DOPAMINERGIC MECHANISMS INVOLVED IN THE ESTROGEN MODULATION OF THE PROLACTIN RESPONSE TO ORPHANIN FQ/NOCICEPTIN

by Brandi Nicole Johnson

The role of estrogen in modulating Orphanin FQ/Nociceptin (OFQ/N) induced prolactin secretion and the involvement of hypothalamic dopaminergic neurons involved in this response were investigated. In ovariectomized Sprague-Dawley rats, estrogen treatment produced an increase in basal circulating prolactin levels and pituitary prolactin content. The prolactin response to OFQ/N was 6-8 times greater in estrogen-replaced rats compared to placebo-treated controls. Also, OFQ/N produced a rapid, transient inhibition of tuberoinfundibular dopaminergic (TIDA) neuronal activity which preceded the prolactin secretory response in the estrogen-replaced rats only. The results of this study indicate that estrogen is necessary for the prolactin response to OFQ/N and estrogen mediates this effect at the pituitary and hypothalamic levels by increasing pituitary prolactin content and sensitizing TIDA neurons to OFQ/N-induced inhibition of activity. These results suggest that estrogen is responsible for the gender differences in the prolactin response to OFQ/N.
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A Thesis

Submitted to the faculty of Miami University in partial fulfillment of the requirements for the degree of Master of Science Department of Zoology

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Introduction

**Prolactin Regulation:** Prolactin is a 197-199 amino acid peptide hormone with a molecular weight of 23 kDa and is secreted by lactotroph cells in the anterior pituitary gland [1, 2]. Prolactin has numerous functions, including roles in reproduction, lactation, homeostasis, growth, metabolism, immunocompetence, and the stress response [2]. Many factors contribute to the regulation of prolactin secretion; however, it is primarily under the tonic inhibitory control of hypothalamic dopamine [1]. Hypothalamic neurons release dopamine into the portal blood vessels and it travels to the anterior pituitary gland where it tonically inhibits prolactin secretion from the lactotroph cells [3].

There are three subpopulations of hypothalamic dopaminergic neurons that regulate the prolactin secretion [4]. The tuberohypophyseal dopaminergic (THDA) neurons are located in the rostral arcuate nucleus and their axons terminate in the neural and intermediate lobes of the pituitary. The periventricular hypothalamic dopaminergic (PHDA) neurons are located in the periventricular nucleus and terminate in the intermediate lobe of the pituitary. THDA and PHDA neurons have a role in the regulation of prolactin release during lactation [4]. No sex differences have been detected in the basal activity of these neurons and they are not influenced by ovarian steroids [1]. The tuberoinfundibular dopaminergic (TIDA) neurons are located in the dorsomedial arcuate nucleus and their axons terminate in the external zone of the median eminence [4]. There are distinct sex differences in the function of these neurons. The number of nerve terminals in the median eminence is the same in male and female rats, but the activity of these neurons is much higher in female rats [3], and ovarian steroids modulate these neurons [4].

Prolactin can also regulate its own secretion through a short-loop negative feedback mechanism. Increases in circulating levels of prolactin stimulate dopamine synthesis in TIDA neurons in rats [5, 6], which results in inhibition of prolactin synthesis and secretion from the anterior pituitary gland.
**Estrogen:** Estrogen can modulate prolactin secretion at the pituitary and hypothalamic levels [4]. In the pituitary, estrogen stimulates prolactin gene expression in lactotrophs [7, 8, 9, 10], leading to an increase in basal circulating prolactin levels. Estrogen also decreases the number of dopamine receptors on the pituitary, making lactotrophs less sensitive to dopaminergic inhibition [11]. At the hypothalamic level, estrogen can have stimulatory, as well as inhibitory effects on the TIDA neurons, depending on concentration, time of administration, and duration of action [12]. Acutely, estrogen decreases the activity of tyrosine hydroxylase in the TIDA neurons [13]. Chronic estrogen treatment in ovariectomized rats increases basal activity of TIDA neurons [4]. Estrogen also increases prolactin receptor expression in the TIDA neurons [14], making them more sensitive to prolactin short-loop feedback. It is unknown if estrogen exerts direct effects on the TIDA neurons or if it acts through other neurotransmitters or neuropeptides. Estrogen does, however, decrease the activity of tyrosine hydroxylase, the rate-limiting enzyme in dopamine synthesis in cultured hypothalamic neurons, [12], suggesting that estrogen has direct effects on hypothalamic dopaminergic neurons.

One of the mechanisms through which estrogen modulates cell function is genomic. Estrogen enters the cell and binds estrogen receptors (ER), ERα and ERβ, to regulate gene transcription [15]. ER dimerizes and binds an estrogen response element on the promoter region of the responsive gene [16]. In the rat, ERα is primarily expressed in the pituitary gland [17]. ERα and ERβ are both expressed in the hypothalamus but have distinct expression patterns [18, 19].

**Opioids:** Opioids form the major class of strong analgesics. There are three major families of “classic” endogenous opioids; dynorphin, β-endorphin and enkephalins. These peptides elicit their actions by acting primarily on the κ [20, 21], μ ([22, 23, 24], and δ [24, 25, 26] opiate receptor subtypes, respectively. The opiate receptors are coupled to a Gi protein that inhibits adenylyl cyclase [27]. Inhibition of cAMP formation in neurons results in inhibition of voltage-gated calcium channels and activation of G-protein coupled inwardly rectifying K⁺ current [28, 29, 30].
Opioids are localized throughout the central nervous system and play many important roles in central nervous system function [31], including a role in prolactin regulation [32, 33, 34, 35]. Opioids stimulate prolactin secretion, at least in part, by inhibiting TIDA neuronal activity [33, 36, 37, 38].

Orphanin FQ/ Nociceptin (OFQ/N) is a 17 amino acid peptide that makes up the fourth family of endogenous opioids. It has high sequence homology with the other endogenous opiates, especially Dynorphin A [27]. OFQ/N acts on the OP₄/ORL₁ receptor which is a distinct opiate receptor subtype, but has high structural and functional homology with μ, κ, and δ opiate receptors. OFQ/N peptide and mRNA are localized throughout the hypothalamus, especially in the arcuate nucleus [39]. OP₄/ORL₁ receptor mRNA has also been localized in the arcuate nucleus [40, 41].

Although there are only a few reports of the neuroendocrine role of OFQ/N, it has been shown to be a potent stimulus for prolactin secretion in male and female rats in a dose and time related manner [42]. The magnitude of the prolactin response to OFQ/N in female rats was similar to that of the suckling induced prolactin response [32]. This response in female rats was mediated by transient inhibition of TIDA neurons at one minute following intracerebroventricular (icv) injection of OFQ/N [43]. Also, DOPAC levels in the hypothalamus were decreased after administration of OFQ/N [44].

**Gender Differences:** The magnitude of the OFQ/N induced prolactin response in females is 3-4 times higher than in males [42]. By using ovariectomized rats that received estrogen replacement or a placebo, results from our laboratory demonstrated that this gender difference is due to the modulation of estrogen and that OFQ/N inhibits tyrosine hydroxylase (TH) activity, as indicated by TH phosphorylation expression levels in the hypothalamus of estrogen replaced rats [45].

Although there are only a few reports of the neuroendocrine role of OFQ/N, work from our laboratory demonstrates that OFQ/N is a potent stimulus for prolactin secretion in male and female rats in a dose and time related manner [42] and this response is not
mediated by the classic μ, δ or κ opiate receptor subtypes [46]. The magnitude of the OFQ/N induced prolactin response in females is 3-4 times higher than in males [42]. This response in female rats is mediated by transient inhibition of TIDA neurons at one minute following intracerebroventricular (icv) injection of OFQ/N [43]. To determine if estrogen specifically modulates the response of the TIDA neurons to OFQ/N, dopamine turnover was quantified in the median eminence following OFQ/N administration in ovariectomized (OVX), female rats +/- estrogen replacement. In addition, anterior pituitary prolactin expression levels as well as circulating prolactin levels were determined as an index of synthesis and secretion, respectively.
Materials and Methods

**Animals:** Adult, virgin female Sprague-Dawley rats (200-225 g; 14-16 weeks old) (Harlan Laboratories, Indianapolis, IN) were housed in a temperature (21°C) and light-controlled (12L:12D, lights on at 0600) room. Animals were housed two per cage until after surgery, when they were individually housed. Food and water were provided *ad libitum*. All animal protocols were approved by the Miami University Institutional Animal Care and Use Committee in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

**Ovariectomy (OVX):** Female rats were ovariectomized under isoflurane (Abbott Laboratories, Chicago, IL) anesthesia two weeks prior to experiments. At this time animals were implanted subcutaneously with pellets containing either sesame oil (placebo) or 1.7 mg 17-β-estradiol (Innovative Research of America, FL) with an estimated release rate of 0.05 mg/day.

**Intracerebroventricular cannulation:** Five to seven days before being used in an experiment, animals were implanted with chronic intracerebroventricular (icv) cannulae into the right lateral ventricle of the brain following the coordinate system of Pellegrino, et al [47] as previously described [48] under ketamine (80 mg/kg, intramuscular, Phoenix Scientific, St. Joseph, MO) and xylazine (14 mg/kg, intramuscular, Phoenix Scientific, St. Joseph, MO) anesthesia.

**Experimental Procedure:** Experiments were conducted two weeks following ovariectomy. They were performed between 0830-1030 h to minimize any effects of circadian variations in hormone levels. After a 30 minute acclimation period, animals were injected with OFQ/N (55 pmol or 550 pmol, icv in a 5 µl volume) or an equal volume of saline (control). Animals were sacrificed 1, 3 or 10 minutes after injection and blood was collected in heparinized (1000 U/ml) tubes. Brains were quickly removed and placed in ice cold saline. The entire pituitary gland was removed from the sella turcica and placed on a cold plate under a stereomicroscope. The neurointermediate lobe (NIL) was separated from the anterior lobe of the pituitary and both were collected. The median eminence was removed from the rest of the hypothalamus following the guidelines of Palkovits and Brownstein [49]. Upon collection, all tissues were immediately placed in separate 1.5 ml eppendorf tubes and frozen in liquid nitrogen. Tissues were stored at -
80°C until they were analyzed for amine and metabolite content (median eminence and NIL) or for prolactin expression levels (anterior pituitary gland). Trunk blood was centrifuged (3,000 Xg, 5 min) and the plasma was collected. Plasma was stored at -20°C until prolactin and 17β-estradiol levels were determined by RIA. The uterus was dissected and weighed post-mortem.

**Radioimmunoassay:** Circulating plasma prolactin levels were measured in triplicate samples by double antibody radioimmunoassay using reagents purchased from the National Hormone and Peptide Program and Dr. A.F. Parlow. Circulating levels of 17-β estradiol were quantified using a double antibody RIA kit (MP Biomedicals, Irvine, CA) following the manufacturer’s instructions.

**High Performance Liquid Chromatography/ Electrochemical Detection:** Detection of amines and their metabolites in the median eminence and NIL was performed as previously described [50]. Briefly, microdissected tissue was placed in 200 µl (median eminence) or 50 µl (NIL) of mobile phase (0.2 mM EDTA, 50 mM monobasic NaH2PO4, 1.2 mM Heptanesulfonic acid) without methanol and sonicated (Kontes Micro Ultrasonic Cell Disrupter, Vineland, NJ). The homogenate was centrifuged for 10 minutes at 15,000 Xg at 4°C. The supernatant was collected, and the pellet was solubilized in 1.0 N NaOH and stored at -20°C until assayed for protein [51]. An aliquot of the supernatant was injected onto a C-18, reverse phase column (Varian, Walnut Creek, CA) for separation of amines and their metabolites. The sample was pumped through a Waters 2695 separations module (Waters, Milford, MA) at a flow rate of 1 ml/min in mobile phase containing 6% methanol. The following amines and metabolites were quantified: 3,4-dihydroxyphenylacetic acid (DOPAC), dopamine, 5-hydroxyindole-3-acetic acid (5HIAA), and serotonin. Column effluent was monitored by a coulometric detector (ESA, Chelmsford, MA), with the first electrode set at -250mV, the second set at +250mV, and the guard cell set at +350mV. Data were collected and analyzed using Waters Millennium Chromatography Manager Software (Waters, Milford, MA). Unknown amine concentrations were calculated based on known standards (Sigma Chemical Co., St. Louis, MO). Data are expressed as ng of amine per mg protein.
**Western Blot Analysis:** The anterior pituitary gland was sonicated in 100µl of homogenizing buffer (1M Tris, 1% SDS and protease inhibitor cocktail (Sigma Aldrich, St. Louis, MO)). The protein content was determined in a 10µl aliquot using a BCA protein assay kit (Pierce, Rockford, IL). Samples containing 5, 10 or 20 µg of homogenized tissue were separated on a 12% SDS-polyacrylamide gel for 2 hours at 200V. Protein was transferred to Immobilon-P transfer membranes (Millipore Corp, MA) at 100V for 55 minutes. Membranes were blocked with 8% nonfat milk for 3 hours at room temperature. Membranes were then incubated for 15 hours at 4°C with primary antibodies (1:2,000,000 rabbit anti-rat prolactin antibody (NHPP, Dr. A.F. Parlow) and 1:3000 rabbit anti-rat actin (Sigma, St. Louis, MO)) diluted in Tris buffer containing 0.05% tween (TBST). Following four washes with TBST, membranes were incubated in goat anti-rabbit horseradish-peroxidase-conjugated secondary antibody (Chemicon, Temecula, CA) diluted 1:5,000 in TBST for two hours at room temperature. After washing membranes in TBST, prolactin and actin were detected using SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) and developed on Kodak biomax film (Fisher, Pittsburgh, PA). Optical density of prolactin and actin bands was quantified using ImageQuant 5.2 analysis software. Actin was used as an internal control and data were expressed as a ratio of prolactin to actin.

**Statistical Analysis:** Effects of estrogen treatment on pituitary prolactin content, estrogen levels, uterine weight and body weight were analyzed using a two-sample t-test with unequal variances. The effect of estrogen on basal prolactin levels was analyzed using a chi-square test. The effects of OFQ/N on prolactin levels and dopamine turnover were analyzed using one-way analysis of variance, followed by a Dunnett multiple comparisons test.
**Results**

Estrogen replacement in OVX rats produced a significant increase in circulating estrogen levels (Table 1). Estrogen replacement also produced a significant decrease in body weight and an increase in uterine weight (Table 1). Basal circulating prolactin levels (Fig. 1), and pituitary prolactin content (Fig. 2) were significantly elevated in the estrogen replaced females.

The magnitude of the prolactin response to OFQ/N was 6-8 times greater in estrogen replaced animals (Fig. 3a) than in placebo animals (Fig. 3b). In estrogen treated animals, the prolactin secretory response to 55 pmol OFQ/N occurred rapidly, i.e. within 3 minutes (Fig. 3a). This same dose had no effect on prolactin levels in the placebo treated animals (Fig. 3b). Following the 550 pmol dose, there was a small, albeit significant, increase in prolactin levels by 1 minute in the estrogen-replaced animals and this increase persisted through 10 minutes (Fig. 3a). The only significant effect of OFQ/N in the placebo-treated animals occurred at 10 minutes after administration of 550 pmol of OFQ/N (Fig. 3b).

The DOPAC/dopamine ratio in the median eminence was used as an indicator of dopamine turnover and thus activity of the TIDA neurons. In the estrogen-replaced animals, dopamine turnover was only inhibited in the animals receiving 55 pmol OFQ/N and this inhibition was rapid and transient, occurring within one minute and returning to control levels by 3 minutes (Fig 4a). The 550 pmol dose did not inhibit dopamine turnover during the time course examined in these studies (Fig. 4a). Dopamine turnover was unchanged in the placebo animals in response to either dose of OFQ/N (Fig. 4b).
Table 1. Physiological effects of estrogen in ovariectomized rats +/- estrogen replacement. Values are means ± SEM. *Significantly different from OVX+Placebo animals, p<0.0001

<table>
<thead>
<tr>
<th></th>
<th>Circulating Estradiol (ng/ml)</th>
<th>Body Weight (g)</th>
<th>Uterine Weight (g)</th>
<th>% Uterine Weight/Body Weight</th>
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</thead>
<tbody>
<tr>
<td>OVX + Placebo</td>
<td>33.42 ± 2.93</td>
<td>218.9 ± 2.7</td>
<td>0.125 ± 0.0043</td>
<td>0.058 ± 0.002%</td>
</tr>
<tr>
<td>OVX + Estrogen</td>
<td>529.02 ± 57.24 *</td>
<td>186.0 ± 2.0 *</td>
<td>0.560 ± 0.015 *</td>
<td>0.3081 ± 0.008% *</td>
</tr>
</tbody>
</table>
Figure 1. The effect of estrogen replacement on basal circulating prolactin levels in ovariectomized (OVX) rats. Basal prolactin levels were significantly elevated in estrogen-replaced, ovx rats (OVX + E) compared to placebo-replaced animals (OVX + Placebo). Values are mean ± SEM.

*Significantly different from placebo group, p< 0.001.
Prolactin (ng/ml)

- **OVX+Placebo**
- **OVX+E**

- n=18
- n=19
Figure 2. The effect of estrogen replacement on anterior pituitary prolactin content in OVX rats. (A) Representative image of a western blot of prolactin and actin protein expression in the anterior pituitary of OVX + Placebo (lanes 1, 2 and 3: 5, 10 and 20 µg) and OVX + E (lanes 4, 5 and 6: 5, 10 and 20 µg) rats. (B) Quantification of the prolactin/actin ratio in the anterior pituitary gland of OVX + E treated rats as a percentage of placebo controls. Prolactin levels were significantly elevated in OVX + E rats.

* Significantly different from placebo group, p< 0.001.
A.

- **OVX + Placebo**
  - Lane 1
  - Lane 2
  - Lane 3

- **OVX + Estrogen**
  - Lane 4
  - Lane 5
  - Lane 6

- Actin (42 kD)
- Prolactin (23 kD)
Figure 3. Prolactin response to OFQ/N administration in (a) OVX + E and (b) OVX + Placebo rats. (a) Prolactin levels were significantly elevated 3 minutes after injection of 55 pmol OFQ/N and by 1 minute after injection of 550 pmol OFQ/N. The response to both doses remained elevated through 10 minutes. (b) The only significant effect of OFQ/N administration in the placebo animals occurred at 10 minutes following 550 pmol dose. Values are mean ± SEM.

* Significantly different from saline injected group at the same time point, p<0.05,
†p<0.0005.
A.

- Prolactin (ng/ml)
- Saline
- 55 pmol OFQ/N
- 550 pmol OFQ/N

1 Minute    10 Minutes  3 Minutes

*     *     *

n=6  n=11  n=7
n=7  n=9   n=7
n=6  n=7   n=5

†
B.

[Graph showing prolactin levels in response to saline and two doses of OFQ/N over 1, 3, and 10 minutes, with n values indicated for each group.]
Figure 4. TIDA neuronal activity in response to OFQ/N injection in (a) OVX + E and (b) OVX + Placebo rats. (a) The 55 pmol dose caused inhibition of TIDA neuronal activity only at 1 minute following the injection. The 550 pmol dose did not affect dopamine turnover at any of the times examined. (b) Neither dose of OFQ/N produced any change in dopamine turnover in the median eminence in the placebo animals. Values are mean ± SEM.

*Significantly different from saline injected group at the same time point, p< 0.0005.
DOPAC/DA

Saline

55 pmol OFQ/N

550 pmol OFQ/N

1 Minute    10 Minutes  3 Minutes

A.
B.

- **Saline**
- **55 pmol OFQ/N**
- **550 pmol OFQ/N**

DOPAC/DA

<table>
<thead>
<tr>
<th>Time</th>
<th>Saline</th>
<th>55 pmol OFQ/N</th>
<th>550 pmol OFQ/N</th>
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</thead>
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<tr>
<td>1 Minute</td>
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<td></td>
<td></td>
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<tr>
<td>10 Minutes</td>
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</table>

- n=6
- n=6
- n=6
- n=5
- n=6
- n=7
- n=5
- n=6
- n=4
Discussion

The results of this study demonstrate that estrogen is an important modulator of the stimulatory effect of OFQ/N on prolactin secretion. Estrogen modulates the prolactin response to OFQ/N at both the pituitary and the hypothalamic levels. Ovariectomized females that received estrogen replacement had increased pituitary prolactin content and an increase in basal circulating prolactin levels, confirming estrogen’s stimulatory effect on pituitary lactotrophs [4]. At the level of the hypothalamus, OFQ/N-induced inhibition of TIDA neuronal activity occurred only in estrogen-replaced, ovx females. In these animals, the 55 pmol dose of OFQ/N transiently inhibited TIDA neurons and this inhibition preceded the OFQ/N-induced stimulation of prolactin secretion. The modulatory effects of estrogen at the pituitary and hypothalamic levels are responsible for a prolactin response to OFQ/N that was 6-8 times greater in estrogen-replaced animals than placebo-replaced animals. Previous results from our laboratory demonstrated that OFQ/N-induced prolactin secretion was 3-4 fold greater in intact females than males [42].

The results of the current study demonstrate that estrogen is an important factor in modulating the prolactin secretory response to OFQ/N, and that this modulation affects the TIDA neurons. The prolactin response to OFQ/N in estrogen replaced animals is, at least in part, due to rapid and transient inhibition of TIDA neurons. We have shown that the TIDA neurons were inhibited by one minute following injection of 55 pmol OFQ/N and that activity returned to control levels by 3 minutes. TIDA neuronal inhibition preceded the prolactin increase that occurred 3 minutes after OFQ/N injection. In contrast, administration of a 10-fold higher dose of OFQ/N did not cause inhibition of the TIDA neurons in estrogen-replaced animals, which may be due to the time course of these experiments. Because the TIDA neuronal inhibition in response to 55 pmol was rapid and transient, and prolactin levels were already significantly increased by 1 minute after injection of 550 pmol OFQ/N, the TIDA neuronal inhibition may have occurred even sooner than 1 minute after OFQ/N injection. This seems likely because TIDA neuronal inhibition is a major mechanism mediating stimulus-induced prolactin secretion.
[1], and our studies to date [43] indicate that OFQ/N, like the other endogenous opiates and opiate agonists, stimulate prolactin secretion by inhibiting TIDA neurons [1, 4].

In this study, prolactin levels remained elevated for the duration of the experiments, i.e. at least 10 minutes following the OFQ/N injection. In previous studies, OFQ/N administration to intact female rats produced elevated levels of prolactin for 20-30 minutes, depending on the dose [42, 46, 50]. Prolactin levels remain elevated even after TIDA neuronal activity returned to control levels [43]. In addition to dopamine inhibition, there are many other factors that regulate prolactin secretion [1], some of which have been shown to be involved in opiate induced prolactin secretion, and are probably involved in OFQ/N-induced prolactin release. Netti, et al, [52] showed that when hypothalamic catecholamines were depleted with α-methyl-p-tyrosine (α-MPT), OFQ/N administration still increased prolactin levels in male rats. It is important to note; however, that α-MPT administration produces an increase in basal prolactin levels because dopamine is depleted. Therefore, the OFQ/N was likely stimulating prolactin releasing factors under conditions when TIDA neurons were already severely inhibited due to the α-MPT.

A number of releasing factors that have been implicated in opiate-induced prolactin regulation may also be involved in OFQ/N stimulation of prolactin secretion. Histamine, a prolactin releasing factor, is involved in the prolactin response to morphine [53, 54], and VIP stimulates prolactin release by acting on the endogenous opioids [55]. Although serotonin, and its precursor, 5-hydroxytryptophan, induce prolactin secretion [56, 57] and serotonin is involved in the prolactin response to suckling [58, 59], serotonin does not appear to be involved in mediating the prolactin response to OFQ/N. In this study, serotonin turnover did not change following estrogen treatment or OFQ/N administration (See Appendix). In addition, previous results from our lab demonstrate that pharmacological blockade of serotonergic receptors did not attenuate the prolactin response to OFQ/N [50]. Another stimulatory factor that may be responsible for sustaining the prolactin response to OFQ/N is oxytocin. Oxytocin stimulates prolactin secretion in vivo and in vitro [1]; furthermore, ORL-1 mRNA is expressed in the
magnocellular paraventricular nucleus and the supraoptic nucleus [40]. Based on our results and those of Netti, et al [52], it is likely that OFQ/N administration produces a rapid, transient inhibition of TIDA neuronal activity, followed by a more sustained effect on other non-dopaminergic mechanisms, e.g. prolactin releasing factors. The releasing factors that are necessary to sustain the prolactin response are currently being investigated.

There was no change in the dopaminergic activity of either the THDA or PHDA neurons in response to estrogen treatment or OFQ/N administration (See Appendix). Although PHDA and THDA neurons play a role in regulating prolactin secretion, in contrast to the TIDA neurons, they are not regulated by gonadal steroids [1, 4]. Also, ORL-1 mRNA is not highly expressed in the periventricular nucleus [40], indicating that the OFQ/N receptor may not be highly expressed in PHDA neurons and therefore, the neurons may be less sensitive to OFQ/N than the TIDA neurons.

The results of this study demonstrated that estrogen is an important modulator of the stimulatory effects of OFQ/N on prolactin release. ERα and ERβ are abundant in the arcuate nucleus [19], the area containing the cell bodies of TIDA neurons [1, 4]. Thus, it is possible that estrogen elicits direct effects on TIDA neurons. Also, ORL-1 mRNA levels are high in the arcuate nucleus [40]. One possible mechanism of action for estrogen is that it may increase the sensitivity of the TIDA neurons to OFQ/N by increasing ORL-1 expression. Estrogen modulation of the OFQ/N system is further supported by studies showing the colocalization of ORL-1 with estrogen receptor-β in the paraventricular nucleus [60]. Furthermore, there are estrogen dependent sex differences in ORL-1 expression in the trigeminal nucleus [61] and in the ventromedial hypothalamus [62], offering additional evidence that estrogen modulates OFQ/N sensitivity in target tissues.

In conclusion, the results of this study demonstrate that estrogen is necessary for OFQ/N to inhibit TIDA neuronal activity and produce a significant increase in circulating levels of prolactin in female rats. Estrogen mediates this effect at both the pituitary and
hypothalamic level by increasing pituitary prolactin content and sensitizing TIDA neurons to OFQ/N-induced inhibition of activity. These results suggest that estrogen makes the female more sensitive to OFQ/N stimulation and is responsible for the gender differences in the prolactin secretory response to OFQ/N administration.
References


Appendix

This appendix contains figures showing negative data relevant to the study.
Figure 5. Serotonin activity in the median eminence in response to OFQ/N (a) OVX + E and (b) OVX + Placebo rats. Serotonin turnover was unchanged in the in (a) OVX + E and (b) OVX + Placebo rats in response to either dose of OFQ/N. Values are mean ± SEM.
A.

- Saline
- 55 pmol OFQ/N
- 550 pmol OFQ/N

<table>
<thead>
<tr>
<th>Time (Minutes)</th>
<th>Saline</th>
<th>55 pmol OFQ/N</th>
<th>550 pmol OFQ/N</th>
</tr>
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<tbody>
<tr>
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<tr>
<td>10</td>
<td>n=6</td>
<td>n=7</td>
<td>n=4</td>
</tr>
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B.

![Graph showing the effect of Saline, 55 pmol OFQ/N, and 550 pmol OFQ/N on 5HIAA/5HT levels at 1 Minute, 3 Minutes, and 10 Minutes. The graph includes error bars for each condition.]
Figure 6. PHDA and THDA neuronal activity in response to OFQ/N in (a) OVX + E and (b) OVX + Placebo rats. Dopamine turnover was unchanged in the (a) OVX + E and (b) OVX + Placebo rats in response to either dose of OFQ/N. Values are mean ± SEM.
A.

![Graph showing DOPAC/DA levels over time with different treatment groups: Saline, 55 pmol OFQ/N, and 550 pmol OFQ/N. The graph includes data points for 1 Minute, 3 Minutes, and 10 Minutes with sample sizes indicated for each time point.

- **Saline**
  - 1 Minute: n=5
  - 3 Minutes: n=7
  - 10 Minutes: n=6

- **55 pmol OFQ/N**
  - 1 Minute: n=10
  - 3 Minutes: n=6
  - 10 Minutes: n=6

- **550 pmol OFQ/N**
  - 1 Minute: n=8
  - 3 Minutes: n=8
  - 10 Minutes: n=6

The graph indicates a trend of increased DOPAC/DA levels with 550 pmol OFQ/N compared to the other groups over time.
B.

- Saline
- 55 pmol OFQ/N
- 550 pmol OFQ/N

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<thead>
<tr>
<th>Time</th>
<th>Saline</th>
<th>55 pmol OFQ/N</th>
<th>550 pmol OFQ/N</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Minute</td>
<td>n=2</td>
<td>n=5</td>
<td>n=5</td>
</tr>
<tr>
<td>3 Minutes</td>
<td>n=3</td>
<td>n=6</td>
<td>n=8</td>
</tr>
<tr>
<td>10 Minutes</td>
<td>n=2</td>
<td>n=4</td>
<td>n=3</td>
</tr>
</tbody>
</table>
Figure 7. Serotonin activity in the neurointermediate lobe in response to OFQ/N in (a) OVX + E and (b) OVX + Placebo rats. Serotonin turnover in the neurointermediate lobe was unchanged in (a) OVX +E and (b) OVX + Placebo rats in response to either dose of OFQ/N. Values are mean ± SEM.
A.

- Saline
- 55 pmol OFQ/N
- 550 pmol OFQ/N

5HT/5HIAA

1 Minute 3 Minutes 10 Minutes

n=6 n=10 n=9 n=7 n=7 n=9 n=7 n=8 n=6
B.  

Graph showing the effect of Saline, 55 pmol OFQ/N, and 550 pmol OFQ/N on 5HIAA/5HT levels over time (1 Minute, 3 Minutes, and 10 Minutes) with sample sizes indicated for each group.