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ABSTRACT

THE EFFECTS OF ORPHANIN FQ/NOCICEPTIN (OFQ/N) DELETION ON THE HYPOTHALAMIC-PITUITARY-ADRENAL (HPA) AXIS ACTIVITY AND PROLACTIN RESPONSE TO STRESS

Kelly Zullig

These studies were conducted to examine the role of Orphanin FQ/Nociceptin (OFQ/N) in regulating hypothalamic pituitary adrenal (HPA) axis activity and modulating prolactin (PRL) secretion in response to stress. First, studies were conducted to characterize knockout mice under resting conditions and explore possible compensatory mechanisms that may have occurred in OFQ/N knockout animals. There were no differences between knockout and wild-type animals in circulating corticosterone (CORT) or PRL levels, in prolactin receptors (PRL-R) in the choroid plexus, or hypothalamic levels of nociceptin (NOP) or delta receptors (DOP). Knockout males had significantly less mu receptor (MOR) mRNA but this did not translate into a protein difference. Knockout males and females had significantly less kappa receptor protein (KOP) than wild-type animals. OFQ/N injection still produced a significant increase in PRL secretion in knockout animals, indicating that they have functional NOP. There were no genotype differences in HPA axis activation, as indicated by increased CORT levels, or in the PRL secretory response to restraint, orbital shaker or platform shaker stress. This indicates that OFQ/N is not essential for the neuroendocrine response to stress. Further, in males OFQ/N did not affect the animals' ability to habituate to platform shaker stress, signifying that OFQ/N is also not crucial for adaptation to a homotypic stressor. There were clear differences between CORT and PRL in the magnitude and time course of response to the stressors applied. Stressor dependent differences were also seen within the individual CORT and PRL responses. This was not surprising, given the complexity of the stress response and the numerous pathways involved. The magnitude, time course, and even the experimental environment of stressors must be carefully considered when interpreting results of studies involving stress paradigms.

THE EFFECTS OF ORPHANIN FQ/NOCICEPTIN (OFQ/N) DELETION ON THE HYPOTHALAMIC-PITUITARY-ADRENAL (HPA) AXIS ACTIVITY AND PROLACTIN RESPONSE TO STRESS

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DEDICATION

This dissertation is dedicated to my parents Wayne and Linda Prince and to my husband Keith, who always knew that I could do it, even when I sometimes did not.

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Background and Significance:

Prolactin (PRL) regulation:

The primary source of endogenous PRL is secretion by the lactotroph cells in the anterior pituitary gland. Although the lactotrophs have a high level of spontaneous secretory activity, PRL secretion is held under tonic inhibitory control by factors secreted by hypothalamic neurons. By far, the major inhibitory factor is dopamine, which is synthesized in and secreted from hypothalamic tuberoinfundibular dopaminergic (TIDA) neurons (Freeman et al., 2000; Ben-Jonathan and Hnasko, 2001). PRL also affects its own secretion through a negative feedback loop. High levels of PRL in the circulation will stimulate the TIDA neurons resulting in increased dopamine release which inhibits PRL secretion and returns circulating levels to basal values (Freeman et al., 2000; Ben-Jonathan and Hnasko, 2001). A clear gender difference in PRL regulation is evident, due to the effects of estrogen, which influences PRL synthesis and secretion by acting on the anterior pituitary gland and the hypothalamic TIDA neurons (Lamberts and Macleod, 1990; Torner and Neumann, 2002). For example, females have higher circulating PRL levels than males (Yamaji et al., 1976), and have increased expression of PRL in the pituitary (González-Parra et al., 1996). In addition to being affected by estrogen levels, the activity of the TIDA neurons is regulated by other neural factors including the endogenous opioid peptides (EOP). EOP suppress TIDA neuronal activity, which results in increased PRL secretion (Freeman et al., 2000; Ben-Jonathan and Hnasko, 2001). Administration of OFQ/N, the most recently discovered opiate peptide (Civelli et al., 1998), also produced an increase in PRL secretion in male and female rats (Bryant et al., 1998) and is involved in PRL regulation during lactation (Chesterfield et al., 2006).

Endogenous opioid peptides (EOP):

EOP are widely distributed throughout the central nervous system (CNS) and are involved in many biological actions. There are three classic opiate peptide families, the enkephalins (ENK), endorphins (END) and dynorphins (DYN). These opiates act through three distinct G-protein coupled receptors, the delta (DOP), mu (MOP) and kappa (KOP) opiate receptors. While there is cross reactivity among these opiates and

their receptors, the preferential ligand-receptor combinations are: ENK- DOP or MOP, END- MOP or DOP and DYN- KOP (Reisne, 1995).

OFQ/N shares high sequence homology with the classic EOP, especially dynorphin (Meunier et al., 1995; Reinscheid et al., 1995), but it has its own distinct receptor, the nociceptin (NOP) receptor (Reinscheid et al., 2000). OFQ/N binds to NOP with high affinity, and does not bind to the other classic opiate receptors. Additionally, the classic EOP do not bind to NOP (Mogil and Pasternak, 2001). NOP is found in high concentrations in the hypothalamus and is colocalized in a number of brain regions with other opioid receptors and peptides (Neal et al., 1999b). This, combined with OFQ/N's localization to areas such as the median eminence of the hypothalamus (Neal et al., 1999a), indicates that OFQ/N is involved in regulating neuroendocrine processes.

Stress:

A stressor is defined as a stimulus that poses a real or perceived threat to an individual's homeostasis (Drolet et al., 2001; Herman et al., 2003). The stress response involves a very complex set of reactions, including activation of the sympathetic nervous system, which produces the well-characterized "fight or flight" response. In addition, other neuroendocrine responses are essential for maintaining homeostasis and ensuring survival of the individual during stress (Herman et al., 2003), including activation of the HPA axis (Figure 1). The HPA axis begins with stimulation of corticotrophin releasing factor (CRF) neurons in the paraventricular nucleus (PVN) of the hypothalamus, which release CRF. CRF stimulates corticotrophs in the anterior pituitary gland to synthesize and secrete adrenocorticotropic hormone (ACTH). ACTH enters the circulation and stimulates the adrenal cortex to secrete glucocorticoids, e.g. CORT. The glucocorticoids, such as CORT stimulate metabolism of fat in adipose tissue and glycogen in skeletal muscle and promote glucoenogenesis in the liver, providing increased blood glucose levels (Herman and Cullinan, 1997). The magnitude of the HPA axis response varies depending upon the type of stress experienced by the subject, and CORT levels tend to differ following stressors of various potencies (Djordjević et al., 2003). In fact, Herman et al. (2003) recently classified stressors into one of two categories, *real* and *predicted*. A real stressor is one that poses an actual physical threat to the individual (such as

hemorrhage), and stimulates direct pathways from the brainstem to the PVN, which is the central integration point of inputs that influence the HPA axis (Figure 2). A predicted stressor is one that is a perceived threat (such as a novel environment), which requires processing through higher brain centers. Predicted stressors trigger indirect pathways to the PVN, e.g. through the limbic system (Herman et al., 2003).

Activation of the HPA axis is essential for survival of stress, and an acute, novel stress always activates this axis. In cases of chronic stress, however, negative feedback mechanisms exist that inhibit the activity of the HPA axis, and help to prevent the deleterious effects of stress (Englemann et al., 2004). When HPA axis activation is no longer sustained during chronic stress, habituation or adaptation has occurred (Herman et al., 2003; Simpkiss and Devine, 2003). Importantly, it is chronic exposure to the same (homotypic) stress that leads to adaptation (Bhatnagar et al., 2002).

PRL is also implicated in the stress response; stress causes an increase in circulating PRL levels. This is due, at least partially, to activation of EOP neurons that act to suppress TIDA neuronal activity (Freeman et al., 2000; Ben-Jonathan and Hnasko, 2001) Although PRL is a peptide, circulating PRL enters the CNS via specific receptors in the choroid plexus in the brain (Walsh et al., 1987; Smith et al., 2004). In fact, increased circulating PRL levels cause upregulation of PRL-Rs in the choroid plexus (Fujikawa et al., 1995), which allows for a greater amount of PRL to be transported into the brain (Mangurian et al., 1992). As a result, PRL-R are also upregulated in the hypothalamus (Fujikawa et al., 2004). PRL also stimulates the increase of CRF mRNA in the hypothalamus (Fujikawa et al., 2004), which provides evidence that PRL is stimulatory to the HPA axis. Additionally, levels of brain opiates that are increased during stress (Valentino and Van Bockstaele, 2001) are stimulatory, at least initially, to the HPA axis (Pechnick, 1993). Finally, the glucocorticoids, released from the adrenal cortex due to stimulation by ACTH, increase blood glucose levels which provide energy resources necessary for survival (Charmandari et al., 2005).

The mechanisms responsible for PRL release and the role of PRL in mediating the stress response are not clear. Antagonizing CRH receptors (CRH-R) significantly attenuated the PRL response to the elevated plus-maze trials (Keck et al., 2003), which would indicate that the HPA is also stimulatory to PRL secretion. PRL has been shown

to have protective effects in response to stress, such as prevention of hypocalcemia and ulcerogenesis (Fujikawa et al., 1995; Fujikawa et al., 2004) It has been suggested that PRL has an immunoregulatory function that helps protect an individual from the deleterious consequences of stress, and it may help maintain homeostatic balance during periods of stress (Freeman et al., 2000).

OFQ/N has been shown to elicit anxiolytic effects, or a decreased response to stress (Jenck et al., 1997; Griebel et al., 1999; Le Cudennec et al., 2002). OFQ/N knockout mice also display behaviors of increased anxiety (Reinscheid and Civelli, 2002), have elevated glucocorticoid levels, and show impaired adaptation to stress when compared to wild type mice (Köster et al., 1999). Conflicting evidence, however, suggests that OFQ/N acts as an anxiogenic; intracerebroventricular (ICV) injections of OFQ/N in rats increased CORT levels of animals at rest (Devine et al., 2001) and caused increased anxiety behaviors (Fernandez et al., 2004). Although these findings support a role for OFQ/N in regulating the stress response, its specific role and its mechanism of action remains unknown.

The purpose of these studies was to investigate the possible role of OFQ/N in regulating the activity of the HPA axis, and as a modulator of PRL secretion in response to stress. Understanding these processes is crucial to our uncovering the mechanisms involved in the stress response and will ultimately lead to a better understanding and treatment of stress-related disorders.

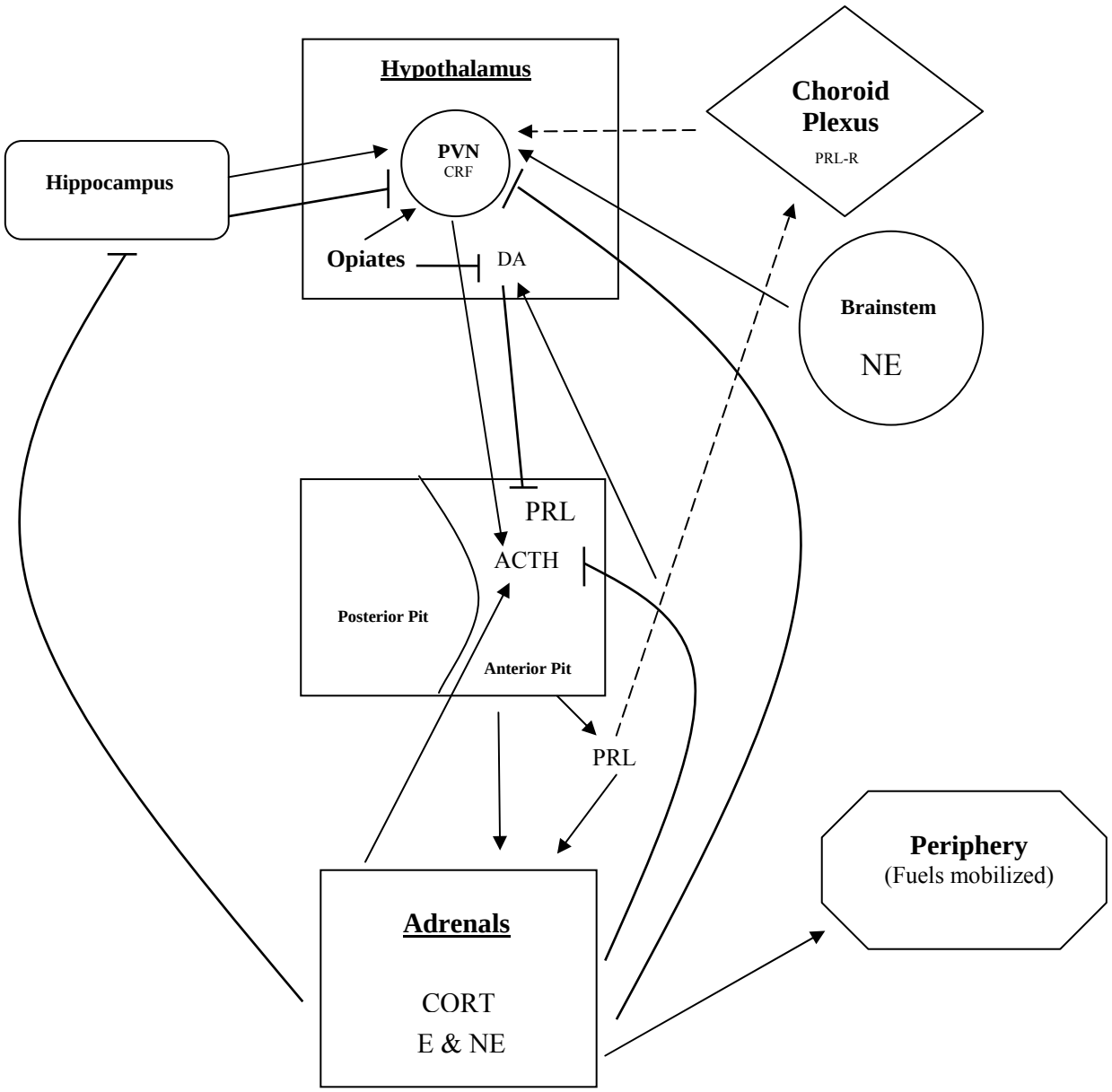

Figure 1. Major components and pathways of the neuroendocrine stress response

The middle three boxes of the diagram represent the structures that are directly part of the hypothalamic-pituitary-adrenal (HPA) axis. The paraventricular nucleus (PVN) of the hypothalamus releases corticotropin releasing factor (CRF), which stimulates the anterior pituitary gland to release adrenocorticotropic hormone (ACTH). ACTH travels through the general circulation and stimulates the adrenal glands to release glucocorticoids, such as corticosterone (CORT). CORT circulates in the periphery, acting at target tissues to mobilize stored fuels and increase blood glucose. Negative feedback effects on the HPA axis are exerted by CORT acting at both the hypothalamic and pituitary levels, as well as higher brain areas. Direct pathways, e.g. through the brainstem and indirect pathways, e.g. from the hippocampus and other limbic structures either stimulate or inhibit the HPA

axis. Epinephrine (E) and Norepinephrine (NE) released from the adrenals or the brainstem are stimulatory to the HPA axis. Opiates are stimulatory to the HPA axis and also inhibit dopamine (DA), which removes the inhibitory control over prolactin (PRL) secretion. PRL released from the anterior pituitary gland travels in the circulation and sensitizes the adrenal glands to ACTH. Additionally, PRL is transported into the brain through prolactin receptors (PRL-R) in the choroid plexus, where it can then act as a stimulatory agent to the HPA axis. Lines with an arrowhead signify stimulatory pathways. Lines ending in a T signify inhibitory pathways. Dotted lines indicate the pathway of PRL that is transported into the brain.

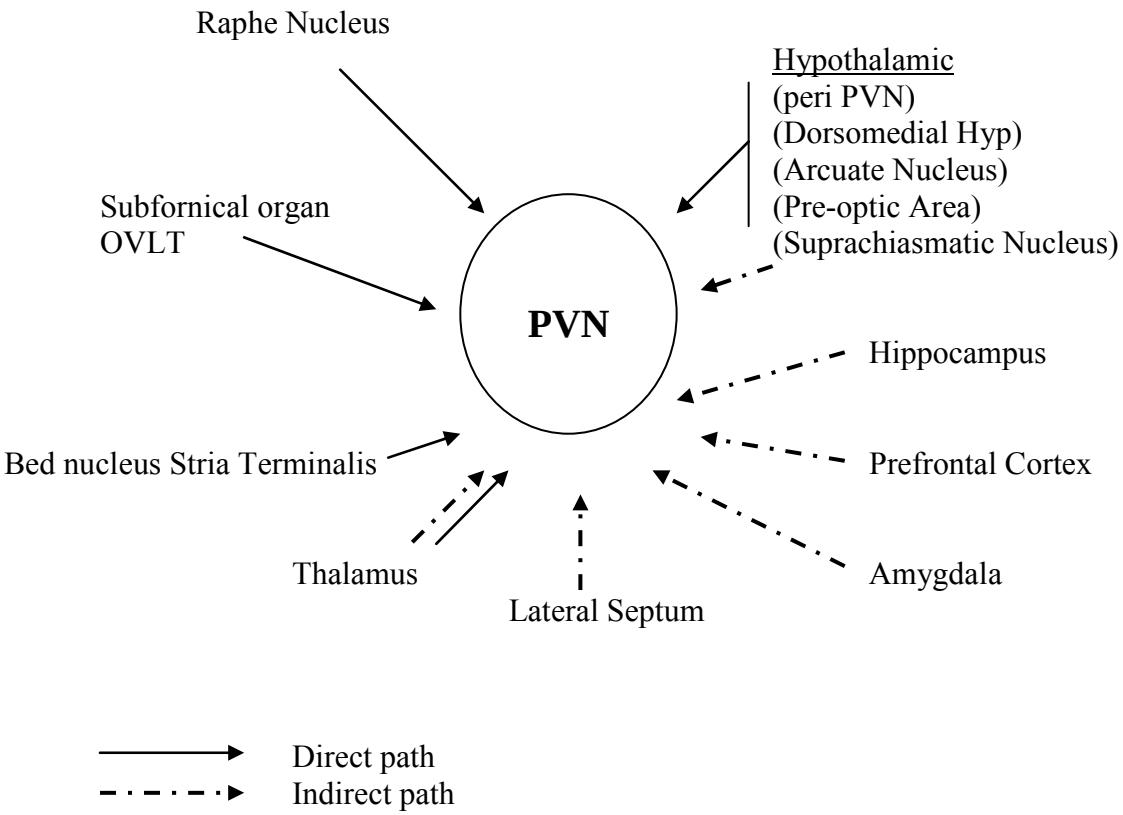

Figure 2. Inputs to the paraventricular nucleus (PVN) of the hypothalamus that have implications in the stress response. The PVN is the center of integration for inputs that affect the HPA axis. The peri-PVN is the area of neurons immediately surrounding the PVN.

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Effect of Orphanin FQ/Nociceptin (OFQ/N) and Isoflurane on the Prolactin Secretory Response in OFQ/N Knockout Mice

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Abstract

The prolactin secretory response to subcutaneous injection of orphanin FQ/nociceptin (OFQ/N) was measured in wild-type and OFQ/N knockout mice. These injections were given with and without isoflurane anesthesia, to determine if isoflurane would affect the prolactin secretory response. OFQ/N injection significantly increased prolactin levels in males and females, regardless of genotype, with a more robust response in females. Isoflurane pretreatment did not affect prolactin levels in controls or in animals injected with OFQ/N. This is the first report that exogenously administered OFQ/N stimulates prolactin secretion in mice and that brief isoflurane exposure does not significantly affect this response.

Key words: opiates, opioid, anesthesia, gender, stress

1. Introduction

Orphanin FQ/Nociceptin (OFQ/N) shares high sequence homology with the classic opiate peptides, especially dynorphin [11,17]. However, it does not bind to the traditional opiate receptors but binds with high affinity to its own receptor, ORL-1 [18]. ORL-1 is found in high concentrations in the hypothalamus [11], including the median eminence [13], indicating that OFQ/N is likely involved in regulating neuroendocrine processes.

Like other endogenous opiates, OFQ/N administration increased prolactin (PRL) secretion in the rat [2]. However, OFQ/N does not always produce an opiate-like effect, and there is conflicting evidence regarding its function. OFQ/N knockout mice display anxiety related behaviors and elevated corticosterone (CORT) levels [10]. Also, OFQ/N has been shown to attenuate the anxiety and fear response when given to rodents via intracerebroventricular (ICV) injection [8]. However, this was challenged by Fernandez et al. [5], who demonstrated that OFQ/N given by ICV injection to rodents at similar doses acted as an anxiogenic, not an anxiolytic. Furthermore, ICV OFQ/N increased

corticosterone (CORT) levels in unstressed rats [4], indicating activation of the hypothalamic-pituitary-adrenal axis.

The stress caused by injections and handling may further complicate interpretation of the physiological functions of OFQ/N [12]. To minimize these possible complications, administration of a mild inhalant anesthesia, such as isoflurane, which is quickly eliminated from an animal via the lungs [1], may decrease the stress response associated with handling [15]. The purpose of the current study was to examine the effects of OFQ/N on circulating PRL levels in male and female OFQ/N knockout mice and to determine if pretreatment with isoflurane anesthesia affects the PRL secretory response.

2. Methods

2.1 Animals

OFQ/N knockout mice were generated as described previously [10]. Briefly, nonlitter F1 and F2 mice were intercrossed to obtain F2 and F3 mice, respectively, on a 129/Ola x C57BL/6 hybrid background. Experiments were performed using mice obtained from a breeding colony that was established at Miami University with initial breeding pairs provided by R. Reinscheid. Male and female knockout and wild-type mice (2-3 months old, 17-32g) were provided food and water *ad libitum*. Animals were housed 2-3 per cage under controlled temperature (21°C) and light (12h light: 12h dark). All experiments were conducted following the guidelines of the Animal Welfare Act and were approved by Miami University's Institutional Animal Care and Use Committee (IACUC).

2.2 Anesthesia and injections

Animals were placed in an anesthetic chamber and exposed to 15 sec of isoflurane anesthesia. Immediately after removing mice from the chamber, they were injected subcutaneously with 30 μg OFQ/N (Sigma, St. Louis, MO) or an equal volume of saline (0.1 ml) or they were not injected. Animals were sacrificed 10 minutes after receiving an

injection and/or isoflurane exposure. Control animals remained in their home cages until sacrificed at times corresponding to those of treated animals. Basal PRL levels were determined in control animals that did not receive any treatment before sacrifice. At the time of sacrifice, trunk blood was collected and centrifuged (3000Xg) to obtain plasma. Plasma was stored at -20°C until assayed for hormone concentrations.

2.3 Radioimmunoassay

Plasma PRL concentrations were measured in duplicate samples by double antibody radioimmunoassay (RIA). Reagents for the RIA were obtained from NIDDK's National Hormone and Pituitary Program (NHPP) and Dr. A. F. Parlow. PRL was iodinated using $Na^{125}I$ (Perkin-Elmer, MA) as described by Greenwood and Hunter [7]. PRL levels are expressed in ng/ml and were determined using a standard curve of mouse PRL reference prep-3. The upper and lower limits for the PRL assay were 400 and 0.8 ng/ml, respectively. The intraassay coefficient of variation was 10% and the interassay coefficient of variation was 16.5% across three assays.

2.4 Statistics

A three factor ANOVA model was used to analyze the PRL levels. In order to make the variability within each sex-treatment-genotype subgroup comparable (as is required in ANOVA), the PRL levels were log transformed. Means of the transformed data were compared using t statistics. For both sexes, the Bonferroni multiple comparison technique was used to ensure that the probability of a type I error occurring anywhere in the set of comparisons for that sex was no more than 0.05.

3. Results

Subcutaneous injection of OFQ/N alone significantly increased circulating PRL levels in both female (Figure 1A) and male (Figure 1B) knockout and wild-type mice ($p<0.0001$), with a more robust response in females. Isoflurane pretreatment did not affect the PRL

secretory response to OFQ/N. Regardless of treatment, there was no significant difference in the PRL response to OFQ/N between knockout and wild-type mice of the same sex. In addition, animals given isoflurane before saline injections did not have significantly different levels of circulating PRL than either control animals (basal) or animals injected with saline only.

4. Discussion

This is the first report demonstrating that subcutaneous OFQ/N administration increases circulating levels of PRL in mice. In fact, to our knowledge, this is the first report that OFQ/N stimulates PRL release in mice, regardless of route of administration. Opioid peptides have previously been shown to be transported across the blood-brain barrier by specific transporters [6]. Although less than 1% of a peptide injected into the circulation crosses the blood-brain barrier, it is enough to cause physiological changes [14]. Indeed, Kastin et al. [9], demonstrated that morphine and a potent analog of Met-enkephalin (injected intraperitoneally) crossed the blood-brain barrier in male rats as indicated by changes in cortical electroencephalographic readings. In our experiments, the injection itself was not sufficient to stimulate PRL release because neither wildtype nor knockout controls had elevated PRL levels following the saline injection.

Regardless of sex or genotype, no difference in resting PRL levels was detected between groups exposed to isoflurane anesthesia prior to injection and those that were not pretreated with isoflurane. Furthermore, this method of anesthesia does not appear to affect the PRL secretory response to OFQ/N. These data are in agreement with Reburn and Wynne-Edwards [15], who reported that isoflurane did not affect basal PRL secretion in dwarf hamsters. Administration of OFQ/N to OFQ/N knockout mice produced a significant increase in plasma PRL levels within 10 minutes. Because OFQ/N acts at a specific ORL1 receptor and it does not bind to other opiate receptor subtypes [16], our results indicate that the OFQ/N knockout mice have functional ORL-1 receptors, even in the absence of the peptide . In fact, OFQ/N knockout mice have been shown to upregulate ORL-1 receptors in areas of the brain such as the hypothalamus, suggesting that OFQ/N receptors are responding to the loss of OFQ/N [3].

In summary, OFQ/N injected subcutaneously produces a significant increase in circulating PRL levels in OFQ/N male and female wild-type and knockout mice, indicating that the knockout mice do not lose their sensitivity to OFQ/N stimulation. Furthermore, pretreatment with isoflurane anesthesia did not affect the PRL secretory response to OFQ/N administration, nor did it affect basal levels of PRL. Because basal levels of PRL were not affected by isoflurane, this rapidly metabolized anesthetic may be useful in minimizing any stress associated with handling when investigating PRL regulation by OFQ/N.

Acknowledgements

This work was supported by NIH grant DK061956 to PC.

Figure 1a.

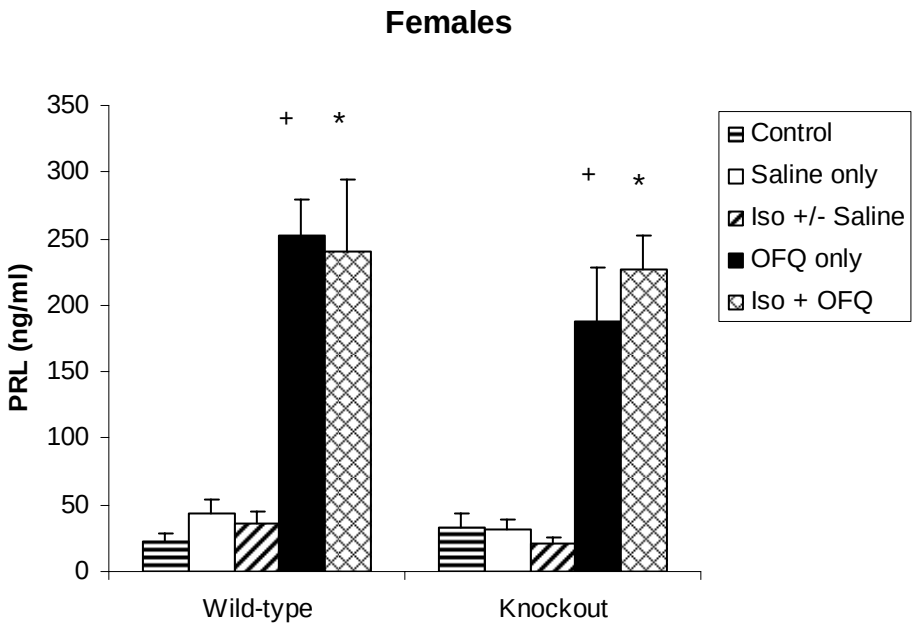

Figure 1a. Effects of OFQ/N with and without isoflurane anesthesia pretreatment on the prolactin secretory response in female wild-type and knockout mice. Groups of animals that were not exposed to isoflurane were injected with OFQ/N (30 μg, sc in 0.1 ml saline) (OFQ only) or an equal volume of saline (0.1ml) (Saline only). Groups of animals briefly exposed to isoflurane were given the same dose of OFQ/N (Iso + OFQ), an equal volume of saline or no injection. There was no difference in the prolactin levels between animals pretreated with isoflurane that received saline or no injection, so these values were pooled (Iso +/- Saline). Control animals did not receive any treatment. Values are means $\pm$ SEM.

Control (WT=8; KO=8); Saline only (WT=8; KO=7); Iso +/- Saline (WT=15; KO=16); OFQ only (WT=7; KO=6); Iso + OFQ (WT=4; KO=6).
+ Significantly different from Saline only, within the same genotype ($p<0.0001$).
* Significantly different from Iso +/- Saline, within the same genotype ($p < 0.0001$).

Figure 1b.

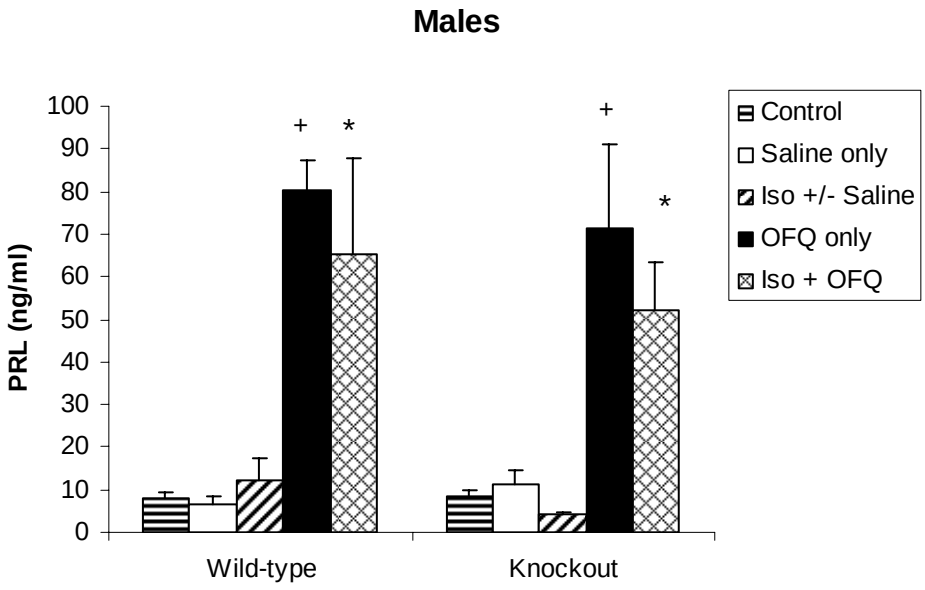

Figure 1b. Effects of OFQ/N with and without isoflurane anesthesia pretreatment on the prolactin secretory response in male wild-type and knockout mice. Groups of animals that were not exposed to isoflurane were injected with OFQ/N (30 μg, scin 0.1 ml saline) (OFQ only) or an equal volume of saline (0.1ml) (Saline only). Groups of animals briefly exposed to isoflurane were given the same dose of OFQ/N (Iso + OFQ), an equal volume of saline or no injection. There was no difference in the prolactin levels between animals pretreated with isoflurane that received saline or no injection, so these values were pooled (Iso +/- Saline). Control animals did not receive any treatment. Values are means $\pm$ SEM.

Control (WT=8; KO=8); Saline only (WT=9; KO=8); Iso +/- Saline (WT=17; KO=23); OFQ only (WT=6; KO=8); Iso + OFQ (WT=6; KO=7).
+ Significantly different from Saline only, within the same genotype ($p<0.0001$).
* Significantly different from Iso +/- Saline ($p < 0.0001$), within the same genotype.

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Effect of Orphanin FQ/Nociceptin (OFQ/N) and isoflurane on the corticosterone secretory response in OFQ/N knockout mice

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Abstract

The effect of Orphanin FQ/Nociceptin (OFQ/N) administration on corticosterone (CORT) secretion was determined in wild-type and OFQ/N knockout male and female mice. Additionally the effect of pretreating animals with isoflurane anesthesia to minimize the potential stress of injection was examined. OFQ/N did not significantly increase CORT levels in males or females of either genotype, but CORT levels were increased in all groups that received any injection or were exposed to isoflurane. These results demonstrate that OFQ/N does not elevate circulating CORT levels; however the injection process itself results in an increase in CORT secretion. Pretreatment with isoflurane did not significantly diminish the CORT response to injection, except in wild-type males. In fact, the isoflurane exposure itself increased CORT levels above basal values. Additionally, a gender difference was evident; females displayed a greater change of plasma CORT levels than males. Finally, because even saline injection increased CORT levels, we closely investigated another possible non-specific stress effect, i.e. the effect of transporting animals from their home environment in the animal facility to the laboratory on the day of the experiment. Although basal CORT levels were similar to values reported in other studies, circulating CORT levels were elevated in animals transported to the laboratory, even after a 30 minute acclimation period. These results indicate that the experimental protocol that is followed when conducting stress experiments needs to be carefully considered.

Key words: opiates, opioid, anesthesia, gender, stress

1. Introduction

Orphanin FQ/Nociceptin (OFQ/N) is an endogenous opiate heptadecapeptide found in high concentrations in the hypothalamus [13], including the median eminence [15]. Localization of OFQ/N and its receptor in these areas suggests that OFQ/N may be involved in neuroendocrine mechanisms. Despite sharing high sequence homology with other endogenous opiate peptides (EOP), OFQ/N has its own unique, high affinity

receptor, ORL-1 [18], referred to as NOP. Importantly, OFQ/N does not appear to bind to any of the classic opiate receptors, nor do the other EOP bind to NOP [18].

There is conflicting evidence regarding the function of OFQ/N. Similar to the other EOP [5], OFQ/N increases prolactin (PRL) levels in rats [2] and mice [20]. Further, Jenck et al. [7] reported that intracerebroventricular (ICV) injection of OFQ/N attenuated anxiety and fear in rats and mice, i.e. it had an opiate-like effect. Consistent with an anxiolytic effect, OFQ/N knockout animals have been shown to display increased anxiety-related behaviors and increased corticosterone (CORT) levels following stress [9]. Contrary to these studies, Fernandez, et al. [4] reported that OFQ/N produced anxiogenic effects in rodents. Additionally, ICV injections of OFQ/N increased CORT in unstressed rats [3], indicating a stimulatory, anxiogenic effect of OFQ/N on the hypothalamic-pituitary-adrenal (HPA) axis. However, other studies have described attenuating effects of OFQ/N on plasma CORT levels [10] or differential effects on diurnal CORT secretion after administration of an NOP specific antagonist [11]. The purpose of this study was to determine the effect of OFQ/N on plasma CORT levels in male and female wild-type and OFQ/N knockout mice and to determine if pretreatment with isoflurane could minimize any potential stress effects due to handling and/or injection. Finally, basal CORT levels were compared between animals that had blood samples taken in their home quarters in the animal facility and those that were transported from that facility to the laboratory and allowed to acclimate for at least 30 minutes. These studies will help to clarify the role of OFQ/N in HPA axis modulation and determine experimental and environmental factors that may confound such studies.

2. Methods

2.1. Animals

OFQ/N knockout mice were generated as described previously [9]. All animals were genotyped using standard PCR (polymerase chain reaction). Ear punches (2mm in diameter) were taken for identification purposes, while the animals were under isoflurane anesthesia. Tissues were subjected to DNA extraction using the HotShot method [19].

Isolated DNA was used in a PCR reaction containing 2 mM $MgCl_2$, 200 µM each dNTP and 0.4 µM of each of the following primers: OFQ/N fwd: GACCCAGAGCTTGTGTCAGC; OFQ/N rev: CTCATAAACTCACTGAACCGC, and neomycin cassette primer in the transgenic mice: CCGGAGAACCTGCGTGCAATCC. Cycling parameters were as follows: 94°C for 3 min, followed by 31 cycles of: 94°C for 30 sec, 60°C for 30 sec, 72°C for 45 sec, and a final extension of 5 min at 72°C. Following gel electrophoresis, bands were scored as follows: 250 bp product only = wild-type, 550 bp product only = knockout, 250 and 550 bp products = heterozygote.

Male and female knockout and wild type mice (2-3 months old, 17-32g) were housed 2-3 per cage under controlled temperature (21ºC) and light (12h light: 12h dark) conditions and were given food and water *ad libitum*. Experiments were conducted following the guidelines of the Animal Welfare Act and all protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at Miami University.

2.2. Treatment groups and sampling

2.2.1. Basal sampling

Basal CORT levels were measured in two separate groups of untreated animals. One group was transported from the animal facility to the laboratory and allowed to acclimate for at least 30 minutes. The laboratory was maintained as a quiet, stress-free environment. These animals were sacrificed by rapid decapitation and trunk blood was collected. A second group was sampled in the animal facility by tail clip. Before blood collection, animals were removed from their home cage and placed in a plexiglass chamber. The tip of the tail was snipped and blood was collected into capillary microfuge tubes. Blood collections were completed in less than one minute to minimize any effects of stress associated with handling or blood sample withdrawal. Plasma was collected following centrifugation (13000 x g) and stored at -20ºC until CORT levels were measured by radioimmunoassay (RIA).

2.2.2. Experiment 1

All animals were transported to the laboratory and allowed to acclimate for at least 30 minutes prior to receiving any treatment. In one experimental group, animals were placed in an anesthetic chamber and exposed to 15 sec of isoflurane anesthesia. Immediately following removal from the chamber, mice were subcutaneously (sc) injected with either 30 μg OFQ/N in 0.1 ml saline or an equal volume of saline. Additionally, some animals that were exposed to isoflurane were not injected. We have previously shown that this dose of OFQ/N significantly increased circulating prolactin levels [20]. Animals in the second group were not exposed to isoflurane prior to being injected with either 30 μg OFQ/N, sc in 0.1 ml saline, or an equal volume of saline. In both cases, animals were sacrificed 10 minutes after receiving the injection. Animals that were not injected were sacrificed 10 minutes after isoflurane exposure. To control for chamber exposure, a separate group of animals was placed in the anesthetic chamber for 15 sec without isoflurane and sacrificed 10 min later. Trunk blood was collected at the time of sacrifice, centrifuged (13000 x g) and plasma was stored at -20ºC until CORT levels were measured by RIA.

2.2.3. Experiment 2

This experiment was conducted in the animal facility to examine any potential effects of transporting animals to the laboratory. In this experiment, basal blood samples were taken via tail clip. Twenty-four hours later, animals were divided into three groups. In one group, animals were injected with 30 μg OFQ/N, sc in 0.1 ml saline. In the second group, animals were injected with 0.1 ml saline. Animals in a third group were placed in an anesthetic chamber and exposed to 15 sec of isoflurane anesthesia and returned to their home cage. Animals were sacrificed 10 minutes after injection or isoflurane exposure. At the time of sacrifice, trunk blood was collected, centrifuged (13000x g) and plasma was stored at -20ºC until CORT levels were measured.

2.3. Radioimmunoassay

Plasma CORT concentrations were measured in duplicate samples by double antibody radioimmunoassay (RIA) using CORT RIA kits purchased from MP Biomedicals (Irvine, CA) following manufacturer's instructions. Interassay variability was < 8% and intraassay variability was < 3%.

2.4 Calculation of fold change

To determine any gender differences in the magnitude of the CORT response to treatment, the change in circulating CORT levels after saline, OFQ/N or isoflurane (Experiment 2) was quantified. Basal CORT levels were averaged by gender and genotype and the CORT level following treatment for each individual was divided by the appropriate mean basal CORT level.

2.5. Statistics

CORT levels did not lend themselves to ANOVA models because the variance differed widely from group to group even after transformation. Tests were adjusted so that the overall Type I error probability for each set of comparisons was no more than 0.1. The Bonferroni multiple comparison technique was used for each set of data analyzed. Data in Figures 1 and 2 were analyzed using two-tailed, two-sample t-tests, with respective p-values of < 0.025 (4 comparisons per gender) and < 0.0071 (14 comparisons per gender) before two means were declared significantly different.

The data presented in Figure 3a were transformed by taking the inverse square root for each value. All comparisons were made between basal values and levels after treatment using one-tailed, paired t-tests, except for OFQ/N-injected knockout females. We were able to obtain a basal sample from only 2 of the animals in this group. Therefore, we calculated the mean of the basal CORT levels in all knockout females and compared the CORT levels after OFQ/N injection with that basal value. CORT levels in treatment

groups, i.e. in the OFQ/N vs. Saline treated groups, were compared using two-tailed, two sample t-tests. For the data presented in Figure 3b, the CORT levels in the basal vs. treated groups were compared using one-tailed, paired t-tests with the exception of wild-type animals treated with OFQ. This comparison was made using a sign test for median. Wild-type OFQ vs. Saline was compared using a Mann-Whitney test, and Knockout OFQ vs. Saline was compared with a one-tailed, two sample t-test. Since a total of 4 comparisons per gender within a genotype were made, p-values had to be < 0.05 to reach significance. To determine gender differences, the fold change in CORT levels was determined (Table 1). These data were log transformed and then analyzed using one-tailed, two sample t-tests or Mann-Whitney tests. Because a total of 6 comparisons were made, the p-value had to reach < 0.033 to declare significance.

3. Results

3.1. Basal sampling

Basal CORT levels were not different between genotypes in either male or female mice, but animals that were transported from the animal facility to the laboratory had elevated CORT levels, even after allowing them at least 30 minutes to acclimate (Figure 1).

3.2. Experiment 1

Regardless of genotype, animals that received any injection had significant increases in CORT levels (Figure 2) above basal values (dashed line in Figure 2). OFQ/N did not increase CORT levels above saline-injected animals in either female (Figure 2a) or male (Figure 2b) mice. Simply placing animals in the anesthetic chamber without any isoflurane (Chamber) did not significantly affect CORT levels. In every group, except the knockout females, isoflurane exposure significantly increased CORT levels above basal values and above levels in animals that were placed in the chamber without isoflurane. In fact, except for wild-type males (Figure 2b), isoflurane did not

significantly reduce CORT levels compared to animals that received either saline or OFQ/N injection. Overall, isoflurane pretreatment did not seem to reduce the effects of injection in either wild-type or knockout animals of either gender.

3.3. Experiment 2

To eliminate the possibility that OFQ/N failed to increase CORT levels because CORT levels were already elevated after being transported to the laboratory, we determined the CORT response to injections of saline or OFQ/N or to isoflurane exposure in animals that remained in the animal facility. Similar to the results in the first experiment, injecting animals with either saline or OFQ/N or exposing them to isoflurane resulted in a significant increase of CORT levels in both males and females of both genotypes (Figure 3). The magnitude of the CORT response was similar regardless of the treatment, but overall, females had a greater CORT response to injection and to isoflurane exposure than males. The change was statistically significant in the wild-type females exposed to isoflurane and knockout females that were injected with saline or OFQ/N (Table 1).

4. Discussion

There was no significant difference in basal CORT levels between wild-type and knockout mice whether basal samples were collected in the animal facility or in the laboratory, but transporting animals to the laboratory did increase overall basal CORT levels (Figure 1). This result is in contrast to the results of Köster et al. [9] who reported that male OFQ/N knockout mice had higher basal CORT levels than their wild-type littermates. This discrepancy may be due to differences in sampling methods and/or experimental conditions, such as prior handling, housing or presence/absence of low-level environmental stressors such as noise and light conditions. Clearly, the results of our study indicate that such factors are important considerations when studying the stress response. Although animals were allowed to acclimate for at least 30 minutes after being transported from the animal facility, CORT levels were significantly higher when

compared to animals that were not transported. This difference in basal levels was likely due to the transport itself and/or the exposure to a novel environment, even though the laboratory is maintained in a quiet, stress-free condition. Importantly, the basal levels in animals that were transported were still similar to those reported by others (one group reported 70 – 90 ng/ml in males [6]; another group reported ~35 ng/ml in males [1]). In spite of that similarity, and because transported animals had significantly elevated CORT levels, we conclude that transport to a new location produced significant stress to these animals. Perhaps allowing for a longer acclimation time would reduce CORT levels. For example, after 2 hours, Bilkei-Gorzo, et al. [1] reported basal CORT was ~35 ng/ml [1], but this was still higher than the levels we quantified in animals that were not transported. Collectively, these studies demonstrate that, when using CORT levels as an indication of HPA axis activation, it is particularly important to understand the effects of experimental conditions on basal hormone levels. This is especially true when using OFQ/N knockout animals because, if they are more susceptible to anxiety [9], then low-level environmental stressors, such as sampling methods or handling, may have a greater effect on the knockout animals, leading to the conclusion that resting levels are higher in knockout mice.

OFQ/N administration did not significantly stimulate CORT release beyond levels of animals injected with saline (Figures 2 and 3). This was true whether experiments were conducted on animals that were transported to the laboratory or on animals that remained in the animal facility. These results are contradictory to results from other studies in rodents in which OFQ/N has been reported to variously decrease [10] or increase [3] CORT levels. However, the route of OFQ/N injection, the animal species studied [14], and, the time the sample was taken likely influence the results. In our study, blood samples were taken at one time point, 10 minutes after injection, when PRL levels have been shown to be increased [20]. It is possible that CORT levels might be elevated at later time points. Also, because CORT levels were already increased in animals that were transported, it is possible that the effect of an OFQ/N injection might not be detectable if maximum hormone plasma levels were already achieved. This does not seem likely, however, for three reasons. First, saline and OFQ/N both increased CORT levels in a similar manner. Second, we can detect even higher CORT levels in

wild-type and OFQ/N knockout mice subjected to various stressors in our laboratory (data not shown). Third, even when basal levels were very low, i.e. in animals that remained in the animal facility, there was no difference in the magnitude of the CORT response to saline or OFQ/N injection. Therefore, OFQ/N did not specifically activate the HPA axis in either males or females of either genotype, but injecting the animals was sufficiently stressful to elicit HPA axis activation.

In order to try to minimize the potential effects of stress caused by injection, we administered isoflurane to lightly anesthetize animals prior to injection. Isoflurane pretreatment did not affect basal prolactin levels [20], but did significantly increase basal CORT levels in all groups except knockout females. Further, only wild-type males pretreated with isoflurane showed a significant decrease in CORT when compared to wild-type males injected with OFQ/N (Figure 2b, groups 4 & 5). Nonetheless, pretreated wild-type males injected with OFQ/N still had much higher CORT levels than basal values (dashed line in figure 2b). To control for any possible effect of the anesthetic chamber, we quantified CORT levels in animals that were placed in the anesthetic chamber without any isoflurane. Levels in males and wild-type females were not significantly different from basal, control values (dashed line in Figure 2). This result suggests that the CORT increase following isoflurane exposure was due to the isoflurane and not due to placement in the chamber, contradicting the results of Reburn and Wynne-Edwards [17], who did not detect increases in cortisol levels in dwarf hamsters after isoflurane exposure. Finally, the increase in CORT levels following injection or isoflurane exposure was greater in females than in males (Table 1). In three groups of females, the gender difference was significant, i.e. in wild-type, isoflurane treated females and in the knockout females injected with saline or OFQ/N. Gender-specific differences in the stress response are well-characterized and have been reported by others [8, 12, 16].

In summary, there was no difference in basal circulating CORT levels between wild-type and OFQ/N knock-out male or female mice, indicating that, under resting conditions, OFQ/N does not affect the HPA axis. OFQ/N given subcutaneously does not have an effect on CORT levels in male or female wild-type or knockout mice. Additionally, isoflurane alone increased CORT levels and should not be used when

examining neuroendocrine parameters related to stress. Finally, investigators must carefully consider experimental protocols, when measuring CORT levels. Our results clearly demonstrate that transporting animals, even to a quiet, stress-free environment in the laboratory with an acclimation period, significantly elevated basal CORT levels. Possible non-specific stress effects must be considered when conducting stress experiments.

Acknowledgments

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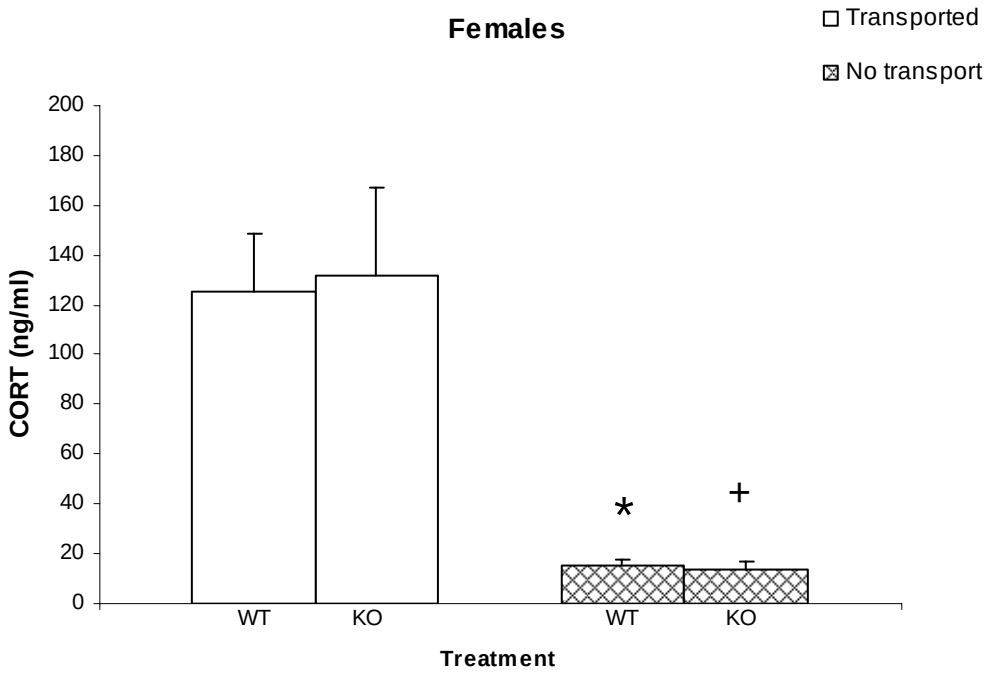
Figure 1a.



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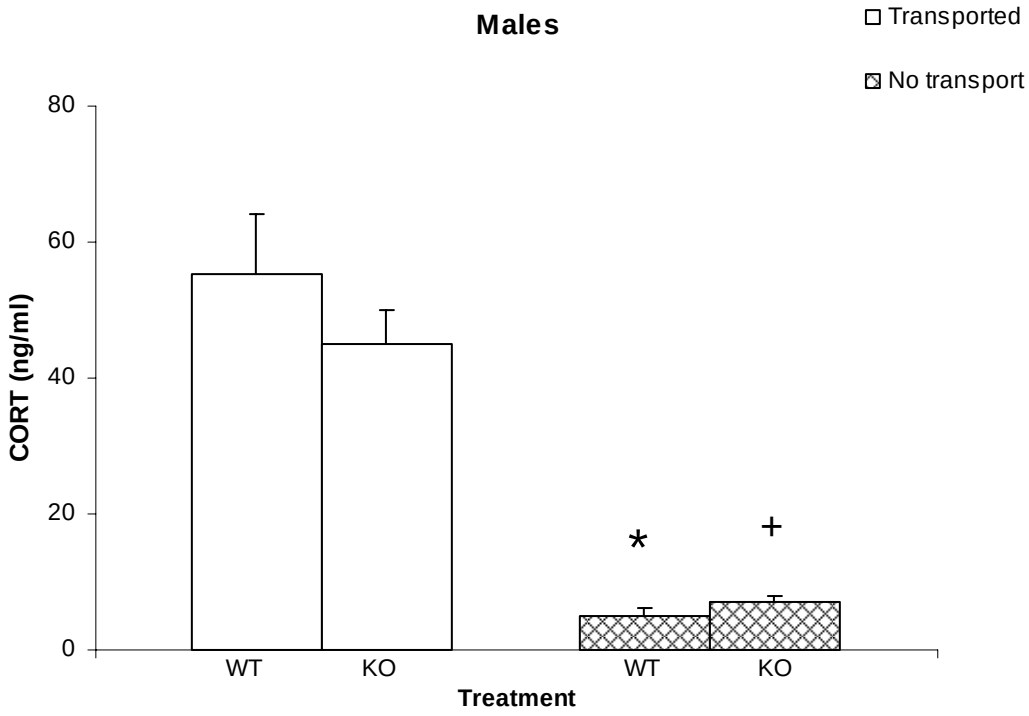

Figure 1. Basal corticosterone (CORT) levels in female (a) and male (b) wild-type and knockout mice. Animals were either transported from the animal facility (Transported) and sampled after at least 30 minutes of acclimation (open bars) or remained in the animal facility for blood collection (No transport, hatched bars). Values are means ± S.E.M. The numbers of animals per group were: Transported (females: WT = 15, KO = 6; males: WT = 10, KO = 9); No transport (females: WT = 28, KO= 28; males: WT = 30, KO = 30).

(a) * Significantly different from Transported WT ($p < 0.0005$)
+ Significantly different from Transported KO ($p = 0.011$)
(b) * Significantly different from Transported WT ($p < 0.0005$)
+ Significantly different from Transported KO ($p < 0.0005$)

Figure 2a.

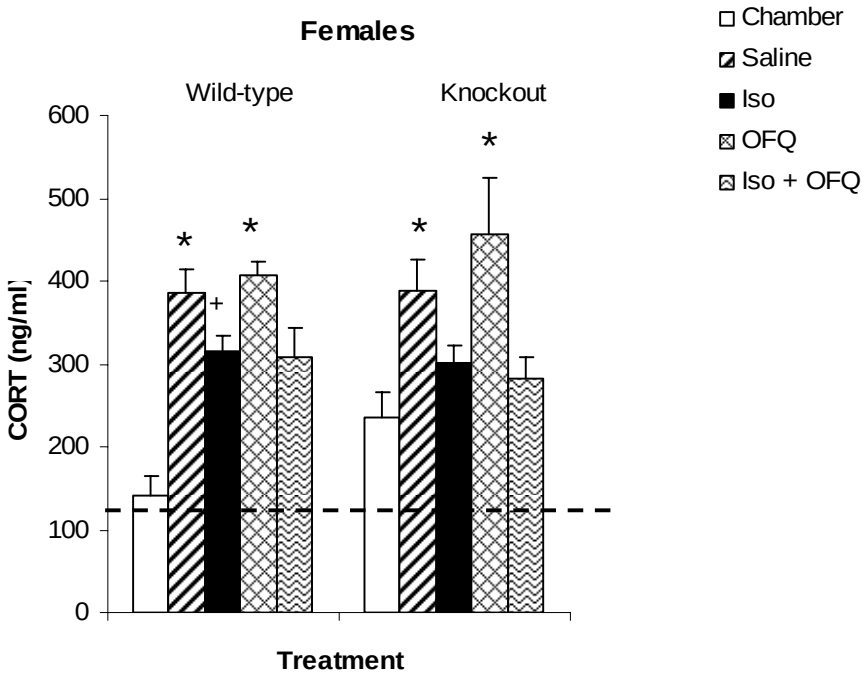

Figure 2b.

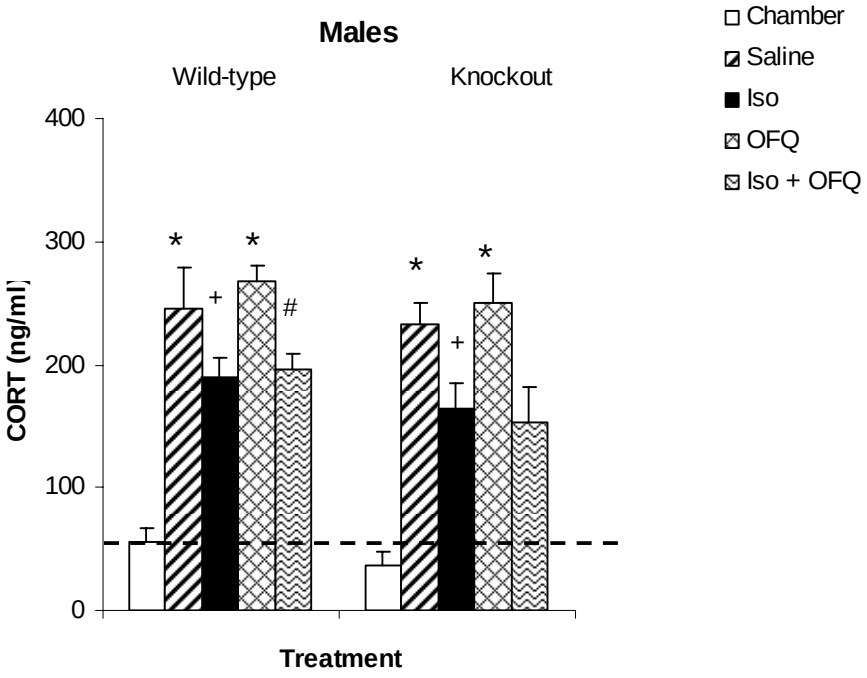

Figure 2. Effects of OFQ/N or saline with and without pretreatment of isoflurane anesthesia on the CORT secretory response in female (a) and male (b) wild-type and OFQ/N knockout mice transported to the laboratory. Animals were divided into 5 groups: (1) Animals were briefly placed in an anesthetic chamber without any isoflurane (Chamber). (2) Animals were injected with saline (0.1 ml, sc) (Saline). (3) Animals were exposed to isoflurane and were then either injected with 0.1 ml saline (sc) or did not receive any injection. Since there was no effect of the injection in these animals, values were pooled (Iso). (4) Animals were injected with OFQ/N (30 µg, sc in 0.1 ml saline), but were not pretreated with isoflurane (OFQ). (5) Animals were exposed to isoflurane for 15 seconds and then injected with 30 µg, sc of OFQ/N (Iso + OFQ). Values are means ± S.E.M. The dashed line represents the mean basal CORT level for animals transported to the laboratory (basal levels were determined as pooled values by gender, since there was no difference between genotypes). The SEM for basal CORT levels for females is ± 18.98 and for males is ± 5.60. The numbers of animals per group were: Chamber (females: WT = 8, KO = 8; males: WT = 5, KO = 5); Saline (females: WT = 9, KO = 7; males: WT = 10, KO = 8); Iso (females: WT = 16, KO = 13; males: WT = 14, KO = 16); OFQ (females: WT = 9, KO = 7; males: WT = 6, KO = 8); Iso + OFQ (females: WT = 9, KO = 8 females; males: WT = 8, KO = 8).

(a) * Significantly different from basal levels ($p < 0.0005$)
+ Significantly different from WT Chamber ($p < 0.0005$)
(b) * Significantly different from untreated animals ($p < 0.0005$)
+ Significantly different from Chamber ($p < 0.0005$)
# Significantly different from WT OFQ ($p = 0.003$).

Figure 3a.

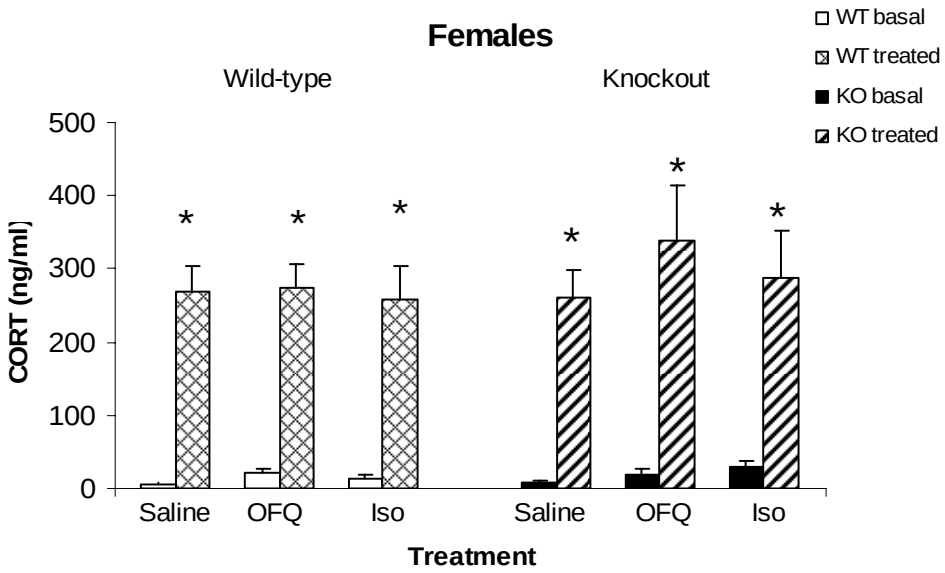

Figure 3b.

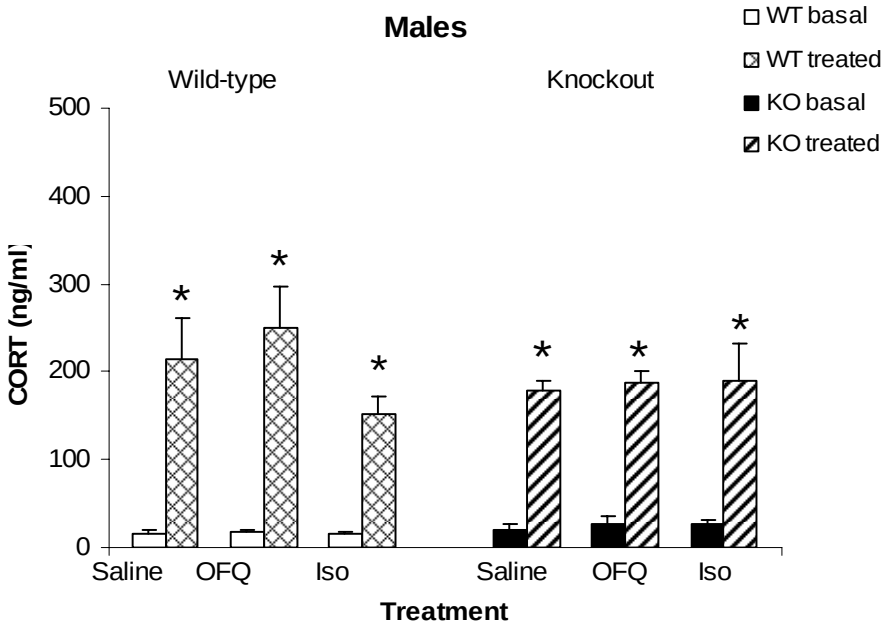

Figure 3. Effects of saline or OFQ/N injection or 15 seconds of isoflurane exposure on the CORT secretory response in female (a) and male (b) wild-type and OFQ/N knockout mice. These animals remained in the animal facility during the experiment. Animals were divided into 3 groups: (1) Animals were injected with saline (0.1 ml, sc) (Saline). (2) Animals were injected with 30 μg, sc of OFQ/N (OFQ). (3) Animals were exposed to isoflurane for 15 sec (Iso). Basal samples were taken by tail clip 24 hours prior to treatment. Values are means ± S.E.M. Saline (females: WT = 6, KO = 4; males: WT = 4, KO = 5); OFQ (females: WT = 6, KO = 5; males: WT = 6, KO = 5); Iso (females: WT = 6, KO = 5; males: WT = 7, KO = 5).
In Saline and OFQ treated females (a), we were not able to collect basal samples from every animal that was subsequently injected. The number of animals used for basal samples are: Saline (WT = 4; KO = 3), OFQ (WT = 5; KO = 2).

(a) *Significantly higher than basal (WT Saline, $p = 0.003$; WT OFQ, $p = 0.010$; WT Iso, $p = 0.002$; KO Saline, $p = 0.019$; KO OFQ, $p < 0.0005$; KO Iso, $p = 0.015$)
(b) *Significantly higher than basal (WT Saline, $p = 0.025$; WT OFQ, $p = 0.03$; WT Iso, $p < 0.0005$; KO Saline, $p < 0.0005$; KO OFQ, $p = 0.001$; KO Iso, $p = 0.029$)

Table 1. Fold change from basal CORT levels after injection with saline, OFQ/N or isoflurane exposure in the animal facility.

	Saline	OFQ/N	Isoflurane
Wild-type Female	18.52 ± 2.56	18.89 ± 2.38	17.78 ± 3.32*
Wild-type Male	13.42 ± 2.91	15.59 ± 2.96	9.53 ± 1.17
Knockout Female	13.03 ± 1.76*	16.81 ± 3.75*	14.29 ± 3.17
Knockout Male	7.36 ± 0.83	7.70 ± 0.50	7.74 ± 1.80

*Significantly different from male (WT Iso, $p = 0.0082$; KO Saline, $p = 0.016$; KO OFQ, $p = 0.032$)

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Hypothalamic opiate receptor and prolactin receptor levels in the choroid plexus in Orphanin FQ/Nociceptin (OFQ/N) knockout mice

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Abstract

The purpose of this research was to determine if compensatory changes occur in Orphanin FQ/Nociceptin (OFQ/N) knockout mice that might affect neuroendocrine function. Basal levels of endogenous opiate receptor mRNA and protein in the hypothalamus and prolactin receptor (PRL-R) mRNA levels in the choroid plexus of OFQ/N wild-type and knockout mice were quantified by qRT-PCR and western blot analysis. Both male and female knockout mice had significantly lower kappa receptor (KOP) protein levels, and male knockout mice also had reduced KOP mRNA levels compared to wild-type mice. Mu receptor (MOP) mRNA expression was also reduced in knockout males, but this was not translated into a difference in protein. There were no genotype differences in PRL-R mRNA in the choroid plexus. These results indicate that the only compensation affecting opiate receptors in OFQ/N knockout mice is a decrease in KOP expression levels. The physiological and behavioral consequences of this compensation remain to be determined.

Introduction

Endogenous opiates (EOP) are widely distributed throughout the central nervous system (CNS) and are involved in many biological actions [1, 2] There are three classic opiate peptide families, the enkephalins, endorphins and dynorphins. These opiates act through distinct G-protein coupled receptors, the delta (DOP), mu (MOP) and kappa (KOP) receptors. While there is cross reactivity among these opiates and their receptors, the preferential ligand-receptor combinations are: enkephalin-DOP or MOP, endorphin-MOP or DOP and dynorphin-KOP [3].

Orphanin FQ/Nociceptin (OFQ/N) shares high sequence homology with the classic EOP, especially dynorphin [4, 5], but it has its own distinct receptor, the nociceptin/orphanin FQ peptide receptor (NOP) [6]. OFQ/N binds to NOP with high affinity, but does not bind to the other classic EOP receptors. Additionally, the classic EOP do not bind to NOP [7]. NOP is found in high concentrations in the hypothalamus and is colocalized in a number of brain regions with other opioid receptors and peptides

[8]. This, combined with OFQ/N's localization to areas such as the median eminence of the hypothalamus [9], indicates that OFQ/N may be involved in regulating neuroendocrine processes.

Although the OFQ/N knockout mouse does not produce the OFQ/N peptide [10], it does have NOP [11] that respond to administration of OFQ/N [12], and it has become a useful model for examining the physiological role of OFQ/N [6, 10, 13]. However, as with all knockout models, one must consider the possibility that compensatory mechanisms may mask effects due to the loss of the gene of interest. The purpose of these experiments was to investigate possible compensation(s) that may have occurred during development. Specifically, the possibilities examined were altered expression levels of opiate receptors. Since EOP receptors are localized to the hypothalamus, and are involved in neuroendocrine function [14, 15], we quantified hypothalamic EOP receptor levels and compared knockouts to wild-type mice.

OFQ/N knockout mice have been reported to display increased anxiety following stress [10, 16]. When an individual is stressed, corticotropin releasing factor (CRF) is released from neurons in the paraventricular nucleus of the hypothalamus and activates the hypothalamic-pituitary-adrenal (HPA) axis [17-19]. Prolactin (PRL) is also released during stress [20], at least, in part, due to modulation by the classic EOP [20, 21]. Additionally, recent studies in our laboratory have shown that OFQ/N is necessary for the PRL response to acute immobilization stress in male OFQ/N knockout mice [22]. Systemic PRL is transported into the cerebrospinal fluid by PRL-receptors (PRL-R) in the choroid plexus [23] and can influence neural activity in brain areas that are sensitive to PRL, i.e. areas that express PRL-R. Increased circulating levels of PRL, due either to exogenous PRL administration [24] or to a stress-induced PRL secretory response [25, 26], upregulate the long form of PRL-R in the choroid plexus. Increased PRL-R expression allows for more PRL transport into the brain. Changes in PRL sensitivity could affect HPA axis activation because PRL has been shown to stimulate CRF secretion in vitro [24]. We compared PRL-R expression levels in the choroid plexus as a potential marker for susceptibility to stress. We hypothesized that if OFQ/N knockout mice were more anxious under resting conditions, they would have higher levels of PRL-R expression levels in the choroid plexus.

Methods

OFQ/N knockout mice were generated as described previously [10]. All animals were genotyped using standard PCR (polymerase chain reaction). Ear punches (2 mm in diameter) were taken for identification purposes, while the animals were under isoflurane anesthesia. Tissues were subjected to DNA extraction using the HotShot method [25]. Isolated DNA was used in a PCR reaction containing 2 mM $MgCl_2$, 200 µM each dNTP and 0.4 µM of each of the following primers: OFQ/N fwd: GACCCAGAGCTTGTGTCAGC; OFQ/N rev: CTCATAAACTCACTGAACCGC, and neomycin cassette primer in the transgenic mice: CCGGAGAACCTGCGTGCAATCC. Cycling parameters were as follows: 94°C for 3 min, followed by 31 cycles of: 94°C for 30 sec, 60°C for 30 sec, 72°C for 45 sec, and a final extension of 5 min at 72°C. Following gel electrophoresis, bands were scored as follows: 250 bp product only = wild-type, 550 bp product only = knockout, 250 and 550 bp products = heterozygote.

Male and female knockout and wild type mice (2-3 months old, 17-32g) were housed 2-3 per cage and given food and water *ad libitum*. All animals were housed under controlled temperature (21ºC) and light (12h light: 12h dark). Experiments were conducted following the guidelines of the Animal Welfare Act and were approved by the Miami University Institutional Animal Care and Use Committee (IACUC).

Animals were sacrificed by decapitation under basal, resting conditions. Brains were rapidly removed from the cranial vault and placed in ice cold saline. Whole hypothalamus was microdissected following the guidelines of Palkovits [26] and either snap frozen (for western blotting or radioimmunoassay) or immediately stored in 500 µl RNA later solution (Ambion, TX) for future RNA isolation.

Hypothalamic RNA was isolated by homogenizing tissue in 500 µl Tri-Reagent (Molecular Research Center, OH) using an Omni tissue homogenizer (Omni International, GA). An additional 500 µl of Tri-Reagent was added and the sample was vortexed. After 5 min of incubation, 200 µl of chloroform was added. Following centrifugation (12,000 x g, 4°C, 15 min) the aqueous layer was transferred into a new 1.5 ml microfuge tube. One volume of 70% ethanol was added, and the solution was gently

mixed. This mixture was applied to a Micro RNeasy column, and extraction proceeded according to Qiagen's instructions. RNA was eluted with dd H_2O. All RNA was DNAse treated using Turbo DNAse (Ambion, TX) per Ambion's instructions.

Reverse transcription (RT) was performed under the following conditions: 0.5 µg RNA, 2 µl Takara random hexamers at 0.2 µg/µl (Promega, WI) and dd H_2O up to 12 µl. This mixture was heated to 70°C for 10 min, and then cooled to 4°C. The following components were added: Promega RT buffer (to 1X), dNTPs (final [667 µM]), $MgCl_2$ (final [3.3 mM]), and dd H_2O up to 30µl, followed by 1 unit of Improm II reverse transcriptase enzyme (Promega, WI). Negative controls contained all reaction components except for the RT enzyme. The reaction was heated to 25°C for 5 min, then 42°C for 1 hour, with a final enzyme deactivation at 70°C for 15 min. All RT reactions were purified using the Qiaquick nucleotide removal kit (Qiagen, CA) and eluted from the column with 60 µl of elution buffer. The cDNA was divided into aliquots for storage at -20°C until used for real-time PCR.

Semi-quantitative real time PCR was run following the standard curve method [27, 28] using sample cDNA to generate a standard curve. Samples were diluted 1:50 in dd H_2O to ensure that they would fall within the standard curve. All standards were run in duplicate and each sample was run in triplicate. Negative controls (no RT enzyme) were included once for each sample. All reactions were conducted using a Rotorgene 3000 (Corbett Life Science, Sydney, Australia). Reactions for PRL-R incubated at 95°C for 15 min, followed by 48 cycles of 95°C for 5 sec, 60°C for 10 sec, and 72°C for 15 sec. Reactions for all of the opiate receptors were incubated at 95°C for 15 minutes, followed by 48 cycles of 95°C for 10 sec, 58°C for 15 sec and 72°C for 15 sec. Reactions for the internal standard L7 were run at the same time as the gene of interest. All products were subjected to melting curve analysis to ensure that only one product was being generated. Values obtained for each transcript were normalized to values for L7.

Sequences for all primers are listed in Table 1. Primers were obtained from the following sources: PRL-R primers (long form of the receptor) were taken from Ling, et al. [29], KOP primers were from Primer Bank [30] (ID#24111248a2). L7 primer sequences were a generous gift from Paul Bushdid, University of Cincinnati Children's Hospital. All other primers were generated from Genbank sequences [31] (specific

accession numbers are: MOP: #U26915, NOP: #X91813, and DOP: #NM_013622) using software provided online by IDT and the BLAT alignment program developed at the University of California Santa Cruz [32].

For western blotting, frozen hypothalamic tissue was sonicated in homogenizing buffer containing a protease inhibitor cocktail (1:100) (Sigma, MO). Following centrifugation (13,000 x g, 4°C, 6 min), protein concentration was determined in an aliquot of the supernatant using a BCA protein assay (Pierce, IL), The remainder of the supernatant was diluted 1:1 in Laemmli buffer [33] and the proteins were resolved on a 10% polyacrylamide gel and then transferred to a PVDF membrane at 100 V for 45 min. The membrane was blocked in 8% nonfat dry milk in Tris Buffered Saline + Tween (TBST) for 2 hr and then probed with primary antibodies for either MOP or KOP with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal standard. Specific conditions for each protein are listed in Table 2. Following incubation, membranes were rinsed and probed with horseradish peroxidase (HRP) congugated secondary antibody (Goat anti-rabbit λ-globulin, Chemicon, CA) at a concentration of 1:5000 for 1.5 hours. Supersignal West Pico (Pierce, IL) was used for chemiluminescent detection of bands. Densitometric analysis of protein bands was conducted using ImageQuant software (GE Healthcare, NJ).

Statistical analysis

Protein and mRNA levels in wild-type animals were standardized to 100% and data from knockout animals were expressed as a percentage of wild-type levels. These data were analyzed via one sample t-tests with a p-value of 0.05 required to declare significance.

Results

There were no significant differences in the mRNA levels of any of the opiate receptors in the hypothalamus of OFQ/N knockout females when compared to wild-type (Fig 1a). In males, however, knockout mice had significantly reduced KOP and MOP

mRNA levels (Fig 1b). In knockout males, levels of KOP and MOP mRNA were 88.6 ± 3.8% and 71.6 ± 7.5% of wild-type, respectively. KOP protein levels were significantly reduced in both female (Fig 2a) and male (Fig 2b) knockout mice. Protein levels of KOP were 60.2 ± 3.1% of wild-type in knockout females and 68.4 ± 3.2% of wild-type in males. In contrast to the reduced levels of KOP protein, MOP protein levels were not decreased in knockout males (Fig 2b).

Levels of PRL-R mRNA expression in the choroid plexus did not differ between genotypes in males or females. Levels in OFQ/N knockout males were 95.2 ± 2.4% of wild-type and levels in females were 94.4 ± 5.5% of wild-type (data not shown).

Discussion

Male and female OFQ/N knockout mice had reduced KOP protein levels, but exhibited few other differences in opiate receptor expression levels. Although MOP mRNA was significantly reduced in knockout males, this was not translated into reduced protein levels. The relationship between the amount of mRNA transcribed and the amount that is actually translated into protein is very complex [34], and the genotype difference we detected in mRNA levels may not be physiologically relevant. These results demonstrate that little compensation in the endogenous opiate system can be detected under basal, resting conditions. It is possible that greater differences might be detected when animals are challenged under physiological conditions known to involve the EOP system, such as stress. The EOP and their receptors are found in high concentrations in brain areas involved in stress circuits, and opiates are activated by stress [1]. A number of pharmacological studies have shown that opiate receptor agonists stimulate the HPA axis [35-38]. However, the response of the HPA axis to stress was not diminished in transgenic mice that do not express any of the three classic opiate receptors [39], indicating that either the opiates are not *necessary* for the HPA response, or that compensatory mechanisms have occurred in those knockout animals. In a more recent study, each of the classic EOP were selectively deleted, and the CORT response to a zero maze test was significantly affected by each individual deletion [40]. In this study, enkephalin knockouts had lower basal CORT levels than wild-type mice as well as

a smaller but longer-lasting CORT response to stress. Dynorphin knockouts had a faster and longer-lasting CORT response to stress than wild-type animals, and β-endorphin knockouts had a smaller CORT response that lasted as long as the wild-type response. Such discrepancies in findings further emphasize the importance of characterizing knockout animals before subjecting them to different physiological conditions.

The major change in opiate receptor expression in our study was that OFQ/N knockout males had significantly less hypothalamic KOP mRNA and protein. This reduction in KOP protein and mRNA is in contrast to the results of Clarke et al. [11] who reported that KOP protein levels were not significantly different in OFQ/N knockouts, but that NOP was upregulated. One possible explanation for the discrepancy in results may be due to differences in detection methods. Clarke et al. [11] quantified receptor expression using ligand autoradiography binding; however, we used real-time PCR and western blot analysis to quantify receptors. Real-time PCR is the most sensitive technique currently available for measuring mRNA levels, and is able to detect very small changes in transcript expression [28]. In our study, the decrease in KOP was detected in male mice at the mRNA level and then confirmed at the protein level. In female mice, however, we did not see a reduction in mRNA, but did detect reduced KOP protein. Lack of correlation between mRNA and protein levels have been reported by others [34, 41] and, because the receptor protein binds the opiate, its level is probably more physiologically relevant. Although our results do not differentiate between intracellular (e.g. recycling or newly synthesized) and extracellular membrane-bound receptor protein, it is clear that the total pool of available KOP protein is significantly reduced in both genders of OFQ/N knockout mice. This may either affect acute signaling properties of KOR-expressing neurons or their ability to respond to chronic activation. Receptor binding studies would be an additional, reliable method to determine the density of cell surface opiate receptors [42-44] and would be a potential way to confirm the results of our study.

KOP receptors preferentially bind dynorphin, which, of all the opiate peptides, shares the highest sequence homology with OFQ/N. Because both male and female knockout animals had reduced KOP protein levels, these animals may have higher concentrations of hypothalamic dynorphin. Downregulation of opiate receptors is known

to occur when opiate receptors are exposed to increased concentrations of their respective endogenous ligands, e.g. in models of addiction (for reviews see [45, 46]). To date, hypothalamic opiate peptide levels have not been determined in OFQ/N knockout mice, therefore, it is possible that increased dynorphin produced KOP downregulation. This possibility is currently being examined.

Similar to the other opiate peptides [1, 47, 48], OFQ/N is likely involved in mediating the stress response [49-52], but its role is not well understood. It is clear that one of the effects of the opiates is to stimulate PRL secretion, which, in turn, causes upregulation of PRL-R in the choroid plexus. This mechanism allows for increased PRL uptake into the brain during stress [53, 54] and PRL is known to influence the stress response [53]. We hypothesized that, if OFQ/N knockout mice have increased susceptibility to stress [10], they may have higher PRL-R expression levels in the choroid plexus. However, we found that PRL-R expression levels in the choroid plexus were not different between the genotypes, indicating that, under resting conditions, this mechanism of transporting PRL into the brain [54] is probably the same in both genotypes. This result is also consistent with the finding that basal levels of PRL are not different between genotypes [12].

In summary, OFQ/N knockout mice have decreased levels of KOP mRNA and receptor protein but levels of the other opiate receptors are similar between genotypes. The physiological consequences of decreased KOP are not known, but the neuroendocrine response to stress is not affected by this downregulation (CH 4). No genotype difference was detected in PRL-R mRNA levels in the choroid plexus under resting conditions, indicating that PRL transport into the brain in knockout mice is not different from wild-type mice. Taken together, these results indicate that, with the exception of KOP downregulation, there is no compensation in opiate receptor expression in OFQ/N knockout mice.

Table 1. Primer sequences for real-time PCR

Gene	5' Primer	3' Primer
PRL-R	AAGCCAGACCATGGATACTGGAG	AGCAGTTCTTCAGACTTGCCCTT
MOP	CCCAGTTCTTTATGCGTTCCTG	CAGTTAGGGCAATGGAGCAGTT
NOP	TCCTGCCTGCCTTTCTGCTAA	GCTGCCATACAAGACCTCCCA
KOP	CCGCTGTCTACTCTGTGGTAT	AGTAACCAAAGCATCTGCCAAA
DOP	TCATGTTTGGCATCGTCCGGTA	AACGGCCACGTTTCCATCAA
L7	GAAGCTCATCTATGAGAAGGC	AAGACGAAGGAGCTGCAGAAC

Table 2. Specific conditions for western blotting

Protein (kD)	***% Acrylamide***	***Transfer Conditions***	***Primary Antibody***
MOP (45)	8	Overnight, 4°C- 2000mA total current	Rabbit anti-MOP (Chemicon); 1:90,000; 2hr
KOP (46)	10.5	45 min - 100V	Rabbit anti-KOP (Affinity Bio-Reagents); 1:70,000; overnight, 4°C
GAPDH (35)	Same as protein of interest	Same as protein of interest	Rabbit anti-GAPDH; 1:50,000 (with MOP); 1:200,000 (with KOP) (Novus)

Fig 1a.

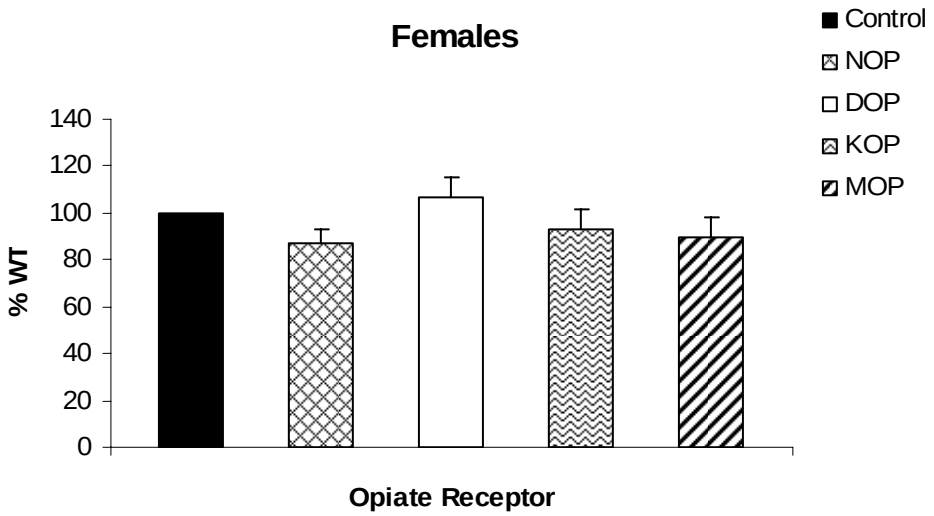

Fig 1b.

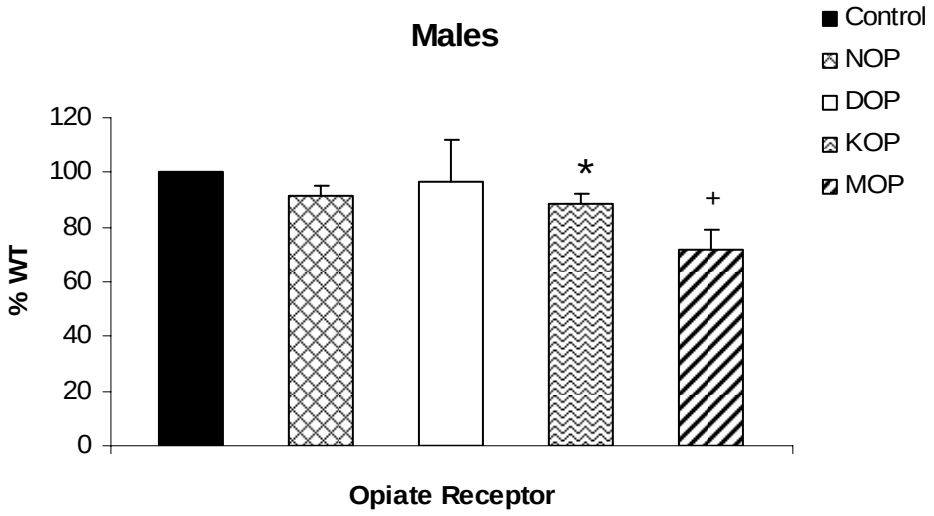

Figure 1. Hypothalamic mRNA levels of NOP, DOP, KOP and MOP were quantified by real-time PCR in wild-type and knockout female (a) and male (b) mice (n=6 each gender and genotype). Wild-type levels for each opiate receptor were set to 100%, (black bar, control) and knockout levels were expressed as a percent of wild-type. There was a significant decrease in both KOP and MOP mRNA in knockout males.
* Significantly less than wild-type, p = 0.03; + Significantly less than wild-type, p = 0.01).

Fig 2a.

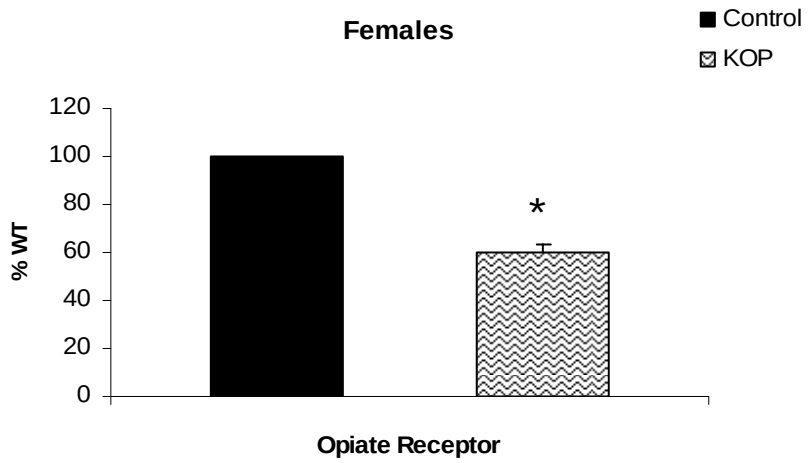

Fig 2b.

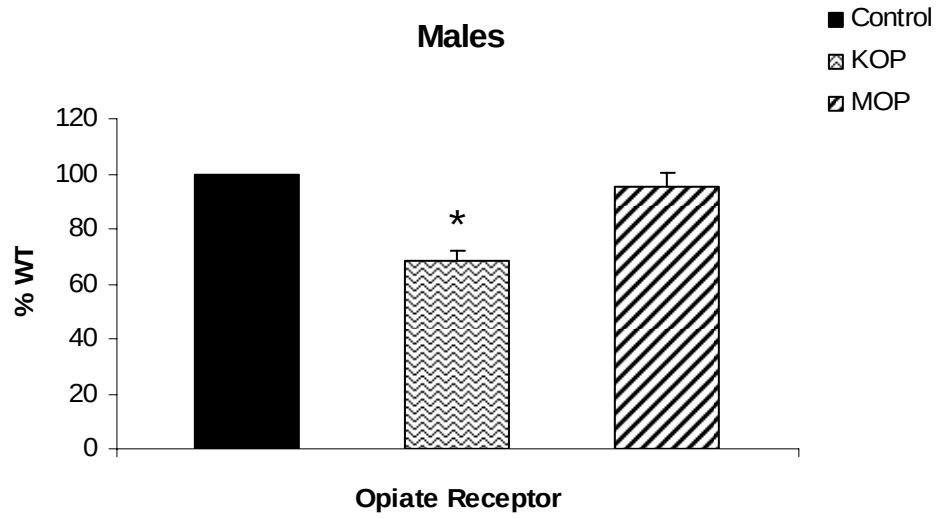

Figure 2. Hypothalamic protein levels of KOP were quantified by western blot analysis in wild-type and knockout female (a) (n = 5 WT; n = 6 KO) and male (b) (n = 4 WT; n = 4 KO) mice. Levels of MOP were measured in males only (b) (n= 6 WT; n = 5 KO). Wild-type levels for each opiate receptor were set at 100% (black bar, control) and knockout levels are expressed as a percent of wild-type. There was a significant decrease in KOP receptor protein in both female and male knockout mice.

* Significantly less than wild-type, p = 0.006 for females (a) and 0.01 for males (b).

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Corticosterone and prolactin response to various stressors in Orphanin FQ/Nociceptin (OFQ/N) knockout mice

Abstract

These studies were conducted to examine the role of Orphanin FQ/Nociceptin (OFQ/N) in modulating hypothalamic-pituitary-adrenal (HPA) axis activity and the prolactin (PRL) secretory response to stress. Wild-type and OFQ/N knockout male and female mice were subjected to restraint, orbital shaker or platform shaker under acute, repeated and chronic conditions. All stressors tested resulted in increased HPA axis activation, as indicated by increased plasma corticosterone (CORT) levels, and increased PRL secretion, although the secretory profile of the two hormones were different. Additionally, the loss of OFQ/N did not affect the ability of males to habituate to repeated shaker stress. Females did not show similar signs of habituation, suggesting there is a gender difference in this response. There were few genotype differences in the the stress-induced CORT and PRL increases, indicating that OFQ/N is not necessary for these neuroendocrine responses. Both the CORT and PRL secretory responses were stressor and time course dependent, with unique response profiles for each hormone. Therefore, these factors must be carefully considered when interpreting results from stress experiments.

Introduction

The stress response is very complex containing multiple levels of neural control, and it is essential for survival, activating physiological processes that reestablish homeostasis after stress [1-3]. The activation of the hypothalamic-pituitary-adrenal (HPA) axis is a key neuroendocrine response to stress and corticotropin releasing factor (CRF) is the main regulator of the HPA axis [2]. CRF stimulates corticotrophs in the anterior pituitary gland to secrete adrenocorticotropic hormone (ACTH), which stimulates the release of glucocorticoids from the adrenal cortex. Glucocorticoids, such as corticosterone (CORT), mobilize metabolic stores to increase blood glucose levels.

Herman et al. [1] have classified stressors based on the nature of the stress, as well as the neurocircuitry involved in mediating the response. *Predicted stress* occurs

when a threat is perceived, e.g. in an open field test or a novel environment, rather than a direct threat to the individual. Cognitive processing is necessary to elicit a physiological response, and this type of emotional, psychogenic stress activates limbic system pathways [1, 3]. The second type of stress is *real*, which represents an actual physical threat to the organism, e. g. hypoxia, hemorrhage or pain (reviewed in Herman et al. [1]). While both types of stress activate CRH neurons in the hypothalamus, a real stressor triggers neural pathways that act directly on the hypothalamus via efferent pathways from the brainstem (reviewed in Herman et al. [1]). While activation of the HPA axis is critical during stress, it is also important to terminate the response to avoid deleterious effects [1, 3, 4]. Therefore, this response is tightly controlled in order to execute efficient termination. Additionally, exposure to a repeated homotypic (same) stressor results in adaptation or habituation of the HPA axis [1, 5].

The endogenous opioid peptides (EOP) are clearly involved in the stress response [6-9] . Although, in general, the opiates act as anxiolytic peptides [10], the role of Orphanin FQ/Nociceptin (OFQ/N) remains controversial. OFQ/N has been shown to have an anxiolytic role [11, 12], while other studies have shown OFQ/N to act as an anxiogenic agent [13, 14]. In addition to the EOP, prolactin (PRL) has also been implicated in the stress response. Stress causes an increase in circulating PRL levels [15, 16] due, at least partially, to activation of EOP neurons that act to suppress hypothalamic tuberoinfundibular dopaminergic (TIDA) neuronal activity [15, 17]. This increased circulating PRL causes upregulation of prolactin receptors (PRL-R) in the choroid plexus [18] and increases PRL transport into the brain [19] via these specific PRL-R [20, 21]. PRL, acting centrally, has been shown to have protective effects in response to stress, such as prevention of hypocalcemia and ulcerogenesis [18, 22]. It has also been suggested that PRL has an immunoregulatory function that helps protect an individual from the deleterious consequences of stress, and it may help maintain homeostatic balance during periods of stress [15]. The mechanisms responsible for PRL release and the role of PRL in mediating the stress response are not clear, but antagonizing CRH receptors (CRH-R) significantly attenuated the PRL response to the elevated plus-maze [23], indicating that HPA axis activation stimulates PRL secretion. The purpose of these studies was to further investigate the role of OFQ/N in activating the HPA axis and in

stimulating PRL release following stress. Because the type and duration of the stress exposure can influence the neuroendocrine response [24], a variety of stressors of different potencies following different time courses were used. Additionally, we examined the effect of OFQ/N deletion on adaptation to repeated, homotypic stress. We predicted that, if OFQ/N exerts an anxiolytic role, the deletion of this peptide would hinder the ability of animals to adapt to the stress.

Animals

Male and female OFQ/N knockout and wild type mice (2-3 months old, 17-32g) were housed 2-3 per cage under controlled temperature (21ºC) and light (12h light:12h dark) conditions and were given food and water *ad libitum*. Experiments were conducted following the guidelines of the Animal Welfare Act and all protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at Miami University.

OFQ/N knockout mice were generated as described previously [11]. All animals were genotyped using standard PCR (polymerase chain reaction). Ear punches (2mm in diameter) were taken for identification purposes, while the animal was under isoflurane anesthesia. The tissue was subjected to DNA extraction using the HotShot method [25]. Isolated DNA was used in a PCR reaction containing 2mM $MgCl_2$, 200µM each dNTP and 0.4µM of each of the following primers: OFQ/N fwd: GACCCAGAGCTTGTGTCAGC; OFQ/N rev: CTCATAAACTCACTGAACCGC, and neomycin cassette in the transgenic mice: CCGGAGAACCTGCGTGCAATCC. Cycling parameters were as follows: 94°C for 3 min, followed by 31 cycles of: 94°C for 30 sec, 60°C for 30 sec, 72°C for 45 sec, and a final extension of 5 min at 72°C. Following gel electrophoresis, bands were scored as follows: 250 bp product only = wild-type, 550 bp product only = knockout, 250 and 550 bp products = heterozygote.

Experiment 1– CORT and PRL response to various stressors

Female and male wild-type and knockout mice were randomly assigned to one of 5 groups. Group 1 (control) was handled daily for 8 days but not subjected to any

stressor. They were sacrificed by rapid decapitation on the last day of the experiment. Animals in Group 2 (Acute Restraint: AR) were sacrificed immediately following 30 minutes of restraint stress to determine the animals' stress response to a one-time only 30 minute bout of restraint (control for Group 3). Group 3 (Chronic restraint: CR) was restrained for 30 min every day for 8 days, a paradigm known to induce habituation in rats [5], and were sacrificed after the final bout of restraint. Group 4 (Acute Shaker: AS) was subjected to orbital shaker stress for 30 minutes and immediately sacrificed (control for Group 5). Group 5 (Chronic Restraint + Acute Shaker: CR + AS) was restrained for 30 min every day for 8 days and subjected to a novel, acute stress of 30 min on the orbital shaker on day 9 and immediately sacrificed.

Experiment 2 – Time course of the CORT and PRL response to acute orbital shaker stress

We further examined the time course of the CORT and PRL responses to orbital shaker stress. Female and male wild-type and knockout mice were subjected to orbital shaker stress using a BioShaker (Molecular Technologies, Inc.; St. Louis, MO) set to maximum speed for 5 (Group 1), 15 (Group 2) or 30 (Group 3) minutes. Animals were sacrificed immediately following the stress bout.

Experiment 3 – CORT response to acute platform shaker stress and after 3 and 5 days of stress

Since animals failed to habituate to the chronic restraint paradigm in Experiment 1, we further examined habituation in wild-type and knockout males by subjecting them to platform stress for different periods of time. Basal blood samples were collected by tail clip under resting conditions 24 hours prior to any exposure to stress. Briefly, animals were removed from their home cage and placed in a plexiglass chamber. The tip of the tail was snipped and blood was collected into capillary microfuge tubes. Blood collections were completed in less than one minute to minimize any effects of stress associated with handling or blood sample withdrawal. Animals were then divided into three groups. In one group, animals were subjected to 5 minutes of shaker stress

(Eberbach platform shaker, 150 cycles/min) [26]. Blood samples were taken by tail clip immediately following this acute exposure and 20 minutes after the termination of stress. Separate groups of animals were subjected to repeated bouts of 5 minutes of shaker stress at 30 minute intervals for 3 or 5 days.

Experiment 4 – CORT and PRL response to variable bouts of platform shaker stress

Based on the results of experiment 3, mice habituated to platform shaker stress by 3 days, as indicated by CORT levels. To determine the time course of habituation, female and male wild-type and knockout mice were individually housed and randomly assigned to 1 of 5 groups. Group 1 (control) were untreated; Group 2 (acute) were subjected to 1 bout of shaker stress (Eberbach platform shaker 150 cycles/min) [26] for 5 min and immediately sacrificed; Group 3, (3x); Group 4 (6x); and Group 5 (30x) were subjected to 3, 6 or 30 bouts, respectively, of 5 minutes shaker stress at 30 minute intervals (25 minutes of rest between each bout). Animals were sacrificed immediately following their final stress bout.

Radioimmunoassay (RIA)

At the time of sacrifice, trunk blood was collected from all animals and centrifuged (13,000x g, 5 min). Plasma was collected and stored at -20°C until CORT and/or PRL levels were measured by double antibody radioimmunoassay (RIA).

Plasma CORT concentrations were measured in duplicate samples using CORT RIA kits purchased from MP Biomedicals (Irvine, CA) following manufacturer's instructions. CORT levels are expressed in ng/ml. The interassay coefficient of variation was < 8% and the intraassay coefficient of variation was < 4%.

Plasma PRL concentrations were measured in duplicate samples using reagents obtained from NIDDK's National Hormone and Pituitary Program (NHPP) and Dr. A. F. Parlow. PRL was iodinated using $Na^{125}I$ (Perkin-Elmer, MA) as described by Greenwood and Hunter [27]. PRL levels are expressed in ng/ml. The upper and lower

limits for the PRL assay were 400 and 0.8 ng/ml, respectively. The intraassay coefficient of variation was 10% and the interassay coefficient of variation was 12%.

Statistical analysis

Data in different treatment groups did not appear to be normally distributed and the groups did not have similar variances. Appropriate Box-Cox transformations were applied so the resulting data could be assumed to be normally distributed. Groups were compared by analyzing the transformed data using either paired or two sample t-tests. Tests were adjusted by using the Bonferroni multiple comparison technique to ensure that the overall type I error probability for each set of comparisons was no more than 0.1. In Figure 1, data were analyzed using pairwise contrasts, except for the comparison between acute (AR) and chronic restraint (CR), which was analyzed with a two-tailed two-sample t-test. CORT data were square root transformed for females and log transformed for males, with respective p-values < 0.0058 (17 comparisons) and < 0.0053 (19 comparisons) required to declare significance. PRL data were log transformed for females and inverse transformed for males, with a p-value < 0.0125 (8 comparisons) required to declare significance. In Figure 2, data were analyzed using pairwise contrasts. CORT data were square root transformed, with a p-value < 0.011 required to declare significance. PRL data were log transformed, with a p-value < 0.011 required to declare significance. The data in Figures 3 and 4 were analyzed using pairwise contrasts following log (Figure 3) or inverse (Figure 4) transformation and a p-value < 0.0167 was required to declare significance. In Figure 5, possible genotype differences in CORT levels were analyzed using two-tailed two-sample t-tests. Comparisons of CORT levels between treatment groups were conducted using one-tailed two-sample t-tests. CORT data were square root transformed for females and log transformed for males, with p-values < 0.01 (10 comparisons) required to declare significance. PRL data were analyzed using two-tailed two-sample t-tests for genotype differences and one-tailed two-sample t-tests for comparison of stressed groups to control, with p-values < 0.0077 (13 comparisons) required to declare significance.

Results

Experiment 1 – CORT and PRL response to various stressors

All stressors examined produced a significant increase in circulating CORT levels in both female (Figure 1a) and male (Figure 1b) mice, regardless of genotype. The only significant difference between genotypes was that CORT levels in knockout females were significantly lower than wildtype subjected to acute orbital shaker stress following chronic restraint stress (CR + AS, Figure 1a). Also, the wild-type females in this group (CR + AS) had significantly greater CORT levels than animals that were only chronically restrained (CR). The only significant difference among treatment groups in males was that the chronically restrained (CR), knockout males had significantly higher CORT levels than the knockout mice that were acutely restrained (AR) (Figure 1b). The PRL response to stress was different from the CORT response. Neither acute orbital shaker stress (AS), nor the chronic plus acute shaker stress (CR +AS) produced any increase in PRL levels in females (Figure 1c) or males (Figure 1d) of either genotype. Similar to the CORT response, there was no significant effect of genotype.

Experiment 2 – Time Course of the CORT and PRL Response to Acute, Orbital Shaker Stress

Because 30 minutes on the orbital shaker did not stimulate PRL secretion, we more closely examined the time course of the response to determine if there was any transient increase in PRL release. Similar to the results from experiment 1, acute shaker stress produced significant increases in circulating CORT levels in both females (Figure 2a) and males (Figure 2b), regardless of genotype. In fact, CORT levels continued to increase in a time-dependent manner in all groups, except knockout males. Knockout males stressed for 15 or 30 minutes had significantly greater CORT levels than those stressed for only 5 minutes, but there was no significant difference between the levels at 15 and 30 minutes. Moreover, after 15 minutes of orbital shaker stress, CORT levels in the knockout males were significantly greater than the CORT levels in wild-type males at the same time (Figure 2b).

The PRL response to acute stress on the orbital shaker was markedly different than the CORT response. PRL levels in the knockout females were significantly higher

at 5 minutes compared to both 15 and 30 minutes, but, in the wild-type females, the PRL levels after 5 minutes were significantly greater than those in the 30 minute group only (Figure 2c). Further, the knockout females had higher PRL levels than the wild-type females at 5 minutes (Figure 2c). The males, regardless of genotype, did not respond to acute, orbital shaker stress (Figure 2c).

Experiment 3 – CORT Response to Platform Shaker Stress

To examine possible effects of OFQ/N deletion on stress adaptation, we measured CORT levels after acute and repeated stress. Regardless of genotype, acute stress produced a significant increase in CORT levels and the increase persisted for at least 25 minutes (Figure 3). Following 3 or 5 days of shaker stress (5 minutes of stress, every 30 minutes), there was no difference in CORT levels compared to basal values in either wild-type or knockout males (Figure 4).

Experiment 4 – CORT and PRL response to platform shaker stress

Because neither genotype exhibited any evidence of a stress response after 3 days of shaker stress, the CORT and PRL responses to shaker stress were determined at earlier time points. Female (Figure 5a) and male (Figure 5b) animals stressed on the platform shaker had significantly greater CORT levels than control, regardless of the number of bouts, and there were no significant difference between genotypes in any group. In males, (Figure 5b), but not females (Figure 5a), the animals exposed to 30 bouts of stress had significantly lower CORT levels than the animals exposed to 3 bouts.

There was no significant effect of stress or genotype on PRL levels in females (Figure 5c) or males (Figure 5d). Knockout females stressed for one bout showed a trend towards increased PRL levels compared to control, but it was not significant ($p = 0.033$).

Discussion

The results of these studies indicate that the deletion of the OFQ/N peptide did not abolish either the CORT or PRL secretory response to stress in males or females.

Additionally, the absence of this peptide did not affect the ability of males to habituate, at least to the shaker stress paradigm used in this study. Gender differences, especially in the PRL response, were detected in both the magnitude of the response, which was stressor dependent, and in the time course of habituation. Further, the magnitude of the CORT and PRL responses was both stressor and time dependent, and was different depending on the stressor, with acute shaker stress producing a significant increase in circulating CORT levels that persisted for at least 30 minutes, but only a rapid and transient increase in PRL levels.

Even in the absence of the OFQ/N peptide, all of the stressors used in the current study activated the HPA axis, as indicated by increased circulating CORT levels in both males and females. Restraint and orbital shaker stress also produced an increase in circulating PRL levels. Because both genotypes responded to the stress, it is clear that OFQ/N is not *necessary* to evoke either of these neuroendocrine responses. It was surprising, however, that there was very little difference between genotypes regardless of the type or duration of the stress stimulus. Some investigators have reported that OFQ/N acts as an anxiogenic peptide [13, 14, 28], while others report that OFQ/N, similar to the other endogenous opiates [10], is anxiolytic [11, 12, 29-31]. Additionally, in another study in which the classic EOP were selectively deleted, the CORT response to a zero maze test was significantly affected by each individual deletion [32]. Enkephalin knockout mice had lower basal CORT levels than wild-type mice, as well as a smaller, but longer-lasting, CORT response to stress, suggesting an anxiogenic effect. Dynorphin knockouts had a faster and longer-lasting CORT response to stress suggesting that dynorphin is anxiolytic, and β-endorphin knockouts had a smaller CORT response that lasted as long as the wild-type response, indicating some anxiogenic effect of this opiate. Although it seemed unlikely that the removal of one peptide would abolish the stress response, based on previous reports, we predicted that the magnitude or duration of the CORT and/or PRL response would vary between genotypes.

In our study, one significant effect of OFQ/N deletion was that knockout females, subjected to chronic restraint followed by a novel stress (orbital shaker), had significantly lower CORT levels than wild-type females (Figure 1a). Also, the wild-type females in this group had CORT levels that were significantly greater than the CR only-treated

females. These results suggest a possible anxiogenic role for OFQ/N in females. The increased response in wild-type animals is in agreement with Bhatnagar et al. [33], who reported that animals had a greater CORT response to a novel stress after being exposed to chronic stress. On the other hand, Ostrander et al. [34] demonstrated a blunted HPA response to a novel predicted (psychogenic) stressor, but this followed exposure to chronic variable stress, not the chronic, homotypic stress paradigm that was used in the current study.

A second genotype difference occurred in males after 15 min of orbital shaker stress (Figure 2b) with knockouts having a significantly greater CORT response than wild type mice. This faster rate of HPA axis activation could indicate a possible anxiolytic role for OFQ/N, but by 30 minutes, the CORT response was the same, so it seems unlikely that this is important physiologically.

The only significant effect of OFQ/N gene deletion on PRL secretion was that the PRL response to orbital shaker stress was significantly greater in knockout females (Figure 2c), suggesting an anxiolytic role for OFQ/N. This result contradicts the results reported by Petraglia et al. [35] who demonstrated that blocking classic EOP or their receptors significantly attenuated the PRL response to footshock stress in rats. However, the discrepancy in results may be due to the fact that these pharmacological studies were conducted in males, using a completely different stressor.

In the current study, we used different types and durations of stress to investigate the role of OFQ/N in the neuroendocrine response. Overall, our results demonstrate that OFQ/N is not critical for the stress response. Additionally, depending on the stress, we detected evidence for an anxiogenic and an anxiolytic effect or no effect at all. As seemingly contradictory as these results appear, they are consistent with other reports in the literature. OFQ/N has been reported to act as an anxiogenic peptide [13, 14, 28]. In these studies, OFQ/N was shown to increase anxiety behaviors in rats [14] and to increase circulating CORT levels after ICV injection [13, 14], a response that was blocked by an NOP antagonist [28]. These studies were performed in males only, and have been contradicted by others. In several reports, OFQ/N has been shown to have an anxiolytic role, with OFQ/N knockout mice displaying increased anxiety [11, 12], and OFQ/N attenuating anxiety in rats and mice [30], as well as reducing defensive behaviors

in mice during predator stress [31], and causing a decrease in CORT after ICV injection in mice [29]. Further, other studies, that used mice in which all three classic opiate receptors (mu, delta, and kappa) were genetically deleted, indicated that the HPA axis to stress was not diminished in the absence of opiate receptor activation [36]. Taken together, the role of the endogenous opiates in regulating the stress response is still not clear, but the type and duration of the stress, as well as the gender of the animal are factors that must be considered. While additional studies need to be conducted to elucidate the role of OFQ/N in mediating the neuroendocrine stress response, it seems improbable that a single neural factor or receptor will be essential for this response. Launching these neuroendocrine responses to stress is absolutely vital to the individual's survival [2] and it is highly unlikely that such a critically important response is dependent on one factor.

OFQ/N deletion also did not affect habituation. Although the EOP contribute to an individual's ability to adapt to a stressor [10], their specific roles and mechanisms of action are not clear, especially for OFQ/N. In our study, all males adapted to platform shaker stress in as few as 3 days (Figure 4), and showed signs of adaptation even after 30 bouts of stress (Figure 5b), but there was no difference between genotypes. Therefore, OFQ/N is not *essential* for adaptation, at least in males. We did not test females after 3-5 days, and females, in contrast to males, did not show signs of adaptation after 30 bouts of shaker stress (Figure 5a). This is in agreement with Haleem et al. [37] who reported that female rats did not adapt to a repeated restraint paradigm, while males did.

Results of the current study confirm that HPA axis activation is influenced by the type of stressor [1]. The magnitude of the CORT response to the orbital shaker was greater than the response to either chronic or acute restraint in females, but in males, the CORT response to chronic restraint and the orbital shaker was similar, with the response to acute restraint being lower. Additionally, there was even a difference depending on the type of shaker stress; the magnitude of the CORT response to the orbital shaker (Figure 2; 5 min) was greater than the response to the platform shaker (Figures 3, 4, and 5). This difference may be due to the strength of the stressor. The orbital shaker produced a faster, fuller (360^{o}) rotation while the platform shaker moved more slowly and laterally only.

In agreement with other studies, different stress paradigms evoked different CORT and PRL responses [24, 38, respectively]. This is not surprising because the neural pathways that control HPA axis activation [1-3] and PRL secretion [15, 17] are different and depend upon the type of stress [1]. All stressors produced an increase in CORT, but not PRL levels. Neither 30 minutes of orbital shaker stress nor 5 minutes on the orbital shaker produced any change in circulating PRL levels in males or females of either genotype (Figures 1 and 2). There was, however, a PRL increase in females subjected to the orbital shaker, but it was rapid, occurring only after 5 minutes, and transient, i.e. returned to basal values by 15 minutes (Figure 2c). On the other hand, both chronic and acute restraint stress elicited a robust PRL secretory response. The type of stressor clearly plays a role in the time course and magnitude of the neuroendocrine response.

It is interesting that in females, orbital shaker stress appeared to be a more potent stressor than restraint, as indicated by higher CORT levels (Figure 1a), but none of these animals maintained a PRL response for the 30 minute duration. They did, however, maintain the PRL response during the 30 minutes of restraint. PRL (for reviews see Ben Jonathan and Hnasko [17] and Freeman et al. [15]) and CORT (reviewed in Charmanadri et al. [2], Englemann et al. [3] and Herman et al. [1]) responses are controlled via different neural pathways which likely produces different patterns in the response. A clear example of this difference was the response to orbital shaker stress (Figure 2), because circulating CORT levels continued to increase as the time and stress persisted, but the PRL response had already returned to basal values by 15 minutes.

The physiological significance of the stress-induced PRL increase is still not clear, but HPA axis activation has been reported to stimulate PRL secretion. For example, administration of a CRH antagonist blocked the PRL response to restraint in female rats [39] and to the elevated plus maze in male rats [23]. Further, PRL has been shown to increase CORT secretion from adrenal cells *in vitro* [40, 41], which supports a stimulatory role for PRL. Studies by Torner et al. [42] suggest PRL plays an anxiolytic role; PRL administration decreased anxiety behaviors in male and female rats, and antisense treatment of PRL-R increased anxiety, but, in both cases, the treatment was chronic. Additionally, PRL administration prevented gastric ulcers and hypocalcemia in

chronically stressed rats [22], and knockdown of PRL-R in the PVN increased both of these deleterious consequences of chronic stress [43].

In summary, neither HPA axis activation, as indicated by increased CORT levels, nor the PRL secretory response to stress, was eliminated by OFQ/N deletion. These results indicate that OFQ/N is not necessary for either of these neuroendocrine responses to stress. It is unlikely that removal of one peptide would abolish such an important physiological response. Further, although females did not show any signs of habituation indicating a gender difference, males did habituate to repeated, homotypic, shaker stress regardless of genotype. This provides evidence that OFQ/N is not necessary for habituation in males. We have previously determined that there is little change in opiate peptide or receptor expression in OFQ/N knockout animals [44], so it is unlikely that compensation mechanisms, at least in the endogenous opiate pathways, eliminate the effects of OFQ/N deletion. The evidence for any significant role of OFQ/N in the stress response remains controversial, indicating that additional studies need to be performed. Because both the CORT and PRL responses to stress were time and stressor dependent, these variables must be carefully considered when interpreting results.

Figure 1a.

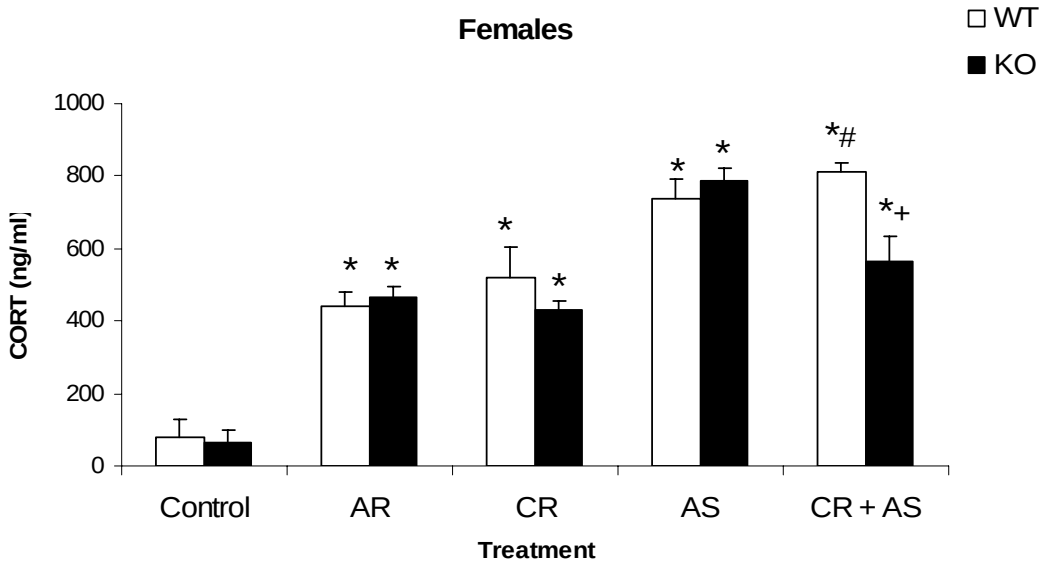


Figure 1b.

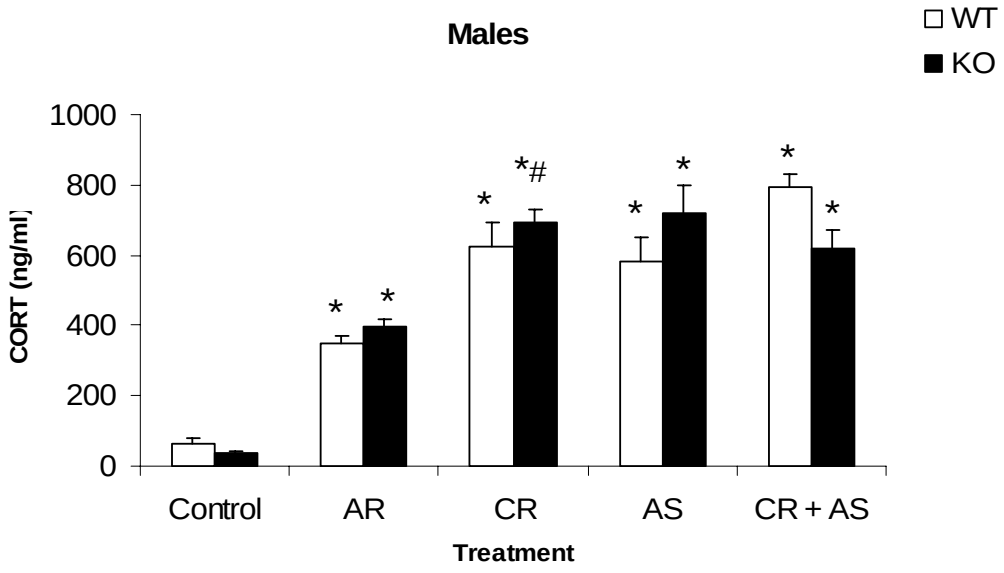


Figure 1c.

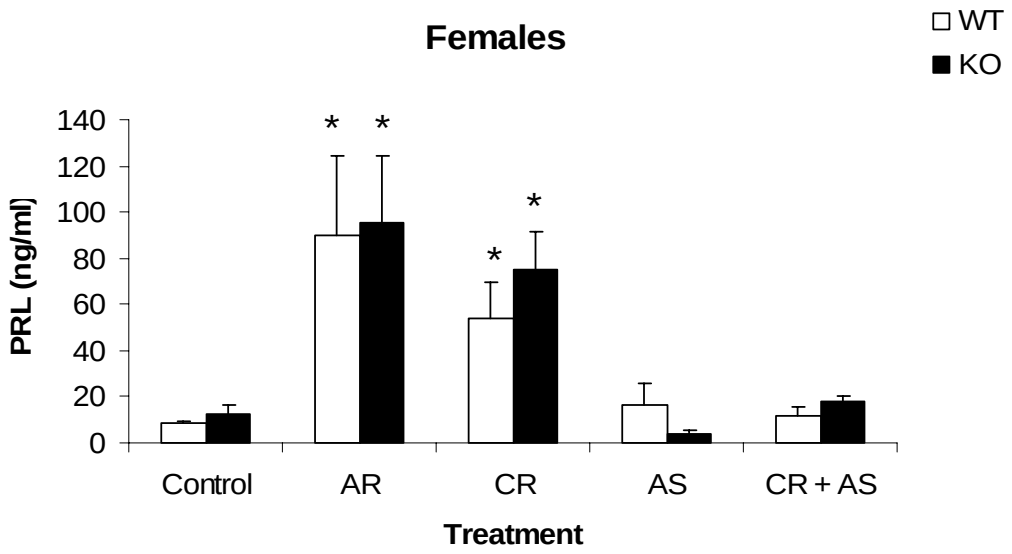


Figure 1d.

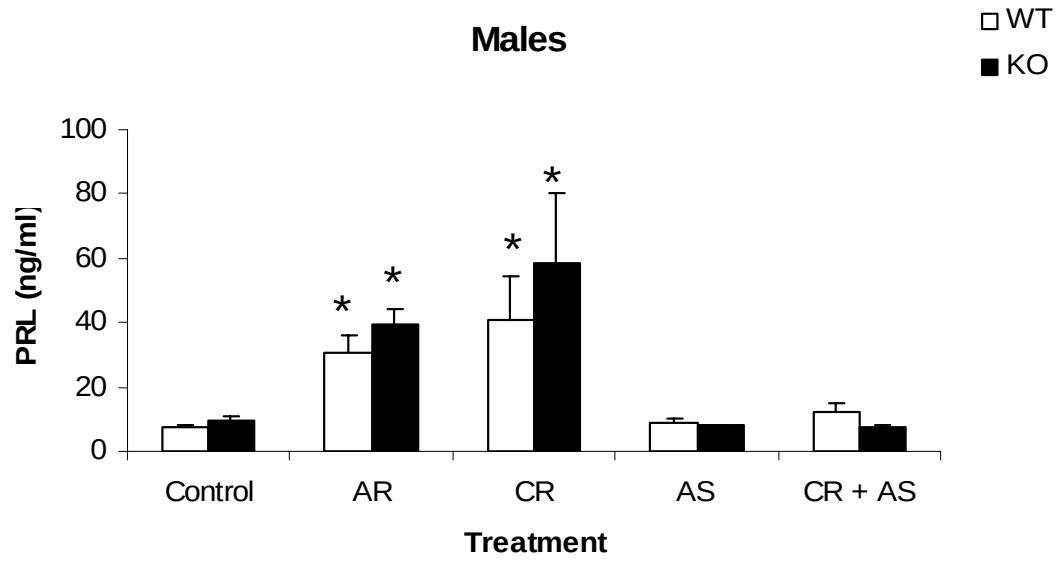


Figure 1. CORT (a, b) and PRL (c, d) levels were measured in wild-type (WT) and knockout (KO) females (a, c) and males (b, d). In group 1, control animals were not subjected to any stress, but were handled daily for 8 days. Animals in group 2 (acute restraint stress, AR) were handled daily for 7 days and restrained for 30 minutes on day 8 immediately prior to sacrifice. Animals in group 3 (chronic restraint, CR) were restrained for 30 min day for 8 days and sacrificed following the final bout of restraint. In Group 4, animals were treated with acute orbital shaker stress (AS). They were handled daily for 8 days and then, on day 9, they were subjected to orbital shaker stress for 30 minutes and immediately sacrificed. In group 5 (Chronic Restraint + Acute Shaker stress group: CR + AS), animals were restrained 30 min each day for 8 days. On day 9, animals were placed on the orbital shaker to expose them to a novel, acute stress, and were sacrificed after 30 minutes. Values are means ± S.E.M. The numbers of animals per group were: Control (females: WT = 5, KO = 6; males: WT = 6, KO = 4); AR (females: WT = 5, KO= 5; males: WT = 6, KO = 6); CR (females: WT = 5, KO = 4; males: WT = 6, KO = 6); AS (females: WT = 10, KO = 5; males: WT = 6, KO = 5); CR + AS (females: WT = 5, KO = 4; males: WT = 6, KO = 6).

(a) * Significantly greater than control ($p < 0.0001$)
+ Significantly less than WT ($p = 0.0056$)
# Significantly greater than CR ($p = 0.0004$)
(b) * Significantly greater than control ($p < 0.0001$)
Significantly greater than AR ($p = 0.0001$)
(c) * Significantly greater than control (AR: WT, $p = 0.0004$; KO, $p = 0.0006$; CR: WT, $p = 0.0046$; KO, $p = 0.0021$)
(d) * Significantly greater than control (AR: $p < 0.0001$; CR: WT, $p < 0.0001$; KO, $p = 0.0003$)

Figure 2a.

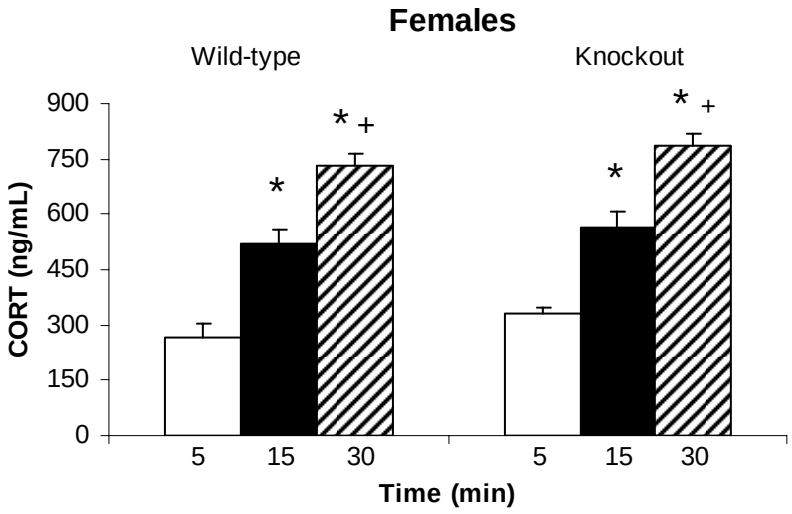


Figure 2b.

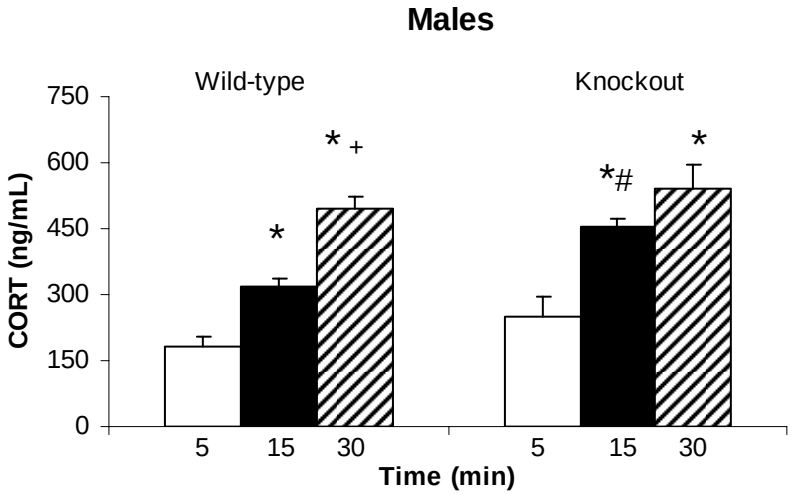


Figure 2c.

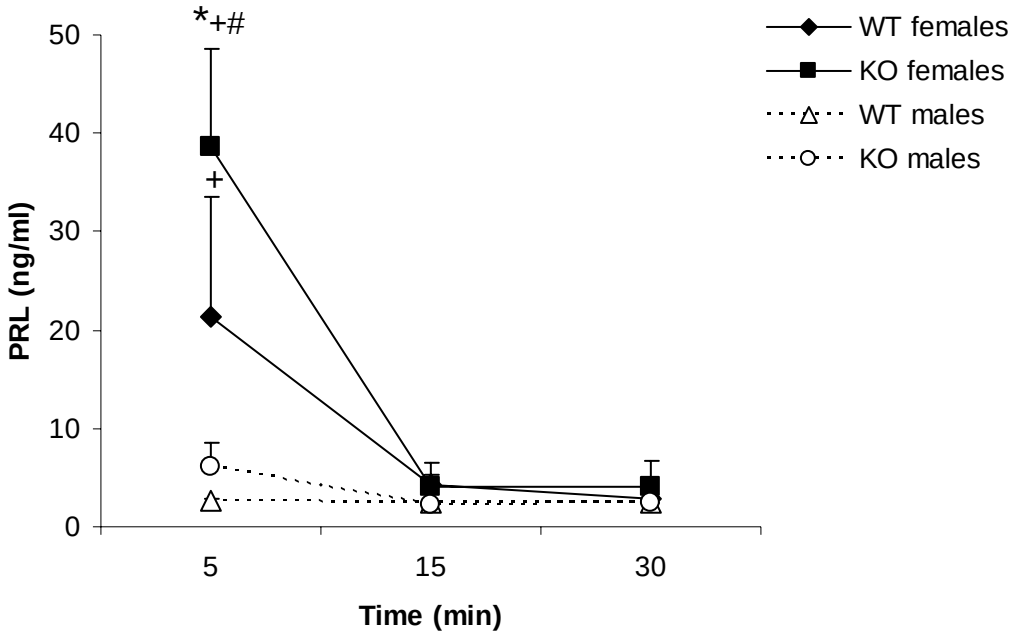


Figure 2. CORT (a, b) and PRL (c) levels were measured in wild-type (WT) and knockout (KO) females (a, c) and males (b, c) subjected to 5, 15, or 30 min of orbital shaker stress. Animals were sacrificed immediately after stress (n = 6, all groups). Values are means ± S.E.M.

(a) * Significantly greater than 5 min ($p < 0.0001$)
+ Significantly greater than 15 min ($p < 0.0005$)
(b) * Significantly greater than 5 min (15 min: WT, $p = 0.0019$; KO, $p < 0.0001$; 30 min: $p < 0.0001$)
+ Significantly greater than 15 min ($p = 0.0018$)
# Significantly greater than WT ($p = 0.01$)
(c) * Significantly greater than 15 min ($p = 0.0003$)
+ Significantly greater than 30 min (WT, $p = 0.031$; KO, $p = 0.0005$)
# Significantly greater than WT ($p = 0.0312$)

Figure 3.

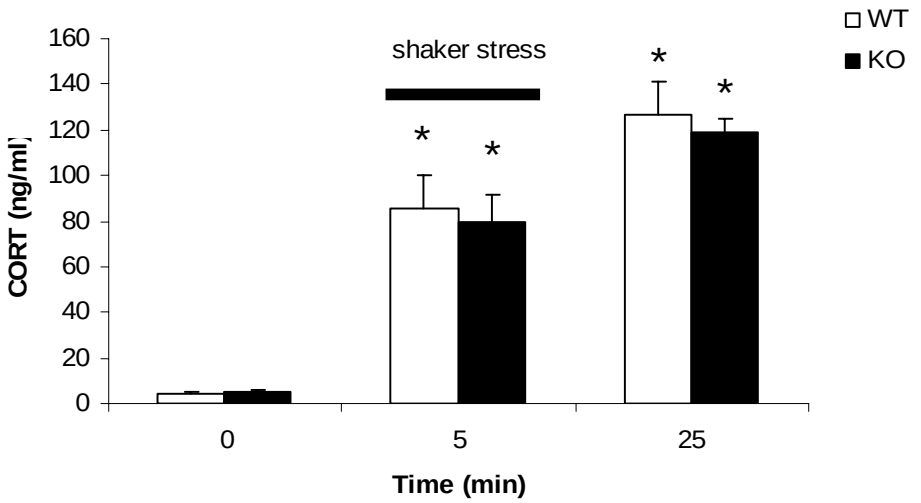


Figure 3. CORT levels were quantified in serial samples from wild-type (WT) and knockout (KO) males (n = 6 per genotype) at resting conditions (Basal), following 5 min of shaker stress (Immediate), and 20 min after the stress ended (+ 20). Values are means ± S.E.M.

* Significantly greater than basal levels in the same genotype ($p < 0.0001$)

Figure 4.

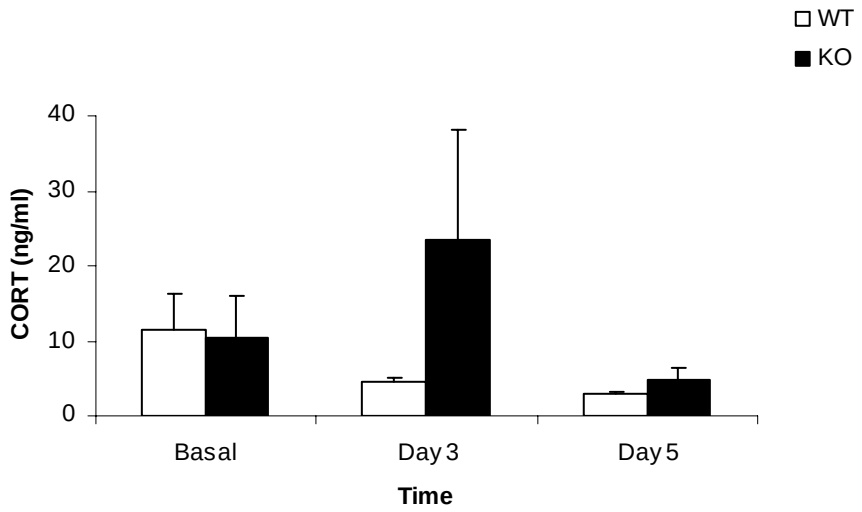


Figure 4. CORT levels were measured in serial blood samples taken from wild-type (WT) and knockout (KO) males (n = 5 per genotype) under resting conditions (Basal) and following 3 (Day 3) and 5 days (Day 5) of platform shaker stress. Shaker stress was given in 5 minute bouts every 30 minutes. Values are means ± S.E.M. There were no significant differences among any groups.

Figure 5a.

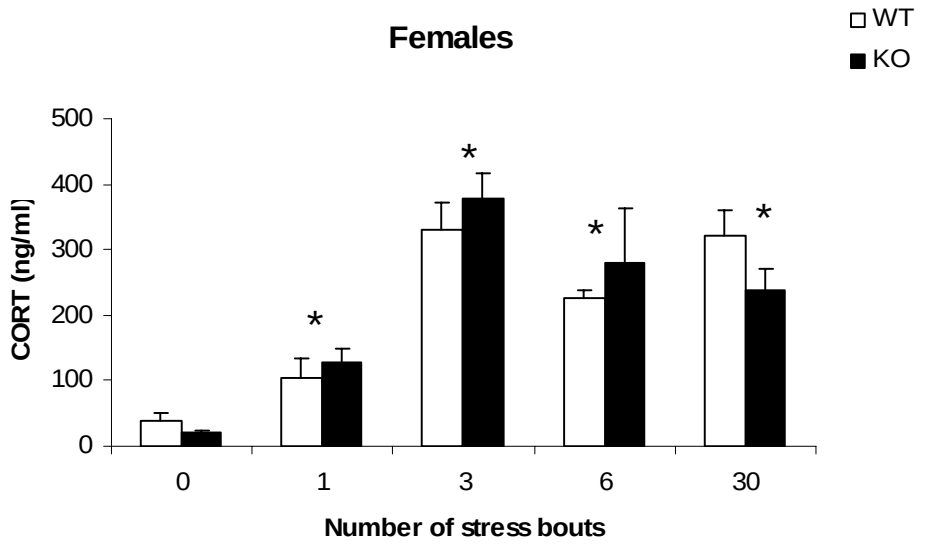


Figure 5b.

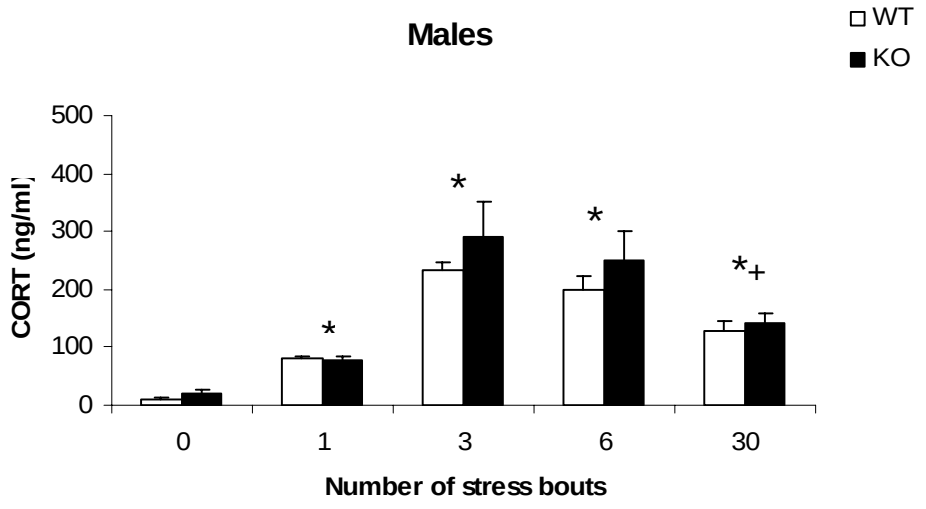


Figure 5c.

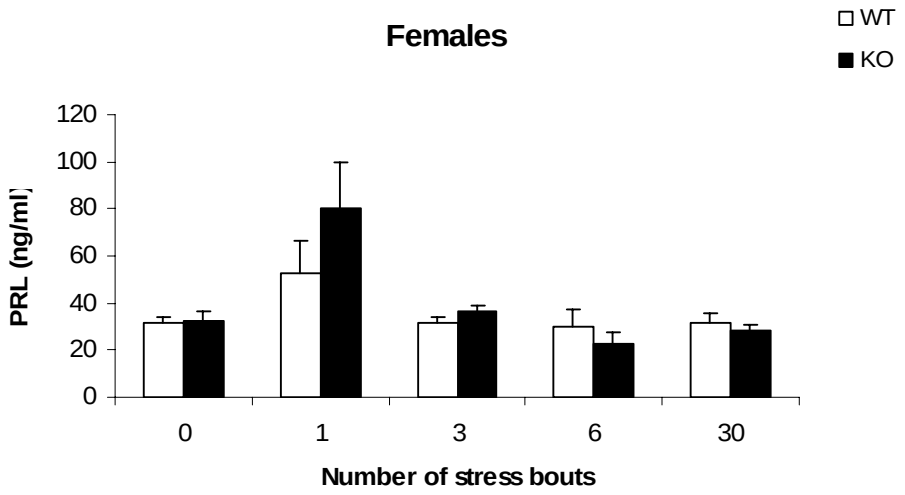
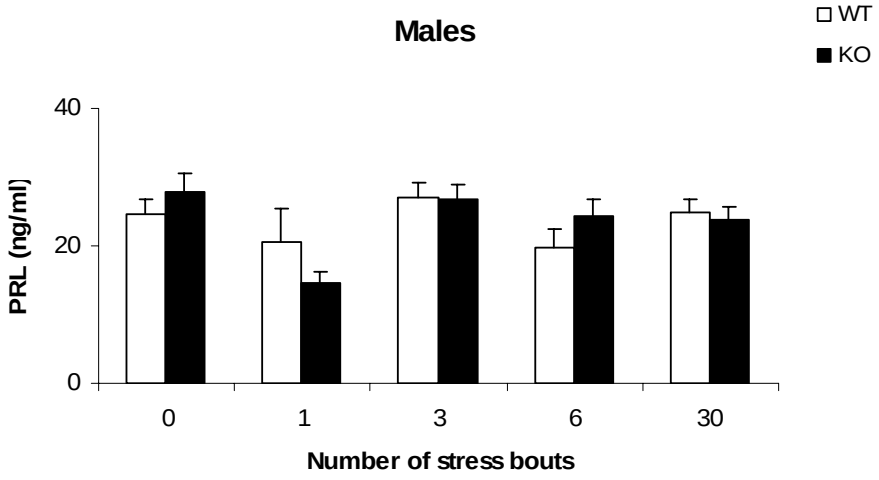


Figure 5d.



5. CORT (a, b) and PRL (c, d) levels were measured in wild-type (WT) and knockout (KO) females (a, c) and males (b, d) that remained in their home cage (control) or were subjected to 1, 3, 6 or 30 bouts of platform shaker stress (5 minutes) at 30 minute intervals and then sacrificed immediately after the final bout of stress. Values are means ± S.E.M. The numbers of animals per group were: Control (females: WT = 15, KO = 17; males: WT = 18, KO = 18); Acute (females: WT = 6, KO = 6; males: WT = 6, KO = 5); 3x (females: WT = 16, KO = 17; males: WT = 18, KO = 18); 6x (females: WT = 6, KO = 6; males: WT = 6, KO = 6); 30x (females: WT = 17, KO = 16; males: WT = 18, KO = 18). Because there was no significant difference between genotypes in any group, CORT levels of both genotypes were pooled for comparisons between groups.

(a) * Significantly greater than control ($p < 0.0001$)
(b) * Significantly greater than control ($p < 0.0001$)
+Significantly less than the same genotype subjected to 3 bouts of stress ($p < 0.0001$)

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Conclusions:

These studies are the first to critically examine neuroendocrine responses under basal and stressed conditions in OFQ/N knockout animals. While CORT levels have been measured in OFQ/N knockouts (Köster et al., 1999), no other studies to date have so thoroughly investigated the response of both the HPA axis and PRL under resting conditions and in response to various stressors. Under basal conditions, PRL (Chapter 1) and CORT (Chapter 2) levels were not different between the genotypes. This finding was in marked contrast to initial reports that knockouts had increased CORT under resting conditions (Köster et al., 1999). In my studies, I found that the experimental conditions, including treatment of the animals, were critical. Transporting animals from the animal facility did produce an increase in CORT levels, but there was still no difference between the genotypes (Chapter 2). Handling the animal, providing time for the animal to acclimate to a novel environment, and the control treatment itself, e.g. injecting a vehicle for a drug, are all potential sources of stress, and knockout animals may be more susceptible to these "nonspecific" types of stressors. Perhaps this accounts for the difference between our results and those reported by Köster, et al., (1999). Also, there was no genotype difference in PRL-R levels in the choroid plexus (Chapter 3), indicating that PRL transport into the brain at rest is similar. If OFQ/N knockout animals are indeed more anxious (Köster et al., 1999; Ouagazzal et al., 2003), it is not due to increased PRL transport into the brain under resting conditions.

My results also demonstrate that knockout mice can respond to OFQ/N injections with a robust PRL response (Chapter 1), indicating that the NOP are functional. This was consistent with the finding that hypothalamic mRNA levels for the NOP receptor were not significantly different between the two genotypes (Chapter 3). Interestingly, there was a significant genotype difference in hypothalamic KOP mRNA levels in both males and females (Chapter 3). These receptors preferentially bind dynorphin (Reisne, 1995), the endogenous opiate that is most similar to OFQ/N (Meunier et al., 1995; Reinscheid et al., 1995). One possible explanation for this decrease in KOP mRNA is that there are increased dynorphin levels to compensate for OFQ/N deletion, and this is currently under investigation.

The neuroendocrine response to stress is vital for the survival of the organism and its regulation is extremely complex and dependent on a number of different neural pathways and factors (Herman et al., 2003). While behavioral assays have provided evidence that OFQ/N can act as an anxiolytic (Jenck et al., 1997; Griebel et al., 1999; Köster et al., 1999; Ouagazzal et al., 2003) or an anxiogenic (Devine et al., 2001; Fernandez et al., 2004) peptide, my results indicate that when the peptide was genetically deleted, crucial neuroendocrine responses were not abolished. Every type and variety of stress (Chapter 4) examined produced activation of the HPA axis and two stressors increased PRL secretion, a strong indication of the importance of these neuroendocrine responses. Further, males of both genotypes were able to habituate to homotypic shaker stress, which indicates that OFQ/N is also not necessary for adaptation to occur. Females did not show signs of adaptation, suggesting that there is a gender difference in the time course for habituation to a homotypic stressor.

Overall, there were few genotype differences in the CORT or PRL response to stress. Knockout females had significantly lower CORT levels than wild-type when exposed to a novel orbital shaker stress following 8 days of chronic restraint (Chapter 4). These results suggest a possible anxiolytic role for OFQ/N in females. In contrast, knockout males had significantly higher CORT levels than wild-type after 15 minutes of acute orbital shaker stress, but this difference did not persist, so it is unlikely that this is an important physiological difference. Knockout females had significantly greater PRL levels than wild-type females after 5 minutes of acute orbital shaker stress, indicating an anxiolytic role for OFQ/N in females. These results are consistent with the current literature, as multiple studies have indicated either anxiolytic or anxiogenic roles for OFQ/N.

In my study, CORT levels of males and females that were injected with either OFQ/N or saline were significantly greater than basal (Chapter 2). Therefore, OFQ/N itself did not cause an increase in CORT, but the stress of injection did. Additionally, animals that were transported from their home environment to the laboratory had significantly greater CORT levels than animals sampled in the home environment, even when those transported had an acclimation period of 30 minutes (Chapter 2). This indicates that the transported animals were stressed from the move to a novel

environment, and CORT levels did not have ample time to return to normal. Also, CORT and PRL both had unique response levels and time courses depending upon the type of stressor applied to the animals (Chapter 4). This is not surprising, given the complexity of the neural network involved in modulating the stress response, and that different pathways are activated depending upon the nature of the stressor. Clearly, the magnitude of the stress and the time(s) that samples are collected are extremely important factors to consider. Further, even just removing animals from their home environment applies a stressor, and must be taken into consideration. Studies to date have varied in nature, that is, some were behavioral, others used pharmacological approaches, and some included quantifying stress hormone levels. When reviewing results of these studies, one must consider these differences. In fact, many factors need to be taken into consideration when planning and/or interpreting a study involving stress trials or measuring stress hormones, because the HPA axis is extremely sensitive.

Finally, several studies have provided evidence that OFQ/N influences behavioral responses to stress (Jenck et al., 1997; Köster et al., 1999; Devine et al., 2001; Ouagazzal et al., 2003; Fernandez et al., 2004), which I did not examine. It is possible that behavioral indices of stress would be different between the genotypes because the neural pathways that regulate these responses are different from the pathways that regulate the neuroendocrine pathways (Herman et al., 2003; Englemann et al., 2004). Therfore, although OFQ/N deletion does not abolish critical neuroendocrine responses, its removal can still have a significant impact on other neural pathways in the brain. It is important to remember that it is the neuroendocrine response, particularly the activation of the HPA axis, is necessary for survival (Herman et al., 2003). While altered behavior may have negative consequences, the animal still has the metabolic responses necessary to handle the physiological demands of stress.

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