ABSTRACT

THE ROLE OF ORPHANIN FQ/NOCICEPTIN IN MEDIATING THE PROLACTIN RESPONSE TO STRESS

By Tiffany Derringer

The role of OFQ/N in mediating the prolactin response to acute and repeated stress was investigated. Male and female wild type and OFQ/N knock-out mice were subjected to 5 minutes of restraint stress (acute) or 4 bouts of restraint stress at 1 hour intervals (repeated) prior to sacrifice. Plasma prolactin and corticosterone levels, as well as pituitary prolactin and estrogen receptor alpha content, were determined. There was a significant gender difference in the prolactin, but not the corticosterone, response to stress. OFQ/N mediates the prolactin response to acute stress in males, but not females. Although stress did not affect pituitary levels of prolactin, female OFQ/N knock-out mice had higher pituitary prolactin content, but not a greater stress response than wild type mice. OFQ/N does not appear to mediate pituitary estrogen receptor alpha expression. These results suggest that OFQ/N is involved in mediating the prolactin secretory response to stress in males.
THE ROLE OF ORPHANIN FQ/NOCICEPTIN IN MEDIATING THE PROLACTIN RESPONSE TO STRESS

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Background

Regulation of Prolactin Secretion

Prolactin (PRL), a protein hormone secreted and released by the anterior pituitary gland, plays many important biological roles in reproduction and homeostasis in mammalian systems [15]. PRL is best characterized for its role in lactation, but also affects angiogenesis, osmoregulation, luteal function in rodents and immune function [17]. While PRL secretion is regulated through complex interactions among many different hypothalamic factors, the primary regulator of PRL release is hypothalamic dopamine (DA) [2]. Hypothalamic DA holds PRL under tonic inhibitory control, suppressing anterior pituitary biosynthesis, storage, and secretion [2]. Antagonism of anterior pituitary DA D_2 receptors removes this tonic inhibitory control over the lactotrophs, the PRL secretory cells of the anterior pituitary gland [15].

The hypothalamus contains three dopaminergic systems involved in the regulation of PRL secretion. The tuberohypophyseal dopaminergic (THDA) neurons originate in the rostral portion of the dorsomedial arcuate nucleus and terminate in the neurointermediate lobe of the pituitary gland [2]. The periventricular hypothalamic dopaminergic (PHDA) neurons originate in the periventricular nucleus of the hypothalamus and terminate in the intermediate lobe of the pituitary. The tuberoinfundibular dopaminergic (TIDA) neurons originate in the A12 region of the dorsomedial arcuate nucleus and terminate in the median eminence of the hypothalamus. While all three dopaminergic systems are involved in the regulation of PRL release, it is the TIDA neurons that play the principal role in PRL regulation [2,15]. However, regulation of PRL also involves a number of other factors, including opiates and estrogen. Modulation of PRL secretion by estrogen contributes to gender differences in the regulation of PRL release [2].

Regulation of Prolactin Secretion: Opiates

There are four endogenous opiate peptide families. Three of these families are termed the “classic” opiate peptides, i.e. the enkephalins, dynorphin, and β-endorphin. These peptides bind to the classic opiate receptors: δ (OP_1) [13,26,45], μ (OP_2) [29,31,45], and κ (OP_3) [6,43], respectively. Orphanin FQ/Nociceptin (OFQ/N), the
fourth endogenous opiate, exhibits high amino acid sequence homology to the classic endogenous opiates, especially dynorphin [30,39]. However OFQ/N lacks the N-terminus region necessary to bind to the classic opiate receptors, and binds to its own unique receptor, ORL1 (OP₄) [30,39]. Similarly, the classic opiate peptides do not demonstrate high affinity for the OP₄ receptor [33]. OFQ/N has a wide distribution in the nervous system, and thus a number of physiological effects of OFQ/N have been reported, ranging from modulation of nociception, to impairment in spatial learning, to attenuation or sensitization of the stress responses [32].

Members of the opiate family are known neuromodulators of the endocrine system, specifically of anterior pituitary function, including PRL secretion. Activation of the classic opiate receptors results in suppression of dopaminergic activity, followed by an increase in PRL release [12]. While the neuromodulatory role of OFQ/N is not well known, OP₄ receptors are expressed in high levels in the hypothalamus, including the median eminence and on the hypothalamic dopaminergic neurons [21,36], indicating that OFQ/N, like the other endogenous opiates, is involved in regulating neuroendocrine function. Several studies support this hypothesis. For example, OFQ/N administration stimulated PRL secretion in both male and female rats [5] and this effect was not mediated by actions at the µ, δ or κ receptor subtypes [4]. While the mechanism of action for OFQ/N remains unclear, OFQ/N administration produced a decrease in the levels of DOPAC, a metabolite of DA, in the median eminence of the hypothalamus [42].

Regulation of Prolactin Secretion: Gender Differences and Estrogen

In females, basal TIDA neuronal activity is 2-3 times higher than in males [9]. This gender difference is due, in part, to mediation by estrogen. Ovariectomized female rats have suppressed TIDA neuronal activity, which is reversed by estrogen replacement [2]. In contrast, androgens inhibit TIDA neuronal activity in males. Orchidectomy increases TIDA neuronal activity in male rats, with androgen therapy reversing this effect [15]. These studies provide evidence that the difference in TIDA neuronal activity, and, thus PRL regulation, between males and females is due to modulation by the gonadal steroids.
Estrogen is one of the most potent regulators of PRL synthesis and secretion [2]. There are multiple estrogen receptor subtypes currently identified [44], including estrogen receptor α (ERα) [18] and estrogen receptor β (ER β) [35]. ERα is highly expressed in the anterior pituitary gland, where it is localized in over 90% of the secretory cells [37]. In mice, there are no detectable levels of ERβ mRNA in the pituitary [7]. In transgenic studies, ERα knock-out mice displayed a marked decrease in PRL mRNA [38,41], however, no change in PRL mRNA in ERβ knock-out mice was detected [38] supporting the finding that ERβ is not expressed in the mouse pituitary. Therefore, ERα is the estrogen receptor subtype most likely involved in PRL regulation.

While ERβ is not directly implicated in PRL regulation, ERβ mRNA has been co-localized with both OFQ/N and OP4 in the rat hypothalamic paraventricular nucleus (PVN) [23]. Relevant to this study, the PVN is an area strongly implicated in the stress response, indicating ERβ may play a role in mediating the hypothalamic-pituitary-adrenal (HPA) axis response to stress. Furthermore, increased circulating PRL levels result in increased uptake of systemic PRL into the brain and this PRL activates the HPA axis [16].

Stress

Stress is generally defined as the condition resulting from events that disrupt homeostasis [12]. The neuroendocrine system responds to acute stress through activation of different pathways, including the hypothalamic-pituitary-adrenal axis (HPA), to create a physiological response necessary for survival of the stressor. Once activated, the HPA axis stimulates the release of corticotrophin releasing hormone (CRH) from the paraventricular nucleus (PVN) of the hypothalamus, which stimulates the rapid release of adrenocorticotrophin (ACTH) from the anterior pituitary gland. ACTH then acts on the adrenal cortex to stimulate the release of glucocorticoids [22]. The stress pathways are modulated by gonadal steroids, creating a gender difference in the stress response. The HPA stress response is enhanced by estrogen and inhibited by testosterone [22].

Stress also stimulates PRL secretion due, in part, to inhibition of hypothalamic dopaminergic activity [15]. Furthermore, this stress-induced PRL increase is due to decreased TIDA neuronal activity in females, but not in males [8]. For many years, this
increase in PRL secretion was thought to be physiologically irrelevant. Recently, however, it has been shown that the increased PRL levels that occur in response to stress act within the central nervous system to activate the CRH neurons in the PVN and to protect against some of the detrimental effects of stress [16]. Although gonadal steroids modulate PRL secretory responses, the effects of steroids on the stress-induced PRL increase is unknown.

**Stress and OFQ/N**

Several studies have suggested that OFQ/N plays a role in mediating the stress response. Acute restraint stress in rats caused a decrease in OFQ/N concentrations in the forebrain which were returned to physiological levels after the stress response was diminished [10], suggesting a direct role for OFQ/N in mediating the stress response. Intracerebroventricular (i.c.v.) administration of OFQ/N has been reported to have both anxiolytic [20,24,25,34] and anxiogenic effects [11,14]. In acute behavioral studies, OFQ/N knockout male and female mice exhibited higher levels of anxiety [34], further supporting the idea that OFQ/N plays a role in the stress response. Finally, OFQ/N has been reported to play a role in the adaptation to repeated stress. Repeated exposure to the same stressor reduces the physiological response to that stimulus, i.e. adaptation occurs to return the system to its homeostatic state. Consequently, repeated stress induces numerous physiological effects that promote adaptation and reestablish homeostasis. OFQ/N may play a role in mediating adaptation because OFQ/N knockout male mice subjected to repeated exposure to stressful stimuli did not show behavioral adaptation to the stimuli [27]. The effects of OFQ/N in adaptation to stress in the female remain unknown.

**Significance**

Little is known about the role of OFQ/N in the neuromodulation of the endocrine system, but it is clearly a potent stimulus for PRL release [5]. The purpose of this study was to investigate the importance of OFQ/N in mediating the PRL-secretory response to acute and repeated stress. Understanding the regulation and control of prolactin secretion by OFQ/N will allow us to better understand reproductive and stress-related disorders.
1. Introduction

Orphanin FQ/Nociceptin (OFQ/N) is a heptadecapeptide that exhibits high amino acid sequence homology to the classic endogenous opiates, especially dynorphin [30,39]. However, OFQ/N lacks the N-terminus region necessary to bind to the classic opiate receptors, μ, δ, or κ, and binds to its own unique receptor, OP₄ [30,39], although this receptor does not bind the classic opiates [33]. OP₄ receptors are expressed at high levels in the hypothalamus, including the median eminence and the hypothalamic dopaminergic neurons [21,36]. OFQ/N administration stimulates PRL secretion in both male and female rats [5] and this effect is not mediated by action at the μ, δ or κ receptor subtypes [4]. While the mechanism of action for OFQ/N induced PRL secretion is not clear, OFQ/N administration produced a decrease in DOPAC levels in the median eminence [42].

Stress stimulates PRL secretion [15], and this increased PRL acts within the central nervous system to protect against some of the detrimental effects of stress [16]. The mechanism for the stress induced PRL secretion is, in part, due to inhibition of hypothalamic dopaminergic activity [15]. In addition, there is a gender difference in the magnitude of dopaminergic inhibition [8] and this is mediated by estrogen [2]. Estrogen is one of the most potent regulators of PRL synthesis and secretion [2]. Of the multiple estrogen receptor subtypes currently identified [44], only estrogen receptor α (ERα) is highly expressed in the pituitary gland of mice [7,37].

Several studies have suggested that OFQ/N plays a role in mediating the stress response, however, the results are conflicting. In response to acute stress, OFQ/N has been reported to have both anxiolytic [20,24,25,34] and anxiogenic effects [11,14]. In transgenic studies, OFQ/N knockout male mice failed to habituate to repeated stress [27], while the effects of OFQ/N in adaptation to stress in the female remain unknown.

Little is known about the role of OFQ/N in the neuromodulation of the endocrine system, but it is clearly a potent stimulus for PRL release [5]. The purpose of this study was to investigate the importance of OFQ/N in mediating the PRL-secretory response to acute and repeated stress.
2. Materials and Methods

2.1 Animals

OFQ/N knock-out mice were generated from C57BL/6 x 129/Ola strains, as previously described [27]. Heterozygote breeder pairs were the generous gift of Dr. R. Reinscheid (UC, Irvine). Animals were maintained in a colony in the Miami University Animal Care Facility. Upon weaning, same-sex offspring were housed 2-3 per cage under controlled temperature (21°C) and lighting (12 h light and 12 h dark) conditions, in a quiet, stress free environment, with food and water provided ad libitum. All procedures were reviewed and approved by the Miami University Institutional Animal Care and Use Committee (IACUC) and adhere to NIH guidelines.

2.2 Genotyping Mice

All mice in the colony were genotyped using Polymerase Chain Reaction (PCR) following the guidelines described by Koster, et al. [28]. The primers (Integrated DNA Technologies, Coralville, IA) were:

Primer OFQ 282: 5’ GACCCAGAGCTTGTGTCAGC,  
Primer OFQ 530: 5’CTCATAAAACTCACTGAACCGC,  
and Primer NEO: 5’CCGGAGAACCTGCGTGCAATCC.

The PCR running conditions were: 3 min 94°C; 31 x (30 s 94°C; 30 s 60°C; 45 s 72°C); 5 min 72°C. PCR products were separated on a 1.5% agarose gel. Genotypes were determined as follows: wild type = 250 bp, OFQ/N gene expressed; heterozygote = 550 cassette and 250 bp, 1 copy OFQ/N gene expressed, 1 copy cassette; knock-out = 550 cassette only, no OFQ/N gene.

2.3 Stress Paradigm

All experiments were performed between 0800-1200h. Mice were weighed on the day of the experiment, and allowed to acclimate to the experimental environment for approximately 15 minutes. Male and female wild type (wt) and knock-out (ko) mice were subjected to 15-20 seconds of isoflurane exposure (IsoFlo, Abbott Laboratories, Abbott Park, IL) followed by 5 minutes of immobilization in a plexiglas restraint.
chamber (Braintree Scientific, Braintree, MA). For repeated stress, animals were subjected to 4 bouts of 5 minutes of restraint at 1 hour intervals, and sacrificed after the fourth round of stress. A separate group of animals was sacrificed after only a single stress exposure to determine their acute response to immobilization stress. A control group remained in their home cage until sacrifice.

Animals were sacrificed by rapid decapitation and trunk blood was collected. The brain was rapidly removed and placed in ice cold saline. The pituitary was immediately removed from the sella turcica and frozen. The hypothalamus was microdissected from the rest of the brain. Tissues were snap-frozen in liquid nitrogen and stored at -80°C until used. Blood samples were centrifuged at 3,000Xg for 5 minutes, and plasma was collected and stored at -20°C. Post-mortem vaginal smears were taken to determine the stage of the estrous cycle at the time of experimentation [1]. Smears were stained with 1% Toluidine Blue and the stage of the estrous cycle was recorded. All females were in either the proestrous or estrous stage at the time of experimentation.

2.4 Radioimmunoassay

Double antibody radioimmunoassay was used to determine both plasma PRL and corticosterone (CORT) levels. PRL was measured in duplicate samples using reagents purchased from the National Hormone and Peptide Program and Dr. A. F. Parlow. Secondary antibody (Goat-anti-rabbit gamma globulin) was purchased from Antibodies, Inc. (Davis, CA). Iodination was performed using the Chloramine T method [19] modified for PRL. Iodinated PRL was eluted from a Sephadex G-25 column (Amersham Pharmacia Biotech, Piscataway, NJ) using 1% PBSA. Intra-assay variability was less than 10%. Plasma corticosterone levels were detected using ImmunoChem™ Corticosterone 125I RIA kit (MP Biomedicals, Orangeburg, NY), as per manufacturer instructions. Intra-assay variability was less than 8%.

2.5 Western Blot method

Pituitary and hypothalamic tissue was sonicated in 100 µl buffer [(1M Tris, 1% SDS and protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO))]. The homogenate was centrifuged (14,000xg, 6 min) and the supernatant was collected. A 10 µl sample of
the supernatant was analyzed for protein content using a BCA protein assay kit (Pierce, Rockford, IL). The remainder of the sample was diluted 1:1 in Laemmli buffer and stored at -20°C.

Homogenized pituitary protein was loaded onto a 12% bis-acrylamide gel and separated by SDS poly-acrylamide gel electrophoresis. Separated proteins were electrophoretically transferred to a PVDF membrane (Millipore, Billerica, MA) with amperage totaling 2000mA. Following transfer, membranes were blocked in a solution of tris-buffered saline containing 0.05% tween (TBST) and 8% nonfat dry milk for 3 hours at room temperature. To detect PRL expression levels in the pituitary gland, rabbit anti-mouse PRL antiserum (NHPP) was diluted to 1:120,000 or 1:80,000 in TBST for pituitaries from female and male mice, respectively. Actin antiserum (Sigma-Aldrich, St. Louis, MO) was diluted to 1:20,000 in TBST to detect the level of actin, which was used as the internal standard in the same tissue. Membranes were incubated with primary antibodies overnight at 4°C. After TBST rinse, membranes were incubated for 90 minutes with secondary antibody (Goat-anti-rabbit gamma globulin (GARGG)) (Chemicon, Temecula, CA), diluted to 1:5000 in TBST. Membranes were rinsed prior to detection by chemiluminescence.

After detection, membranes were rinsed 3 times in TBST and antibodies were stripped at room temperature for 1 hour (ImmunoPure IgG Elution Buffer, Pierce, Rockford, IL). For ERα expression, membranes were rinsed 3 times in TBST before being blocked in a solution of 5% bovine serum albumin/8% nonfat dry milk/TBST at room temperature for 5 hours. 1:20,000 rabbit polyclonal ERα (Santa Cruz Biotechnology, Santa Cruz, CA) primary antibody was diluted in 5% bovine serum albumin/TBST and incubated overnight at 4°C. After TBST rinse, membranes were incubated for 90 minutes with secondary antibody (Goat-anti-rabbit gamma globulin (GARGG)) (Chemicon, Temecula, CA), diluted to 1:5000 in TBST. Membranes were rinsed prior to detection.

All proteins were detected by chemiluminescence (Pierce, Rockford, IL). Density ratios (ImageQuant, Amersham Biosciences, Piscataway, NJ) of ERα/actin and PRL/actin were used for statistical analysis. To determine differences in protein expression levels between the genotypes, density ratios for wt, unstressed controls was
set to 100% and the ratios in the ko control group were expressed as a percentage of wild type. To determine any changes in protein expression levels due to treatment, the density ratios for unstressed controls within a genotype were set to 100%; ratios from the acute and repeated stress groups were determined as a percentage of the control group from the same genotype.

2.6 Statistical Analysis

PRL and CORT hormone data were analyzed using analysis of variance (ANOVA) which was fit using the general linear models command “PROC GLM” on SAS software (Windows Version 8, SAS Institute, Cary, NC, USA). Due to variability, male PRL data were log transformed prior to analysis. Overall experiment-wise error was held at \( p=0.05 \). This implies that the chance of a type I error occurring anywhere within a set of comparisons is less than 0.05. With 9 comparisons, \( p \leq 0.0056 \) was considered to be statistically significant. For western blot analysis, ratios of protein to actin were compared to the appropriate inter-gel control. One sided, upper tail t-tests were run, except for males, where a two sided t-test was run. For the effect of treatment, four comparisons were made, and \( p \leq 0.0125 \) was considered to be statistically significant. To determine changes in protein expression levels between genotypes, \( p \leq 0.05 \) was considered to be statistically significant.

3. Results

3.1 CORT response to immobilization stress

Acute immobilization stress produced a significant increase in CORT levels in both wt (\( p=0.0003 \)) and ko (\( p=0.0001 \)) males (figure 1A). CORT was also significantly increased following the fourth bout of stress (\( p=0.0015 \) and \( p=0.0012 \), for wt and ko, respectively) indicating sensitivity to the stress was maintained. Similarly in females, immobilization stress produced a significant increase in CORT levels, regardless of genotype (\( p<0.0001 \) and \( p=0.0028 \), for wt and ko, respectively) (figure 2A) and levels
remained significantly elevated in both wt (p<0.0001) and ko (p=0.0005) mice subjected to repeated stress.

3.2 PRL response to immobilization stress

The PRL response to acute stress was significantly increased in wt (p=0.0009), but not ko (p=0.3763) males compared to control levels (figure 1B). Further, the PRL response in wt males was significantly greater than in ko males (p=0.0006). PRL levels were not increased in either wt or ko mice following repeated stress, and PRL levels following repeated stress were significantly lower than the response to acute stress in wt males (p=0.0006).

PRL levels were significantly increased in wt females following acute stress (p=0.0005). The increase in PRL levels in the OFQ/N ko was not significant when compared to control ko females (p=0.0158). Acute stress produced a PRL increase in ko females that was not significantly different from the response in wt females (p=0.9506, figure 2B). Although the PRL response to repeated stress was diminished compared to acute stress in both wt and ko females, this response was not significantly different from the levels in controls or those subjected to acute stress.

3.3 Gender difference in PRL response to repeated immobilization stress

There was a significant difference in the response to repeated stress between males and females. The PRL secretory response appeared to adapt to repeated stress in wt males because PRL levels were not elevated compared to controls and they were significantly decreased following acute stress (p=0.0006, figure 1B). However, neither wt nor ko females (figure 2B) showed any evidence of adaptation.

3.4 Pituitary PRL and ERα expression levels

In response to acute and repeated stress, pituitary PRL content remained unchanged across treatment groups in both males (figure 3A) and females (figure 3B). Female ko mice, however, had a significantly greater level of pituitary PRL expression under basal, resting conditions compared to wt (p=0.018) (figure 4A). There was no difference in ERα pituitary content between wt and ko females (figure 5A).
4. Discussion

The results of this study demonstrate that there are significant gender differences in the PRL, but not the CORT, response to stress and that OFQ/N plays a significant role in mediating the PRL secretory response to acute stress in male, but not female, mice. Acute and repeated stress produced an increase in circulating CORT levels in male and female mice, regardless of genotype, indicating activation of the HPA axis. Further, OFQ/N is not critical to this response, since it occurred even in ko mice. However, the PRL response to stress was different from the CORT response.

Acute stress produced an increase in PRL in wt, but not ko males, indicating that, in males, OFQ/N is necessary for the PRL secretory response to acute stress. Additionally, the PRL response to stress appeared to adapt rapidly in the wt males because after only 4 bouts of stress, PRL levels were not different from unstressed, control values. In contrast, acute stress produced an increase in PRL levels in females of both genotypes, and neither wt nor ko females adapted to the stress. Although resting levels of pituitary PRL were doubled in ko female mice compared to wt, stress did not produce any significant change in anterior pituitary PRL or ERα content. Furthermore, the circulating levels of PRL in ko females were not different from wt, even though ko mice had more pituitary PRL that could potentially be released. These results suggest that pituitary PRL content is probably not a major factor in determining the magnitude of the PRL response to the stress stimulus.

Acute, immobilization stress induced a significant CORT response regardless of gender or genotype. Immobilization stress is a known activator of the limbic-hypothalamic-pituitary-adrenal axis [11]. Devine, et al., reported that immobilization stress and OFQ i.c.v. injection in rats produced activation of the HPA axis, and proposed that OFQ/N and immobilization stress mediate this activation through similar pathways [11]. Koster, et al. [28] suggested that OFQ/N is anxiolytic under resting conditions because basal CORT levels were significantly elevated in ko male mice. They also detected a significantly greater CORT response to a mild stressor in ko male mice. However, we detected no significant difference in CORT levels between ko and wt mice, under either resting conditions or in response to stress. Koster, et al. [27] exposed mice
to a mild stress, i.e. an elevated plus maze, while the mice in this study were physically restrained. Furthermore, Koster, et al. [28], measured CORT levels 5 minutes after the completion of the stress, while samples were taken immediately after completion of the stress in this study. These differences in the stress paradigm, as well as in the time course of the blood sampling, may explain the discrepancy between results.

It is known that PRL is modulatory to the HPA axis activation during stress [40]. PRL receptors are located on the adrenal glands [3] and increased PRL levels lead to stimulation of glucocorticoid secretion [3]. PRL, acting at specific PRL receptors in the brain, also protects against some of the detrimental effects of stress [16]. In this study, PRL levels were not increased following acute stress in ko males, however, CORT levels were elevated, indicating that HPA axis activation occurs even without elevated PRL. It is not clear if this is the case in females, because PRL levels were elevated following acute stress in both wt and ko mice. The PRL secretory response to stress would have to be blocked to determine if PRL is necessary for HPA activation in stress.

In response to repeated stress, the PRL secretory response appears to adapt in wt males. Additionally, CORT levels for all males remained elevated indicating that this adaptation was specific to PRL. However, it is also possible that the attenuated response to repeated stress in wt males was due to negative feedback since high levels of PRL activate TIDA neurons [2, 15]. Perhaps the PRL response to the first bout of stress activated TIDA neurons, thus preventing PRL secretion to a later stress stimulus. Repeated stress in females, on the other hand, did not show adaptation in the PRL secretory response. Taken together, these results indicate a gender difference in the PRL response to repeated stress. The gender difference may be due to the influence of estrogen. Estrogen is a potent stimulus for PRL secretion [2] and is known to play a major role in regulating TIDA neuronal activity [9] and pituitary lactotroph growth and secretion [28].

Although there were no significant differences in the PRL secretory response to stress in females, PRL and ERα expression levels were determined as an indication of pituitary sensitivity. Since estrogen plays a major modulatory role in PRL regulation in females, differences in pituitary ERα expression may alter PRL responsiveness to a number of stimuli. There were no differences in pituitary ERα expression levels in wt or
ko females, indicating pituitary sensitivity to estrogen is probably similar in both genotypes. This is consistent with the similarity in the PRL secretory response in both genotypes. However, the pituitary PRL content in ko females was double the amount in wt under resting conditions, even though the PRL response to stress was not significantly different between genotypes. This indicates that, although the pituitary has larger pools of PRL in the ko, and the potential exists for a significantly greater response to a stimulus, this did not occur. One explanation for the difference in pituitary PRL content is that more PRL is synthesized in ko animals. This is consistent with OFQ/N having an inhibitory effect on TIDA neurons [42]. If the TIDA neurons are not inhibited to the same degree when OFQ/N is absent, then more PRL would be synthesized [2]. Alternatively, PRL degradation could be decreased, leading to increased pituitary PRL content. In either case, PRL release is not affected by OFQ/N in females, because there was no difference in the PRL secretory response to stress between the genotypes. Furthermore, stress did not affect pituitary PRL content in either male or female mice.

In conclusion, this is the first evidence of a role for OFQ/N in modulation of the PRL secretory response to stress. OFQ/N mediates the PRL secretory response to acute stress in males, but not in females. Furthermore, OFQ/N does not appear to play a role in the modulation of the CORT response to this stress paradigm, in contrast to previously reported results [27]. Interestingly, female OFQ/N ko mice have more pituitary PRL content than wt, but the magnitude of the response to the stress was not different from wt. Additional studies investigating the role of OFQ/N in mediating the effects of chronic stress on PRL secretion are currently being conducted.
Fig. 1. Effect of restraint stress on plasma CORT (A) and PRL (B) levels in OFQ/N wild type and knock-out male mice. Control mice were left undisturbed in their home cage until sacrificed. Separate groups of mice were subjected to 1x 5 min (acute) or 4 x 5 min (repeated) of immobilization stress. Values are means ± SEM.
* significantly different from levels in its own genotype control.
† significantly different from levels in acutely stressed wild type animals.
**A**

![Bar graph showing CORT levels for different genotypes and treatment groups.](image)

- **Wild Type**
  - Control: n=3
  - Acute: n=6
  - Repeated: n=4

- **Knock-out**
  - Control: n=5
  - Acute: n=5
  - Repeated: n=6

**B**

![Bar graph showing PRL levels for different genotypes and treatment groups.](image)

- **Wild Type**
  - Control: n=4
  - Acute: n=6
  - Repeated: n=4

- **Knock-out**
  - Control: n=5
  - Acute: n=5
  - Repeated: n=6
Fig. 2. Effect of restraint stress on plasma CORT (A) and PRL (B) levels in OFQ/N wild type and knock-out female mice. Animals were treated as described in the legend in figure 1. Values are means ± SEM.
* significantly different from levels in its own genotype control.
Fig. 3. Effect of restraint stress on male (A) and female (B) pituitary PRL content in OFQ/N wild type and knock-out mice. Animals were treated as described in the legend in figure 1. For details of the western analysis, see methods section. Values, expressed as mean ± SEM, are the ratios of PRL:Actin density in the stressed group as a percent of the unstressed, control. (C) Representative western blot (female pituitaries). Controls: lanes 1-3: 5, 10, 20 µg of protein; Acute stress: lanes 4-6: 5, 10, 20 µg; Repeated stress: lanes 7-9: 5, 10, 20 µg, respectively.
**A** Males

- **Genotype**
  - **Wild Type**
  - **Knock-out**

- **Control**
- **Acute**
- **Repeated**

- **PRL:Actin Density (% of control)**

- **n=2**
- **n=2**
- **n=2**
- **n=3**
- **n=3**
- **n=3**

**B** Females

- **Genotype**
  - **Wild Type**
  - **Knock-out**

- **Control**
- **Acute**
- **Repeated**

- **PRL:Actin Density (% of control)**

- **n=4**
- **n=5**
- **n=4**
- **n=5**
- **n=4**
- **n=5**

**C** Lane:

- **1 2 3 4 5 6 7 8 9**

- **Actin (42kD)**
- **PRL (23kD)**
Fig. 4. Expression levels of pituitary PRL (A) in control (unstressed) OFQ/N wild type and knock-out female mice. Values, expressed as mean ± SEM, are the ratios of PRL:Actin density in the knock-out animals as a percent of the wild-type.
(B) Representative western blot. For details of the western analysis, see methods section.
Wild type: lanes 1, 2 and 5, 6:10, 20 µg, respectively; Knock-out: lanes 3, 4 and 7, 8: 10, 20µg, respectively.
*significantly different vs. wild type.
A

![Graph showing PRL:Actin density (% of wt) for Wild Type and Knock-out genotypes. The graph indicates a significant difference (*).](image)

- **Genotype**
  - Wild Type
  - Knock-out

- **PRL:Actin density (% of wt)**
  - n=5
  - n=10

B

![Western blot analysis showing lanes 1 to 8 with Actin (42kD) and PRL (23kD) proteins.](image)

- **Lane:** 1 2 3 4 5 6 7 8
- **Proteins:**
  - Actin (42kD)
  - PRL (23kD)
Fig. 5. Expression levels of pituitary ERα (A) in control (unstressed) OFQ/N wild type and knock-out female mice. Values, expressed as mean ± SEM, are the ratios of ERα:Actin density in the knock-out animals as a percent of the wild-type.

(B) Representative western blot. For details of the western analysis, see methods section. Wild type: lanes 1, 2 and 5, 6:10, 20 µg, respectively; Knock-out: lanes 3, 4 and 7, 8: 10, 20µg, respectively.
A

Genotype

ER alpha:Actin Density (% of wt)

Wild type
Knock-out

B

Lane: 1 2 3 4 5 6 7 8

ERα (66kD)
Actin (42kD)
Literature Cited


