diffusion patterns of N/OFQ following these injections, rats were injected with ^{3}H-N/OFQ into either the lateral ventricle, amygdala, or BNST. Following intraparenchymal injections, diffusion of N/OFQ was localized to the target structures. Following the ICV injections, N/OFQ diffused throughout the ventricles and into periventricular structures. A number of limbic, hypothalamic, and brainstem structures were identified that could contribute to the effects observed following ICV injections.

The possibility that N/OFQ and NOP receptor gene expression and receptor binding are modified by exposure to acute and repeated social stress was also explored. Rats were exposed to social stress (acute, repeated, repeated plus acute, or no stress) and then their brains were analyzed for mRNA expression and receptor binding. There were no statistically significant differences in prepro-N/OFQ mRNA expression or in receptor binding in any of the groups. However, the rats exposed to acute stress displayed greater NOP receptor mRNA expression in the septum, BNST, amygdala, and PVN, as compared to the expression in the unstressed controls. The rats exposed to repeated stress displayed greater NOP receptor mRNA expression in the BNST and PVN, and those exposed to repeated plus acute stress displayed greater expression in the amygdala and PVN.

Overall, it appears that the N/OFQ-NOP receptor system is involved in the regulation of affective states through actions in limbic structures. Additionally, this may be a dynamic system that can be modified by acute and chronic social stress exposure, especially in limbic regions and the PVN.

CHAPTER 1
INTRODUCTION

Stress and Stress Response

Stress

Stress is defined as a physiological response to a change or threat of change, real or perceived, in an organism's environment (Herman and Cullinan, 1997). The physiological responses include activation of systems involved in preparation for high-energy behavioral responses and the subsequent return to homeostatic balance, such as activation of the autonomic nervous system and the hypothalamic-pituitary-adrenal (HPA) axis. The stimuli that initiate these organismic responses are referred to as stressors and can be classified as systemic or processive. Systemic stressors involve direct threats to tissues and organ systems, including events such as cold exposure or food deprivation (Herman and Cullinan, 1997). These systemic stressors appear to be processed primarily by brainstem inputs to the hypothalamus, and may not require processing by higher order limbic and cortical systems. On the other hand, processive stressors, or "emotional stressors", do seem to require higher order processing involving cortical and limbic inputs to the hypothalamus. These types of stressors do not necessarily represent immediate threats to tissues, but derive their qualitative value relative to the organism's previous experience (Herman and Cullinan, 1997).

There is anatomical evidence for at least some differentiation between these two stressor classifications. For example, ether exposure, a systemic stressor, and novel open field exposure, a processive stressor, produce different patterns of *c-fos* activation, with open field exposure producing greater *c-fos* activation in the lateral septum, medial amygdala, and dorsomedial hypothalamus (Emmert and Herman, 1999). Additionally, lesions of the medial prefrontal cortex (MPFC; Diorio et al., 1993) and of the amygdala (Feldman et al., 1994) alter stress responses to

processive stressors, such as restraint, photic, or acoustic stimuli, but not to ether. Rats with MPFC lesions display prolonged elevations in adrenocorticotropic hormone (ACTH) and corticosterone (CORT) following restraint, but not following ether (Diorio et al., 1993). Rats with central and medial amygdaloid lesions display attenuated corticotrophin releasing hormone (CRH), ACTH, and CORT responses to photic and acoustic stimuli but not to ether (Feldman et al., 1994). These studies provide evidence that limbic structures respond strongly to processive stressors and less so to systemic stressors.

HPA Axis Activation Following Stress Exposure

The HPA axis is one important system involved in the processing of stressor-related information and in controlling physiological responses and emotionally-relevant behaviors. Cortical and limbic structures, as well as brainstem structures, have converging inputs at the paraventricular nucleus of the hypothalamus (PVN) where they are involved in regulation of the HPA axis (Herman et al., 1989). Additionally, there are inputs from cortical, limbic, and brainstem regions to the local surround, the peri-PVN.

The PVN is comprised of subdivisions that can be differentiated in terms of their cytoarchitecture, neurotransmitter content, and projections. The dorsal parvocellular division, the ventral extent of the medial parvocellular division, and the lateral parvocellular division project to the brainstem and the spinal cord and are involved in autonomic regulation. The posterior magnocellular division releases arginine vasopressin (AVP) and oxytocin and projects to the posterior pituitary. The dorsolateral extent of the medial parvocellular division primarily contains and releases CRH, although approximately 50% of these cells coexpress AVP. These factors are released into the hypophyseal portal circulation at the external median eminence and primarily affect the anterior pituitary (for review see Whitnall, 1993; Herman et al., 2002b).

The dorsolateral extent of the medial parvocellular PVN is heavily involved in HPA axis regulation. This region integrates the excitatory and inhibitory inputs from the cortex, limbic structures, brainstem, and peri-PVN to produce a net response (for reviews see Herman et al., 2002a and b). Net activation of the dorsolateral extent of the medial parvocellular PVN results in co-release of CRH and AVP. CRH stimulates the anterior pituitary to release adrenocorticotropic hormone (ACTH) into the bloodstream, and AVP acts as a powerful synergist in this process (Gillies et al., 1982). This effect may be further potentiated after repeated stress as the number of cells coexpressing CRH and AVP increase (for example de Goeij et al., 1992; Aubry et al., 1999). Once ACTH is released into the bloodstream, it induces the adrenal synthesis and release of glucocorticoids. The glucocorticoids, including corticosterone (CORT) in rats and cortisol in humans, increase energy utilization and cardiovascular tone, shift the immune response from pro-inflammatory to anti-inflammatory, and terminate further HPA axis activity.

Anatomy of the Limbic-HPA Axis

HPA axis activation is affected by inputs from a number of cortical, limbic, and brainstem regions. The hippocampus, MPFC, septum, amygdala, and bed nucleus of stria terminalis (BNST) all project to the PVN and /or its surround, a region that is dense with glutamatergic and GABA-ergic projections to the PVN (Sawchenko and Swanson 1983; Whitnall, 1993; Boudaba et al., 1996; Boudaba et al., 1997; Ziegler and Herman, 2001; Herman et al., 2002a). The hippocampus is involved in regulation of HPA axis tone and inputs from this structure provide inhibitory modulation of the PVN (Herman and Cullinan, 1997). The MPFC is involved in negative feedback inhibition of the HPA axis, especially in response to processive stressors (Diorio et al. 1993). The septum provides direct and indirect inputs to the PVN (Sawchenko and Swanson, 1983; Risold and Swanson, 1997). The lateral septum generally provides excitatory

input to the HPA axis while the medial septal input is inhibitory (Dunn, 1987a). The amygdala has few direct inputs to the PVN (Prewitt and Herman, 1998), although the medial amygdala (MeA) does have some inputs to the peri-PVN (Canteras et al., 1995; Prewitt and Herman, 1998). The amygdalar involvement in HPA axis control is complex. While electrical stimulation of the MeA and the basomedial amygdala (BMA) increase levels of circulating CORT and stimulation of the central amygdala (CeA), basolateral amygdala (BLA), and lateral amygdala (LA) decrease CORT (Dunn and Whitener, 1986), lesions of both the CeA and MeA decrease the ACTH and CORT response to processive stressors while not affecting basal levels (Feldman et al., 1994). The CeA and MeA have dense projections to the BNST and to hypothalamic nuclei other than the PVN (LeDoux et al., 1988; Canteras et al., 1995; Prewitt and Herman, 1998; Dong et al., 2001). Therefore, the complex actions on HPA axis activity are likely indirect. The BNST is an important limbic structure as it integrates inputs from the amygdala, the lateral septum, and the hippocampus and has dense inhibitory and excitatory projections to the PVN and peri-PVN (Sawchenko and Swanson, 1983; LeDoux et al., 1988; Cullinan et al., 1993; Whitnall, 1993; Canteras et al., 1995; Boudaba et al., 1996; Cullinan et al., 1996; Boudaba et al., 1997; Risold and Swanson, 1997; Prewitt and Herman, 1998; Dong et al., 2001; Dong and Swanson, 2004). The inputs to the PVN from the BNST are also complex. Electrical stimulation of the anterior and medial regions of the BNST increase levels of circulating CORT (Dunn, 1987a,b), stimulation of the lateral regions of the BNST decrease levels of circulating CORT, and stimulation of the posterior regions produces mixed results (Dunn, 1987b). Additionally, lesions of the anterior regions of the BNST decrease CRH mRNA in the medial parvocellular PVN, while lesions of the posterior regions increase CRH mRNA (Herman et al., 1994). However, lesions of the lateral BNST attenuate the ACTH and CORT

response to stressors (Gray et al., 1993). None of these lesions alter the basal activity of ACTH or CORT (Gray et al., 1993; Herman et al., 1994).

Autonomic Nervous System

Several regions of the PVN, including the dorsal parvocellular, ventromedial parvocellular, and lateral parvocellular neurons, project to brainstem autonomic neurons (for review see Herman et al., 2002b). Sympathetic activation stimulates the release of norepinephrine from sympathetic neurons and epinephrine from the adrenal medulla (Martin and Haywood, 1992; Kvetnansky et al., 2006). This activation further increases cardiovascular tone (Martin and Haywood, 1992), as well as inhibits the inflammatory immune response and stimulates anti-inflammatory responses via innervation at the lymphoid organs (for review see Elankov et al., 2000; Eskandari and Sternberg, 2002).

There is a tight interplay between the HPA axis and the sympathetic nervous system (SNS). Stressors activate both systems (Kvetnansky et al., 2006), and activation of one system typically results in activation of the other (for review see Elenkov et al., 2000). Therefore, there is a dual response from both systems, enhancing the full stress response.

Chronic Stress and Disease

Responses to acute stressors are thought to be adaptive in the short term by increasing access to energy stores and increasing cardiovascular tone. However, the effects of chronic cortisol elevations can be detrimental. In rats chronic stress is associated with increased basal CORT (Blanchard et al., 1998), blunted ACTH responses to stressors (Hauger et al., 1990), altered CRH and AVP levels in the pituitary (Hauger et al., 1990; de Goeij et al., 1991), hippocampal atrophy (Manji et al., 2001), adrenal hypertrophy (Blanchard et al., 1998; Hauger et al., 1990), thymus involution, and depressive-like behaviors such as decreased grooming (Blanchard et al., 1998).

In humans, similar changes are seen in individuals with depression. For example, individuals with depression display alterations in HPA axis activity including elevated levels of CRH in their cerebrospinal fluid (CSF; Nemeroff et al., 1984; Nemeroff et al., 1991; Heuser et al., 1998) and cortisol in their blood plasma (Board et al., 1956; Gold et al., 1986; Arborelius et al., 1999). Individuals with depression also display blunted ACTH responses to CRH (Gold et al., 1986; Holsboer et al., 1986), but normal to elevated cortisol responses (Gold et al., 1986; Holsboer et al., 1986; Juruera et al., 2004). There are also increases in CRH mRNA and in CRH and AVP proteins in the PVN in post-mortem tissues of individuals with depression (Herman and Cullinan, 1997). In addition, these individuals display hippocampal atrophy (Manji et al., 2001), adrenal hypertrophy (Rubin et al., 1995), and immune dysfunction (Weisse, 1992). These symptoms seem to be a function of hypersecretion of CRH (Labeur et al., 1995; Linthorst et al., 1997) as well as impaired negative feedback (Nemeroff et al., 1991; Juruera et al., 2004). This HPA axis dysfunction appears to be a primary factor in causing depressive symptoms. For example, individuals with depression who respond well to anti-depressant treatment often display normalization of the HPA axis (Board et al., 1956; Gold et al., 1986; Amsterdam et al., 1988; Nemeroff et al., 1991; Heuser et al., 1998), as well as normalization of hippocampal and adrenal size (Manji et al., 2001; Rubin et al., 1995) and immune functioning (Weisse, 1992). Other disorders associated with alterations in HPA axis activity include panic disorder, obsessive compulsive disorder, post-traumatic stress disorder, Tourette's syndrome, alcoholism and alcohol withdrawal, anorexia, fibromyalgia, and chronic fatigue syndrome (Crofford, 1998; Arborelius et al., 1999; Juruera et al., 2004).

These physiological changes and the development of psychopathology, particularly depression and anxiety disorders, are further associated with chronic processive stress in humans

(for review see Arborelius et al., 1999). Stress factors associated with chronic depression include unemployment, lack of insurance, lower levels of education, low income, general medical conditions, low quality of life, low social adjustment, and generalized anxiety (Gilmer et al., 2005). The quality of life measures involved questions regarding work, economics, and social and familial relationships (Wisniewski et al., 2005). In another study, depression and generalized anxiety disorder were associated with recent stressful events including injury or illness and relationship problems (including death of a close individual and relationship breakdown). Additionally, both conditions were associated with lifetime stressors, such as a history of bullying or sexual abuse (Jordanova et al., 2007). Therefore, chronic stress exposure leading to HPA axis dysregulation may be implicated in human psychopathology.

Animal Models of Chronic Stress

Chronic Variable Stress

The convergence of the effects of chronic stress in rats and of chronic stress in humans suggests that animal studies may provide important insights into the biochemical basis of stress-induced psychopathology in humans. However, while a number of processive stressors are commonly used in research with rats (e.g., restraint, exposure to a novel environment, and exposure to soiled cages, bright lights, or loud noises), many of these are limited in their long-term effects, because the rats habituate to repeated exposure to these stimuli (for example see Barnum et al., 2007).

One common stress procedure used is the chronic variable stress (CVS) regimen. Many studies have reported increased basal HPA axis activity and the absence of habitutation (for example see Marin et al., 2007). However, as with Marin and colleagues' study, often these CVS regimens include at least one stressor with a strong systemic component, such as cold exposure or food/water deprivation. In our lab, when only processive stressors were used in the

CVS regimen, no increases in baseline HPA axis functioning, particularly in CORT levels, were observed (Simpkiss and Devine, 2003). Additionally, Ostrander and colleagues (2006) found that rats displayed habituation to novel processive stressors after CVS, but maintained responsivity to systemic stressors. Therefore, the utility of these CVS regimens as a model of human chronic processive stress and the development of psychopathology is limited. When only processive stressors are used, CVS does not cause the kinds of chronic, basal changes seen in human populations.

Other Chronic Stress Regimens

Exposure to predatory stress, social separation, and inescapable tail shock have produced more substantial and reliable alterations in behavioral and physiological activities than the CVS regimen has. For example, repeated visual and olfactory exposure to a cat produces increased basal CORT, adrenal hypertrophy and thymus involution, and depressive behaviors such as decreased grooming in rats (Blanchard et al., 1998). Young squirrel monkeys that have been separated from their peers display elevated CORT even several days later (Lyons et al., 1999). Additionally, even a single exposure to inescapable tail shock produces elevated CORT responses 24 hrs later (Deak et al., 1999) and HPA hyperresponsivity as many as 10 days later (Johnson et al., 2002). These models of stress produce changes that look more like those seen in human chronic stress and depression.

Social Defeat

Recently, our lab has begun to work with a model of social stress, the social defeat procedure. In this procedure a small naïve male intruder rat is placed into the cage of a larger conspecific (the resident). This resident has experience with territorial interactions and will typically display dominance behaviors when the intruder is present. These behaviors include pinning the intruder, biting the intruder, and kicking bedding at the intruder. In response to the

resident's dominance behaviors, the intruder typically exhibits submissive behaviors, including freezing and lying on its back with paws up and abdomen exposed (supine posture). These types of social interactions produce activation in limbic-hypothalamic structures of intruder rats as evidenced by increases in *c-fos* expression in the hypothalamus, septum, BNST, amygdala, and relevant brainstem nuclei such as the locus coeruleus and the nucleus of the solitary tract (Martinez et al., 1998; Chung et al., 1999; Nikulina et al., 2004). Additionally, several studies have reported activation of the HPA axis in intruder rats following social defeat exposure, as evidenced by increases in circulating ACTH (Heinrichs et al., 1992; Ebner et al., 2005) and CORT (Heinrichs et al., 1992; Covington and Miczek, 2001; Wommack and Delville, 2003; Ebner et al., 2005). Also, unlike CVS, social defeat does not result in habituation of the HPA axis response, or even of the body temperature response (i.e., induction of fever; Barnum et al., 2007). Additionally, rats exposed to repeated social defeat display increases in basal CORT (de Goeij et al., 1992; Barnum et al., 2007; Stone et al., in preparation). Repeated social defeat also causes other long-term changes, including decreases in thymus and seminal vesicle masses (Buwalda et al., 2001). These results suggest that social defeat affects the functioning of the HPA axis and HPA axis regulation, as well as other organs involved in immune response and reproduction.

In addition to physiological changes, repeated social defeat also induces long-term behavioral changes. Rats that are exposed to social defeat display increased anxiety-related behaviors in the elevated plus maze (Heinrichs et al., 1992; Calfa et al. 2006) and increased behavioral despair in the Porsolt test (Rygula et al., 2005; Stone et al., in preparation). All of these results suggest that repeated social defeat may be a valuable tool in studying potential long-term effects of chronic stress exposure.

Nociceptin/Orphanin FQ

One neurotransmitter system that has been shown to be important in the neural response to stress is nociceptin/orphanin FQ (N/OFQ). 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 (Reinscheid et al., 1995; Shimohigashi et al., 1996; Butour et al., 1997). The NOP receptor is a 7-transmembrane, G-protein coupled receptor (Bunzow et al., 1994; Chen et al., 1994; Wang et al., 1994; Wick et al., 1994; Lachowicz et al., 1995; Reinscheid et al., 1996) that is negatively linked to adenylate cyclase (Mollereau et al., 1994; Lachowicz et al., 1995; 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; Mollereau et al., 1994; Wang et al., 1994; Wick et al., 1994; Lachowicz et al., 1995), although it does not bind any of the opioids with high affinity (Bunzow et al., 1994; Wang et al., 1994; Lachowicz et al., 1995; Butour et al., 1997). This low affinity between N/OFQ and opioid receptors and between NOP receptors and opioid peptides suggests that the N/OFQ-NOP receptor system is functionally distinct from the opioid system.

N/OFQ, the NOP receptor, and their mRNAs are widely distributed throughout the brain, spinal cord, and periphery (Bunzow et al., 1994; Chen et al., 1994; Mollereau et al., 1994; Wang et al., 1994; Wick et al., 1994; Lachowicz et al., 1995; Nothacker et al., 1996; Neal et al., 1999a

and b; Devine et al., 2003), consistent with a wide range of functions including pain modulation (Meunier et al., 1995; Reinscheid et al., 1995; Tian et al., 1997), motor performance (Reinscheid et al., 1995; Devine et al., 1996), spatial learning (Sandin et al., 1997; Sandin et al., 2004), and feeding (Pomonis et al., 1996; Nicholson et al., 2002). However, N/OFQ and NOP receptor expression are relatively high in some limbic regions including the hypothalamus, septum, BNST, and amygdala (Bunzow et al., 1994; Wang et al., 1994; Lachowicz et al., 1995; Nothacker et al., 1996; Neal et al., 1999a and b; Devine et al., 2003). This limbic localization is consistent with an additional role of N/OFQ in emotional regulation, particularly stress and anxiety responses. For example, Devine and colleagues (2003) found that N/OFQ is released from forebrain neurons following exposure to acute restraint stress.

Exogenous N/OFQ administration has also been found to increase anxiety-related behaviors and HPA-axis activation in response to stressful/anxiety-provoking stimuli. 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 take advantage of rats' motivation to explore during foraging activities, while, on the other hand, displaying a natural aversion to brightly lit and open spaces. 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 (Crawley and Goodwin, 1980; Costall et al., 1989; Onaivi and Martin, 1989; Chaouloff et al., 1997). 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 (Hughes, 1972; Crawley and Goodwin, 1980; Crawley, 1981; Handley and Mithani, 1984; Pellow et al., 1985; Pellow and File, 1986; Costall et al., 1989; Onaivi and Martin, 1989; Stefanski et al., 1992; Simon et al., 1994; Chaouloff et al., 1997; Fernandez et al., 2004).

In our lab, a modified version of the open field test is used. A start box was attached to one wall of the open field, which allows the use of latency to enter the open field and time spent in the open field as measures of anxiety-related behavior (in addition to thigmotactic behavior that has been reported in previous versions of the open field). The test has been calibrated (lighting, handling, etc.) so that vehicle-treated rats spend approximately 25% of the test time in the open field, allowing observation of both increases and decreases in anxiety-related behaviors. 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 into 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 into the center of the open field (Fernandez et al., 2004). These data provide evidence that the modified open field is a valid and sensitive tool for measuring changes in the expression of anxiety-related behaviors.

Intracerebroventricular (ICV) injections of N/OFQ have been shown to increase anxiety-related behaviors in this open field test, as well as the elevated plus maze and the light-dark test (Fernandez et al. 2004; Green et al., in press). N/OFQ-treated rats, as compared to vehicle-treated rats, display longer latencies to enter and spend less total time in the open area of

the open field test, the open arms of the elevated plus maze, and the lit box in the light-dark test. These behaviors resemble the effects following injections of other anxiogenic drugs, such as FG 7142 and are opposite from the effects following injections of anxiolytic drugs, such as diazepam. 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), and mildly stressed conditions (N/OFQ injections are administered without allowing rats to recover from the stress of handling) they exhibit substantial elevations in circulating ACTH and CORT (Devine et al., 2001; Nicholson et al., 2002; Legget et al., 2006). Elevations of ACTH and CORT are also observed when rats are injected and then exposed to the open field test (Fernandez et al., 2004). Additionally, injections of N/OFQ affect gene regulation in this system, producing increases in CRH mRNA in the PVN and increases in proopiomelanocortin (POMC; the precursor to ACTH) mRNA in the pituitary (Legget et al., 2006). This suggests that N/OFQ is important in the regulation of the HPA axis.

In the following experiments, the role of N/OFQ in stress and anxiety is further explored. The potential role of limbic structures in producing increased anxiety-related behaviors and HPA axis activation is examined, and the diffusion characteristics of N/OFQ following ICV and intraparenchymal injections are verified. Finally, the endogenous regulation of N/OFQ mRNA and NOP receptor mRNA following acute and repeated social defeat exposure is examined.

CHAPTER 2
ANATOMICAL ANALYSIS OF ANXIOGENIC BEHAVIORAL EFFECTS OF EXOGENOUS NOCICEPTIN/ORPHANIN FQ

Background

The N/OFQ-induced elevations in circulating hormone concentrations described in Chapter 1 are mediated by limbic inputs, including the septum, BNST, and amygdala (Misilmeri and Devine, in preparation). Specifically, unstressed injections into these limbic structures produce elevations in circulating ACTH and CORT. Accordingly, limbic structures are implicated in the HPA-axis modulating effects of N/OFQ under unstressed conditions.

In light of the observations that N/OFQ produces anxiogenic behavioral effects and activation of the HPA axis, and that the hormonal alterations produced by N/OFQ are at least partially mediated by limbic structures, we previously explored the role of one limbic structure, the amygdala, in affecting anxiety-related behaviors and HPA axis activation in rats (Green et al., in press). The rats that were injected with N/OFQ into the amygdala displayed longer latencies to enter the open field (Figure 2-1). However, the behavioral effects of these injections into the amygdala were not as potent as the effects after injections into the lateral ventricle (Figure 2-2). Additionally, there were no significant between-groups differences in circulating CORT concentrations when rats were given intra-amygdaloid injections of N/OFQ or vehicle prior to the open field exposure (Figure 2-3). This lack of a hormonal effect might seem surprising at first, since there are elevations in circulating CORT concentrations after intra-amygdaloid N/OFQ when rats are tested under unstressed conditions (Misilmeri and Devine, in preparation). However, in that unstressed experiment, the controls exhibited low basal CORT levels, and the effects of the N/OFQ injections were small. In our behavioral experiment, the rats were injected under stressed conditions (handling) and were exposed to the additional stressor of the novel open field. In this instance the circulating CORT concentrations in the

vehicle-treated rats were much higher. Thus, the stress-induced elevations in CORT could have obscured any HPA axis-activating effect of the amygdaloid injections of N/OFQ. This finding concurs with a previous report in which the mild stress of a novel environment partially obscured the hormonal effects of N/OFQ after an ICV injection (Devine et al., 2001).

Because of these less potent effects, the BNST was examined as a potential site for the anxiogenic effects of N/OFQ. The BNST is another limbic structure known to participate in the regulation of behavioral and hormonal responses to anxiety-inducing stimuli (for examples see Henke, 1984; Rogan et al., 1997; Walker and Davis, 1997). The amygdala and BNST can be differentiated in terms of their roles in fear and anxiety (Lee and Davis 1997; 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 responding to anxiety-provoking stimuli (primarily those stimuli that are long in duration, non-specific, and non-conditioned). For example, lesions of the BNST, but not the amygdala, block light-enhanced startle, while amygdaloid lesions, but not BNST lesions, block fear-potentiated startle (Walker and Davis, 1997). The modified open field test used in the present experiment resembles tests of generalized anxiety more than it resembles tests of fear, as there is no specific or conditioned fear stimulus. In this respect, the BNST may be more involved in the behavioral responses during neophobic tests of anxiety such as the open field test and may be an important potential mediator of the anxiogenic effects that were seen after ICV injections of N/OFQ.

Methods

Animals

Male Long Evans rats (n = 34, 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 in a climate-controlled vivarium (temperature 21-23 C˚ humidity 55-60%) until surgery. After surgery, the rats were singly-housed in the same environment. Standard laboratory chow and tap water were available *ad libitum* throughout the experiment. All procedures in this and subsequent experiments were pre-approved by the University of Florida's Institutional Animal Care and Use Committee, and the experiments were conducted in compliance with the National Research Council's 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 10 ml of ketamine yielding a 12 ml 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 $Mg2^+$, 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 0.5 µl aECF.

Surgery

Each rat (265-407g) 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. AErrane was administered as supplemental surgical anesthesia as necessary. Once the rat was

anesthetized, a stainless steel guide cannula (11mm, 22 gauge) was implanted into the right BNST (0.3 mm posterior to bregma, 3.0 mm lateral from the midline, and 5.4 mm ventral from dura; n = 28). Cannulae were implanted at a 14^{o} angle to minimize intrusion of the cannula into the ventricle. Six additional rats were each implanted with a cannula aimed at extra-BNST sites (anatomical controls). Each cannula was secured with dental cement anchored to the skull with stainless steel screws (0.80 x 3/32"). 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 given at least 7 days to recover from surgery.

Equipment

Anxiety-related behavior was measured in an open field test. 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 (see Figure 2-4). The bottom and sides were constructed of black acrylic. A black acrylic guillotine door separated the start box and the open field. This door was 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 and open field were approximately equal (14-30 lux).

Anxiety-Testing Procedure

After the surgical recovery period, 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 1 µl Hamilton syringe. Each rat then received a 0.5 µl injection of aECF vehicle 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 using a syringe pump, and the injector was left in place for 3 additional minutes to allow diffusion of the injection volume. 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 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 30 minutes elapsed after the start of the injection. At this time, the rat was rapidly decapitated.

Immediately after decapitation, 6 ml of trunk blood was collected into polypropylene tubes containing 600 µl of Na_2EDTA (20 µg/µl) on ice. The tubes were centrifuged at 1000x gravity. The plasma fraction was collected, aliquotted, and frozen at -80ºC. Later, RIA was performed for quantification of plasma concentrations of CORT using a kit from 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.

Additionally, each brain was removed, frozen in 2-methylbutane at -40°C, and stored at -80°C. Later, each brain was sectioned at 30 µm and stained with cresyl violet for cannula

placement verification. Adrenal glands, thymus glands, and spleens were dissected out and weighed for verification of the health status of the rats.

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 and the inner 9 squares defined an inner zone (Fig. 2-4). Between-groups differences in the latency to enter the open field, total time spent in the open field, latency to enter and time spent in the inner zone, and the number of entries into the open field and the inner zone were used as measures of anxiety. An entry into the open field or movement from the periphery to the inner zone was counted when all 4 paws of the rat left one zone and entered a new zone.

Statistical Analyses

In order to analyze differences between the N/OFQ-treated groups and the vehicle-treated group, one-way ANOVAs were calculated for latency to enter the open field and the inner zone, total time spent in the open field and the inner zone, number of entries into the open field and inner zone, plasma CORT concentrations, and organ masses. All significant effects ($p< 0.05$) were further analyzed using Fisher's LSD post-tests.

Differences between anatomical controls and intra-BNST vehicle controls for each of the listed measures were analyzed by individual *t*-tests.

Results

Anxiety-Related Behavior

The intra-BNST N/OFQ-treated rats showed greater expression of anxiety-related behaviors than the aECF vehicle-treated rats did. The N/OFQ-treated rats displayed significantly longer latencies to enter the open field (Fig. 2-5a; $F(3,24) = 4.009$, $p<0.05$) than did the vehicle-treated rats, but the groups did not differ significantly in latency to enter the inner zone

(Fig. 2-5b; $F(3,24) = 2.593$, $p>0.05$). There were also no significant differences between N/OFQ-treated and vehicle-treated rats in time spent in the open field (Fig. 2-5c; $F(3.24) = 2.075$, $p>0.05$) or the inner zone (Fig. 2-5d; $F(3,24) = 2.669$, $p>0.05$). However, the N/OFQ-treated rats displayed significantly fewer entries into the open field (Fig. 2-5e; $F(3,24) = 3.055$, $p<0.05$) and significantly fewer entries into the inner zone (Fig. 2-5f; $F(3,24) = 3.321$, $p<0.05$) than did the vehicle-treated rats.

The BNST placements were fairly evenly distributed throughout the medial and lateral divisions (Fig.2-6). The effects of vehicle injections into these BNST sites were compared against the effects of N/OFQ injections into extra-BNST sites (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. 2-5a and b), time (Fig. 2-5c and d), or entries (Fig. 2-5e and f).

Circulating Corticosterone

Injections of N/OFQ into the BNST produced significant elevations in circulating CORT (Fig. 2-7; $F(3,22) = 3.171$, $p<0.05$) as compared to the CORT concentrations in the vehicle-treated rats. Concentrations of circulating CORT were not significantly different between N/OFQ-treated anatomical controls and vehicle-treated rats.

Organ Masses

There were no significant differences in adrenal weights (Fig. 2-8A), thymus gland weights (Fig. 2-8B), or spleen weights (Fig. 2-8C) between the vehicle-treated and N/OFQ-treated rats and between the vehicle-treated rats and anatomical controls.

Discussion

Anxiety-Related Behaviors and Corticosterone

We previously found that injections of N/OFQ into the right lateral ventricle produce dose orderly elevations in all measured anxiety-related behaviors in the modified open field and elevations in circulating CORT (Fernandez et al., 2004; Green et al., in press). Injections into the right amygdala also elevated anxiety-related behaviors, although the behavioral and hormonal effects were less potent than those seen following ICV injections. Similarly, injections into the right BNST produced elevations in anxiety-related behaviors and elevations in circulating CORT, and once again, these effects were less potent than those observed after ICV injections. The behavioral results are somewhat surprising as the amygdala, and especially the BNST appeared to be ideal candidates for mediating the behavioral actions of N/OFQ. Both structures are involved in responses to emotionally-salient stressors, and both structures have relatively high levels of NOP receptor mRNA expression and binding (Neal et al., 1999b). It is possible, however, that ICV injections are having effects at multiple sites, accounting for the greater potency of this route of administration. Because injections into the amygdala and into the BNST produce partial effects on anxiety-related behaviors, there may be additive or synergistic effects at these and other limbic, cortical, and brainstem structures following ICV injections. For example, the lateral septum may be another structure involved. Like the amygdala and the BNST, the lateral septum is involved in responses to stressors and has at least moderate levels of NOP receptor binding.

The hormonal effects following intraparenchymal injections are less surprising. The modest effects following amygdaloid injections can be expected considering there are only sparse connections between the amygdaloid complex and the PVN (Prewitt and Herman, 1998). Unlike the amygdala, though, the BNST does have many connections to the PVN (for example

see Prewitt and Herman, 1998; Dong et al., 2001; Dong and Swanson, 2004). This anatomical difference is consistent with the greater elevations in circulating CORT observed in stressed (the present experiment) and unstressed rats (Misilmeri and Devine, in preparation) following intra-BNST injections versus intra-amygdaloid injections.

One concern with the smaller effects of our intraparenchymal injections versus ICV injections is the fact that they were done unilaterally (injections were into the right hemisphere in all groups). Previous analysis of the diffusion of N/OFQ after unilateral ICV injections suggested that there was at least some bilateral distribution, primarily at midline periventricular structures (D. P. Devine, personal communication). It is not clear if bilateral injections into the BNST or the amygdala would have increased the effects. To our knowledge, the effects of bilateral BNST injections of anxiolytic or anxiogenic compounds have not been reported with any standard tests of fear or anxiety. Thus it is possible that injections of N/OFQ into the right and left BNST might produce more potent effects. However, in the case of the amygdala, the right hemispheric structure is generally more dominantly involved in emotionally-relevant behavioral responses (Coleman-Mesches and McGaugh, 1995a and b; Andersen and Teicher, 1999; Adamec et al., 2001; Peper et al., 2001; Scicli et al., 2004), or is at least no less involved than the left amygdala (Good and Westbrook, 1995; LaBar and LeDoux, 1996; Izquierdo and Murray, 2004). In fact, bilateral injections of drugs in to the amygdalae 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 see Coleman-Mesches and McGaugh, 1995b). Therefore, in our previous analysis of the effects of unilateral amygdaloid injections it does not seem likely that bilateral injections would have produced more potent effects.

There are reports that N/OFQ and its synthetic analogue Ro64-6198 each exert anxiolytic actions in rats and mice (Jenck et al., 1997; Jenck et al., 2000; Gavioli et al., 2002; Varty et al. 2005). Additionally, some researchers find opposing effects depending on the dose of drug used (Kamei et al., 2004), the number of times the drug is administered (Vitale et al., 2006), or the anxiety tests used (in this case with NOP receptor knockouts; Gavioli et al., 2007). The reasons for these discrepancies are currently unclear; although, there are methodological differences that may account for the contrasting results (see Fernandez et al 2004 for a full discussion of the potential explanations). It is possible that N/OFQ may produce differing anxiogenic and anxiolytic actions depending upon the prior history and/or the current stress status of the animals, but we, in the Devine lab, have so far been unable to detect anxiolytic effects in our studies.

It has been argued that the increases in anxiety-related behaviors may be due to locomotor impairment produced by N/OFQ. At higher doses of N/OFQ there are profound motoric effects that result in postural changes and reduced locomotion (Devine et al., 1996). Recently, Vitale and colleagues (2006) reported reduced exploration of the open arms of the elevated plus maze after a single dose of N/OFQ administered ICV. However, a second dose given 2 hours later resulted in a reversal of this effect. They interpreted the reduced exploration as a locomotor effect, and concluded that the lack of this effect following the second administration was due to tolerance (rats develop tolerance to the locomotor-inhibiting effects of N/OFQ; see Devine et al., 1996). However, anxiogenic actions have been observed at doses that do not produce locomotor effects (Devine et al., 1996; Fernandez et al., 2004; Green et al., in press). Additionally, locomotor controls show that motoric effects are not the likely source of the reduced exploration following N/OFQ injections (Fernandez et al., 2004; Green et al., in press). For example, when the threatening stimulus of a brightly lit environment in the dark-light box is removed, thus

creating a dark-dark test, N/OFQ-treated rats and vehicle-treated rats explored the boxes in a comparable manner (Fernandez et al., 2004). Likewise, when the threatening stimulus of a large open field is removed and replaced with an undersized open field equal in size to the start box, N/OFQ-treated rats and vehicle-treated rats again behaved in a comparable manner (Green et al., in press). The only difference in these conditions is the presence or absence of a threatening stimulus. Thus, it seems likely that behavioral differences observed in the open field test and light-dark box are due to differences in the anxiety states of the rats and not due to locomotor impairment.

The results of the present experiment, as well as the results following injections into the amygdala, provide additional evidence of an anxiety-related effect as opposed to a locomotor effect. The intraparenchymal injections in these experiments were highly specific, with no significant effects observed in the anatomical controls. Given the known roles of the BNST and amygdala, injections into these structures would not be expected to affect locomotor behaviors, but would be expected to affect anxiety-related behaviors.

Organ Masses

In the present study, the adrenal, thymus, and spleen masses were measured to establish that there were no systematic differences in health status or stress exposure between the various groups of rats. Since there were no significant differences in adrenal gland masses, thymus gland masses, or spleen masses between any of the groups tested, it can be concluded that there were no apparent differences in the health or stress history of the rats that can account for the behavioral or hormonal differences.

Summary

The results of the present study show that N/OFQ injections affect anxiety and HPA axis activity through limbic structures including the BNST. In addition, previous work has shown a

role for the amygdala. This suggests the possibility that limbic N/OFQ neurotransmission may be involved in regulation of affect. However, because ICV injections of N/OFQ produced greater, more potent effects than did injections into either the BNST or the amygdala, there may be additive or synergistic actions between the BNST and the amygdala. Additionally, the contralteral structures as well as other relevant limbic, cortical, and brainstem structures may be involved.

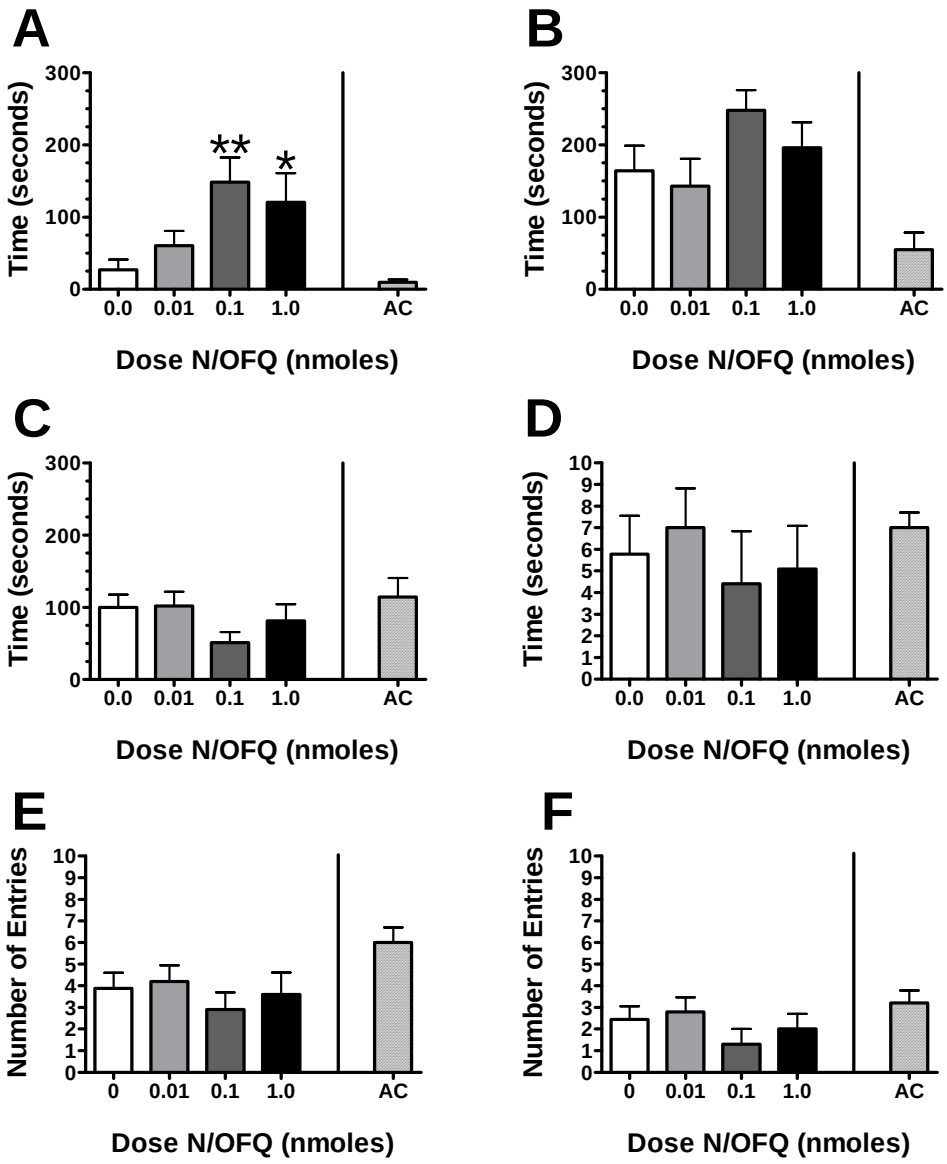

Figure 2-1. Anxiety-related behaviors following intra-amygdaloid injections of N/OFQ. (A) N/OFQ-treated rats exhibited longer latencies to enter the open field. However, injections of N/OFQ into the right amygdala did not significantly alter (B) the latency to enter the inner zone, (C) the time spent in the open field, (D) the time spent in the inner zone, (E) the number of entries into the open field, (F) or the number of entries into the inner zone. 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 are expressed as * $p < 0.05$ and ** $p < 0.01$. AC =anatomical controls.

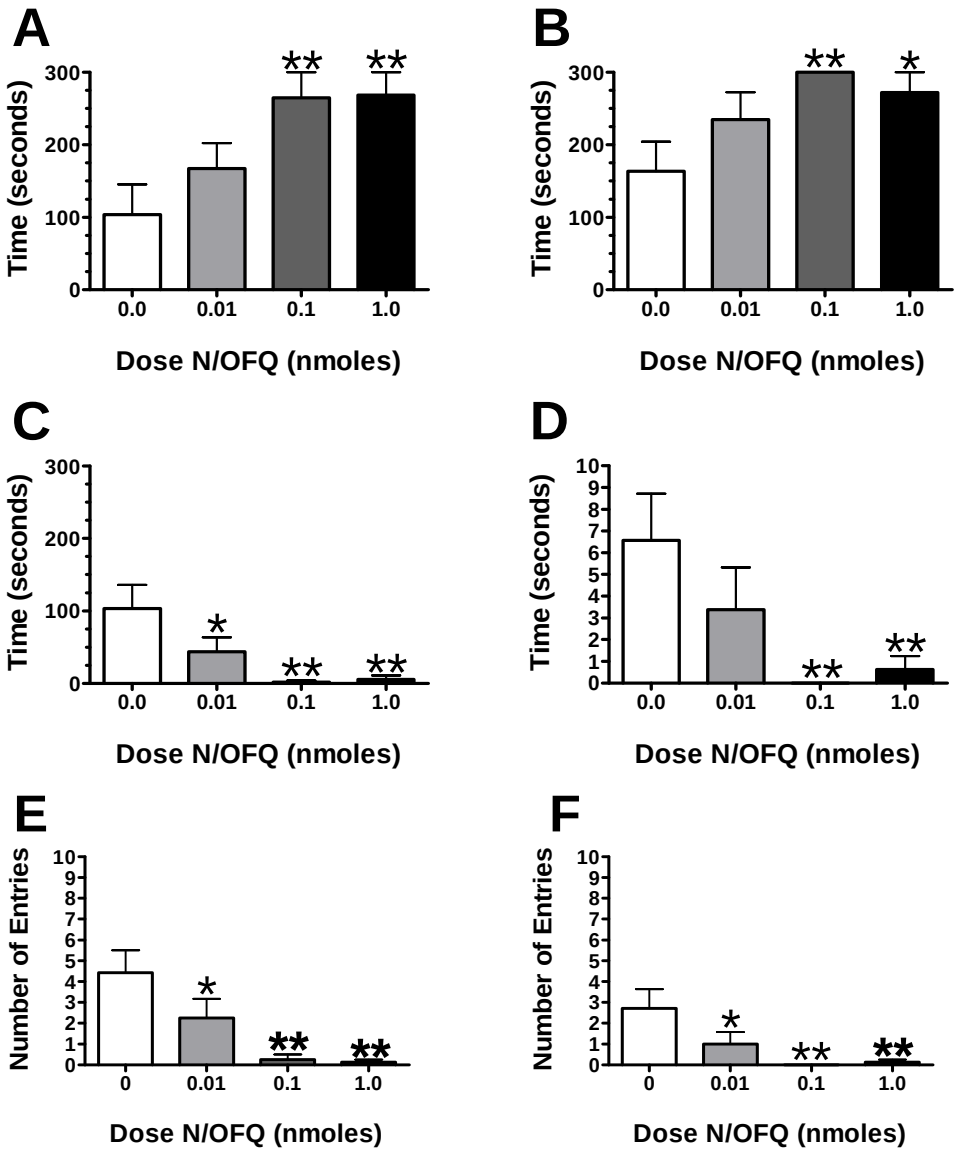

Figure 2-2. Anxiety-related behaviors following ICV injections of N/OFQ. N/OFQ-treated rats exhibited: (A) longer latencies to enter the open field and (B) the inner zone, (C) less total time in the open field and (D) the inner zone, and (E) fewer entries into the open field and (F) the inner zone. Values 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 ** $p < 0.01$.

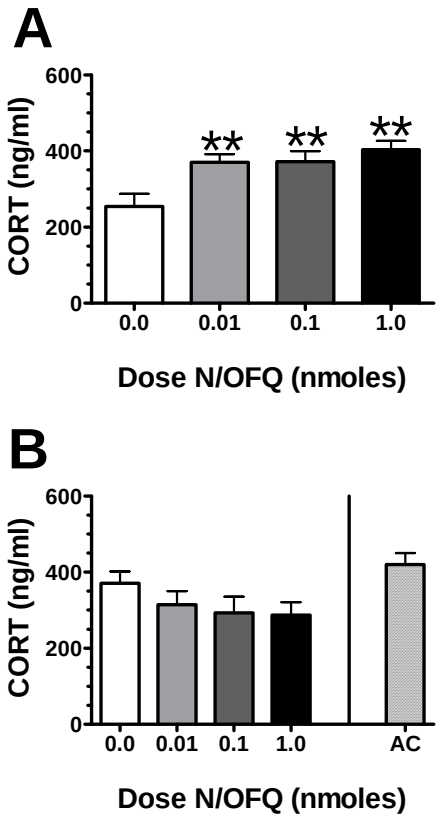

Figure 2-3. Concentrations of circulating corticosterone following ICV and intra-amygdaloid injections of N/OFQ. (A) Injections of N/OFQ into the right lateral ventricle produced elevations in circulating CORT. (B) N/OFQ administration into the right amygdala did not significantly alter the levels of circulating corticosterone.Values expressed are group means ± SEM (n = 7-10 rats per group). Significant differences between the N/OFQ-treated rats and the aECF-treated controls are expressed as ** $p < 0.01$. AC = anatomical controls

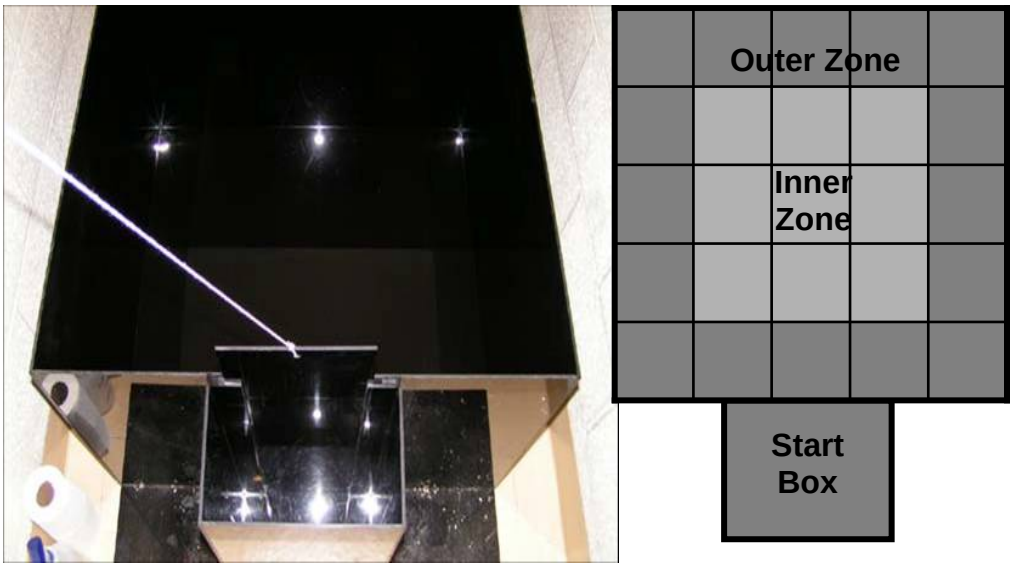

Figure 2-4. Photograph of the open field and a diagram of the zones used for scoring. The rat is placed in the start box for the first minute of the test. After the door opens, the rat may move between the start box and the open field freely. The outer zone represents the periphery of the open field. The inner zone represents the central region of the open field.

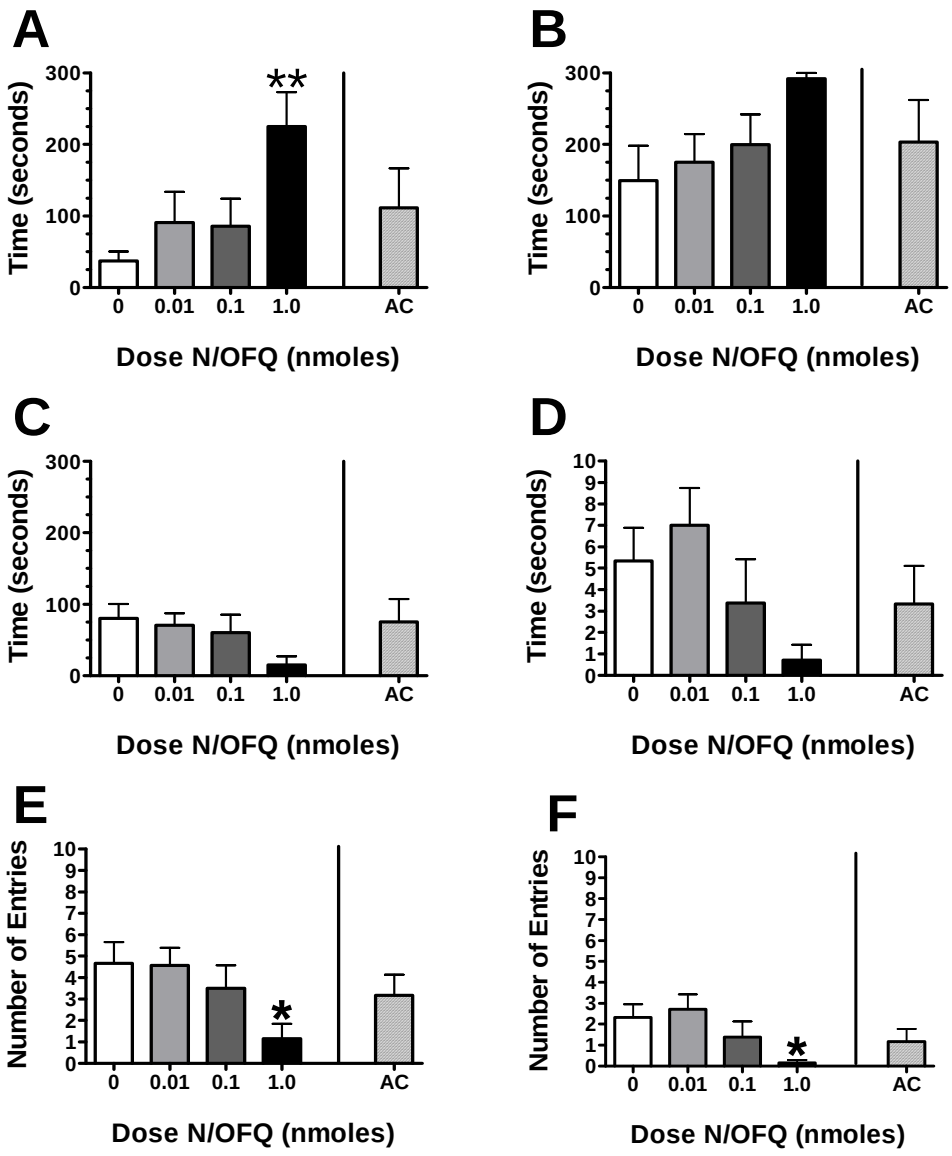

Figure 2-5. Anxiety-related behaviors following intra-BNST injections of N/OFQ. N/OFQ-treated rats exhibited (A) longer latencies to enter the open field and (E) fewer entries into the open field and (F) the inner zone. However, there were no significant differences in (B) latency to enter the inner zone or in (C) time spent in the open field or (D) the inner zone. Values expressed are group means ± SEM (n = 6-8 rats per group). Significant differences between the N/OFQ-treated rats and the aECF-treated controls are expressed as * $p < 0.05$ and ** $p < 0.01$. AC = anatomical controls.

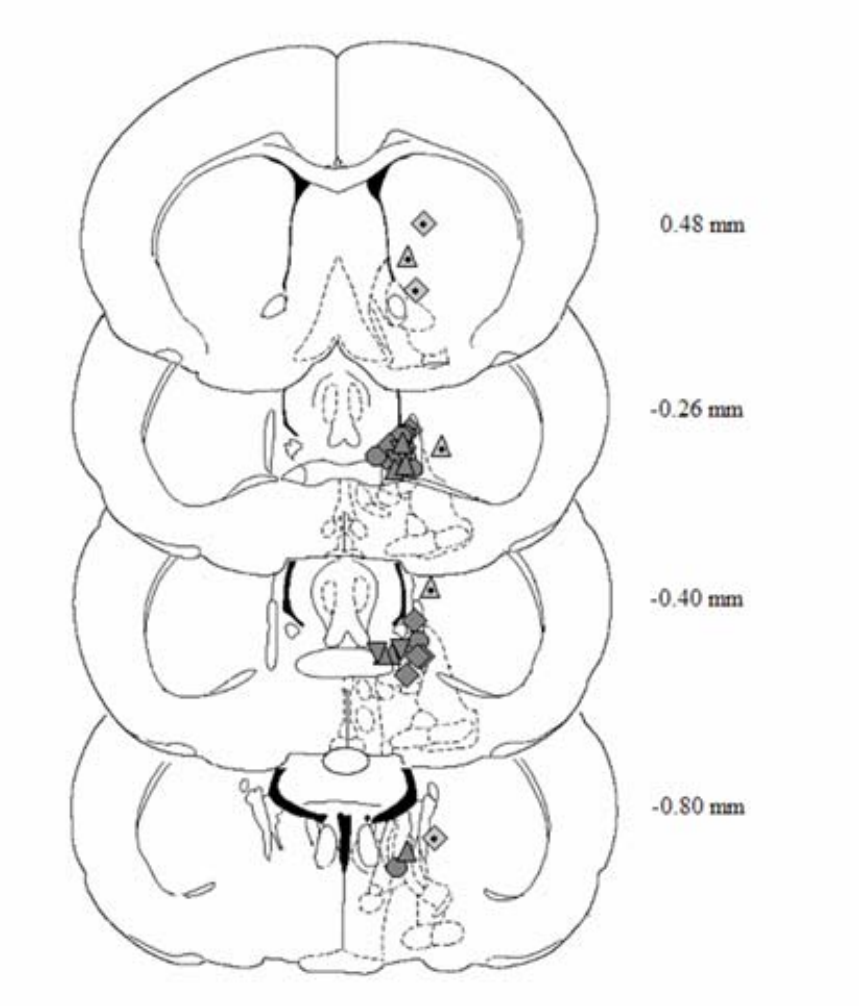

Figure 2-6. Anatomical map of BNST placements. The injector tip placements are illustrated for each rat, including anatomical controls (adapted from Paxinos and Watson, 1998.). ▲=1.0 nmole doses; ♦=0.1 nmole doses; ▼=0.01 nmole doses; ●=aECF controls. Anatomical controls are identified by a black dot placed within the marker.

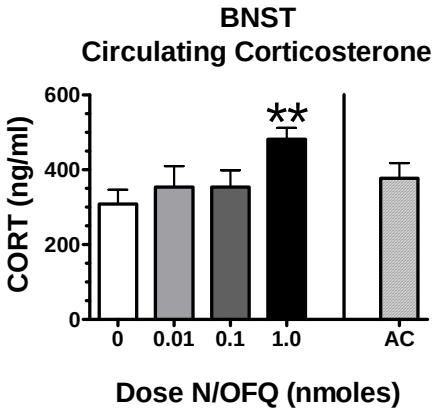

Figure 2-7. Concentrations of circulating corticosterone following intra-BNST injections of N/OFQ. Injections of N/OFQ into the BNST produced elevations in CORT at the highest dose administered. Values expressed are group means ± SEM (n = 6-8 rats per group). Significant differences between the N/OFQ-treated rats and the aECF-treated controls are expressed as ** $p < 0.01$. AC = anatomical controls

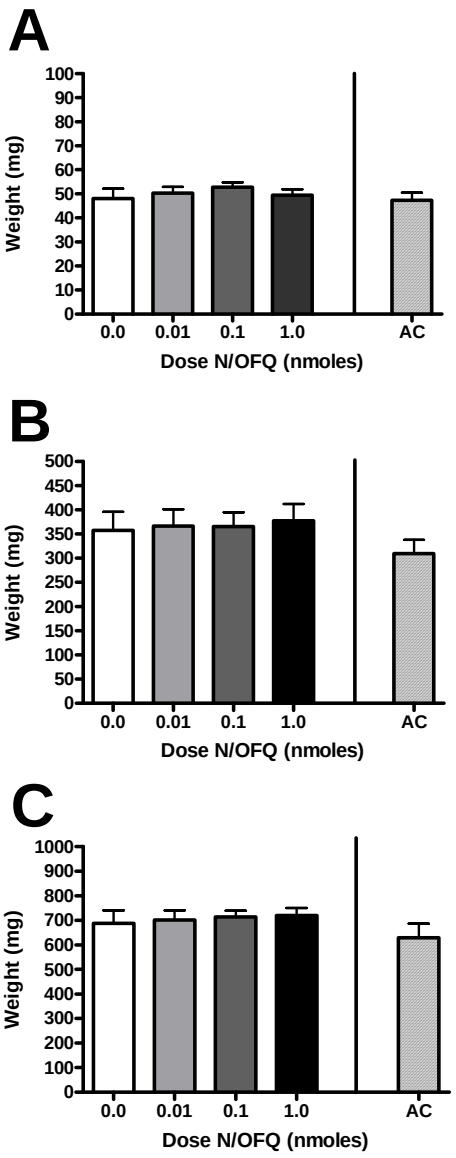

Figure 2-8. Analysis of glandular masses. (A) Adrenal gland masses, (B) thymus gland masses, and (C) spleen masses showed no significant differences between groups. Values expressed are group means ± SEM (n = 6-8 rats per group).

CHAPTER 3
DIFFUSION OF NOCICEPTIN/ORPHANIN FQ AFTER INTRACEREBROVENTRICULAR AND INTRAPARENCHYMAL INJECTIONS

Background

The behavioral and hormonal effects following injections of N/OFQ into the right lateral ventricle, the amygdala, the BNST, and other limbic structures have been described (Fernandez et al., 2004; Green et al., in press; Missilmeri and Devine, in preparation). However, the extent of the diffusion of N/OFQ following these injections is not clear. Data from the anatomical controls in Chapter 2 suggest that diffusion following injections into the BNST and the amygdala was limited to the target structure. To confirm this and compare it with the penetration of N/OFQ into the tissue following ICV injections, ^{3}H-N/OFQ was injected into the lateral ventricle, the BNST, and the amygdala.

Methods

Animals

Male Long Evans rats (n = 15, 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 in a climate-controlled vivarium (temperature 21-23 C° humidity 55-60%) until surgery. After surgery, the rats were singly-housed in the same environment. Standard laboratory chow and tap water were available *ad libitum* throughout the experiment.

Drugs

Ketamine and xylazine were both obtained from Henry Schein (Melville, NY) and were mixed as described in Chapter 2. 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), as described in Chapter 2. N/OFQ was prepared at concentrations of

1.0 nmole per 1.0 µl aECF for ICV injections or per 0.5 µl aECF for intra-amygdaloid and intra-BNST injections. ^{3}H-N/OFQ was prepared by adding 0.01 nmoles ^{3}H-N/OFQ (Phoenix Pharmaceuticals, Belmont, CA) to the 1.0 nmole N/OFQ doses.

Surgery

Each rat (268-319g) 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. AErrane was administered as supplemental surgical anesthesia as necessary. Once the rat was anesthetized, a stainless steel guide cannula (6 or 11mm, 22 gauge) was 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 = 5), the right amygdala (1.8 mm posterior to bregma, 3.9 mm lateral, and 6.2 mm ventral; n = 5) or the right BNST (0.3 mm posterior to bregma, 3.0 mm lateral from the midline, and 5.4 mm ventral from dura; n = 5). The ICV and amygdaloid cannulae were each implanted vertically, and the BNST cannulae were implanted at a 14^{o} angle. Each cannula was secured with dental cement anchored to the skull with stainless steel screws (0.80 x 3/32"). 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 given at least 7 days to recover from surgery.

Injection Procedure

Following recovery from surgery, each of the rats was handled for 5 minutes on one day. Two days later the rats were given ^{3}H-N/OFQ injections. Each rat received a 1.0 µl (ICV, n= 5) or 0.5 µl (intra-amygdala, n= 5, and intra-BNST, n= 5) injection of aECF containing 1.0 nmole N/OFQ plus 0.01 nmole ^{3}H-N/OFQ. These injections were administered over a 2-minute period, and the injector was left in place for 3 additional minutes to allow for diffusion. Each rat was

rapidly decapitated 12 minutes following the start of the ^{3}H-N/OFQ injection. This time point corresponded with the time at which the rats in Experiment 1 were removed from the open field.

Immediately after decapitation (approximately 15 min following the start of the ^{3}H-N/OFQ injection), each brain was removed, frozen in 2-methylbutane at -40°C, and stored at -80°C. Later, each brain was sectioned at 20 μm and placed on x-ray film (Kodak X-Omat) at -80°C for 12 weeks. Following film exposure, the brain sections were stained with cresyl violet for further cannula placement verification and anatomical evaluation of the film images.

Results

Intracerebroventricular Injections

Injections of ^{3}H-N/OFQ into the right lateral ventricle produced differing diffusion patterns depending on whether there was a successful ventricular placement or not. Of the 5 ICV placements, cresyl violet staining revealed 1 clear hit, 3 marginal hits, and 1 miss. In the rat with the clear ventricular placement (Fig. 3-1), ^{3}H signal was seen in all aspects of the right lateral ventricle (Fig. 3-1a and b), continuing in more posterior regions into the dorsal and ventral 3rd ventricle (Fig. 3-1a-d), through the cerebral aqueduct, and into the 4th ventricle (Fig. 3-1e and f). From these locations there was visible diffusion approximately 0.5-1mm into the tissues, reaching the lateral septum; medial and lateral subnuclei of the BNST, striatum; subfornical organ; numerous thalamic nuclei; paraventricular, suprachiasmatic, posterior, and arcuate nuclei of the hypothalamus; medial preoptic area; fimbria; periaqueductal gray; some cerebellar regions (particularly those that emerge near the 4th ventricle); tegmental nuclei; and locus coeruleus. In general, signal was visible around the ventricular spaces throughout the anterior to posterior extent.

In the rats with marginal ventricular hits (e.g., the cannula tip was located at the edge of the corpus callosum near the ventricular wall) there was some visible ^{3}H signal in the right lateral

ventricle, but only faint signal in the dorsal and ventral 3rd ventricle. No signal was visible in the more posterior cerebral aqueduct or 4th ventricle. In these cases the diffusion from the ventricular regions was much lighter, primarily reaching the lateral septum and striatum.

In the rat with a clear ventricular miss (the cannula tip was located in the corpus callosum and did not pierce the ventricle) ^{3}H signal is very faint in the right lateral ventricle and only located in a very limited anterior to posterior span (less than 1mm). In this rat, as well as those with marginal hits, the ^{3}H-N/OFQ was visible primarily as an injection bolus dispersed over the corpus callosum.

In all cases, there was substantial signal along the edges of the cannula track, suggesting that the drug diffused up along the sides of the cannula implant. This generally led to signal affecting the cingulate, M1, and M2 cortices, lateral septum, and striatum.

Intra-Amygdaloid Injections

All 5 of the intra-amygdaloid placements displayed visible signal over the amygdala. In general, substantial signal was visible along the edges of the cannula track with an injection bolus of approximately 1-1.5mm radius ventral to the cannula tip. This bolus was typically visible over the central amygdala, medial amygdala, basolateral amygdala, basomedial amygdala, and internal capsule (Fig. 3-2a). Considering some variability in placement, other structures that occasionally displayed signal included the BNST-intraamygdaloid nucleus, anterior amygdala, reticular thalamus, lateral hypothalamus, and substantia innominata.

As with the ICV placements, all amygdaloid placements displayed signal along the edges of the cannula track, affecting structures such as the S1 cortex and the striatum. To verify that this was occurring following the injections (and not as an effect of the removal of the cannula following decapitation), one intra-amygdaloid implanted brain was rapidly frozen prior to removing the cannula. In this instance there was still substantial signal visible around the

cannula track, suggesting that this was, in fact, occurring during and following the injections (Fig. 3-2b).

Intra-BNST Injections

All 5 of the intra-BNST placements displayed visible signal over the BNST. However, they also all displayed some ventricular involvement, even when the cannula did not apparently pierce the ventricular wall. Observation following cresyl violet staining revealed 1 placement that did not appear to pierce the ventricle, 1 with marginal entry into the right lateral ventricle, and 3 that fully passed through the right lateral ventricle.

With the one placement that did not pierce the ventricle, there was visible signal over the medial BNST (Fig. 3-3a). As was the case with the ICV and intra-amygdaloid placements, there was visible signal along the cannula track edges, reaching structures such as the S1 cortex, the striatum, and some thalamic nuclei. As mentioned above, despite no obvious penetration into the ventricles, there was still visible signal in the lateral and 3rd ventricles (Fig. 3-3b). From the ventricular spaces there was faint diffusion visible into the subfornical organ; septum; and the paraventricular, suprachiasmatic, and arcuate nuclei of the hypothalamus; and the median eminence.

In the case of the more marginal ventricular penetration, there was visible signal over the medial and lateral BNST, anterior commissure, diagonal band, ventral pallidum, substantia innominata, and preoptic areas (Fig. 3-3c). The visible signal along the cannula track was evident over the M1 cortex, the lateral and medial septum, and the striatum. There was signal visible in the right lateral ventricle; however, in more posterior regions the signal was very faint (Fig. 3-3d). Here there was slight visible signal in the cerebral aqueduct with some diffusion into the periaqueductal gray.

In the cases where the cannula passed through the right lateral ventricle, there was visible signal over the medial and lateral BNST and anterior commissure (Fig. 3-3e). In some cases there was signal over the preoptic areas, hypothalamic nuclei (PVN, arcuate, lateral), and ventral pallidum. The signal along the edges of the cannula track was generally visible over the M1 cortex and the striatum. Additional signal greatly resembled ICV injections, with signal visible in the right lateral ventricle, dorsal and ventral 3rd ventricle, cerebral aqueduct, and 4th ventricle. Diffusion from these ventricular regions affected structures such as the septum; subfornical organ; thalamic nuclei; periventricular, paraventricular, arcuate, ventromedial, dorsomedial, and anterior nuclei of the hypothalamus; and the medial preoptic area. In more posterior regions, there was greater variability in the signal displayed. Two of the 3 placements displayed visible signal in the cerebral aqueduct with diffusion into the periaqueductal gray (Fig. 3-3f). One of these continued to display clear signal into the 4th ventricle with diffusion over the emerging cerebellum, the tegmental nuclei, and the locus coeruleus.

Discussion

Injections of ^{3}H-N/OFQ into the right lateral ventricle revealed differing diffusion patterns depending on the accuracy of the placement. In our behavioral experiments, only rats that had a cannula placement that was clearly within the lateral ventricle were used. This was verified by cresyl violet staining and visualization of the cannula tip. Therefore, the diffusion pattern that is depicted in Figure 3-1 is the best representation of the diffusion that occurred in the rats in the behavioral studies. Accordingly, the potent hormonal and behavioral effects seen following ICV injections were likely due to ipsilateral effects in rostral brain regions, with potential bilateral involvement of periventricular, midline structures at more caudal sites. The sites that were labeled with ^{3}H-N/OFQ and are most likely to be involved in anxiety-related responses include the lateral septum, the BNST, the medial preoptic area, the periaqueductal gray, the

tegmental nuclei, and the locus coeruleus. Interestingly, substantial diffusion into the amygdala from the ventricles was not observed, although our behavioral study suggests that the amygdala is involved in anxiogenic responses to N/OFQ. It is important to note that ^{3}H produces a faint signal, requiring months of film exposure. Thus the possibility cannot be ruled out that a small amount of the ICV-injected N/OFQ might diffuse as far as the amygdala.

The size and shape of the diffusion patterns after lateral ventricle, BNST, and amygdala injections are in agreement with previous analyses using other compounds (puromycin, CORT, insulin-like growth factor) that were injected into the hypothalamus, frontal cortex, or lateral ventricle (see Renner et al., 1984; Diorio et al., 1993; Nagaraja et al., 2005). The localization observed following intraparenchymal injections suggests that the behavioral and hormonal effects are primarily due to actions within the target structures. However, with all of the injections (ICV, intra-BNST, and intra-amygdaloid), there was significant diffusion up the cannula track. Again, this is in agreement with previous diffusion studies (for example see Renner et al., 1984). This does not appear, however, to contribute to the behavioral or hormonal effects observed in chapter 2. In those experiments, anatomical controls were included. In all the anatomical controls, there would be diffusion back up the cannula track affecting the same general extra-amygdaloid and extra-BNST sites as the targeted injections did. The lack of behavioral and hormonal effects in the anatomical controls confirms that the effects of the BNST and amygdaloid injections were indeed mediated at the target structures.

On the other hand, the possibility cannot be ruled out that the BNST injections of N/OFQ described in chapter 2 produced some effects via diffusion into the ventricles, since some ventricular diffusion was apparent even when the cannula did not pierce the lateral ventricle. While our analyses of anxiety-related behaviors or circulating CORT did not include data from

rats in which clear penetration of the ventricle was observed, this may not have eliminated all ventricular involvement.

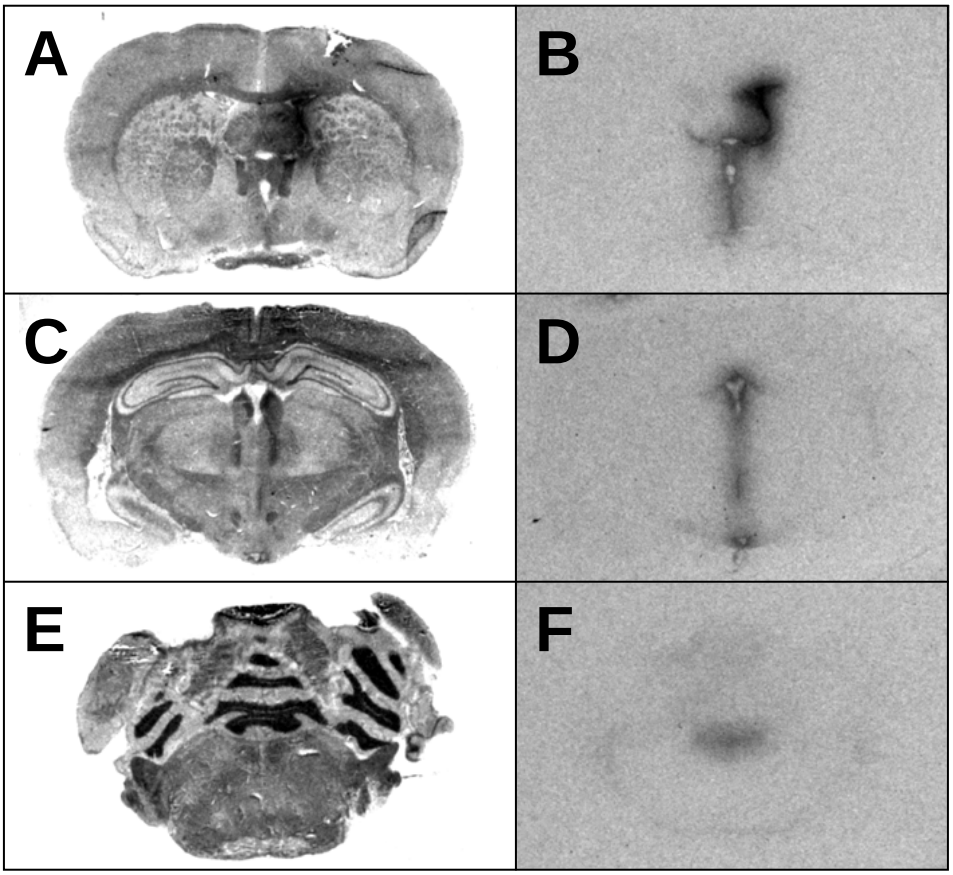

Figure 3-1. Representative x-ray images of ^{3}H-N/OFQ diffusion following ICV injections overlaid on the corresponding cresyl violet stained section (A, C, and E) and x-ray images alone (B, D, and F). (A, B) Injections into the right lateral ventricle resulted in strong ^{3}H signal in the ipsilateral ventricle with some diffusion into the contralateral ventricle and the 3rd ventricle. Further diffusion from these ventricles is most prominent in the periventricular structures within approximately 0.5-1mm from the ventricular wall. Diffusion continues posteriorly through (C, D) the 3rd ventricle and (E,F) into the 4th ventricle.

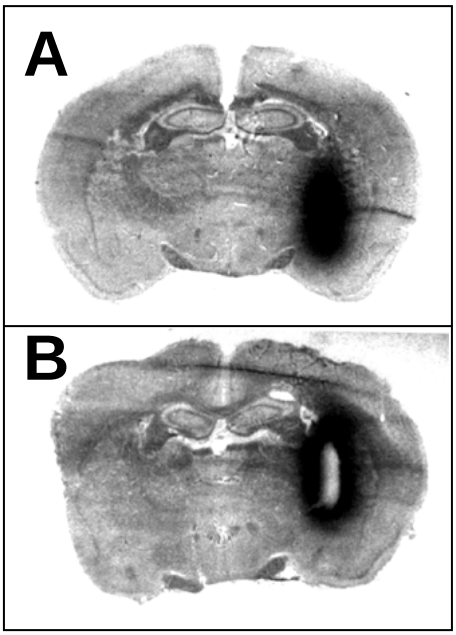

Figure 3-2. Representative x-ray images of ^{3}H-N/OFQ diffusion following intra-amygdaloid injections overlaid on the corresponding cresyl violet stained section. (A) Diffusion of ^{3}H-N/OFQ from these placements is generally localized to the injection site with diffusion throughout the amygdaloid complex. (B) In addition there is substantial diffusion along the cannula track that occurs during the injection procedure, as evidenced by freezing the tissue prior to cannula removal.

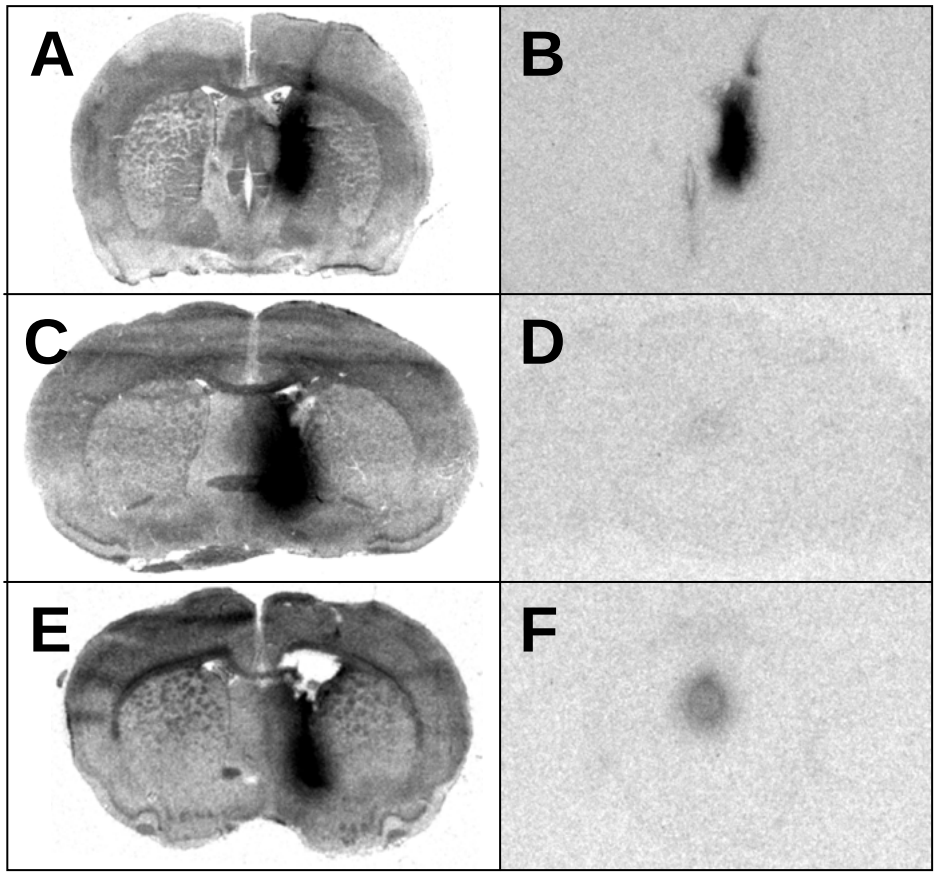

Figure 3-3. Representative x-ray images of ^{3}H-N/OFQ diffusion following intra-BNST injections overlaid on the corresponding cresyl violet stained section (A, C, and E) and x-ray images alone (B, D, and F). (A,B) Diffusion of ^{3}H-N/OFQ from successful BNST placements, with no ventricular piercing, results in ^{3}H signal that is generally localized to the injection site with diffusion across numerous BNST sub-nuclei and along the cannula track. With these placements there is slight diffusion into the ventricles. (C, D) Cannulae that pierce the lateral ventricle or (E, F) fully pass through it result in injections with diffusion into the posterior 4th ventricle and surrounding structures. In our anxiety experiments, rats with placements piercing the ventricle were removed from the experiments.

CHAPTER 4
NOCICEPTIN/ORPHANIN FQ AND NOP RECEPTOR GENE REGULATION AND NOCICEPTIN/ORPHANIN FQ BINDING TO THE NOP RECEPTOR AFTER SINGLE OR REPEATED SOCIAL DEFEAT EXPOSURE

Background

Administration of the NOP receptor antagonists J-113397, [Nphe^1]-nociceptin (1-13)-NH_2 and UFP-101 have antidepressant effects in animal models of depression (Redrobe et al. 2002; Gavioli et al. 2003; 2004). This raises the possibility that the N/OFQ-NOP system may play an important role in stress-associated psychopathology. If N/OFQ neurotransmission is truly implicated in these disorders, it seems reasonable to expect that severe or repeated stress might cause dysregulation of the expression of genes and/or proteins in the N/OFQ-NOP receptor system. In fact, Devine and colleagues (2003) found that the system is tightly regulated. Acute restraint stress caused a decrease in the N/OFQ content in the forebrain of rats. This decrease occurred rapidly and was replenished within a 24 hour period. Thus, it appears that there is a release of N/OFQ from basal forebrain neurons following acute stress exposure and the content is restored rapidly in those neurons. Following from this, the primary aim of this study was to examine the gene regulation of N/OFQ and the NOP receptor in response to acute and repeated social stressors.

N/OFQ is cleaved from a precursor protein, prepro-N/OFQ. In rats, prepro-N/OFQ is 181 amino acids, containing one copy of N/OFQ at the 135-151 amino acid positions (Nothacker et al., 1996). Therefore, the gene regulation of prepro-N/OFQ was examined using *in situ* hybridization with a radiolabelled riboprobe complimentary to the prepro-N/OFQ mRNA. In addition, repeated N/OFQ release might affect gene regulation of the NOP receptor, a 367 amino acid protein (Wang et al., 1994). Therefore, *in situ* hybridization was conducted using a

radiolabelled riboprobe aimed at the NOP receptor mRNA in order to explore changes in the receptor's gene expression.

Changes in receptor mRNA expression, however, may not accurately represent changes in protein synthesis. Post-transcriptional processes could destroy the mRNA, blocking protein synthesis. Additionally, post-translational mechanisms could result in the breaking down of the protein or in the packaging of receptors in vesicles for later use, but not resulting in insertion of the receptors into the membrane. To determine if changes in receptor mRNA expression results in changes in receptor insertion and functionality, a preliminary examination of the binding of N/OFQ to the NOP receptor was conducted using autoradiography with ^{125}I-N/OFQ.

In order to expose rats to social stressors, the social defeat procedure described in chapter 1 was used. This allowed the examination of changes in gene regulation in response to a single, acute social defeat, repeated social defeat with no acute exposure, and repeated social defeat with an acute exposure.

Methods

Animals

Forty-eight male Long Evans rats and 24 female Long Evans rats (Harlan, Indianapolis) were used in this experiment. Twenty-four of the male rats and the 24 female rats were used as resident pairs in the social defeat procedure. These male rats, weighing 400-500g at arrival (600-700g at the time of the experiment), were pair-housed prior to vasectomy surgery, and then singly housed for 1 week during recovery. The females (200-225g at arrival) were pair-housed until the males recovered from surgery, at which time the males and females were housed together. Four of the male rats were used as intruders for the purposes of training the resident males. The remaining 20 males, weighing approximately 300g, were used as experimental intruder rats. The intruder rats were pair-housed throughout the course of the experiment. All of

the rats were housed in 43 x 21.5 x 25.5 cm polycarbonate cages on a 12hr-12hr light-dark cycle (lights on at 7:00 am) in a climate-controlled vivarium (temperature 21-23ºC, humidity 55-60%). Standard laboratory chow and tap water were available *ad libitum*.

Drugs

Ketamine and xylazine were both obtained from Henry Schein (Melville, NY) and mixed as described in Chapter 2. Ketorolac tromethamine (30 mg/ml) and AErrane (99.9 % isoflurane) were also purchased from Henry Schein.

Surgery

Each of the male resident rats was vasectomized 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. AErrane was administered as supplemental surgical anesthesia as necessary. Vasectomy was completed by making a 1 cm ventral midline incision just rostral to the penis. Each vas deferens was isolated using forceps and a 0.5 cm section of each duct was removed using a mini cautery tool. The abdominal wall was sutured with absorbable 4-0 "Ethilon" monofilament nylon non-wicking suture (Ethicon Inc.), and the external incision was closed with stainless steel wound clips (9mm, World Precision Instruments Inc.). Following surgery, each rat was returned to its home cage and allowed 7-10 days for recovery, at which time the wound clips were removed.

Social Defeat Procedure

The resident male and female rats were pair-housed for at least 10 days and each of the male resident rats was trained and tested for territorial behavior prior to introducing the experimental intruder rats. In each training session, resident females were removed from the home cage 10 minutes before resident-intruder encounters. After 10 minutes, the intruder was placed into the cage with the resident. The encounter continued for 5 minutes or until the

intruder displayed submissive behavior defined as entering into a supine posture (Fig. 4-1) 3 times for at least 2 seconds each or freezing for a total of 90 seconds. Encounters were also terminated if either rat was severely bitten, which rarely occurred. The resident rat was considered dominant if the intruder submitted as defined above. The 14 residents that most consistently displayed dominance behaviors at the end of the training period were used for the experiment.

The social defeat procedure with the experimental intruder rats occurred in 2 stages. The first stage was identical to the training session and was terminated by the same criteria (3 submissions, 90 seconds of freezing, 5 minutes maximum, or severe biting). Immediately following termination of stage 1, each intruder rat was placed, individually, into a 10cm x 10cm x 15cm (inner dimensions) double-walled wire mesh cage and placed back into the resident's cage to allow further stress exposure while protecting the rat from potential injury (Fig. 4-2). The intruder remained in the cage until 10 minutes had passed from the start of stage 1. This allowed for equalization of the duration of stress exposure of the intruder rats regardless of how quickly stage 1 was terminated. Following the 10-minute session, the intruder rat was returned to its home cage and the female was returned to the resident's cage. The social defeat procedure was conducted over 6 days wherein specific treatment groups were exposed to differing amounts of repeated and/or acute exposure (Table 1). Group 1 experienced no social defeats on any of the 6 days (No Stress Controls). Group 2 experienced a social defeat encounter on the 6^{th} day only (No Repeated, Acute). Group 3 experienced a social defeat encounter on each of the first 5 days, but did not experience an encounter on the 6^{th} day (Repeated, No Acute), and group 4 experienced social defeat encounters on all 6 days (Repeated, Acute). On the 6^{th} day, all of the

intruder rats were rapidly decapitated at 3 hours following the start of the defeat session, or at the equivalent time in the groups that were not acutely defeated before termination.

Immediately after termination of the rats, each brain was removed, frozen in 2-methylbutane at -40ºC, and stored at -80ºC until use. The thymus glands, adrenal glands, and spleens were dissected out and weighed for examination of the health/stress status of each rat.

The social defeat interactions were recorded and subsequently scored for the number of defeats and total time spent in submissive postures (total time defeated) for each interaction. There was an uneven number of rats per group, with 4 rats included in groups 1 and 4 and 6 rats included in groups 2 and 3. The *in situ* hybridization procedure has limitations in the number of slides that can be processed per experiment; therefore, the number of rats was reduced to 4 per group in all 4 groups. In group 4, there was variability in the number of defeats that each rat received; therefore, the groups were balanced such that each group included some rats that received a high number of defeats and some rats that had a low number of defeats.

In Situ Hybridization

Each brain was sectioned into 15 µm slices and mounted onto polylysine-subbed slides. Every third section was processed for prepro-N/OFQ, NOP receptor mRNA, or NOP receptor autoradiography. The 504 base cDNA fragment corresponding to the 5' end of the coding region of the rat prepro-N/OFQ (Fig. 4-3) was cloned into plasmid pAMP and was provided by Dr. Olivier Civelli. The 529 base cDNA fragment corresponding to 102 bases in the 3' untranslated region through 427 bases of the open reading frame of the NOP receptor (Fig. 4-4) was cloned into BSSK and was provided by Dr. Huda Akil. Antisense and sense riboprobes were generated and labeled with ^{35}S-UTP (>1000 Ci/nmole; GE Healthcare). The pAMP plasmid was linearized with EcoR1 and transcribed with SP6 polymerase to generate the antisense prepro-N/OFQ riboprobe. This plasmid was also linearized with HindIII and transcribed with T7 to yield the

sense probe. The BSSK plasmid was linearized with EcoR1 and transcribed with T7 to generate the antisense riboprobe for NOP receptor mRNA, and this plasmid was also linearized with Xho1 and transcribed with T3 to yield the sense probe for the NOP receptor. The brain sections were prepared for hybridization by soaking in 4% formaldehyde for 1 hour, then rinsing in 3 washes of 2x SSC and one wash of ddH_2O all at room temperature (RT). The sections were soaked in 0.1M Triethanolamine plus 0.25% vol/vol acetic anhydride for 10 min., then rinsed in ddH_2O and dried using graded ethanols (50, 70, 80, 95, 95, 100, and 100%). The radiolabelled riboprobes were prepared in 50% hybridization buffer and were hybridized to the mounted sections. The slides were coverslipped and incubated overnight in humidified boxes at 55ºC. Coverslips were then removed and the sections were treated with RNAse A (to remove single-stranded, nonspecific label), then washed in 2x SSC (RT), 1x SSC (RT), and 0.1x SSC (67ºC) to further remove excess label. The sections were dried in graded ethanols from 50% to 100%. Once the sections and slides were completely dried they were placed on Kodak X-OMAT film in x-ray cassettes and exposed at room temperature for 1 week (NOP receptor) or 2 weeks (N/OFQ). The films were developed then photographed and digitized with an MCID camera and software system (Imaging Research Inc., Canada).

Due to the limitations in the total number of slides that can be processed in any single *in situ* hybridization experiment, separate experiments were conducted for the anterior and posterior halves of the brain for each mRNA probe. This division was between -3.6 (last anterior section) and -4.8 (first posterior section) mm posterior to bregma, according to the Paxinos and Watson (1998) rat brain atlas. Optical densities were measured in selected brain regions that are known to participate in physiological stress responses and/or emotional regulation. Those regions were the MPFC; the dorsolateral, interomediolateral, and ventrolateral septum (DLS, ILS, andVLS,

respectively); the anteromedial BNST, the posteromedial BNST, the lateral BNST, and the ventromedial BNST; the CeA, MeA, BMA, BLA, and LA; the PVN, arcuate, and ventromedial (VMH) hypothalamus; the MPOA; the reticular nucleus and zona incerta of the thalamus; CA1, CA2, CA3, and dentate gyrus regions of the hippocampus; the substantia nigra pars reticularis, pars lateralis, and pars compacta; the periaqueductal gray; the dorsal raphe; and the locus coeruleus.

Autoradiography

All sections from the 4 groups were processed together for autoradiographic analysis of N/OFQ binding to the NOP receptor so that identical radioligand binding conditions were used across groups. Slides were incubated with ^{125}I-[^{14}Tyr]-N/OFQ such that there were approximately 6000 counts per minute per section. Controls for nonspecific binding were also incubated with 1 μM N/OFQ. The iodinated probe was applied in an incubation chamber (RT, 60-80% humidity) in a buffer of 50 mM Tris HCl (pH 7.0), 1.0 mM EDTA, 0.1% BSA with proteinase inhibitor consisting of 0.1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1.0 μg/ml pepstatin, and 1.0 mM iodoacetamide. The incubation was terminated after 1 hour with four 4-minute washes in 50 mM Tris HCl on ice (pH 7.0). The sections were then rinsed in ddH_2O to remove excess salt. Dry sections were placed on Kodak X-OMAT film in x-ray cassettes and exposed at room temperature for 5 days. The films were developed then photographed and digitized with the MCID camera and software system.

The total number of slides did not exceed the number that could be processed at one time, so all sections were processed together. Optical densities were measured in the same regions as in the *in situ* hybridization experiments.

Densitometry and Statistics

Potential differences between the repeated and the repeated plus acute groups in the number of defeats and the total time defeated over the first 5 days were analyzed by a 2x5 repeated measures two-way ANOVA. All significant effects ($p< 0.05$) were further analyzed using Bonferroni post-tests. Additionally, potential differences between the acute and repeated plus acute groups in the number of defeats and the total time defeated on the 6th day were analyzed using T-tests.

Densitometric analysis of radioactive signal was analyzed using the MCID Basic software. A standard outline, using the Paxinos and Watson Rat Brain Atlas (1998), was determined for each region analyzed. Bilateral optical density measures were sampled from each region. If multiple sections were used per region, the optical densities for these sections were integrated to create one data point per rat per region per probe. Background measures were taken from the striatum and cerebellum since these are regions with very low levels of expression of N/OFQ mRNA, NOP receptor mRNA, and NOP receptor protein. Background values were subtracted from values obtained in the analyzed regions to control for potential differences in non-specific binding. The group means were compared by a one-way ANOVA for each region analyzed. All significant effects ($p< 0.05$) were further analyzed using Dunnet's post-tests.

In order to analyze differences in organ masses between the stressed and non-stressed groups, group means were compared by one-way ANOVAs for adrenal gland weights, thymus gland weights, and spleen weights.

Results

Social Defeats

Whereas I attempted to balance the stress-exposed groups by including the data from some rats that were defeated multiple times and some rats that were defeated fewer times, there was

still variability in the number of defeats between groups (Fig. 4-5). In general, over the first 5 days the rats experiencing repeated defeats and those experiencing repeated plus acute defeats were exposed to, on average, 11.25 defeats and 9.75 defeats, respectively. However, these differences were not statistically significant ($F_{(1,24)}$= 1.385, p>0.05). Additionally, there were no significant differences in the number of defeats between days 1 through 5 ($F_{(4,24)}$= 1.068, p>0.05) or in the interaction between stress group and day ($F_{(4, 24)}$= 1.748, p>0.05). On the 6th day the acutely defeated group and the repeatedly plus acutely defeated group received, on average, 3 defeats and 1.25 defeats, respectively. Again, though, these differences were not statistically significant (t(6)= 1.849, p>0.05).

There was also some observed variability in the total time defeated (Fig. 4-6). The repeatedly stressed group experienced an average total of 104.75 sec of defeat over the first 5 days, and the repeatedly plus acutely stressed group experienced an average total of 136.75 sec of defeat over the first 5 days. However, these differences were not statistically significant ($F_{(1,24)}$= 2.796, p>0.05). There were also no significant differences in the time defeated between days 1 through 5 ($F_{(4,24)}$= 1.286, p>0.05) or in the interaction between stress group and day ($F_{(4,24)}$= 1.016, p>0.05). On day 6, the acutely stressed group was defeated for an average 20 sec while the repeatedly plus acutely stressed group was defeated for an average of 12.5 sec. Again, these differences were not statistically significant (t(5)= 0.756, p>0.05).

In Situ Hybridization

Sense controls for prepro-N/OFQ and NOP receptor mRNAs displayed no specific signal. The background levels were approximately equal to those treated with antisense probes, and none of the sampled regions expressed signal substantially higher than background after hybridization with the sense probes (Fig. 4-7). Therefore, signal measured from antisense-treated sections can be considered specific for prepro-N/OFQ or NOP receptor mRNA.

Prepro-N/OFQ. There were no significant differences across groups in prepro-N/OFQ mRNA expression in any of the regions examined. However, there were consistent trends toward elevated expression in several limbic regions following acute social defeat exposure. These elevations occured in the dorsolateral and ventrolateral septal nuclei (Fig. 4-8, 4-10), all the nuclei of the BNST (Fig. 4-9, 4-10), the CeA and MeA (Fig. 4-11, 4-13), the zona incerta and reticular nucleus (Fig. 4-12, 4-13), and the dorsal raphe (Fig. 4-14, 4-15). Signal was low and there were no visible trends in the MPFC, BMA, BLA, LA, PVN, arcuate, VMH, MPOA CA1, CA2, CA3, DG, SNpr, SNpl, SNpc, PAG, or LC (data not shown).

NOP Receptor. There were significant stress-induced increases in NOP receptor mRNA expression in several important limbic regions. In the septum (Fig. 4-16, 4-17), there were significant elevations in NOP receptor mRNA expression following acute stress exposure in both the DLS (Fig. 4-16a; $F_{(3, 15)}= 7.134$, $p<0.01$), and the VLS (Fig. 4-16c; $F_{(3, 15)}= 3.656$, $p<0.05$). The ANOVA for the ILS revealed a significant overall effect that appears to result from a trend toward increases in NOP receptor mRNA expression in the acutely defeated groups, (Fig. 4-16b; $F_{(3, 15)}= 3.52$, $p<0.05$). However, the post-tests revealed no significant differences between any of the stressed groups and the control group for NOP receptor mRNA expression in the ILS. In the BNST, there was a significant elevation in NOP receptor mRNA expression in the ventromedial region following repeated social defeat exposure (Fig. 4-18, 4-19; $F_{(3, 14)}= 6.132$, $p<0.05$). In the amygdala, NOP receptor mRNA was elevated in several regions, particularly following acute social defeat exposure (Fig. 4-20). In the CeA, there were elevations in NOP receptor mRNA following exposure to acute and repeated plus acute social defeat exposure (Fig. 4-20a, 4-21; $F_{(3, 15)}= 6.449$, $p<0.01$). In the MeA, these elevations occured following acute social defeat (Fig. 4-20b; $F_{(3, 15)}= 5.174$, $p<0.05$). There were also significant elevations in NOP

receptor mRNA following acute social defeat in the BMA (Fig. 4-20c, 4-19; $F_{(3, 15)}$= 6.02, p<0.01), the BLA (Fig. 4-20d, 4-21; $F_{(3, 15)}$= 8.66, p<0.01), and the LA (Fig. 4-20e; $F_{(3, 15)}$= 9.802, p<0.01). In the PVN there were significant elevations in NOP receptor mRNA in all stress-exposed groups (Fig. 4-21, 4-22a; $F_{(3, 15)}$= 10.47, p<0.01). Differences in NOP receptor mRNA expression approached significance in the VMH (Fig. 22b, $F_{(3, 15)}$= 3.398, p=0.0536) and in the zona incerta (Fig. 22c, $F_{(3, 15)}$= 3.25, p=0.0599). These differences appear to be due to elevations following exposure to acute and to repeated plus acute social defeat. There were no significant differences in NOP receptor mRNA expression in the reticular nucleus of the thalamus, in any of the hippocampal regions, or in any of the midbrain or brainstem regions.

Autoradiography

Controls for non-specific binding displayed little to no signal and background levels were approximately equivalent to those treated with ^{125}I-[^{14}Tyr]-N/OFQ (Fig. 4-21). Therefore, signal measured from ^{125}I-[^{14}Tyr]-N/OFQ -treated sections can be considered specific for binding to the NOP receptor. However, there were no significant differences in N/OFQ binding between any of the stress-exposed groups for any of the regions analyzed (Figs. 4-24 to 4-30).

Organ Masses

There were no significant differences in adrenal weights (Fig. 4-31a), thymus gland weights (Fig. 4-31b), or spleen weights (Fig. 4-31c) between the non-stressed controls and the socially defeated rats.

Discussion

In general, basal expression of prepro-N/OFQ and NOP receptor mRNAs, as well as NOP receptor binding agrees with previous anatomical examinations (Neal et al., 1999a and b). Stress exposure appears to alter these basal levels, particularly in the expression of NOP receptor mRNA. In the BNST, it appears that the N/OFQ-NOP receptor system plays a role in limbic

plasticity following repeated, severe stress exposure. Additionally, in the PVN, NOP receptor regulation appears to be sensitive to acute and repeated social stressors. This suggests that stressor exposure might produce significant changes in HPA responses to N/OFQ.

Prepro-N/OFQ mRNA

Devine and colleagues (2003) previously found release of N/OFQ following acute stress exposure, and the N/OFQ content was replenished within 24 hours. In order for the peptide content to be replenished, transcription and protein synthesis need to occur. Therefore, it was expected that acute and repeated plus acute social defeat would produce increases in prepro-N/OFQ mRNA. While there were not statistically significant changes, there were consistent trends toward elevated mRNA expression following acute social defeat exposure. These trends were repeated over multiple limbic structures, including the septum, the BNST, and the amygdala. All of these regions are involved in responses to fear- and anxiety-provoking stimuli (for example see Goldstein, 1965; Van de Kar et al., 1991; Gray et al., 1993; Calfa et al., 2006; Menard and Treit, 1996; Lee and Davis, 1997; Walker and Davis, 1997; Vyas et al., 2003).

Several of the regions where trends were present are in the basal forebrain, the same region where Devine and colleagues (2003) found release of N/OFQ. This concordance and the consistency of the trends over multiple limbic structures suggest that the effect is a real one. One potential reason for the lack of statistical significance is the variability in the amount of stress exposure, with some rats experiencing fewer social defeats and less total time in submissive postures. While between groups differences in the number and total duration of defeats were not statistically significant, we do not know if these variables are critical in determining the affective and physiological responses of the intruders (or if the mere presence of the resident is the important factor). It is possible that the variability in residents' behaviors could contribute to variability in the mRNA expression of intruders, reducing statistical power. Another

consideration is that Devine and colleagues (2003) found that N/OFQ content was replenished 24 hours later. It is possible that in the current study the timepoint selected was not during the peak of the mRNA upregulation. It is also possible that there is a relatively small upregulation of mRNA (as we observed in the consistent trend across many forebrain regions), and that N/OFQ is slowly replenished over an extended portion of that 24 hour period.

NOP Receptor mRNA

There were significant changes in NOP receptor mRNA expression in several brain regions following social defeat exposure. NOP receptor mRNA expression was significantly increased in several limbic-hypothalamic regions, including the septum, the BNST, the amygdala, and the PVN. These changes primarily occurred following acute social defeat exposure, although in the amygdala there were also increases in NOP receptor mRNA in the repeated plus acute group and in the BNST there were increases in mRNA in the repeated (but no acute) group. This increase in the BNST following repeated stress is interesting given the most recent hypothesis about the roles of the amygdala and the BNST. It has been shown that the amygdala and the BNST can be differentiated in terms of their roles in fear- and anxiety-like responses, with the amygdala primarily involved in fear-like behaviors and the BNST involved in anxiety-like behaviors (Walker and Davis, 1997; for review see Walker et al., 2003). It has been proposed that the involvement of these structures in the response to stressor exposure is related to the length of the exposure, with the amygdala involved in the early, immediate response and the BNST involved in the later, long-term responses (Walker et al., 2003). Our findings are consistent with this interpretation, with the amygdala showing changes in response to an acute social defeat and the BNST showing changes in response to repeated defeat sessions. These results suggest that the N/OFQ-NOP receptor system may respond differently, depending on the type and amount of stress exposure and the region of the brain.

NOP receptor mRNA was also expressed in greater amounts in the PVN following all social defeat regimens (acute, repeated, and repeated plus acute), as compared to the expression in the no stress control group. It is known that administration of N/OFQ into the ventricles produces activation of the HPA axis in unstressed rats and prolongs the activation in mildly stressed rats (Devine et al., 2001). One likely site for this activation is the PVN, since this structure contains the neurons that synthesize and release CRH, activating the HPA axis. Therefore, NOP receptor upregulation in the PVN could suggest an increased sensitivity of the HPA axis.

The upregulation of NOP receptor mRNA in response to stress exposure is somewhat surprising given the increased release of N/OFQ following stressful events (Devine et al., 2003). In some receptor systems, an increase in neurotransmitter release (or the overconsumption of a drug that acts at the receptor) results in long-term desensitization of the receptor—a mechanism that accounts for many tolerance effects. It has already been observed that rats display rapid tolerance to the locomotor-inhibiting effects of N/OFQ (Devine et al., 1996); thus, the system is capable of tolerance and the receptors may desensitize in response to N/OFQ release. There is evidence that the NOP receptor does, in fact, undergo rapid desensitization (Dautzenberg et al., 2001; Spampinato and Baiula, 2006). However, Spampinato and Baiula (2006) demonstrated that at higher levels of N/OFQ exposure, the NOP receptor undergoes internalization and recycling resulting in resensitization and, even, supersentization. These experiments, however, are in artificial systems and it is not clear how these results compare to stress-induced release of N/OFQ *in vivo*.

NOP Receptor Binding

There were no significant increases in N/OFQ binding in any of the regions analyzed, despite the upregulation of NOP receptor mRNA in several regions. There may be some post-

transcriptional or post-translational mechanism interfering with the synthesis of the receptors or with the insertion of the receptors into the cell membrane. However, it may be more likely that the time point selected to examine binding was not the ideal time point. In the acute groups, binding was examined 3 hours after stressor exposure. At this time point, protein synthesis and receptor insertion may not have yet occurred. In the repeated but no acute groups, binding was examined 27 hours after the last stressor exposure. At this point, receptor levels may have returned to baseline. Devine and colleagues (2003) have already shown the peptide content is replenished within 24 hours. Therefore, the N/OFQ-NOP receptor systems appears to be a tightly regulated system in which changes in response to stressors are transient.

To fully examine the potential changes in receptor synthesis and insertion and potential changes in binding, a more thorough analysis would be required. This would involve a full Scatchard analysis to examine binding, protein analysis by immunohistochemistry under non-permeabilizing and permeabilizing conditions (to assay membrane insertion), and protein quantification by Western blot.

Organ Masses

There were no statistically significant changes in adrenal, thymus, or spleen masses in any of the stress-treated groups. Chronic stress exposure tends to produce thymus and spleen involution and adrenal hypertrophy (for examples see Selye, 1936; Bryant et al., 1991; Watzl et al., 1993; Blanchard et al., 1998; Dominguez-Gerpe and Rey-Mendez, 2001; Hasegawa and Saiki, 2002). The thymus glands appear to be most sensitive to stress exposure. Selye (1936) found that even single exposures to potent systemic stressors caused rapid thymus involution. There was a trend toward decreased thymus weights in all of the groups that received social defeat, including the acute group. Therefore, there is some evidence in this experiment that the social defeat procedure was potent enough to produce changes in thymus glands.

Figure 4-1. Photograph of a social defeat interaction. The intruder is engaging in submissive behaviors by displaying a supine posture while the resident is displaying dominant behaviors by standing over the intruder. One defeat was counted when the rats engaged in this interaction for at least 2 seconds prior to disengaging. This first stage of the social interaction continued until 3 defeats occurred, the intruder froze for 90 total seconds, or until 5 minutes had elapsed.

Figure 4-2. Photograph of stage 2 of the social defeat procedure. Following 3 defeats or 5 minutes, the intruder was placed into a double-walled wire mesh cage placed into the resident's cage. The residents continued to display dominant behaviors by climbing atop the cage and kicking bedding at the intruder. The intruder remained in this cage until 10 minutes had elapsed from the start of the procedure.

Table 1: Groups of intruder rats						
Groups	Repeated stress					Acute stress
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Group 1						
Group 2						
Group 3						
Group 4						

Table 4-1. Social defeat regimen. Four groups of rats were exposed to differing amounts of social defeat. Group 1 was not exposed to any social defeat but was left undisturbed in their cages (No stress controls). Group 2 was exposed to a single social defeat session on day 6 (Acute). Group 3 was exposed to one social defeat session on each of 5 days, but no social defeat on day 6 (Repeated, No Acute). Group 4 was exposed to one social defeat session on each of the 6 days (Repeated, Acute).

```
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481 cggttcagtg agtttatgag gcagtacctg gtcctgagca tgcagtcaag ccaacgccgg
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```

Figure 4-3. Prepro-N/OFQ sequence. The 972 bases for the prepro-N/OFQ gene are displayed (Accession # NM_013007). The highlighted region represents the portion of the sequence that was included in the prepro-NOFQ plasmid insert used for the *in situ* hybridization. Sequencing was performed by the University of Florida DNA Sequencing Core, Interdisciplinary Center for Biotechnology Research.

```
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Figure 4-4. NOP receptor sequence. The 2706 bases for NOP receptor gene are displayed (Accession # NM_031569.2). The highlighted region represents the portion of the sequence that was included in the NOP receptor plasmid insert used for the *in situ* hybridization. Sequencing was performed by the University of Florida DNA Sequencing Core, Interdisciplinary Center for Biotechnology Research.

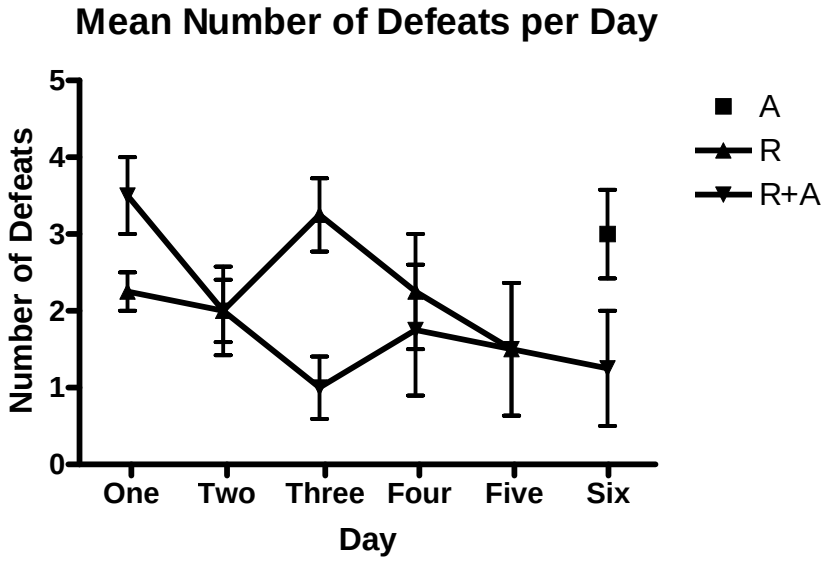

Figure 4-5. Number of social defeats per group per day. Values shown are group means ± SEM (n = 4 rats per group). A = acute stress group, R = repeated stress group, R+A = repeated plus acute stress group.

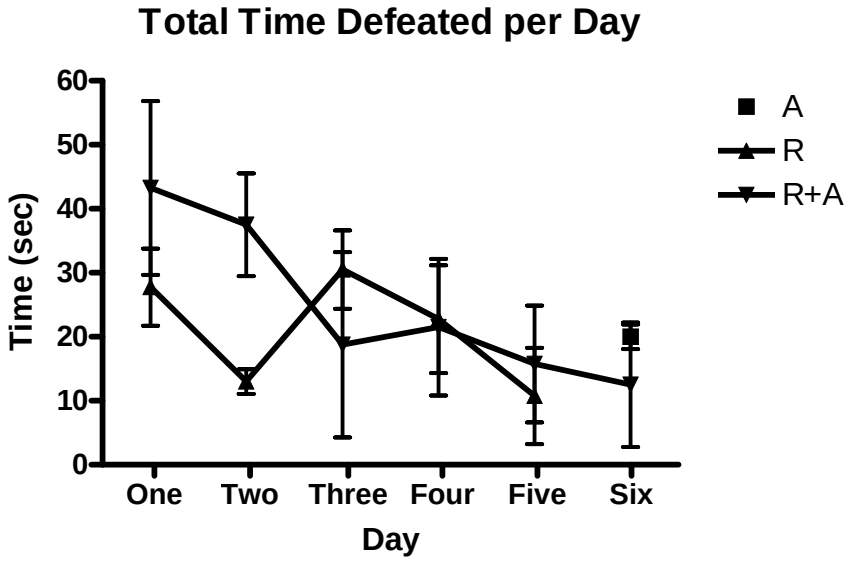

Figure 4-6. Total amount of time the rats were defeated per day. Values shown are group means ± SEM (n = 4 rats per group). A= acute stress, R= repeated stress, R+A= repeated plus acute.

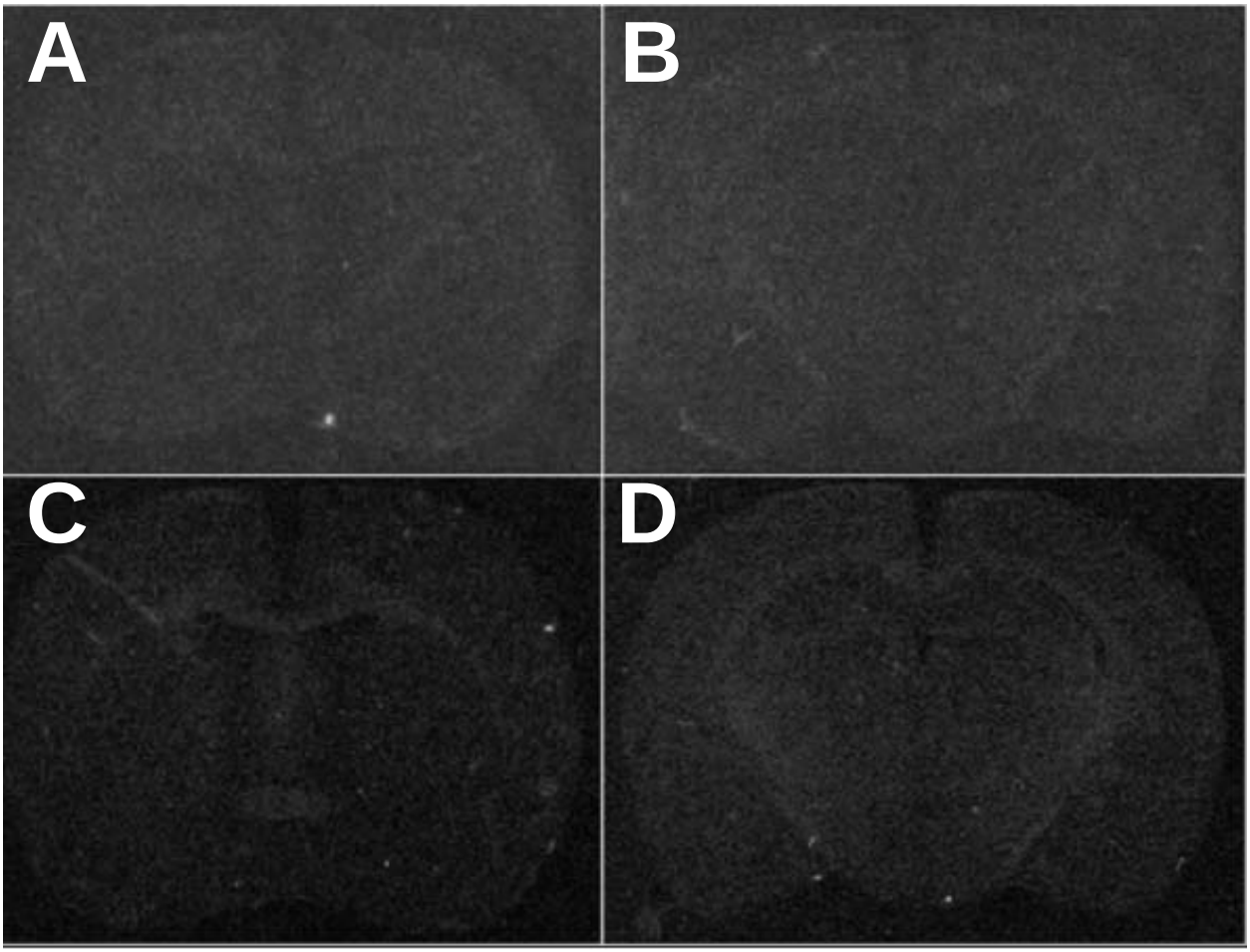

Figure 4-7. Representative x-ray images of brain sections treated with sense riboprobes. Sections treated with (A,B) N/OFQ sense strands and (C,D) NOP receptor sense strands did not display any specific signal, and background levels were comparable to those of antisense-treated sections.

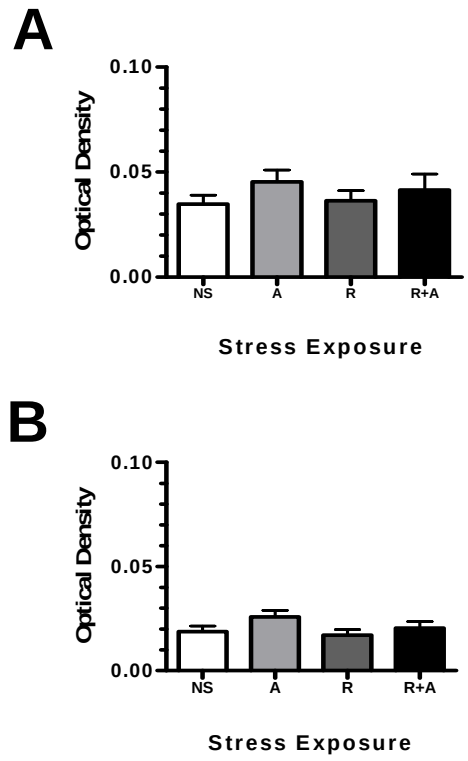

Figure 4-8. Prepro-N/OFQ mRNA expression in the septum. There were trends toward greater mRNA expression in (A) the dorsolateral septum and (B) the ventrolateral septum following exposure to acute social defeat. Values expressed are group means in optical density ± SEM (n = 4 rats per group). NS= no stress, A= acute stress, R= repeated stress, R+A= repeated plus acute.

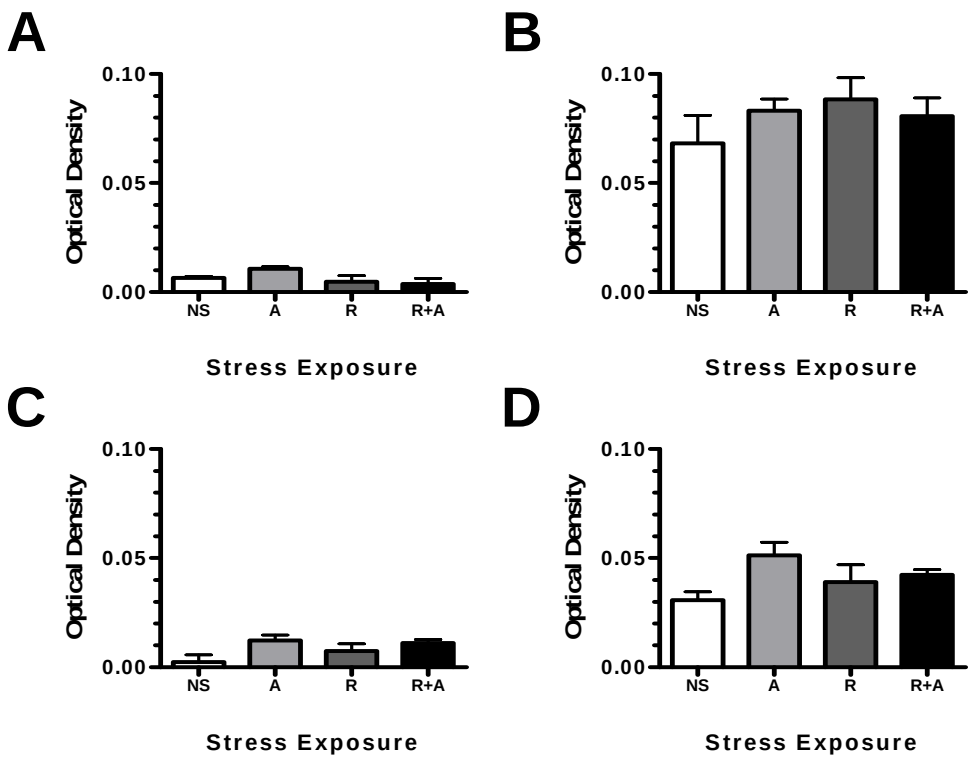

Figure 4-9. Prepro-N/OFQ mRNA expression in the bed nucleus of stria terminalis. There were trends toward greater mRNA expression in the (A) anteromedial BNST, (B) posteromedial BNST, (C) ventromedial BNST, and (D) lateral BNST following exposure to acute social defeat. Values expressed are group means in optical density ± SEM (n = 4 rats per group). NS= no stress, A= acute stress, R= repeated stress, R+A= repeated plus acute.

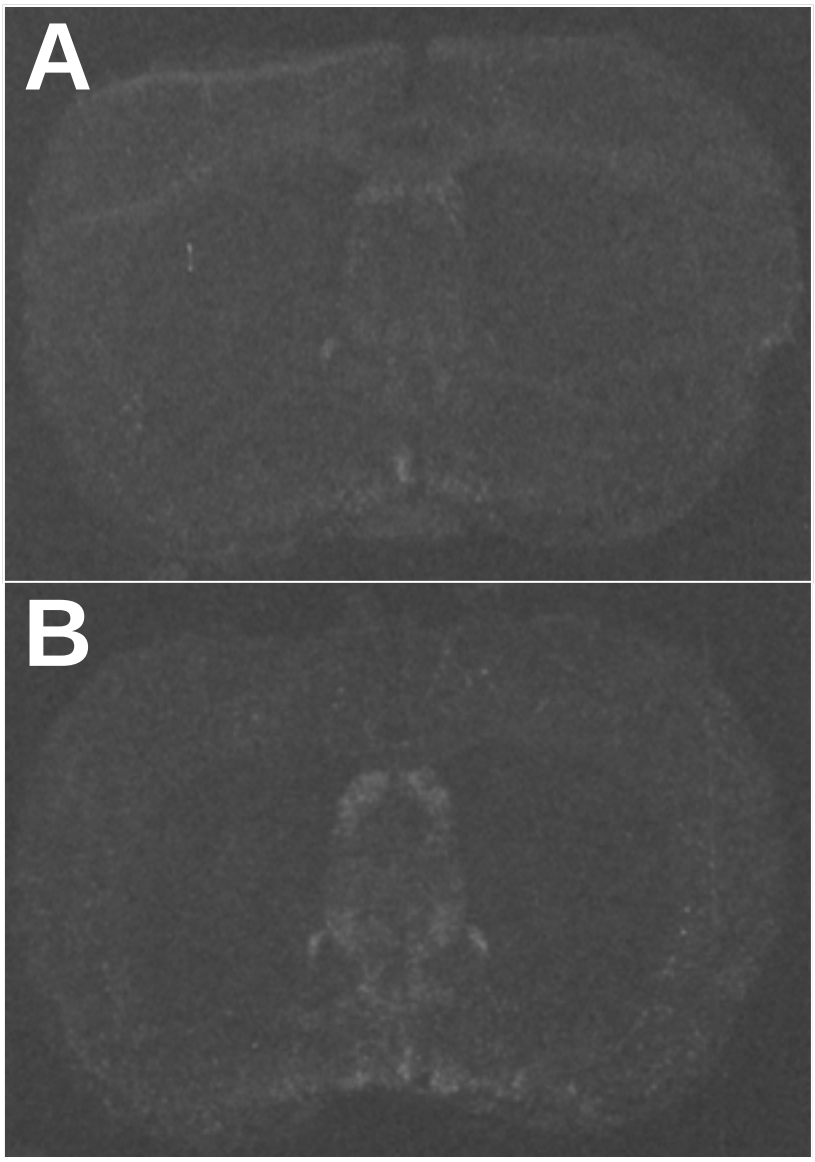

Figure 4-10. Representative x-ray images of brain sections showing prepro-N/OFQ mRNA expression in the septum and BNST. Sections were exposed to ^{125}I-labelled riboprobes complimentary to segments of prepro-N/OFQ mRNA. (A) No stress control rats display low to moderate signal in the dorsolateral septum, intermediolateral septum, ventromedial septum, and lateral BNST. (B) Rats exposed to acute social defeat displayed trends toward increased expression in these regions.

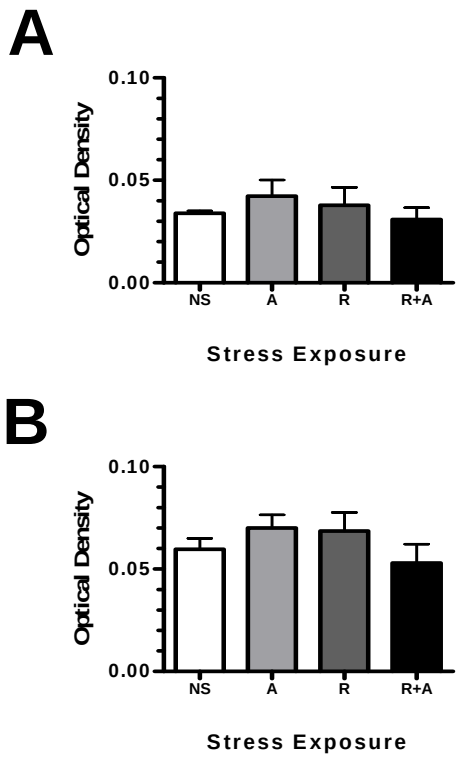

Figure 4-11. Prepro-N/OFQ mRNA expression in the amygdala. There were trends toward greater mRNA expression in (A) the central amygdala and (B) the medial amygdala following exposure to acute social defeat. Values expressed are group means in optical density ± SEM (n = 4 rats per group). NS= no stress, A= acute stress, R= repeated stress, R+A= repeated plus acute.

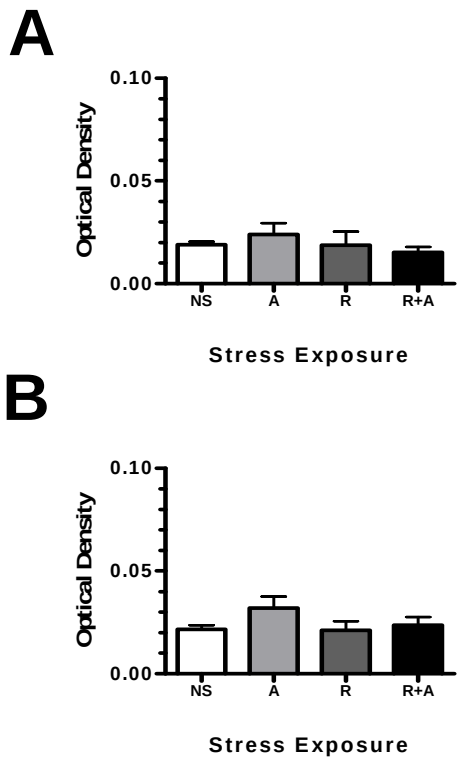

Figure 4-12. Prepro-N/OFQ mRNA expression in the zona incerta and reticular nucleus of the thalamus. There were trends toward greater mRNA expression in (A) the zona incerta and (B) the reticular nucleus following exposure to acute social defeat. Values expressed are group means in optical density ± SEM (n = 4 rats per group). NS= no stress, A= acute stress, R= repeated stress, R+A= repeated plus acute.

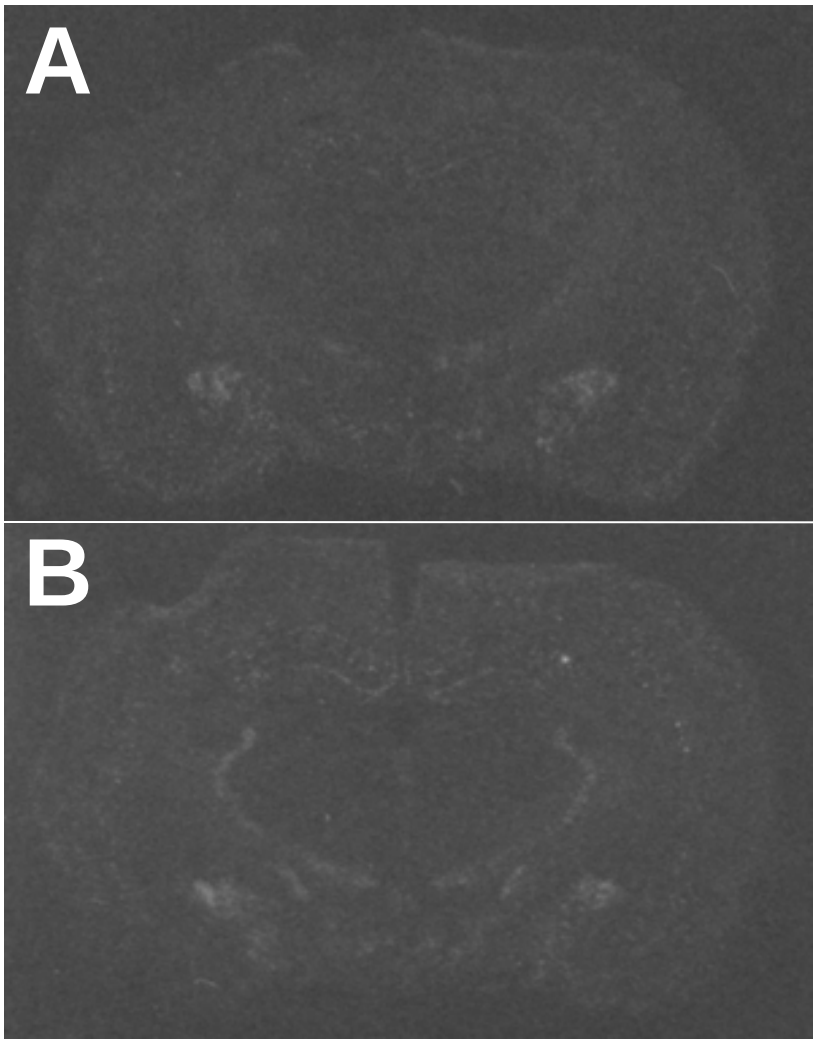

Figure 4-13. Representative x-ray images of brain sections showing prepro-N/OFQ mRNA expression in the amygdala, zona incerta, and reticular nucleus of the thalamus. Sections were exposed to ^{125}I-labelled riboprobes complimentary to segments of prepro-N/OFQ mRNA. (A) No stress control rats display moderate signal in the amygdala and low signal in the zona incerta and reticular nucleus. (B) Rats exposed to acute social defeat displayed trends toward increased expression in these regions. NS= no stress, A= acute stress, R= repeated stress, R+A= repeated plus acute.

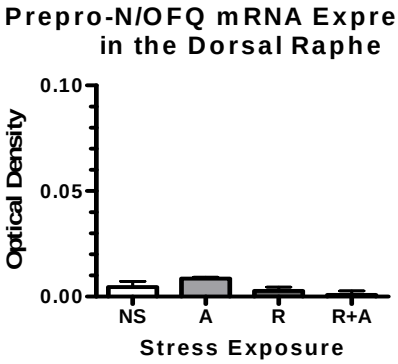

Figure 4-14. Prepro-N/OFQ mRNA expression in the dorsal raphe. There were trends toward greater mRNA expression in the dorsal raphe following exposure to acute social defeat. Values expressed are group means in optical density ± SEM (n = 4 rats per group). NS= no stress, A= acute stress, R= repeated stress, R+A= repeated plus acute.

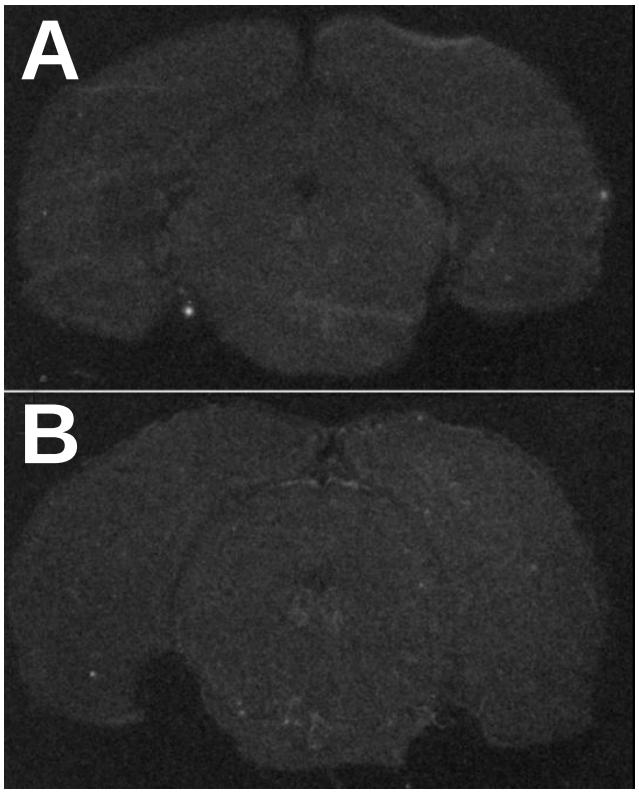

Figure 4-15. Representative x-ray images of brain sections showing prepro-N/OFQ mRNA expression in the dorsal raphe. Sections were exposed to ^{125}I-labelled riboprobes complimentary to segments of prepro-N/OFQ mRNA. (A) No stress control rats display moderate signal dorsal raphe. (B) Rats exposed to acute social defeat displayed trends toward increased expression in these regions. NS= no stress, A= acute stress, R= repeated stress, R+A= repeated plus acute.

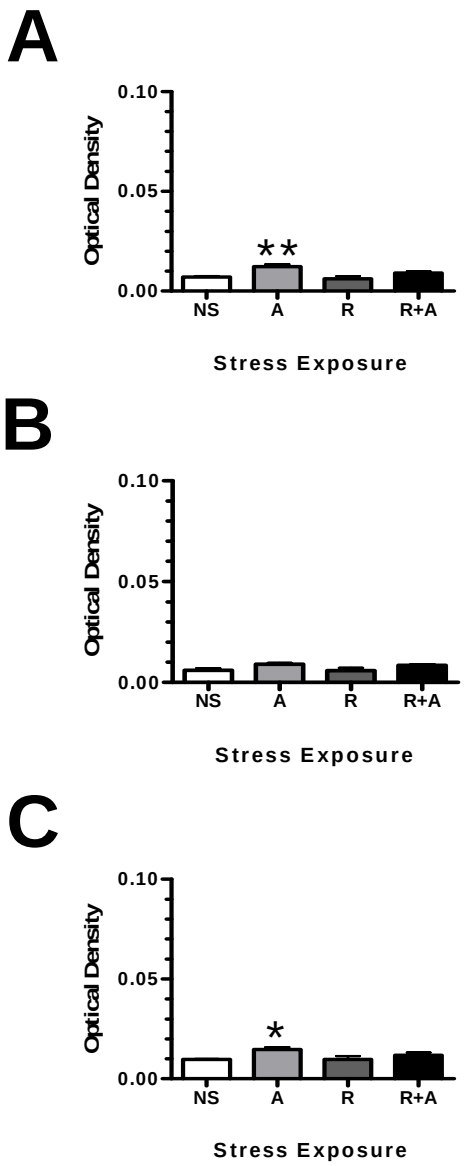

Figure 4-16. NOP rector mRNA expression in the septum. mRNA expression was significantly greater in the (A) dorsolateral septum, (B) intermediolateral septum, and (C) ventrolateral septum following exposure to acute social defeat. Values expressed are group means in optical density ± SEM (n = 4 rats per group). Significant differences between the stress-exposed rats and the no stress controls are expressed as * $p < 0.05$, ** $p < 0.01$. NS= no stress, A= acute stress, R= repeated stress, R+A= repeated plus acute.

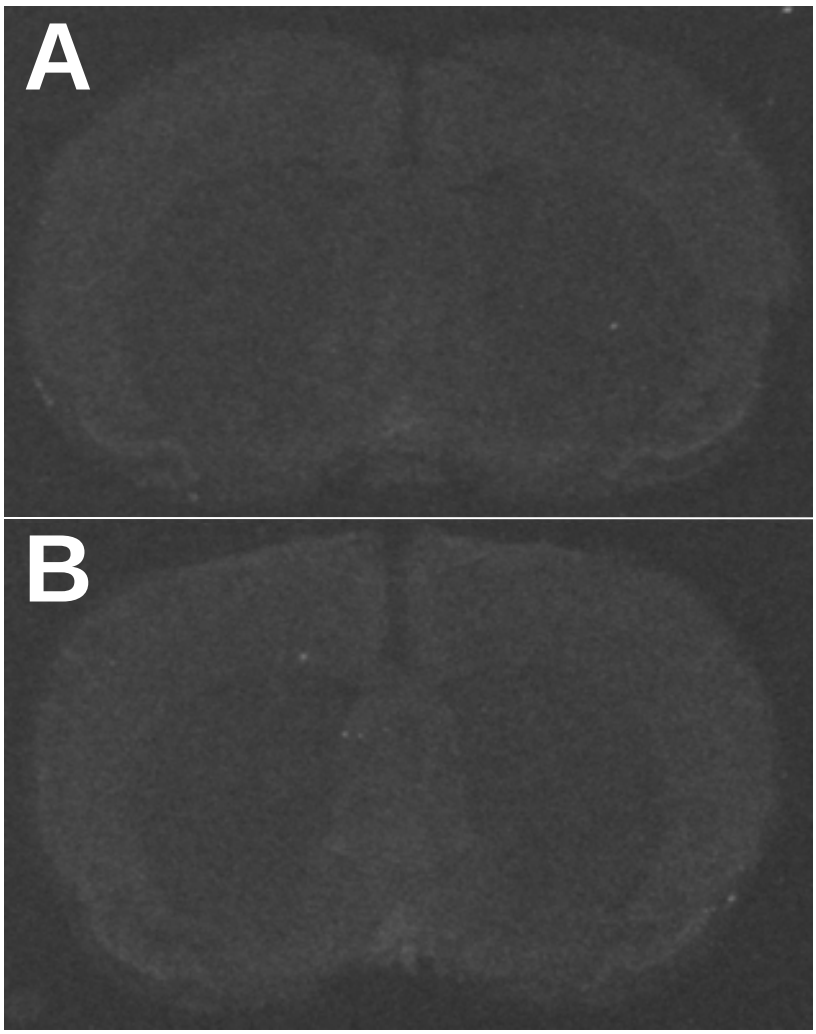

Figure 4-17. Representative x-ray images of brain sections showing NOP receptor mRNA expression in the septum. Sections were exposed to ^{125}I-labelled riboprobes complimentary to segments of NOP receptor mRNA. (A) No stress control rats display low signal in the dorsolateral, intermediolateral, and ventrolateral septum. (B) Rats exposed to acute social defeat displayed significantly greater mRNA expression in these regions regions.

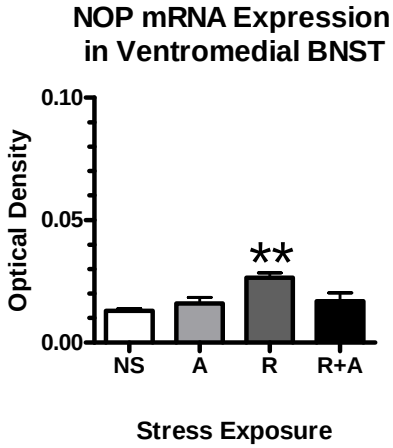

Figure 4-18. NOP receptor mRNA expression in the bed nucleus of stria terminalis. mRNA expression was significantly greater in the ventromedial BNST following exposure to repeated social defeat. Values expressed are group means in optical density ± SEM (n = 4 rats per group). Significant differences between the stress-exposed rats and the no stress controls are expressed as ** $p < 0.01$. NS= no stress, A= acute stress, R= repeated stress, R+A= repeated plus acute.

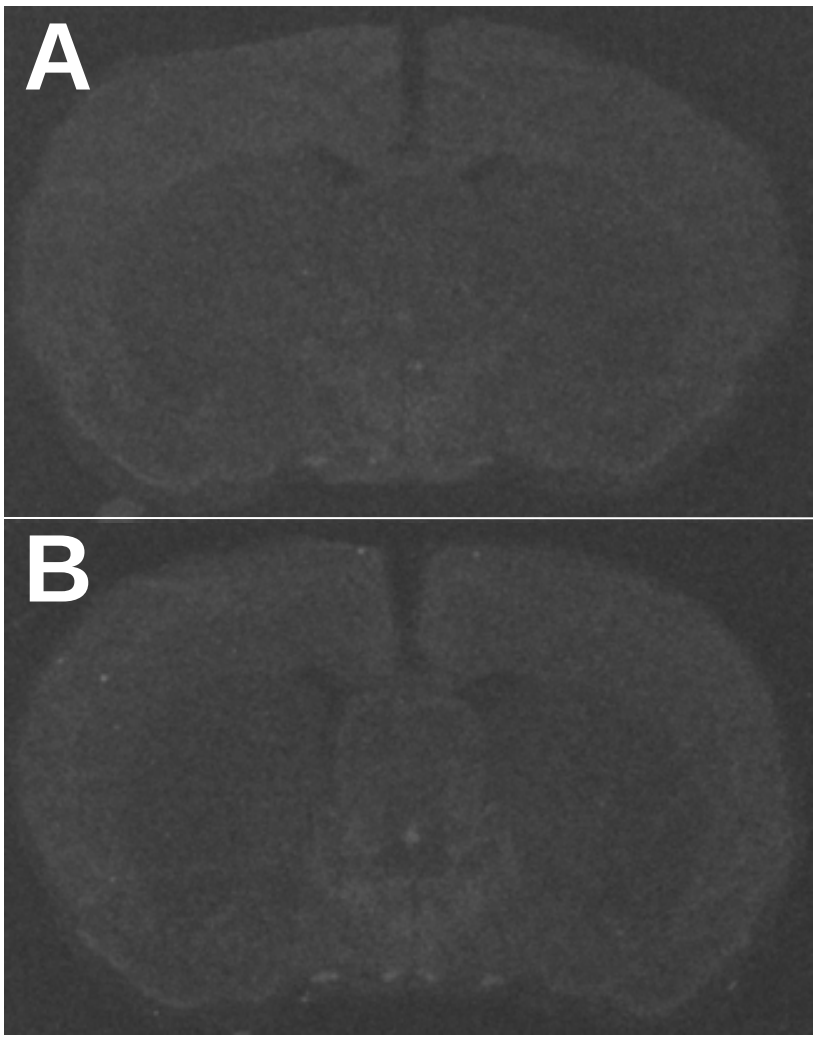

Figure 4-19. Representative x-ray images of brain sections showing NOP receptor mRNA expression in the bed nucleus of stria terminalis. Sections were exposed to ^{125}I-labelled riboprobes complimentary to segments of NOP receptor mRNA. (A) No stress control rats display low signal in the ventromedial BNST. (B) Rats exposed to repeated social defeat displayed significantly greater mRNA expression in the ventromedial BNST.

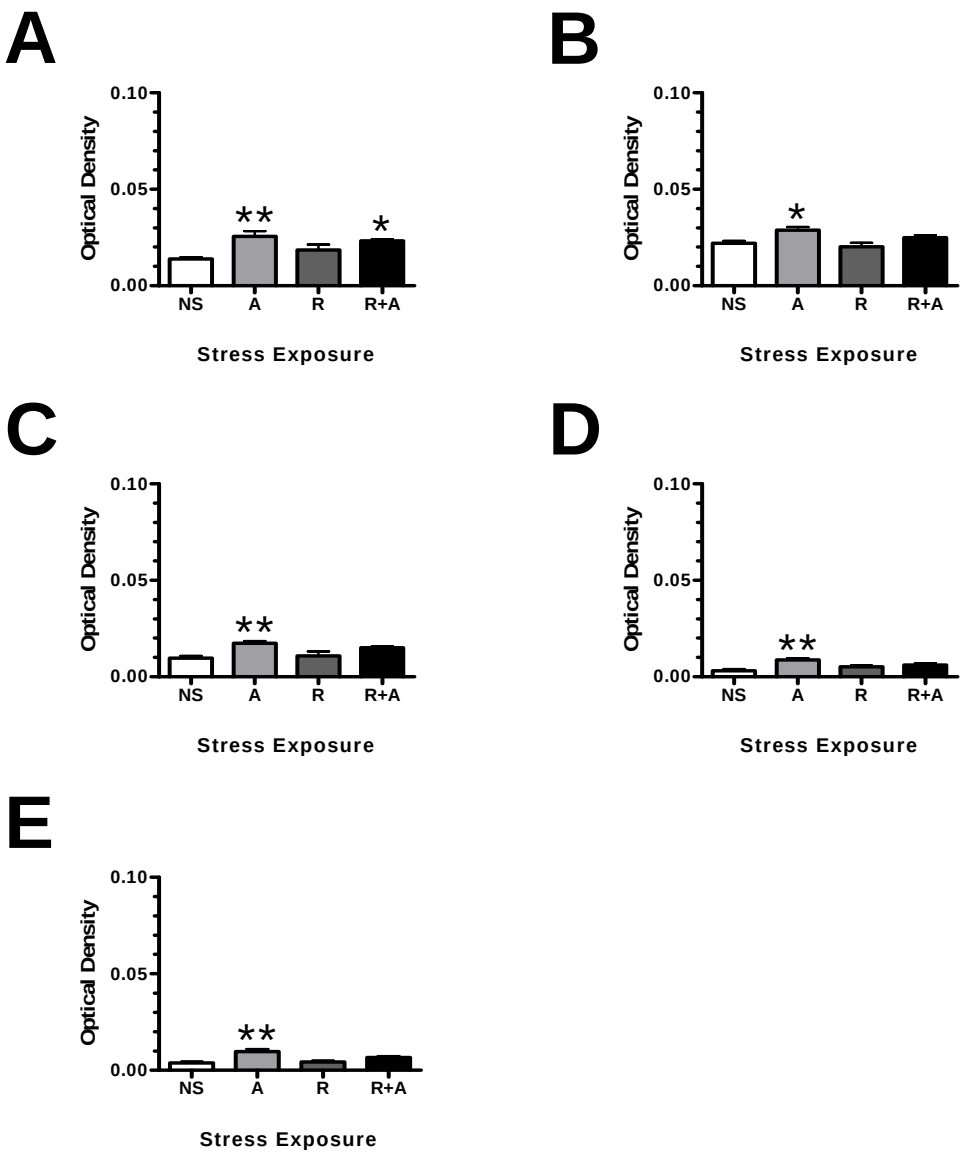

Figure 4-20. NOP receptor mRNA expression in the amygdala. mRNA expression was significantly greater in (A) the central amygdala, (B) the medial amygdala, (C) the basomedial amygdala, (D) the basolateral amygdala, and (E) the lateral amygdala following exposure to acute social defeat. Additionally, there was significantly greater mRNA expression in the central amygdala following repeated plus acute social defeat. Values expressed are group means in optical density ± SEM (n = 4 rats per group). Significant differences between the stress-exposed rats and the no stress controls are expressed as * $p < 0.05$ and ** $p < 0.01$. NS= no stress, A= acute stress, R= repeated stress, R+A= repeated plus acute.

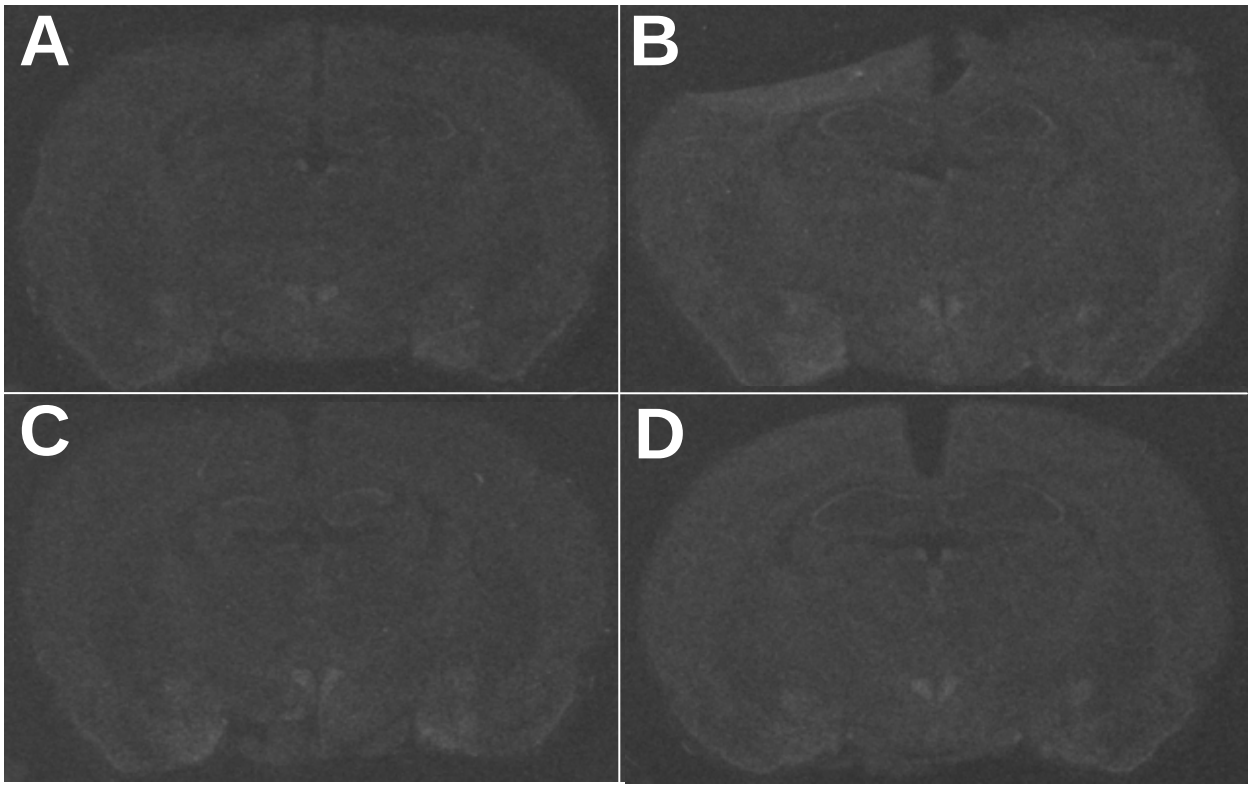

Figure 4-21. Representative x-ray images of brain sections showing NOP receptor mRNA expression in the amygdala and the paraventricular nucleus of the hypothalamus. Sections were exposed to ^{125}I-labelled riboprobes complimentary to segments of NOP receptor mRNA. (A) No stress control rats display low to moderate signal in the amygdala and PVN. Rats exposed to (B) acute and (D) repeated plus acute social defeat displayed greater mRNA expression in the amygdala. Rats exposed to (B) acute, (C) repeated, and (D) repeated plus acute social defeat displayed greater mRNA expression in the PVN.

Figure 4-22. NOP receptor mRNA expression in the paraventricular and ventromedial nuclei of the hypothalamus and in the zona incerta of the thalamus. (A) mRNA expression was significantly greater in the PVN following any exposure to social defeat. In (B) the ventromedial hypothalamus and (C) the zona incerta elevations in mRNA expression following social defeat approached significance. Values expressed are group means in optical density ± SEM (n = 4 rats per group). Significant differences between the stress-exposed rats and the no stress controls are expressed as * $p < 0.05$ and ** $p < 0.01$. NS= no stress, A= acute stress, R= repeated stress, R+A= repeated plus acute.

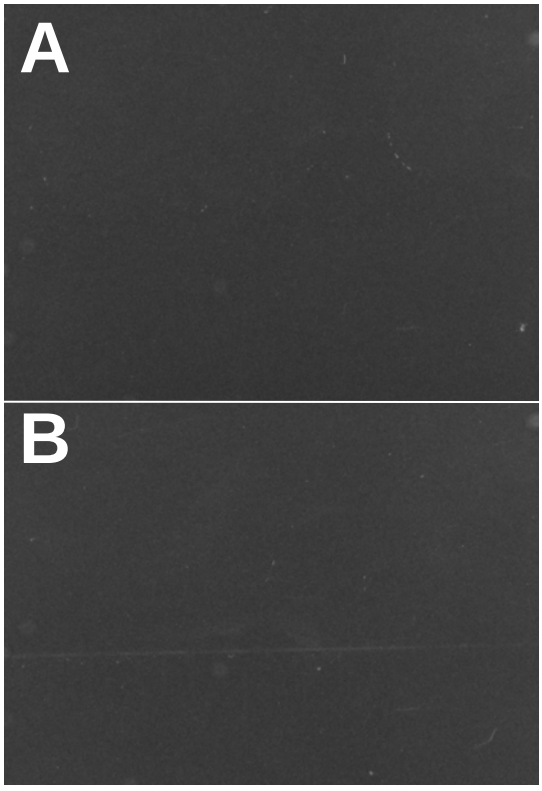

Figure 4-23. Representative x-ray images of brain sections treated with 1 μM N/OFQ plus ^{125}I-[^{14}Tyr]-N/OFQ. Sections treated with 1 μM N/OFQ plus ^{125}I-[^{14}Tyr]-N/OFQ did not display any specific signal, and background levels were comparable to those sections treated with only ^{125}I-[^{14}Tyr]-N/OFQ.

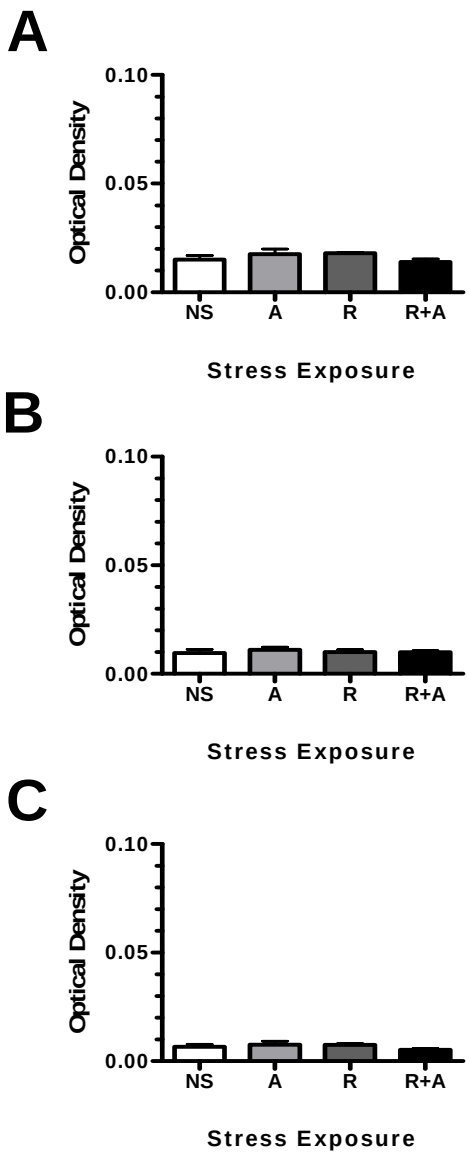

Figure 4-24. ^{125}I-[^{14}Tyr]-N/OFQ binding to the NOP receptor in the septum. There were no significant changes in binding following any stress exposure in (A) the dorsolateral septum, (B) the intermediolateral septum, or (C) the ventrolateral septum. Values expressed are group means in optical density ± SEM (n = 4 rats per group). NS= no stress, A= acute stress, R= repeated stress, R+A= repeated plus acute.

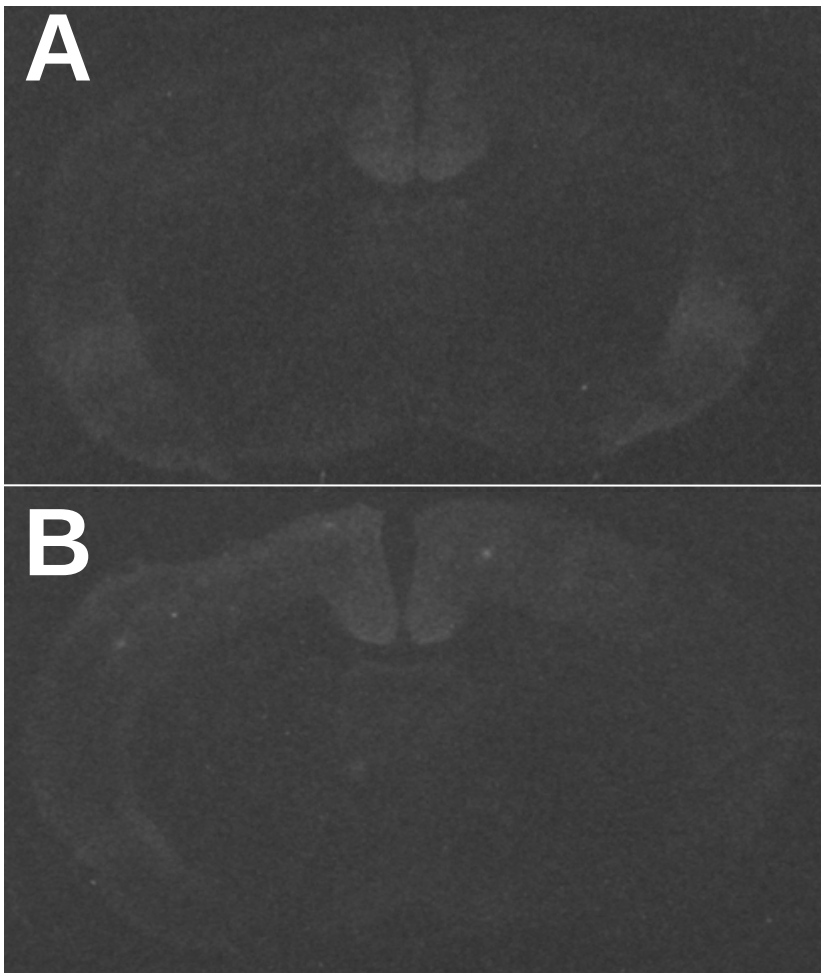

Figure 4-25. Representative x-ray images of brain sections showing ^{125}I-[^{14}Tyr]-N/OFQ binding to the NOP receptor in the septum. Despite an increase in NOP receptor mRNA, (B) the acutely stressed group did not display a significant increase in binding as compared to (A) the no stress controls.

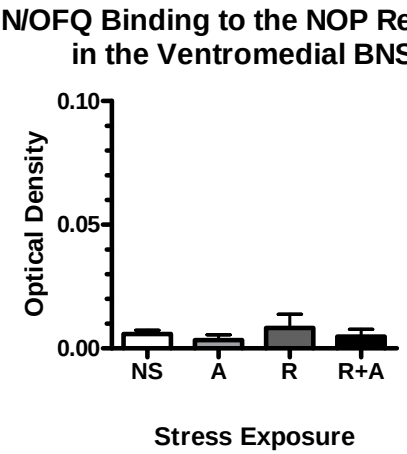

Figure 4-26. ^{125}I-[^{14}Tyr]-N/OFQ binding to the NOP receptor in the ventromedial BNST. There were no significant changes in binding following any stress exposure in the ventromedial BNST. Values expressed are group means in optical density ± SEM (n = 4 rats per group). NS= no stress, A= acute stress, R= repeated stress, R+A= repeated plus acute.

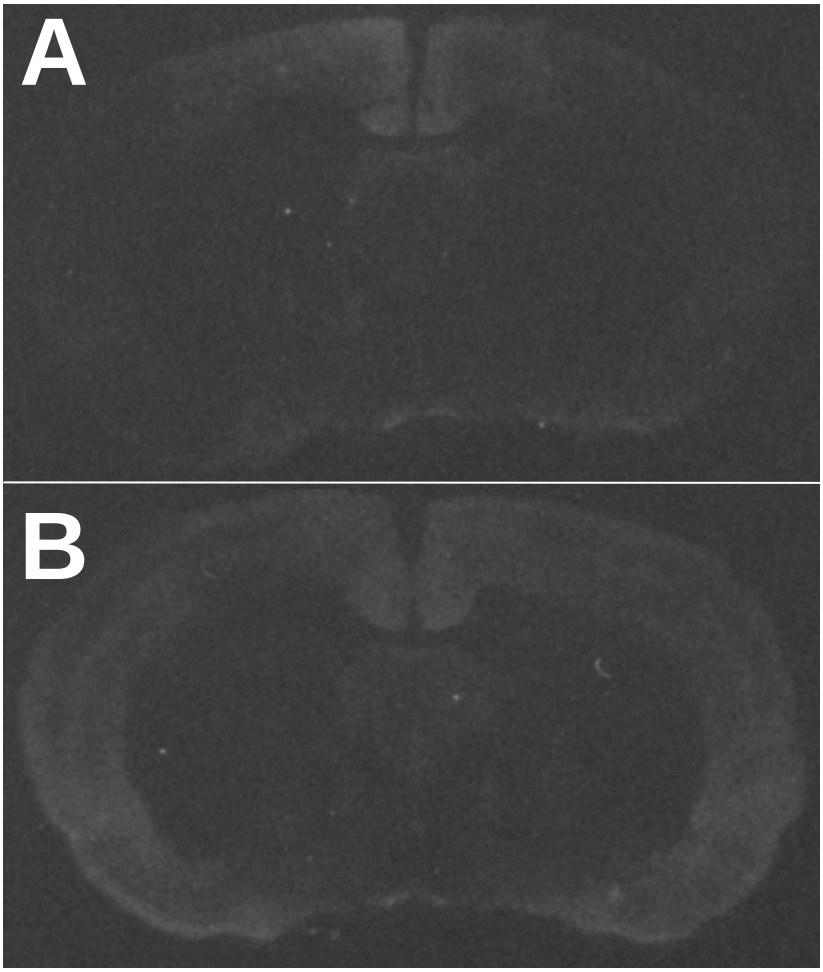

Figure 4-27. Representative x-ray images of brain sections showing ^{125}I-[^{14}Tyr]-N/OFQ binding to the NOP receptor in the BNST. Despite an increase in NOP receptor mRNA, (B) the repeatedly stressed group did not display a significant increase in binding as compared to (A) the no stress controls.

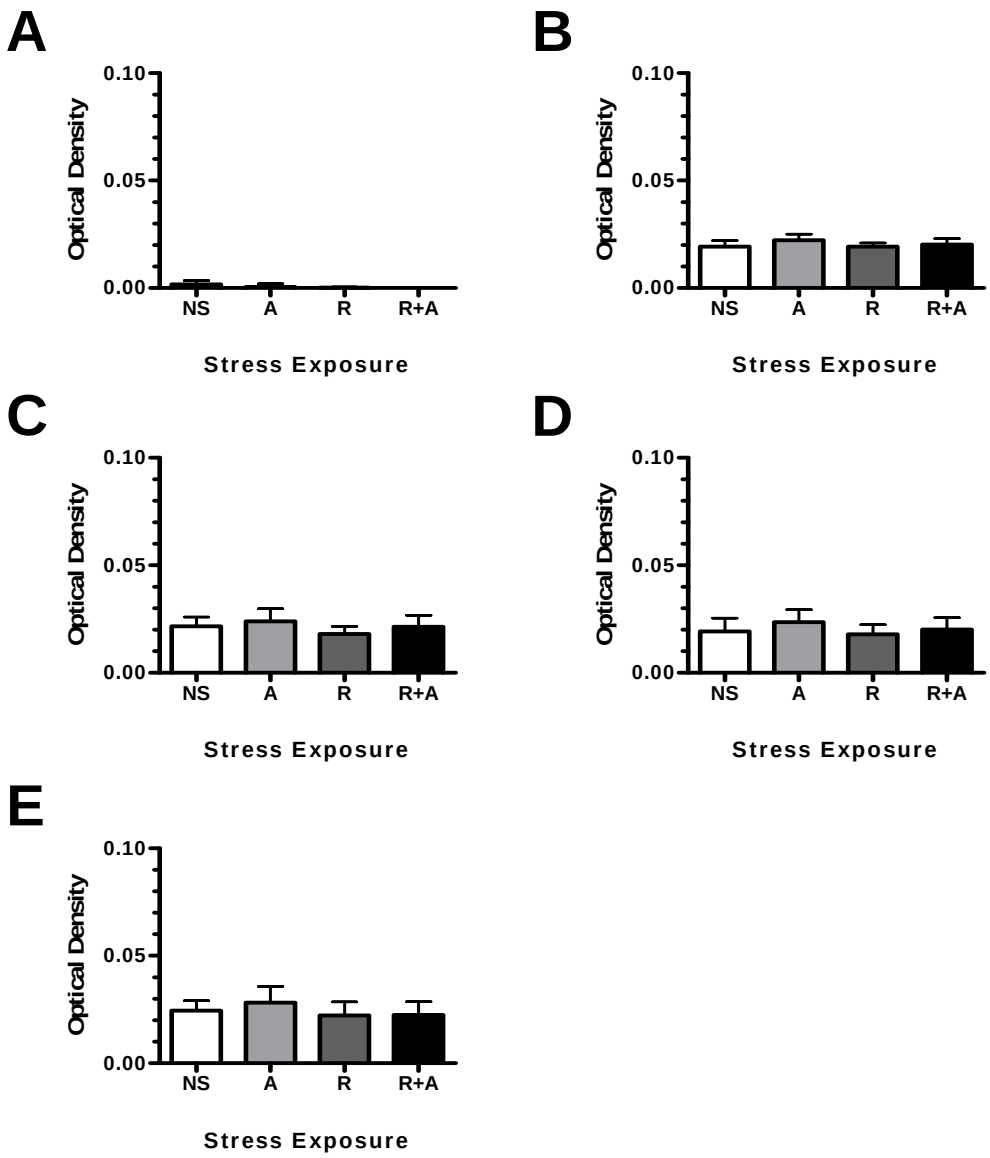

Figure 4-28. ^{125}I-[^{14}Tyr]-N/OFQ binding to the NOP receptor in the amygdala. There were no significant changes in binding following any stress exposure in (A) the central amygdala, (B) the medial amydala, (C) the basomedial amygdala, (D) the basolateral amygdala, or (E) the lateral amygdala. Values expressed are group means in optical density ± SEM (n = 4 rats per group). NS= no stress, A= acute stress, R= repeated stress, R+A= repeated plus acute.

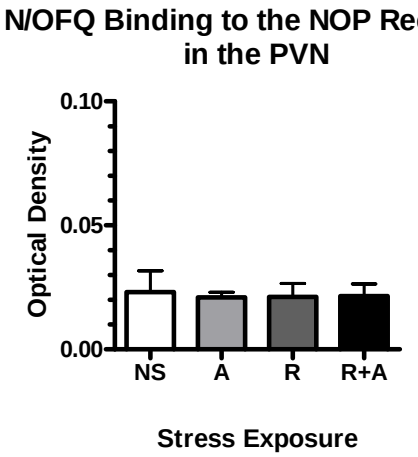

Figure 4-29. ^{125}I-[^{14}Tyr]-N/OFQ binding to the NOP receptor in the PVN. There were no significant changes in binding in the PVN following any stress exposure. Values expressed are group means in optical density ± SEM (n = 4 rats per group). NS= no stress, A= acute stress, R= repeated stress, R+A= repeated plus acute.

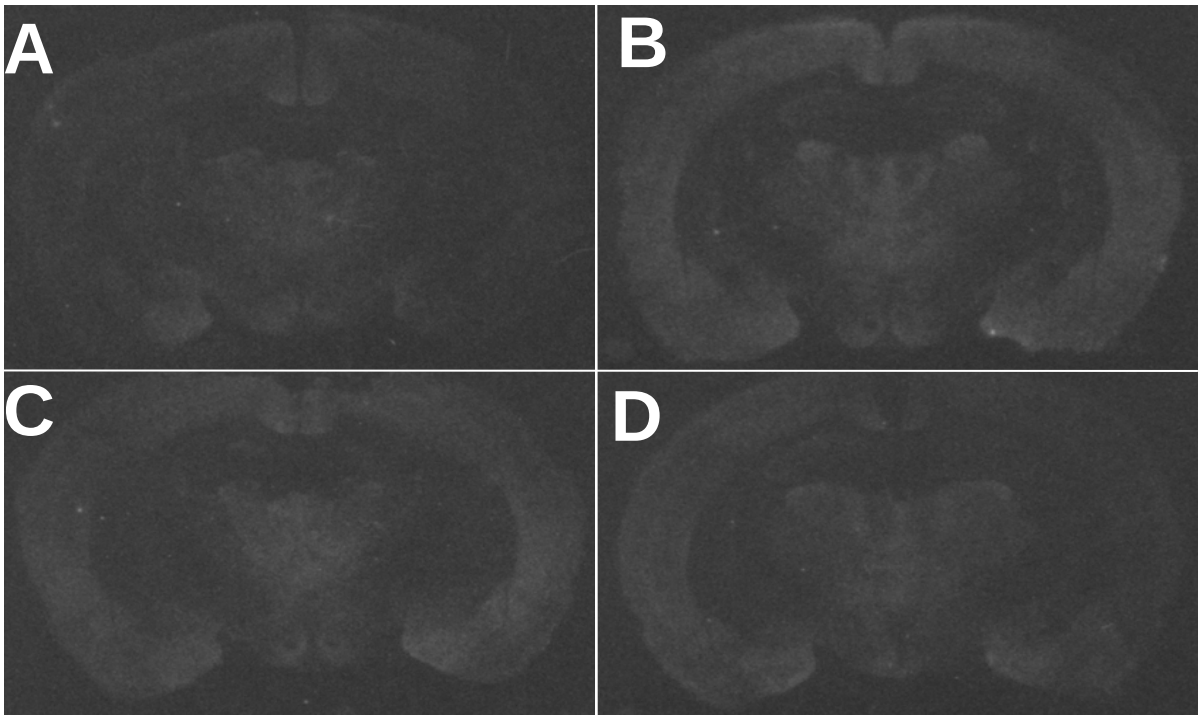

Figure 4-30. Representative x-ray images of brain sections showing 125I-[14Tyr]-N/OFQ binding to the NOP receptor in the amygdala and the PVN. Despite an increase in NOP receptor mRNA in these regions, none of the stressed groups displayed increases in binding. Shown are representative images from (A) the no stress groups, (B) the acutely stressed group, (C) the repeatedly stressed group, and (D) the repeatedly plus acutely stressed group.

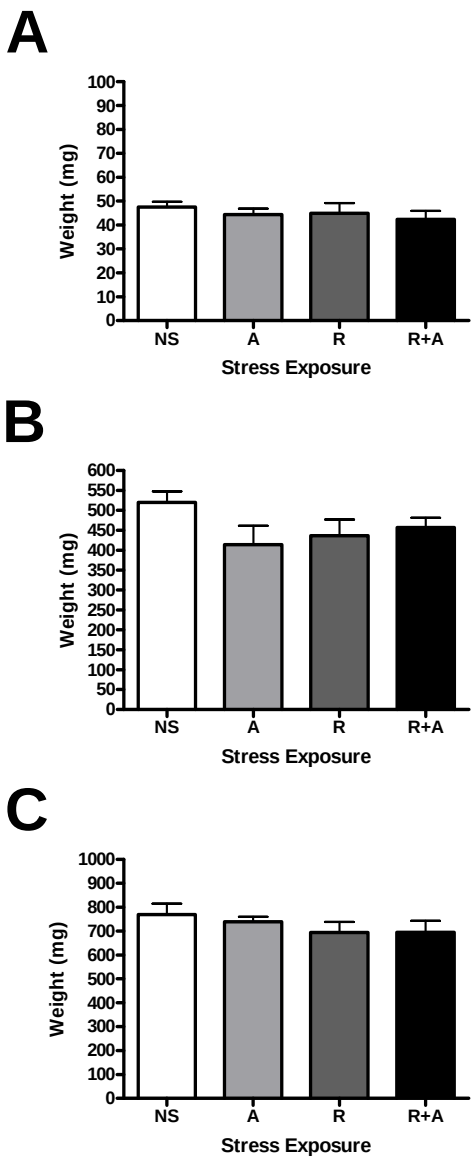

Figure 4-31. Gland masses after social defeat. (A) Adrenal gland masses, (B) thymus gland masses, and (C) spleen masses showed no significant differences between groups. Values expressed are group means ± SEM (n = 4 rats per group).

CHAPTER 5
GENERAL DISCUSSION

There is mounting evidence that N/OFQ is involved in the expression of anxiety-related behaviors and in the activation of the HPA axis. Injections of N/OFQ into the lateral ventricles and into limbic structures increase circulating ACTH and CORT (Devine et al., 2001; Nicholson et al., 2002; Fernandez et al., 2004; Legget et al., 2006; Green et al., in press; Misilmeri and Devine, in preparation) and alter anxiety-related behaviors (Jenck et al., 1997; Jenck et al., 2000; Gavioli et al., 2002; Fernandez et al., 2004; Kamei et al., 2004; Varty et al. 2005; Vitale et al., 2006; Gavioli et al., 2007; Green et al., in press). Additionally, exposure to acute stressors results in release of N/OFQ from forebrain neurons (Devine et al., 2003) and in increases in NOP receptor mRNA expression (present experiments). However, most of these effects are modest and are generally evident in mildly to moderately stressful environments. For example, Devine and colleagues (2001) found that when rats are more profoundly stressed (e.g., restraint), N/OFQ does not alter the HPA axis activation. This has also been observed recently with restraint and with unhandled rats (unpublished).

However, there is some evidence of changes in the regulation of the N/OFQ-NOP receptor system in response to severe chronic stress, particularly in the BNST and the PVN, two regions that are important in affective responses. The upregulation of NOP receptor mRNA in the BNST following chronic stress is consistent with the role of the BNST in long-term responses to ongoing stressors. Additionally, HPA axis regulation in the PVN by the N/OFQ-NOP system may be altered by chronic social stressor exposure.

It remains evident that N/OFQ is an important neuromodulator in a number of functions, including stress response, pain modulation (Meunier et al., 1995; Reinscheid et al., 1995; Tian et al., 1997), motor performance (Reinscheid et al., 1995; Devine et al., 1996), spatial learning

(Sandin et al., 1997; Sandin et al., 2004), and feeding (Pomonis et al., 1996; Nicholson et al., 2002). In general, it appears to be a fairly tightly regulated system that is generally resistant to dysregulation. Thus it seems important in the functioning of normal, nonpathological behavior. Its potential involvement in stress-induced psychopathology is suggested by stress-induced changes in NOP receptor mRNA (especially in the BNST and PVN of chronically-stressed rats). However, the lack of changes in receptor autoradiography indicates that this possibility will need to be studied in more detail.

LIST OF REFERENCES

Adamec RE, Blundell J, Collins A (2001) Neural plasticity and stress induced changes in defense in the rat. Neurosci Biobehav Rev 25:721-744.

Amsterdam JD, Maislin G, Winokur A, Berwish N, Kling M, Gold P (1988) The oCRH stimulation test before and after clinical recovery from depression. J Affect Disord 14: 213-222.

Andersen SL, Teicher MH (1999) Serotonin laterality in amygdala predicts performance in the elevated plus maze in rats. NeuroReport 10:3497-3500.

Arborelius L, Owens MJ, Plotsky PM, Nemeroff CB (1999) The role of corticotrophin-releasing factor in depression and anxiety disorders. J Endocrinol 160:1-12.

Aubry JM, Bartanusz V, Jezova D, Belin D, Kiss JZ (1999) Single stress induces long-lasting elevations in vasopressin mRNA levels in CRF hypophysiotrophic neurons, but repeated stress is required to modify AVP immunoreactivity. J Neuroendocinol 11:377-384.

Barnum CJ, Blandino P, Deak T (2007) Adaptation in the corticosterone and hyperthermic responses to stress following repeated stressor exposure. J Neuroendocinol 19:632-642.

Blanchard RJ, Nikulina JN, Sakai RR, McKittrick C, McEwan B, Blanchard DC (1998) Behavioral and endocrine change following chronic predatory stress. Physiol Behav 63: 561-569.

Board F, Persky H, Hambur DA (1956) Psychological stress and endocrine functions; blood levels of adrenocortical and thyroid hormones in acutely disturbed patients. Psychosom Med 18: 324-333.

Boudaba C, Szabo K, Tasker JG (1996) Physiological mapping of local inhibitory inputs to the hypothalamic paraventricular nucleus. J Neurosci 16: 7151-7160.

Boudaba C, Schrader LA, Tasker JG (1997) Physiological evidenc for local excitatory synaptic circuits in the rat hypothalamus. J Neurophysiol 77: 3396-3400.

Bryant HU, Bernton EW, Kenner JR, Holaday JW (1991) Role of adrenal cortical activation in the immunosuppressive effects of chronic morphine treatment. Endocrinology 128:3253-3258.

Bunzow JR, Saez C, Mortrud M, Bouvier C, Williams JT, Low M, Grandy DK (1994) Molecular cloning and tissue distribution of a putative member of the rat opioid receptor gene family that is not a mu, delta or kappa opioid receptor type. FEBS Lett 347: 284-288.

Butour JL, Moisand C, Mazarguil H, Mollereau C, Meunier JC (1997) Recognition and activation of the opioid receptor-like ORL 1 receptor by nociceptin, nociceptin analogs and opioids. Eur J Pharmacol 321:97-103.

Buwalda B, Felszeghy K, Horvath KM, Nyakas C, de Boer SF, Bohus B, Koolhaas JM (2001) Temporal and spatial dynamics of corticosteroid receptor down-regulation in rat brain following social defeat. Physiol Behav 72:349-354.

Calfa G, Volosin M, Molina VA (2006) Glucocorticoid receptors in lateral septum are involved in the modulation of the emotional sequelae induced by social defeat. Behav Brain Res 172: 324-332.

Canteras NS, Simerly RB, Sawnson LW (1995) Organization of projections from the medial nucleus of the amygdala: a PHAL study in the rat. J Comp Neurol 360: 213-245.

Chaouloff F, Durand M, Mormède P (1997) Anxiety- and activity-related effects of diazepam and chlordiazepoxide in the rat light/dark and dark/light tests. Behav Brain Res 85:27-35.

Chen Y, Fan Y, Liu J, Mestek A, Tian M, Kozak CA, Yu L (1994) Molecular cloning, tissue distribution and chromosomal localization of a novel member of the opioid receptor gene family. FEBS Lett 347:279-283.

Chung KKK, Martinez M, Herbert J (1999) Central serotonin depletion modulates the behavioural, endocrine and physiological responses to repeated social stress and subsequent c-fos expression in the brains of male rats. Neuroscience 92:613-625.

Coleman-Mesches K, McGaugh JL (1995a) Differential involvement of the right and left amygdalae in expression of memory for aversively motivated training. Brain Res 670:75-81.

Coleman-Mesches K, McGaugh JL (1995b) Muscimol injected into the right or left amygdaloid complex differentially affects retention performance following aversively motivated training. Brain Res 676:183-188.

Connor M, Vaughan CW, Chieng B, Christie MJ (1996a) Nociceptin receptor coupling to a potassium conductance in rat locus coeruleus neurones in vitro. Br J Pharmacol 119:1614-1618.

Connor M, Yeo A, Henderson G (1996b) The effect of nociceptin on Ca2+ channel current and intracellular Ca2+ in the SH-SY5Y human neuroblastoma cell line. Br J Pharmacol 118:205-207.

Costall B, Jones BJ, Kelly ME, Naylor RJ, Tomkins DM (1989) Exploration of mice in a black and white test box: Validation as a model of anxiety. Pharmacol Biochem Behav 32:777-785.

Covington HE, Miczek KA (2001) Repeated social-defeat stress, cocaine or morphine. Effects on behavioral sensitization and intravenous cocaine self-administration "binges". Psychopharmacol 158:388-398.

Crawley J, Goodwin FK (1980) Preliminary report of a simple animal behavior model for the anxiolytic effects of benzodiazepines. Pharmacol Biochem Behav 13:167-170.

Crawley JN (1981) Neuropharmacologic specificity of a simple animal model for the behavioral actions of benzodiazepines. Pharmacol Biochem Behav 15:695-699.

Crofford LJ (1998) The hypothalamic-pituitary-adrenal stress axis in fibromyalgia and chronic fatigue syndrome. Z Rheumatol 57: 67-71.

Cullinan WE, Herman JP, Watson SJ (1993) Ventral subicular interaction with the hypothalamic paraventricular nucleus: Evidence for a relay in the bed nucleus of stria terminalis. J Comp Neurol 332: 1-20.

Cullinan WE, Helmreich DL, Watson SJ (1996) Fos expression in forebrain afferents to the hypothalamic paraventricular nucleus following swim stress. J Comp Neurol 368: 88-99.

Danielson PB, Dores RM (1999) Review: Molecular evolution of the opioid/orphanin gene family. Gen Comp Endocrinol 113:169-186.

Dautzenberg FM, Wichmann U, Higelin J, Py-Lang G, Kratzeisen C, Malherbe P, Kilpatrick GJ, Jenck F (2001) Pharmacological characterization of the novel nonpeptide orphanin FQ/nociceptin receptor agonist Ro 64-6198: Rapid and reversible desensitization of the ORL1 receptor in vitro and lack of tolerance in vivo. J Pharmacol & Exp Ther 298: 812-819.

de Goeij DC, Kvetnansky R, Whitnall MH, Jezova D, Berkenbosch F, Tilders FJ (1991) Repeated stress-induced activation of corticotrophin-releasing factor neurons enhances vasopressin stores and colocalization with corticotrophin-releasing factor in the median eminence of rats. Neuroendocrinology 53:150-159.

de Goeij DCE, Dijkstra H, Tilders FJH (1992) Chronic psychological stress enhances vasopressin, but not corticotrophin-releasing factor, in the external zone of the medial eminence of male rats: Relationship to subordinate status. Endocrinology 131:847-853.

Deak T, Nguyen KT, Cotter CS, Fleshner M, Watkins LR, Maier SF, Spencer RL (1999) Long-term changes in mineralocorticoid and glucocorticoid receptor occupancy following exposure to an acute stressor. Brain Res 847: 211-220.

Devine DP, Taylor L, Reinscheid RK, Monsma FJ, Civelli O, Akil H (1996) Rats rapidly develop tolerance to the locomotor-inhibiting effects of the novel neuropeptide orphanin FQ. Neurochem Res 21:1387-1396.

Devine DP, Watson SJ, Akil H (2001) Nociceptin/orphanin FQ regulates neuroendocrine function of the limbic-hypothalamic-pituitary-adrenal axis. Neuroscience 102:541-553.

Devine DP, Hoversten MT, Ueda Y, Akil H (2003) Nociceptin/orphanin FQ content is decreased in forebrain neurones during acute stress. J Neuroendocrinol 15:69-74.

Diorio D, Viau V, Meaney MJ (1993) The role of the medial prefrontal cortex (cingulated gyrus) in the regulation of hypothalamic-pituitary-adrenal responses to stress. J Neurosci 13:3839-3847.

Dominguez-Gerpe L, Rey-Mendez M (2001) Alterations induced by chronic stress in lymphocyte subsets of blood and primary and secondary immune organs of mice. BMC Immunol 2:7.

Dong HW, Petrovich GD, Watts AG, Swanson LW (2001) Basic organization of projections from the oval and fusiform nuclei of the bed nuclei of the stria terminalis in adult rat brain. J Comp Neurol 436:430-455.

Dong HW, Swanson LW (2004) Organization of axonal projections from the anterolateral area of the bed nuclei of the stria terminalis. J Comp Neurol 468:277-298.

Dunn JD (1987a) Differential plasma corticosterone responses to electrical stimulation of the medial and lateral septal nuclei. Neuroendocrinology 46: 406-411.

Dunn JD (1987b) Plasma corticosterone responses to electrical stimulation of the bed nucleus of the stria terminalis. Brain Res 407:327-331.

Dunn JD, Whitener J (1986) Plasma corticosterone responses to electrical stimulation of the amygdaloid complex: cytoarchitectural specificity. Neuroendocrinology 42: 211;217.

Ebner K, Wotjak CT, Landgaf R, Engelmann M (2005) Neuroendocrine and behavioral response to social confrontation: residents versus intruders, active versus passive coping styles. Horm Behav 47:14-21.

Elenkov IJ, Wilder RL, Chrousos GP, Vizi ES (2000) The sympathetic nerve—An integrative interface between two supersystems: The brain and the immune system. Pharmacol Rev. 52: 595-638.

Emmert MH, Herman JP (1999) Differential forebrain c-fos mRNA induction by ether inhalation and novelty: evidence for distinctive stress pathways. Brain Res 845: 60-67.

Eskandari F, Sternberg EM (2002) Neural-Immune interactions in health and disease. Ann NY Acad Sci 966:20-27.

Feldman S, Conforti N, Itzik A, Weidenfeld J (1994) Differential effect of amygdaloid lesions of CRF-41, ACTH, and corticosterone responses followinf neural stimuli. Brain Res 658: 21-26.

Fernandez F, Misilmeri MA, Felger JC, Devine DP (2004) Nociceptin/orphanin FQ increases anxiety-related behavior and circulating levels of corticosterone during neophobic tests of anxiety. Neuropsychopharmacology 29:59-71.

Gavioli EC, Rae GA, Calo G, Guerrini R, DeLima TCM (2002) Central injections of nocistatin or its C-terminal hexapeptide exert anxiogenic-like effect on behaviour of mice in the plus-maze test. Brit J Pharmacol 136:764-772.

Gavioli EC, Marzola G, Guerrini R, Bertorelli R, Zucchini S, De Lima TCM, Rae GA, Salvadori S, Regoli D, Calo G (2003) Blockade of nociceptin/orphanin FQ-NOP receptor signalling produces antidepressant-like effects: Pharmacological and genetic evidences from the mouse forced swimming test. Eur J Neurosci 17:1987-1990.

Gavioli EC, V aughan CW, Marzola G, Guerrini R, Mitchell VA, Zucchini S, De Lima TCM, Rae GA, Salvadori S, Regoli D, Calo G (2004) Antidepressant-like effects of the nociceptin/orphanin FQ receptor antagonist UFP-101: New evidence from rats and mice. Naunyn-Schmiedeberg's Arch Pharmacol 369:547-553.

Gavioli EC, Rizzi A, Marzola G, Zucchini S, Regoli D, Calo G (2007). Altered anxiety-related behavior in nociceptin/orphanin FQ receptor gene knockout mice. Peptides 28: 1229-1239.

Gillies GE, Linton EA, Lowry PJ (1982) Corticotropin releasing activity of the new CRF is potentiated several times by vasopressin. Nature 299: 355-357.

Gilmer WS, Trivedi MH, Rush AJ, Wisniewski SR, Luther J, Howland RH, Yohanna D, Khan A, Alpert J (2005) Factors associated with chronic depressive episodes: A preliminary report from the STAR-D project. Acta Psychiatr Scand 112:425-433.

Gold PW, Loriaux DL, Roy A, Kling MA, Calabrese JR, Kellnor CH, Nieman LK, Post RM, Dicker D, Gallucci W, et al. (1986) Response to corticotrophin-releasing hormone in the hypercortisolism of depression and Cushing's disease: Pathophysiologic and diagnostic implications. N Eng J Med 314: 1329-1335.

Goldstein ML (1965) Effects of hippocampal, amygdala, hypothalamic and parietal lesions on a classically conditioned fear response. Psych Rep 16: 211-219.

Good AJ, Westbrook RF (1995) Effects of a microinjection of morphine into the amygdala on the acquisition and expression of conditioned fear and hypoalgesia in rats. Behav Neurosci 109:631-641.

Gray TS, Piechowski RA, Yracheta JM, Rittenhouse PA, Bethea CL, Ven de Kar LD (1993) Ibotenic acid lesions in the bed nucleus of the stria terminalis attenuate conditioned stress-induced increases in prolactin, ACTH and corticosterone. Neuroendocrinology 57: 517-524.

Green MK, Barbieri EV, Brown BD, Chen KW, Devine DP (in press) Roles of the bed nucleus of stria terminalis and of the amygdala in N/OFQ-mediated anxiety and HPA axis activation. Neuropeptides.

Handley SL, Mithani S (1984) Effects of alpha-adrenoceptor agonists and antagonists in a maze-exploration model of 'fear'-motivated behaviour. Naunyn Schmiedebergs Arch Pharmacol 327:1-5.

Hasegawa H, Saiki I (2002) Psychological stress augments tumor development through β-adrenergic activation in mice. Jpn J Cancer Res 93:729-735.

Hauger RL, Lorang M, Irwin M, Aguilera G (1990) CRF receptor regulation and sensitization of ACTH responses to acute ether stress during chronic intermittent immobilization stress. Brain Res 532: 34-40.

Heinrichs SC, Pich EM, Miczek KA, Britton KT, Koob GF (1992) Corticotropin-releasing factor antagonist reduces emotionality in socially defeated rats via direct neurotropic action. Brain Res 581:190-197.

Henke PG (1984) The bed nucleus of the stria terminalis and immobilization-stress unit activity, escape behaviour, and gastric pathology in rats. Behav Brain Res 11:35-45.

Herman JP, Schafer MK, Young EA, Thompson R, Douglass J, Akil H, Watson SJ (1989) Evidence for hippocampal regulation of neuroendocrine neurons of the hypothalamo-pituitary-adrenocortical axis. J Neurosci 9:3072-3082.

Herman JP, Cullinan WE, Watson SJ (1994) Involvement of the bed nucleus of the stria terminalis in tonic regulation of paraventricular hypothalamic CRH and AVP mRNA expression. J Neuroendocrinol 6: 433-442.

Herman JP, Cullinan WE (1997) Neurocircuitry of stress: Central control of the hypothalamo-pituitary-adrenocortical axis. Trends Neurosci 20:78-84.

Herman JP, Taskers JG, Ziegler DR, Cullinan WE (2002a) Local circuit regulation of paraventricular nucleus stress integration Glutamate–GABA connections. Pharmacol Biochem Behav 71:457-468.

Herman JP, Cullinan WE, Ziegler DR, Tasker JG (2002b) Role of the paraventricular nucleus microenvironment in stress integration. Eur J Neurosci 16:381-385.

Heuser I, Bissette G, Dettling M, Schweiger U, Gotthardt U, Schmider J, Lammers CH, Nemeroff CB, Holsboer F (1998) Cerebrospinal fluid concentrations of corticotrophin-releasing hormone, vasopressin, and somatostatin in depressed patients and healthy controls: Response to amitriptyline treatment. Depress Anxiety 8: 71-79.

Holsboer F, Gerken A, von Bardeleben U, Grimm W, Beyer H, Muller OA, Stalla GK (1986) Human corticotrophin-releasing hormone in depression—Correlation with thyrotropin secretion following thyrotropin-releasing hormone. Biol Psychiatry 21: 601-611.

Hughes RN (1972) Chlordiazepoxide modified exploration in rats. Psychopharmacologia 24:462-469.

Ixart G, Szafarczyk A, Belugou JL, Assenmacher I (1977) Temporal relationships between the diurnal rhythm of hypothalamic corticotrophin releasing factor, pituitary corticotrophin and plasma corticosterone in the rat. J Endocrinol 72:113-120.

Izquierdo A, Murray EA (2004) Combined unilateral lesions of the amygdala and orbital prefrontal cortex impair affective processing in rhesus monkeys. J Neurophysiol 91:2023-2039.

Jenck F, Moreau JL, Martin JR, Kilpatrick GJ, Reinscheid RK, Monsma FJ, Nothacker HP, Civelli O (1997) Orphanin FQ acts as an anxiolytic to attenuate behavioral responses to stress. PNAS 94:14854-14858.

Jenck F, Wichmann J, Dautzenberg FM, Moreau JL, Ouagazzal AM, Martin JR, Lundstrom K, Cesura AM, Poli SM, Roever S, Kolczewski S, Adam G, Kilpatrick G (2000) A synthetic agonist at the orphanin FQ/nociceptin receptor ORL1: anxiolytic profile in the rat. Proc Natl Acad Sci U S A 97:4938-4943.

Johnson JD, O'Connor KA, Deak T, Spencer RL, Watkins LS, Maier SF (2002) Prior stressor exposure primes the HPA axis. Psychoneuronendocrinology 27: 353-365.

Jordanova V, Stewart R, Goldberg D, Bebbington E, Brugha T, Singleton N, Lindesay JEB, Jenkins R, Prince M, Meltzer H (2007) Age variation in life events and their relationship with common mental disorders in a national survey population. Soc Psychiatry Psychiatr Epidemiol 42:611-616.

Juruena MF, Cleare AJ, Pariante CM (2004) The hypothalamic pituitary adrenal axis, glucocorticoid receptor function and relevance to depression. Rev Bras Pisquiatr 26:189-201.

Kamei J, Matsunawa Y, Miyata S, Tanaka S, Saitoh A (2004) Effects of nociceptin on the exploratory behavior of mice in the hole-board test. Eur J Pharmacol 489: 77-87.

Kvetnansy R, Bodnar I, Shahar T, Uhereczky G, Krizanova O, Mravec B (2006) Effect of lesions of A5 and A7 brainstem noradrenergic areas or transaction of brainstem pathways on sympathoadrenal activity in rats during immobilization stress. Neurochem Res 31: 267-275.

Kwak SP, Morano MI, Young EA, Watson SJ, Akil H (1993) Diurnal CRH mRNA rhythm in the hypothalamus: decreased expression in the evening is not dependent on endogenous glucocorticoids. Neuroendocrinology 57:96-105.

LaBar KS, LeDoux JE (1996) Partial disruption of fear conditioning in rats with unilateral amygdala damage: Correspondence with unilateral temporal lobectomy in humans. Behav Neurosci 110:991-997.

Labeur MS, Arzt E, Wiegers GJ, Holsboer F, Reul JMHM (1995) Lon-term intracerebroventricular corticotrophin-releasing hormone administration induces distinct changes in rat splenocyte activation and cytokine expression. Endocrinology 136: 2678-2688.

Lachowicz JE, Shen Y, Monsma FJ, Sibley DR (1995) Molecular cloning of a novel G protein-coupled receptor related to the opiate receptor family. J Neurochem 64:34-40.

LeDoux JE, Iwata J, Cicchetti P, Reis DJ (1988) Different projections of the central amygdaloid nucleus mediate autonomic and behavioral correlates of conditioned fear. J Neurosci 8: 2517-2529.

Lee Y, Davis M (1997) Role of the hippocampus, the bed nucleus of the stria terminalis, and the amygdala in the excitatory effect of corticotrophin-releasing hormone on the acoustic startle reflex. J Neurosci 17: 6434-6446.

Leggett JD, Harbuz MS, Jessop DS, Fulford AJ (2006) The nociceptin receptor antagonist [Nphe(1),Arg(14),Lys(15)]nociceptin/orphanin FQ-NH(2) blocks the stimulatory effects of nociceptin/orphanin FQ on the HPA axis in rats. Neuroscience 141:2051-2057.

Linthorst ACE, Flachskamm C, Hopkins SJ, Hoadley ME, Labeur MS, Holsboer F, Reul JMHM (1997) Long-term intracerebroventricular infusion of corticotrophin-releasing hormone alters neuroendocrine, neurochemical, autonomic, behavioral, and cytokine responses to a systemic inflammatory challenge. J Neurosci 17: 4448-4460.

Lyons DM, Wang OJ, Lindley SE, Levine S, Kalin NH, Schatzberg AF (1999) Separation induced changes in squirrel monkey hypothalamic-pituitary-adrenal physiology resemble aspects of hypercortisolism in humans. Psychoneuroendocrinology 24: 131-142.

Manji HK, Drevets WC, Charney DS (2001) The cellular neurobiology of depression. Nature Med 7: 541-547.

Marin MT, Cruz FC, Planeta CS (2007) Chronic restraint or variable stresses differently affect the behavior, corticosterone secretion and body weight in rats. Physiol & Behav 90:29-35.

Martin DS, Haywood JR (1992) Sympathetic nervous system activation by glutamate injections into the paraventricular nucleus. Brain Res 577: 261-267.

Martinez M, Phillips PJ, Herbert J (1998) Adaptation in patterns of *c-fos* expression in the brain associated with exposure to either single or repeated social stress in male rats. Eur J Neurosci 10:20-33.

Menard J, Treit D (1996) Lateral and medial septal lesions reduce anxiety in the plus-maze and probe-burying tests. Physiol & Behav 60: 845-853.

Meunier JC, Mollereau C, Toll L, Suaudeau C, Moisand C, Alvinerie P, Butour JL, Guillemot JC, Ferrara P, Monsarrat B (1995) Isolation and structure of the endogenous agonist of opioid receptor-like ORL1 receptor. Nature 377:532-535.

Misilmeri MA, Devine DP (in preparation) N/OFQ activates the hypothalamic-pituitary adrenal axis after administration into limbic brain sites.

Moghaddam B, Bunney BS (1989) Ionic composition of microdialysis perfusing solution alters the pharmacological responsiveness and basal outflow of striatal dopamine. J Neurochem 53:652-654.

Mollereau C, Parmentier M, Mailleux P, Butour JL, Moisand C, Chalon P, Caput D, Vassart G, Meunier JC (1994) ORL1, a novel member of the opioid receptor family. Cloning, functional expression and localization. FEBS Lett 341:33-38.

Nagaraja TN, Patel P, Gorski M, Gorevic PD (2005) In normal rat, intraventricularly administered insulin-like growth factor-1 is rapidly cleared from CSF with limited distribution into brain. Cerebrospinal Fluid Res 2: 5.

Neal CR, Mansour A, Reinscheid R, Nothacker HP, Civelli O, Watson SJ (1999a) Localization of orphanin FQ (nociceptin) peptide and messenger RNA in the central nervous system of the rat. J Comp Neurol 406:503-547.

Neal CR, Mansour A, Reinscheid R, Nothacker HP, Civelli O, Akil H, Watson SJ (1999b) Opioid receptor-like (ORL1) receptor distribution in the rat central nervous system: Comparison of ORL1 receptor mRNA expression with 125I-[14Tyr]-Orphanin FQ binding. J Comp Neurol 412:563-605.

Nemeroff CB, Widerlov E, Bissette G, Walleus H, Karlsson I, Eklund K, Kilts CD, Loosen PT, Vale W (1984) Elevated concentrations of CSF corticotropin-releasing factor-like immunoreactivity in depressed patients. Science 226:1342-1344.

Nemeroff CB, Bissette G, Akil H, Fink M (1991) Neuropeptide concentrations in the cerebrospinal fluid of depressed patients treated with electrovonvulsive therapy. Corticotrophin-releasing factor, beta-endorphin and somatostatin. Br J Psychiatry 158: 59-63.

Nicholson JR, Akil H, Watson SJ (2002) Orphanin FQ-induced hyperphagia is mediated by corticosterone and central glucocorticoid receptors. Neuroscience 115:637-643.

Nikulina EM, Covington HE, Ganschow L, Hammer RP, Miczek KA (2004) Long-term behavioral and neuronal cross-sensitization to amphetamine induced by repeated brief social defeat stress: fos in the ventral tegmental area and amygdala. Neuroscience 123:857-865.

Nothacker HP, Reinscheid RK, Mansour A, Henningsen RA, Ardati A, Monsma FJ, Watson SJ, Civelli O (1996) Primary structure and tissue distribution of the orphanin FQ precursor. PNAS 93:8677-8682.

Onaivi ES, Martin BR (1989) Neuropharmacological and physiological validation of a computer-controlled two-compartment black and white box for the assessment of anxiety. Prog Neuropsychopharmacol Biol Psychiatry 13:963-976.

Ostrander MM, Ulrich-Lai YM, Choi DC, Richtand NM, Herman JP (2006) Hypoactivity of the hypothalamo-pituitary-adrenocortical axis during recovery from chronic variable stress. Endocrinology 147:2008-2017.

Paxinos G, Watson C (1998) *The Rat Brain in Stereotaxic Coordinates,* 4th Ed. CD-ROM: Hulasz, P.

Pellow S, Chopin P, File SE, Briley M (1985) Validation of open: closed arm entries in an elevated plus-maze as a measure of anxiety in the rat. J Neurosci Methods 14:149-167.

Pellow S, File SE (1986) Anxiolytic and anxiogenic drug effects on exploratory activity in an elevated plus-maze: A novel test of anxiety in the rat. Pharmacol Biochem Behav 24:525-529.

Peper M, Karcher S, Wohlfarth R, Reinshagen G, LeDoux JE (2001) Aversive learning in patients with unilateral lesions of the amygdala and hippocampus. Biol Psychol 58:1-23.

Pomonis JD, Billington CJ, Levine AS (1996) Orphanin FQ, agonist of orphan opioid receptor ORL1, stimulates feeding in rats. Neuroreport 20:369-371.

Prewitt CMF, Herman JP (1998) Anatomical interactions between the central amygdaloid nucleus and the hypothalamic paraventricular nucleus of the rat: a dual tract-tracing analysis. J Chem Neuroanat 15:173-185.

Redrobe JP, Calo G, Regoli D, Quirion R (2002) Nociceptin receptor antagonists display antidepressant-like properties in the mouse forced swim test. Naunyn-Schmeideberg's Arch Pharmacol 365:164-167.

Reinscheid RK, Nothacker HP, Bourson A, Ardati A, Henningsen RA, Bunzow JR, Grandy DK, Langen H, Monsma FJ, Civelli O (1995) Orphanin FQ: a neuropeptide that activates an opioidlike G protein-coupled receptor. Science 270:792-794.

Reinscheid RK, Ardati A, Monsma FJ, Civelli O (1996) Structure-activity relationship studies on the novel neuropeptide orphanin FQ. J Biol Chem 271:14163-14168.

Renner KJ, Smits AW, Quadagno DM, Hough JC (1984) Suppression of sexual behavior and localization of [3H] puromycin after intracranial injection in the rat. Physiol Behav 33: 411-414.

Risold PY, Swanson LW (1997) Connections of the rat lateral septal complex. Brain Res Rev 24L 115-195.

Rogan MT, Staubli UV, LeDoux JE (1997) Fear conditioning induces associative long-term potentiation in the amygdala. Nature 391:604-607.

Rubin RT, Phillips JJ, Sadow TF, McCracken JT (1995) Adrenal gland volume in major depression. Increase during the depressive episode and decrease with successful treatment. Arch Gen Psychiatry 52: 213-218.

Rygula R, Abumaria N, Flugge G, Fuchs E, Ruther E, Havemann-Reinceke U (2005) Anhedonia and motivational deficits in rats: Impact of chronic social stress. Behav Brain Res 162:127-134.

Sandin J, Georgieva J, Schott PA, Ogren SO, Terenius L (1997) Nociceptin/orphanin FQ microinjected into hippocampus impairs spatial learning in rats. Eur J Neurosci 9:194-197.

Sandin J, Ogren SO, Terenius L (2004) Nociceptin/orphanin FQ modulates spatial learning via ORL-1 receptors in the dorsal hippocampus of the rat. Brain Res 997:222-233.

Sawchenko PE, Swanson LW (1983) The organization of forebrain afferents to the paraventricular and supraoptic nuclei of the rat. J Comp Neurol 218: 121-144.

Scicli AP, Petrovich GD, Swanson LW, Thompson RF (2004) Contextual fear conditioning is associated with lateralized expression of the immediate early gene c-fos in the central and basolateral amygdalar nuclei. Behav Neurosci 118:5-14.

Selye H (1936) Thymus and adrenals in the response of the organism to injuries and intoxications. Brit J Exp Path 17:234-248.

Shimohigashi Y, Hatano R, Fujita T, Nakashima R, Nose T, Sujaku T, Saigo A, Shinjo K, Nagahisa A (1996) Sensitivity of opioid receptor-like receptor ORL1 for chemical modification on nociceptin, a naturally occurring nociceptive peptide. J Biol Chem 271: 23642-23645.

Simon P, Dupuis R, Costentin J (1994) Thigmotaxis as an index of anxiety in mice. Influence of dopaminergic transmissions. Behav Brain Res 61:59-64.

Simpkiss JL, Devine DP (2003) Responses of the HPA axis after chronic variable stress: Effects of novel and familiar stressors. Neuroendocrinol Lett 24:75-81.

Spampinato S, Baiula M (2006). Agonist-regulated endocytosis and desensitization of the human nociceptin receptor. NeuroReport, 17: 173-177.

Stefanski R, Palejko W, Kostowski W, Plaznik A (1992) The comparison of benzodiazepine derivatives and serotonergic agonists and antagonists in two models of anxiety. Neuropharmacology 31:1251-1258.

Stone KL, Naccarato AM, Devine DP (in preparation) Rats exhibit behavioral despair and hormonal alterations following social defeat stress: Implications for stress-induced psychopathology.

Tian JH, Xu W, Fang Y, Mogil JS, Grisel JE, Grandy DK, Han JS (1997) Bidirectional modulatory effect of orphanin FQ on morphine-induced analgesia: antagonism in brain and potentiation in spinal cord of the rat. Brit J Pharmacol 120:676-80.

Van de Kar LD, Piechowski RA, Rittenhouse PA, Gray TS (1991) Amygdaloid lesions: Differential effect on conditioned stress and immobilization-induced increases in corticosterone and rennin secretion. Neurendocrinology 54: 89-95.

Varty GB, Hyde LA, Hodgson RA, Lu SX, McCool MF, Kazdoba TM, Del Vecchio RA, Guthrie DH, Pond AJ, Grzelak ME, Xu X, Korfmacher WA, Tulshian D, Parker EM, Higgins GA (2005) Characterization of the nociceptin receptor (ORL-1) agonist, Ro64-6198, in tests of anxiety across multiple species. Psychopharmacology 182:132-143.

Vaughan CW, Christie MJ (1996) Increase by the ORL1 receptor (opioid receptor-like1) ligand, nociceptin, of inwardly rectifying K conductance in dorsal raphe nucleus neurons. Brit J Pharmacol 117:1609-1611.

Vaughan CW, Ingram SL, Christie MJ (1997) Actions of the ORL1 receptor ligand nociceptin on membrane properties of rat periaqueductal gray neurons in vitro. J Neurosci 17:996-1003.

Vitale G, Arletti R, Ruggieri V, Cifani C, Massi M (2006) Anxiolytic-like effects of nociceptin/orphanin FQ in the elevated plus maze and in the conditioned defensive burying test in rats. Peptides 27:2193-2200.

Vyas A, Bernal S, Chattarji S (2003) Effects of chronic stress on dendritic arborization in the central and extended amygdala. Brain Res 965: 290-294.

Walker DL, Davis M (1997) Double dissociation between the involvement of the bed nucleus of the stria terminalis and the central nucleus of the amygdala in startle increases produced by conditioned versus unconditioned fear. J Neurosci 17:9375-9383.

Walker DL, Toufexis DJ, Davis M (2003) Role of the bed nucleus of the stria terminalis versus the amygdala in fear, stress, and anxiety. Eur J Pharmacol 463:199-216.

Wang JB, Johnson PS, Imai Y, Persico AM, Ozenberger BA, Eppler CM, Uhl GR (1994) cDNA cloning of an orphan opiate receptor gene family member and its splice variant. FEBS Lett 348:75-79.

Watzl B, Lopez M, Shahbazian M, Chen G, Colombo LL, Huang D, Way D, Watson RR (1993) Diet and ethanol modulate immune responses in young C57BL/6 mice. Alcohol Clin Exp Res 17:623-630.

Weisse CS (1992) Depression and immunocompetence: A review of the literature. Psychol Bull 111: 475-489.

Whitnall MH (1993) Regulation of the hypothalamic corticotropin-releasing hormone neurosecretory system. Prog Neurobiol 40:573-629.

Wick MJ, Minnerath SR, Lin X, Elde R, Law PY, Loh HH (1994) Isolation of a novel cDNA encoding a putative membrane receptor with high homology to the cloned mu, delta, and kappa opioid receptors. Brain Res Mol Brain Res 27:37-44.

Wisniewski SR, Rush AJ, Bryan C, Shelton R, Trivedi MH, Marcus S, Husain MM, Hollon SD, Fava M (2005) Comparison of quality of life measures in a depression population. J Nerv Ment Dis 195:219-225.

Wommack JC, Delville Y (2003) Repeated social stress and the development of agonistic behavior: Individual differences in coping responses in male golden hamsters. Physiol Behav 80:303-308.

Ziegler DR, Herman JP (2000) Local integration of glutamate signaling in the hypothalamic paraventicular region: regulation of glucocorticoid stress responses. Endocrinology 141: 4801-4804.

BIOGRAPHICAL SKETCH

Megan K. Green received her Associate of Arts in August 1998 at Okaloosa Walton College. In May 2000, she received a dual Bachelor of Arts in psychology and anthropology from the University of West Florida. Megan began her graduate studies in experimental psychology at the University of West Florida in August 2001. She continued graduate studies at the University of Florida in August 2003, where she completed a Master of Science degree in behavioral neuroscience through the psychology department in December 2005. Currently Megan is employed as a post-doctoral researcher at St. Charles Pharmaceuticals in Alachua, FL.