The Role of Orphanin FQ (OFQ/N) in Mediating Adaptation to Chronic Stress

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by

Jennifer Elaine Kelbley

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ABSTRACT

THE ROLE OF ORPHANIN FQ (OFQ/N) IN MEDIATING ADAPTATION TO CHRONIC STRESS

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Stress causes complex neuroendocrine changes that include activation of the hypothalamic-pituitary-adrenal (HPA) axis, ultimately increasing levels of corticosterone (CORT) in the bloodstream. The purpose of this study was to determine the role of the opiate Orphanin FQ/Nociceptin (OFQ/N) in mediating adaptation to chronic stress. To do this, CORT levels were measured under resting and stress conditions in OFQ/N knockout mice. There was no significant difference in the basal CORT levels of knockout mice compared to their wild type littermates. An intermittent shaker stress was used to elicit the stress response in these mice. The shaker was automatically controlled to deliver 5 minute bouts of shaking every half hour. Knockout and wild type mice were exposed to 1, 3, 6 or 30 of these bouts of stress. All treatment groups were statistically higher than the controls which received no stress (p<0.01). The 3 bout group was the only treatment group in which knockout CORT levels were statistically higher than wild type levels (p=0.03). The CORT levels for both genotypes began to decline in the 6 bout and 30 bout groups. These results suggest that the HPA axis in OFQ/N knockout mice is more sensitive to stress, but adaptation proceeds normally.

Western blot analysis was used to quantify expression levels of the µ-opioid receptor to determine if other endogenous opiates were compensating for the lack of OFQ/N in knockout mice. The western blot results were extremely variable in all treatment groups, and no conclusion could be made.
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by Jennifer Elaine Kelbley

Approved by:

_________________________, Advisor
Dr. Phyllis Callahan

_________________________, Reader
Dr. James Janik

_________________________, Reader
Kelly Zullig

Accepted by:

_________________________, Director, University Honors Program
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Introduction

Stress is a part of everyday life that has become an epidemic in today’s fast paced society. Stress and anxiety disorders affect millions of Americans and account for billions of dollars in health care costs (National Institute of Mental Health, 2001).

When a stressor is applied to the body, multiple neural and hormonal effects take place. These neuroendocrine changes are essential for survival of the individual, and death can occur without them. One of the main physiological responses to stress is activation of the hypothalamic-pituitary-adrenal (HPA) axis. Upon activation, the hypothalamus secretes corticotropin-releasing hormone (CRH) which is synthesized in the paraventricular nucleus. CRH travels via the hypothalamo-pituitary portal vessels to the anterior lobe of the pituitary gland where it stimulates secretion of adrenocorticotrophic hormone (ACTH). ACTH is a key regulator in the secretion of glucocorticoid hormones from the adrenal cortex. These glucocorticoids (mainly corticosterone or CORT in mice and rats and cortisol in humans) are responsible for maintaining homeostasis in response to the stressor and for the ultimate survival of the individual (see Carrasco, et al., 2003; Herman et al., 1997; Heuser et al., 2003 for reviews).

There have been some discrepancies in the formal definition of stress. One operational definition is “anything that induces increased secretion of glucocorticoids” (Heuser, et al., 2003). Therefore, increases in circulating CORT levels can be used as an indicator of stress. However, it has been suggested that CORT could only be used as a marker of stress intensity if low to middle intensity stressors are used (Martí, et al.,
1998). This is due to the ceiling effect of circulating ACTH on the adrenal responsiveness.

CORT has many roles in the stress response. Under resting conditions, CORT is needed to maintain metabolic balance and appropriate blood glucose levels between meals. Increased CORT levels in response to stress heighten these effects to promote survival. This hormone primarily increases metabolism in order to mobilize fuels. Proteins are broken down into their constituent amino acids which are then converted to glucose via gluconeogenesis to provide energy for the brain and muscles. These amino acids are also available to use for tissue repair should injury occur. CORT also stimulates the breakdown of stored triglycerides into fatty acids which can be used as alternative fuel sources for the other cells of the body. Additionally, nonessential functions such as reproduction and growth are temporarily inhibited so the body can focus on the immediate threat. CORT also exerts negative feedback control of the HPA axis by reducing synthesis and secretion of CRH and reducing the response of ACTH-secreting cells to CRH (see Aguilera, 1994 for review).

Activation of the HPA axis is triggered by increases in opiates. Opiates are morphine-like peptides that are endogenously produced in the brain. There are four families of opiates that include β-endorphin, dynorphins, and enkephalins and the most recently discovered of these opiates, Orphanin FQ/Nociceptin (OFQ/N) (Meunier, et al., 1995; Reinscheid, et al., 1995). OFQ/N is a 17 amino acid peptide structurally related to the other opioid peptides, particularly dynorphin A (Meunier, et al., 1995; Reinscheid, et al., 1995). The primary structure of the OFQ/N precursor also displays structural
homology to other opioid precursors, especially preprodynorphin and preproenkephalin (Nothacker, et al., 1996). It is important to note, however, that OFQ/N is a distinct neuropeptide; it is encoded by its own gene and binds to its own distinct receptor, the Opioid Receptor Like (ORL-1) receptor (Meunier, et al., 1995; Reinscheid, et al., 1995). It has many of the same localizations of the other opiates, e.g. it is widely expressed in the hypothalamus (Neal et al., 1999).

Opiates produce their effects by binding to specific receptors on the surfaces of target cells. There are four major types of opiate receptors: the µ, δ, κ, as well as a distinct receptor for OFQ/N, ORL-1 (Henderson et al., 1997). These opioid receptors are coupled to G-proteins and share a high degree of homology (Reinscheid et al., 1995). They act by inhibiting adenylyl cyclase activity which in turn inhibits voltage-gated calcium channels. This also activates inward rectifying potassium channels (see Hawes, et al., 2000 for review). Although ORL-1 shares homology with the other opiate receptor subtypes, especially the κ receptor, OFQ/N does not bind other opiate receptors, and other opiates do not bind ORL-1 (Meunier, et al., 1995; Reinscheid, et al., 1995). This indicates that OFQ/N is a distinct neuropeptide, although it may have shared a common ancestral gene with other opiate peptides.

ORL-1 mRNA has a diffuse distribution in the brain which supports the idea that OFQ/N plays a role in a multitude of central nervous system functions (Neal, et al., 1999). These include motor control and balance, reinforcement and reward, nociception, the stress response, sexual behavior and aggression. The physiological significance of
OFQ/N is not entirely known at this time since it is still a relatively newly discovered peptide.

OFQ/N has been shown to act as an anxiolytic in acutely stressed animals (Jenck, et al., 1997). Intracerebroventricular administration of OFQ/N lowers the increase in plasma CORT following exposure to a mild stressor when compared to animals injected with saline (Le Cudennec, et al., 2002). To better study the function of OFQ/N, a genetic approach was taken in creating OFQ/N deficient mice by targeted disruption of the OFQ/N gene (Köster, et al., 1999). These knockout mice display increased anxiety when exposed to a novel and threatening environment and appear to have impaired adaptation to repeated stress. Basal and post-stress levels of CORT are elevated in the knockout mice compared to wild type littermates (Köster, et al., 1999). Additionally, these OFQ/N knockout mice show region-specific up-regulation of ORL-1 which suggests this neuropeptide may regulate receptor expression (Clarke, et al., 2003).

Although OFQ/N and ORL-1 are structurally similar to the other opiates and their receptors, respectively, OFQ/N produces pharmacological effects that may differ from or even oppose those of the other opioid peptides (Meunier, 1997). In fact, OFQ/N has been shown to act as an opioid antagonist that reverses the opioid-mediated stress-induced antinociception (Mogil, et al., 1996). OFQ/N also reduces analgesia induced by selective μ, δ, and κ receptor agonists (Mogil, et al., 1996). Therefore, OFQ/N may play a role in opioid modulation and thus the stress response. One mechanism for this anti-opioid effect of OFQ/N is heterodimerization of ORL-1 with the opioid receptors since they
share such a high degree of homology (Wang, H., et al., 2005). This heterodimerization would effectively impair the opioid receptor activated signaling pathways in the brain.

The µ-opioid receptor is of specific interest in this study because it has been shown to be involved in modulating the stress response. Acute activation of µ-receptors increases the release of HPA neurohormones, i.e. CORT, while chronic treatment with µ-receptor agonist results in habituation of the HPA axis (degli Uberti, et al., 1995). The µ-receptor is widely distributed in the brain (Mansour, et al., 1994) with highest expression levels in the midbrain-hypothalamic region (Buzas, et al., 1997). These receptors exhibit a high affinity for β-endorphin, enkephalin, and endomorphin (Okada, et al., 2002).

The purpose of this study was twofold. The first aim was to investigate the role of OFQ/N in regulating the stress response of the HPA axis, particularly in mediating adaptation to chronic stress. To examine this OFQ/N knockout mice that were exposed to varying amounts of shaker stress were used. CORT levels in these knockout mice were compared to wild type controls which received the same stress. The second aim was to determine if the µ-opioid receptors have a compensatory effect in knockout mice as a result of the lack of OFQ/N. µ-opioid receptor mRNA expression has been shown to vary in response to stress, according to the duration of the stress (Yamamoto, et al., 2003). However, it is unknown how the expression of these protein receptors changes in response to stress and how this is related to OFQ/N. Therefore, western blot analysis was used to quantify expression levels of the receptor in the hypothalamus of these animals.
Methods

Animals

OFQ/N transgenic mice were bred in our colony. Ear punch tissue samples were taken from animals under isoflurane anesthesia and used for genotyping by PCR (polymerase chain reaction). Animals were housed in a clean, stress free environment, under controlled lighting (12 h light:12 h dark), and controlled temperature (21°C). Animals had access to food and water ad libitum. They were separated by sex and date of birth and housed with no more than three mice to a cage. Male mice 2 to 5 months old were used in these studies. All procedures were approved by the Miami University Institutional Animal Care and Use Committee (IACUC).

Stress Paradigm

A shaking platform equipped with a specially designed caging system was used to deliver shaker stress (Bernatova, et al., 2002). The shaker was automatically controlled and programmed to turn on for 5 minutes every half hour. Wild type and knockout mice were divided into five groups (n=6 for each genotype in each group). A control group received no stress and was housed under the same conditions as the stress groups. An acute group received 1 bout of the shaker stress. The 3x, 6x, and 30x groups received 3, 6, and 30 bouts of the shaker stress, respectively.
Tissue Collection

Blood samples were collected by tail bleeding one day prior to the first exposure to stress to determine basal CORT levels. Blood samples were collected within one minute of removing the animal from its home cage to eliminate any nonspecific stress effects. Animals were subjected to stress the next day and were sacrificed immediately after the final exposure to stress. Control mice remained under the same housing conditions, but without shaking and were sacrificed at time intervals during the experimental procedure. At the time of sacrifice, trunk blood was collected into heparanized eppendorf minifuge vials. The brain was rapidly removed from the cranial vault and immediately placed in ice cold 0.9% saline. The brain was placed on a cold plate and the hypothalamus was rapidly microdissected and snap frozen in liquid nitrogen. The blood samples were centrifuged and the plasma was collected and stored at -20°C until used in a CORT radioimmunoassay.

Radioimmunoassay (RIA)

Plasma CORT levels were quantified using a radioimmunoassay kit from MP Biomedicals (Orangeburg, NY). The assay was performed per their instruction using 5µl of plasma.

Western Blot

Western blot analysis was used to quantify the expression of the µ-opioid receptor protein in the hypothalamus. The hypothalamus was sonicated in 150 µl of buffer (1 M
Tris, 1% SDS and protease inhibitor cocktail (1:100 containing aprotinin, leupeptin, bestatin, pepstatin A, E-64 and AEBSF from Sigma; St. Louis, MO). The homogenate was centrifuged (12,000Xg, 4°C, 6 minutes) and the supernatant was collected. Protein content was determined in a 10 µl aliquot of the supernatant using BCA protein kits (Pierce; Rockford, IL). The remainder of the protein was mixed with Laemmli buffer (Laemmli, et al., 1970) and run on a 10.5% polyacrylamide electrophoresis gel along with a pre-stained protein ladder. After electrophoresis, protein was transferred to a PVDF membrane (100V, 3A for 42 minutes). The membranes were blocked in 8% milk overnight at 4°C to prevent non-specific binding. Primary antibodies to the µ-opioid receptor (CHEMICON International; Temecula, CA) and GAPDH (Novus Biologicals; Littleton, CO), the internal control protein, were incubated with the membrane for 2 hours. The membranes were rinsed in Tris Buffered Saline containing 0.05% Tween (TBST) and incubated with secondary goat anti-rabbit horseradish peroxidase-coupled antibody (CHEMICON International; Temecula, CA) for 1.5 hours. Supersignal West Pico chemiluminescent substrate (Pierce; Rockford, IL) was used to visualize the µ-receptor and GAPDH protein bands, and the membranes were developed on film. The densities of the bands were determined using ImageQuant 5.2 software and the density of the µ-opioid receptor protein was expressed as a ratio to total GAPDH density in the sample in order to normalize the data.
Statistical Analysis

A one-tailed Student’s t-test of unequal variance was used to determine significance. For the hormone data, CORT levels in the knockout animals were compared to the CORT levels in the wild type animals subjected to the same treatment. For the μ-receptor western blot data, expression levels for each treatment group within a genotype were compared to their own control.
Results

There was no significant difference in basal CORT levels between knockout and wild type mice prior to stress exposure (Figure 1; p=0.1). All groups exposed to stress, regardless of genotype, had significantly higher CORT levels compared to the control groups exposed to no stress (Figure 2; p<0.01). The 3x treatment group was the only group in which the CORT levels of the knockout mice were statistically different than the wild type mice (p=0.03). All other groups showed no statistical difference between wild type and knockout mice.

Initial experiments using western blot analysis of μ-opioid receptor expression revealed a single band of 47kD molecular weight. A representative film of these initial westerns is shown (Figure 3). When μ-opioid receptor expression was determined in this study, additional antibody was purchased. As a result, a different lot of the μ antibody was used. Although this antibody was from the same company, multiple bands appeared when probed for the μ-receptor (Figure 4). The quantitative analysis of the western blots yielded extremely variable results within each treatment group (Figure 5). The 3x wild type group was the only group that was statistically different from the controls (p=0.04).
Discussion

The results of this study indicate that there was no difference in the basal CORT levels between wild type and knockout mice. This result contrasts with a previous report that OFQ/N knockout mice have higher resting levels of CORT (Köster, et al., 1999). This discrepancy between the results may be due to the fact that CORT levels are extremely variable depending on how the animals are handled and the method in which the samples are taken. The method used in this study was very fast. The animals were not out of their home cage for more than one minute and handling was minimal. This brief time would not be long enough for any sort of stress response to be activated, including increases in circulating CORT levels. Also, CORT levels fluctuate throughout the day (Coffigny et al., 1978). To control for this fluctuation, all samples in this study were collected early in the morning. Finally, the resting levels reported here are lower than those reported by Koster, et al., (1999), which is another indicator that these CORT levels represent accurate resting levels of this hormone.

One of the purposes of this study was to investigate the role of OFQ/N in mediating adaptation to chronic stress. However, the distinction between acute and chronic stress is rather arbitrary. Results of previous studies have indicated that it takes at least seven days of chronic stress exposure before adaptation to the stress occurs. The marker for stress adaptation was that CORT levels returned to basal values or at least became partially attenuated (Bernatova, et al., 2002; Zelena, et al., 2003; Bhatnagar, et al., 2002). However, a pilot study was performed in which the mice were shaken for 5 minutes every half hour for 7 days. The CORT response to stress was determined at 3, 5
and 7 days after the onset of the stress. Following this stress paradigm, CORT levels in both wild type and knockout mice had already returned to basal values by day 3 (results not shown). Therefore, it was evident that the time course for activation and adaptation of the HPA axis for this particular stress paradigm was much quicker than expected.

The relatively rapid decline in the hormone response observed may be due to the specifics of the stress paradigm. Chronic stress can be classified into three groups; continuous, intermittent, and chronic variable (see Martí, et al., 1998 for review). The shaker stress paradigm used would be considered intermittent since the time between exposures was always the same. Since the time between shaking was rather brief (only 25 minutes), it is feasible that the mice became familiarized with the stress and anticipated the return of the stressor. This reduced emotional activation may be one explanation for the decline in the stress response of the mice after three days. Therefore, a decreased number of exposures to stress was administered in this study (1, 3, 6 or 30 exposures) to see a more complete profile of the stress response.

The post-stress CORT levels displayed a bell-shaped curve, which may be a result of the timing of the HPA axis response to stress. The levels in the control groups for both genotypes were low, which was expected from no stress exposure. The levels for both genotypes were higher in the acute groups since the HPA axis had been activated, and CORT was starting to be released and starting to circulate in the blood. Although these CORT levels following 5 minutes of acute stress were higher than the controls, CORT levels continue to rise for 30-60 minutes after acute stress exposure (Zullig, personal communication). Although the HPA axis was activated and CORT levels were starting to
rise, five minutes was not adequate time for CORT to reach its maximum concentration in the blood.

The CORT levels peaked in the 3x treatment groups for both wild type and knockout mice. The CORT levels for these groups were higher than the acute for two reasons. First, the CORT levels were accumulating in the blood from the previous exposure and second, the exposure to previous stress sensitized the CORT and ACTH response to subsequent HPA activation (Johnson, et al., 2002). The levels in the knockout mice were statistically higher than those in the wild type in this group. This indicates that these mice are at a critical time in the stress response. Since the CORT response was higher in knockouts, it is evident that the HPA axis in these mice is more sensitive to repeated stress. This suggests that OFQ/N is an important factor in regulating the stress response and that it may be important in the initial stages of adaptation, i.e. it may be necessary to attenuate the CORT response.

CORT levels began to decline in the 6x and 30x treatment groups for both genotypes. This decline may be due to deactivation of the HPA axis. As animals begin to adapt to the stress, the HPA axis adapts to the stimulus and CORT levels decline. This decline happened at the same time in wild type and knockout mice. The regulation of the HPA axis is complex and under multiple regulatory signals and processes, including negative feedback of CORT on CRH and ACTH secretion. Similar sensitivity to negative feedback may be one reason for the decline in CORT occurring at the same time in both genotypes. If this is the case, then it seems that OFQ/N is not an important factor in determining the rate of adaptation.
Another possible explanation for this adaptation may involve the opioids. Adaptation has been reported to involve opioid receptor down-regulation (Martí, et al., 1998). In this study, down-regulation of the opioid receptors could have been caused by OFQ/N because OFQ/N has been reported to exhibit anti-opioid effects (Mogil, et al., 1996; Wang, H., et al., 2005). For this reason, it was important to investigate receptor expression in these animals.

Expression of the µ-opioid receptor was extremely variable within each treatment group. Regardless of treatment or genotype, some animals had a very large increase compared to the controls, some had a very large decrease, and some had no change. This large range of expression levels resulted in very high standard errors. Therefore, based on statistical analysis of the data, no conclusion could be made regarding the effect of OFQ/N on µ-receptor expression. There are two possible explanations for the extreme variability of this data. First, the primary antibody used to probe for the µ-receptor in these experiments yielded multiple bands. This antibody to the µ-receptor had been used in previous experiments, and it produced clean films in which there was one distinct band for this protein (Figure 3). However, because all of the antibody had been used in previous experiments, new antibody was ordered. Western analysis of µ-receptor expression using new lots of the antibody yielded films with multiple bands (Figure 4).

There is much discrepancy in the literature about the molecular weight of the µ-receptor. The µ-receptor has been reported to be 47kD (Sarramegna, et al., 2005), 55kD (Wang, S., et al., 2005) and 65kD (Arvidsson, et al., 1995). Kivell et al. (2004) reported that there are two isoforms of the receptor, one located at 50kD and another at 70kD.
Based on these different reports, it appears that the unmodified translational product for the \( \mu \)-opioid receptor is 47kD, which is the band used for comparisons in this study. Other bands at larger sizes may be due to glycosylation, splice variant differences, use of alternative promoters, multimer forms of the receptor, or heterodimer formation (Kivell, et al., 2004; Sarramegna, et al., 2005). Importantly, the antibody used in this study recognized these multiple forms. Therefore, the lack of specificity of the antibody for the unmodified form of the receptor may have detracted from its full binding potential and yielded results that did not reflect the full expression of the 47kD \( \mu \)-opioid receptor.

The variability in the western blot data could also be due to a time related effect. Opioid receptors are proteins, and it takes time for proteins to be made or removed following a stimulus. This timing may vary from one animal to the next. Therefore, animals within each treatment group may have been at different points in the translational process when their hypothalamus was removed. Alternatively, the time course of each individual animal’s response to stress may vary, which would also affect protein expression. Since this may be a time related phenomenon, future studies are needed to investigate more time points.

In conclusion, OFQ/N knockout mice were used to determine the role of this neuropeptide in mediating adaptation to chronic stress. Using CORT levels as an indication of stress, it was found that there was no difference between knockout mice and their wild type littermates under resting conditions. A shaker stress model was used to deliver chronic stress to these animals. It appeared that the HPA axis in knockout mice was more sensitive to stress than wild type mice because the CORT response was greater
in knockouts. This indicates that OFQ/N plays a role in keeping the stress response in check. However, the knockout mice adapted to the stress at the same rate as the wild type mice, suggesting that OFQ/N does not have a large role in the timing of HPA axis regulation. Finally, it is still unknown whether other opiate receptors have a compensatory effect in knockout mice because the results were so variable. Future studies are needed to test this hypothesis.
Figure 1. Basal CORT levels in wild type and knockout mice. Levels are expressed as mean ± SEM in ng/ml of plasma. There was no statistical difference between genotypes (p=0.1).
Figure 2. Plasma CORT levels after exposure to acute or 3, 6, or 30 bouts of stress.

Levels are expressed as mean ± SEM in ng/ml of plasma. All treatment groups were significantly higher than the controls, regardless of genotype (p<0.01). Knockout mice in the 3x group had significantly higher CORT levels than the wild type mice in that group (* p=0.03).
Figure 3. A representative western blot from a previous experiment. 5, 10 and 20 µg of total protein was loaded for each animal. The µ-opioid receptor antibody used in this experiment produced a single distinct band for the receptor at 47kD.
Figure 4. A representative western blot from this experiment. 5, 10 and 20 µg of total protein was loaded for each animal. The µ-opioid receptor antibody used in this experiment was purchased from the same company as that used in Figure 3. However this came from a different production lot number. This antibody produced multiple bands of various sizes. The arrow points to the bands that were quantified.
Figure 5. Western blot analysis of μ-opioid receptor expression in the hypothalamus following acute or 3 or 30 bouts of stress. Levels are expressed as mean percent of control ± SEM. The acute wild type group was the only group statistically different from its control (* p=0.04).
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