The role of Orphanin FQ/Nociceptin (OFQ/N) was investigated during the post-partum period. Female OFQ/N knock-out and wild-type mice were bred. Pups were monitored for survival, weighed, and the presence of milk bands collected from post-partum day 0 until day 1, from day 0 through day 6, and during cross-fostering. Knock-out females experienced a significant pup loss on day 0 until day 1, from day 0 through day 6, and when given pups to cross-foster. Knock-out reared pups had fewer milk bands and significantly less weight gain. Knock-out females had significantly higher pituitary prolactin levels on day 1 and following suckling (0, 5-10 minutes). Knock-out females had significantly lower circulating prolactin on day 1. No difference was observed in maternal behavior, pituitary estrogen receptor alpha content, or circulating prolactin levels following suckling. These data indicate that OFQ/N is necessary for pup survival during the post-partum period.
NEURAL AND ENDOCRINE IMPACT OF MATERNAL ORPHANIN FQ/NOCICEPTIN

A Thesis

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Background

Prolactin Secretion

Prolactin (PRL), a protein hormone synthesized in and secreted from the lactotrophs of the anterior pituitary gland, functions in more than 300 biological processes in mammals, including reproduction and homeostasis (For reviews see Freeman, et al., 2000; Goffin, et al., 2002). PRL is best characterized for its role in lactation (For reviews see Freeman, et al., 2000; Goffin, et al., 2002); it is necessary for proper mammary gland development (Brisken, et al., 1999) and, through actions on the brain, is responsible for the onset of maternal behavior (Lucas, et al., 1998; Bridges, et al., 2001). The regulation of PRL release is complex, but is primarily under tonic inhibitory control by hypothalamic dopamine (DA) neurons. Acting through D2 receptors on the anterior pituitary lactotrophs, DA suppresses the biosynthesis, storage and secretion of PRL (For review see Ben-Johnathan and Hnasko, 2001).

There are three hypothalamic, dopaminergic pathways involved in the regulation of prolactin secretion: the tuberohypophyseal dopaminergic (THDA), the periventricular hypothalamic dopaminergic (PHDA), and the tuberoinfundibular dopaminergic (TIDA) pathways. All three of the pathways function to inhibit PRL secretion, but the TIDA pathway clearly plays the major role in regulating PRL secretion (For review see Ben-Johnathan and Hnasko, 2001). Suckling, the most potent, physiological stimulus for PRL secretion, produces a significant increase in circulating levels of PRL within 1-3 minutes and peaks at 10 minutes (For review see Freeman, et al., 2000). This suckling-induced increase is due, at least in part, to a decrease in the activity of TIDA neurons (For review see Grattan, 2001). In addition, other neural (hypothalamic) factors are involved in the regulation of prolactin secretion. Of the numerous regulatory factors known to be involved in PRL regulation (see Freeman, et al., 2000 for Review), the endogenous opioid peptides (EOP) (reviewed in Andrews and Grattan, 2003) and estrogen (reviewed in Ben-Jonathan and Hnasko, 2001) are both important during the post-partum period.

Endogenous Opioids

There are three families of classic, endogenous opioid peptides (EOP) i.e., dynorphin, β-endorphin, and the enkephalins. These opioid peptides elicit their effects by preferentially
binding to one of three different G-protein-coupled receptors, the κ, μ, or δ, respectively (Freeman, et al., 2000). The EOP are important neuromodulators of the endocrine system, and are involved in regulating anterior pituitary hormonal secretions, including prolactin secretion. The EOP stimulate prolactin release, at least in part, through TIDA neuronal inhibition. This regulation is particularly important in the regulation of prolactin secretion during the reproductive cycle and lactation in females and in the stress response (For reviews see Freeman, et al., 2000, Ben-Jonathan and Hnasko, 2001, Drolet, et al., 2001).

Orphanin FQ/Nociceptin

Orphanin FQ/Nociceptin (OFQ/N) is the fourth endogenous opioid neuropeptide that exhibits a high amino acid sequence homology with the classic endogenous opiates, especially dynorphin. OFQ/N is a distinct neuropeptide (Nothacker, et al., 1996) that lacks the N-terminus region necessary for binding to the classic opioid receptors; OFQ/N binds to its own, distinct receptor, termed the Opioid Receptor Like (ORL1 or OP₄) receptor (Meunier, et al., 1995).

OFQ/N shares a number of characteristics with the classic opiate peptides. Similar to the other endogenous opiates, high levels of the OFQ/N peptide and mRNA, as well as the ORL₁ receptor, are localized throughout the hypothalamus (Neal, et al., 1999a; Neal, et al., 1999b; Norton, et al., 2002). Additionally, OFQ/N, like the other opiates, seems to function as a neuromodulator of the endocrine system. For example, Bryant, et al., (1998) reported that OFQ/N stimulated prolactin secretion in male and female rats, with the magnitude of the response being significantly greater in females. The increase in PRL was not due to activation of the κ, μ, or δ receptor, suggesting OFQ/N acts at its own receptor subtype (Bryant, et al., 2002). More recently, Chesterfield, et al., (In review) reported that OFQ/N does indeed stimulate prolactin release through its own receptor subtype and regulates prolactin secretion during lactation in post-partum rats.

Post-Partum Period

During mammalian pregnancy and lactation, many physiological and behavioral changes occur in the mother’s body and brain to prepare the female for parturition and the post-partum period (For review see Grattan, et al., 2001). Prolactin plays a major role in many of these changes including mammary gland lobulo-alveolar duct development, which is necessary for
milk production (Brisken, et al., 1999), and the synthesis and secretion of milk from the mammary gland (Bole-Feysot, et al., 1998). During this hyperprolactinemic state, PRL has been implicated in the organizational changes that occur in the brain, inducing the onset of maternal behavior (Bridges, 1994). Behaviors include, but are not limited to, nest building, nursing, licking of pups, pup retrieval, and crouching over the nest to provide warmth (Lucas, et al., 1998; Chiang, et al., 2002). Proper maternal behavior is essential for mammalian offspring survival (Lucas, et al., 1998). Studies have shown that maternal behavior is significantly reduced when PRL secretion is suppressed (Bridges, et al., 1997) or when PRL receptor antagonists are administered (Bridges, et al., 2001). Thus, PRL must be secreted into the circulation in order for the onset of normal maternal behavior to occur in the brain.

The suckling stimulus activates endogenous opioid neurons and opiate receptors which inhibits dopamine release from the TIDA neurons (Andrews and Grattan, 2003) and a robust increase in prolactin release (Callahan, et al., 2000). The TIDA neuronal inhibition also causes lactotrophs to become more sensitive to releasing factors which may include estrogen, oxytocin and serotonin (Reviewed in Freeman, et al., 2000). The combination of TIDA neuronal inhibition and activation of releasing factors, results in sustained prolactin secretion in response to suckling (Demarest, et al., 1983).

Prolactin gains access to the brain by carrier-mediated transport in the choroid plexus (Walsh, et al., 1987). This region of the brain contains the highest concentration of prolactin receptors and prolactin receptor mRNA (Pi and Grattan, 1998) which allow this polypeptide hormone to gain access to the cerebrospinal fluid (Torner, et al., 2002) and exert actions in the brain. During pregnancy and lactation, an up-regulation of prolactin receptors occurs in the choroid plexus and hypothalamus (Pi and Grattan, 1999) suggesting that elevated circulating PRL levels may access the brain to regulate neuroendocrine processes throughout this period (Augustine, et al., 2003).

To date, little knowledge has been obtained regarding the role of OFQ/N in the regulation of prolactin secretion or maternal behavior, during the post-partum period. However, studies from our laboratory have demonstrated that OFQ/N administration stimulates prolactin secretion in male and female rats (Bryant, et al., 1998). Furthermore, this increase in PRL secretion is due to OFQ/N acting on the ORL1 receptor (Chesterfield, et al., In review) and causing inhibition of the TIDA neurons and OFQ/N is involved in the suckling-induced prolactin increase
(Chesterfield, et al., In review). The results of these studies, along with its localization in the hypothalamus (Nothacker, et al., 1996), indicate that OFQ/N is an important, physiological regulator of PRL secretion.

**Significance**

The purpose of this study was to investigate the role of OFQ/N in regulating PRL secretion in the post-partum female. Using OFQ/N knock-out mice and their wild-type littermates as controls, these studies examine the regulation of PRL secretion in a physiologically, relevant state, i.e. lactation. These results indicate that OFQ/N is important in prolactin regulation during lactation and offspring survival is decreased when OFQ/N is not expressed in post-partum dams.
1. Introduction

OFQ/N is a heptadecapeptide that exhibits high amino acid sequence homology to the other opiate peptides, particularly dynorphin A (Meunier, et al., 1995; Reinscheid, et al., 1995). OFQ/N is the endogenous ligand for the opioid like orphan receptor ORL1 (Lan, et al., 1997; Meunier, et al., 1995; Mollereau, et al., 1995, Reinscheid, et al., 1995). Although the OFQ/N receptor exhibits structural (Dooley and Houghten, 1996) and functional homology with the other opiate receptors (See Hawes, et al., 2000 for review), it does not bind to the other opiate receptors, nor do the other opiate peptides bind to the OFQ/N receptor (Mogil and Pasternak, 2001).

In situ hybridization and immunocytochemical studies have revealed that the OFQ/N peptide and its mRNA are localized throughout the hypothalamus, particularly in the arcuate nucleus (Neal, et al., 1999b), and high levels of ORL1 receptor mRNA have been localized in the arcuate nucleus as well (Neal, et al., 1999a; see Mollereau and Mouledous, 2000 for review). The cell bodies of the tuberoinfundibular dopaminergic (TIDA) neurons are also located in the arcuate nucleus and opiates stimulate prolactin secretion, at least in part, by inhibiting these TIDA neurons. These localization studies provide anatomic evidence that OFQ/N may also affect TIDA neuronal activity. Supporting this hypothesis, Shieh and Pan (2001) reported decreased DOPAC levels in the hypothalamus following OFQ/N administration. In addition, we reported that OFQ/N stimulates prolactin secretion in both male (Bryant et al., 1998) and female rats (Bryant, et al., 1998, 2002; Janik, et al., 2003). Additionally, OFQ/N does inhibit TIDA neuronal activity in a time-related manner (Chesterfield, et al., in review), but this inhibition does not persist during the peak of the prolactin response (Kraska, et al., 2005), suggesting prolactin releasing factors are also involved.

Prolactin, a hormone best characterized for its role in lactation (For reviews see Freeman, et al., 2000 and Goffin, et al., 2002), including its role in proper mammary gland development (Brisken et al., 1999) and the onset of maternal behavior (Lucas, et al., 1998; Bridges, et al., 2001), is regulated by numerous factors, including the endogenous opioid peptides (EOP) (see Freeman, et al., 2000 for review). During lactation, EOP stimulate prolactin secretion (Callahan, et al., 2000), at least in part, by inhibiting TIDA neurons (For reviews see Freeman, et al., 2000).
The purpose of this study was to determine the physiological role of endogenous OFQ/N in the regulation of prolactin secretion during the post-partum period. Using post-partum OFQ/N knock-out (KO) and wild-type (WT) (control) mice, we determined the effect of OFQ/N gene deletion on anterior pituitary prolactin content and secretion, as well as on maternal behavior and offspring viability.

2. Materials and Methods

2.1 Animals

Orphanin FQ/Nociceptin wild-type and transgenic female mice (*Mus musculus*), previously generated from C57BL/6 x 129/Ola, were bred with males of the same genotype. Breeder pairs were maintained under controlled light conditions (12:12 LD, lights on at 0600), temperature (21°C) and had *ad libitum* access to food and water. All procedures were approved by the Miami University Institutional Animal Care and Use Committee (IACUC) and adhere to the National Institutes of Health guidelines.

Male and virgin female mice of reproductive age, i.e. at least 6 weeks old for females and 8 weeks old for males and less than 6 months old, were bred. Females were individually housed after pregnancy was determined. Experiments were conducted between the hours of 0600 and 1100 to avoid circadian variations in hormone levels.

2.2 Genotyping

Animals were genotyped using the Polymerase Chain Reaction (PCR) as described by Koster, et al., 1999. Ear punches (~3mm diameter) or tail clips (~3mm) were collected from the animals while they were under Isoflurane USP (Phoenix Pharmaceutical Inc., St. Joseph, MO) anesthesia. The DNA primers were:

Primer OFQ 282: 5’ GACCCAGAGCTTGTGTCAGC,
Primer OFQ 530: 5’CTCATAAAACTCAGTCAGC, and
Primer NEO: 5’CCTTGTGACACCTGCAATCC.

Conditions for the PCR were: 94°C for 3 minutes followed by 31 cycles of 94°C for 30 seconds, 60°C for 30 seconds and 72°C for 45 seconds, followed by 5 minute extensions at 72°C. The PCR products were run on a 1.5%
agarose gel and genotypes were determined as follows: wild type = 250bp band OFQ gene, heterozygous = 250bp OFQ gene and 550 cassette, and knock-out = 550 cassette only, no gene.

2.3 Pup Survival Monitoring

On post-partum day 0, the total number of pups delivered and the number of live pups present were recorded for each litter. Pups were individually weighed (to 0.01g) using a Mettler PM2000 scale (Mettler-Toledo Inc.) and the presence or absence of milk bands in the pups was recorded. The number of live pups present, the body weights of each live pup, and the presence or absence of milk bands in the pups was collected again on post-partum day 1. Following sacrifice, the uterus was removed from each dam and the number of uterine implantation sites was recorded.

Pup survival was monitored in a subset of animals from post-partum day 0 until post-partum day 6. In this group of animals, the number of live pups present and the pup body weights were recorded daily. Knock-out dams were sacrificed on the day when no live pups remained in their litters or on post-partum day 6 if pups were present. Wild-type dams were match paired to knock-out dams.

2.4 Maternal Behavior – Post-Partum Day 1

To examine the possibility that knock-out mothers were not caring for their offspring, dams of both genotypes were separated from their pups for 2 hours on post-partum day 1. Dams were returned to their pups and following a 5 minute acclimation period, maternal behaviors were observed over the next 10 minutes. The amount of time (seconds) the dam was engaged in different maternal behaviors was recorded. The behaviors were: nursing, pup licking, pup retrieval, nest building, time spent crouching over the nest to provide warmth, and time spent not in contact with their pups (Lucas, et al., 1998; Chiang, et al., 2002).

2.5 Suckling Experiments

On post-partum day 1, dams were separated from their pups for 2 hours. Following separation and before dam return, the number of live pups present, the body weights of each live pup, and the presence or absence of milk bands in the pups were recorded. The amount of time (seconds) the dams were suckled was recorded. Dams were sacrificed immediately following the
suckling bout and grouped according to the amount of time the dams were suckled. In one group (designated 0 minutes), the pups did not suckle. In the second group, the pups spent at least 1 minute, but less than 5 minutes suckling (designated 1-5 minutes). Pups in the third group spent at least 5 minutes, but less than 10 minutes suckling (designated 5-10 minutes). The body weights and the presence or absence of milk bands were recorded for pups after suckling.

2.6 Cross-fostering Experiments

On post-partum day 0, all litters were culled to 6 pups. Three pups were removed from their birth mother and replaced with three pups from a litter born to a dam with the opposite genotype (Table 1). The number of live pups present and the pup body weights were recorded daily until weaning age, i.e. post-partum day 21.

2.7 Mammary Gland Histology

The mammary glands were removed after sacrifice and the sample was fixed in 10% neutral buffered formalin. Whole-mount slide preparations for the mammary glands were histological examined for developmental differences between the wild-type and the OFQ/N knock-out mice by A. Skowronek, Ph.D., DVM (Battelle Memorial Institute, Columbus, OH).

2.8 Western Blot Analysis

Western blot analysis was preformed to quantify prolactin and estrogen receptor-α (ERα) protein levels in the pituitary gland. The pituitary was sonicated (Branson Sonifier 250, Danbury, CT) in 100 µl homogenizing buffer (1M Tris, 1% SDS and protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO)). The homogenate was centrifuged and 10 µl of the supernatant was collected and analyzed for protein content using a BCA protein assay kit (Pierce, Rockford, IL). The remaining supernatant was diluted 1:1 in a laemmli buffer and stored at -20°C until later use.

Protein was separated on an 8% or a 12% bis-acrylamide resolving gel for ERα, or PRL, respectively, and separated by SDS poly-acrylamide gel electrophoresis (200V). After separation, proteins were transferred for 55 minutes (100V) to a PVDF membrane (Millipore, Billerica, MA). Membranes were blocked in a tris-buffered saline solution (TBST) containing 0.05% Tween and 8% nonfat dry milk for 3 hours at room temperature for PRL and 8% nonfat
dry milk with 4% Bovine Serum Albumin overnight at 4°C for ERα. Rabbit anti-mouse PRL antiserum (National Hormone and Peptide Program and Dr. A.F. Parlow) was diluted to 1:300,000 and rabbit anti-mouse ERα (Santa Cruz Biotechnology, Santa Cruz, CA) was diluted to 1:10,000. Actin, the internal control, was probed with rabbit anti-mouse actin (Sigma, St. Louis, MO) at a 1:10,000 dilution. All primary antibodies were diluted in TBST and membranes were incubated overnight at 4°C. The membrane was incubated in secondary antibody (goat anti-rabbit gamma globulin, 1:5,000 in TBST) (Chemicon, Temecula, CA) for 90 minutes. Membranes were again rinsed with TBST, and proteins were detected by chemiluminesence (Pierce, Rockford, IL). PRL/Actin and ERα/Actin density ratios were quantified for statistical analysis (ImageQuant, Amersham Biosciences, Piscataway, NJ).

2.9 Hormone assays

Plasma PRL concentrations were determined by radioimmunoassay (RIA) (pup survival - post-partum day 0 through day 1 and pup survival - post-partum day 0 through day 6 experiments) or by Nb2 lymphoma (suckling experiment) assay. Circulating prolactin levels in the post-partum day 0 through day 6 experiment were pooled from all animals sacrificed on post-partum days 2 through 6. For the RIA, samples were assayed in duplicate using reagents obtained from the National Hormone and Peptide Program and Dr. A. Parlow. The secondary antibody was purchased from Antibodies, Inc. (Davis, CA). PRL was iodinated using the Chloramine T method (Greenwood & Hunter, 1963) or purchased from Perkin-Elmer (Shelton, CT).

Quantification of circulating prolactin levels using the Nb2 lymphoma bioassay has been previously described (Zinger et al., 2003). Briefly, Nb2 lymphoma cells were starved then plated in a 96-well plate (30,000 cells/well) with conditioned medium. Standards were performed in triplicate using human prolactin standard for iodination (National Hormone and Peptide Program and Dr. A. Parlow) and samples were assayed in duplicate. Following a 3 day incubation period, Nb2 activity was determined by the resazurin method. The amount of PRL was calculated based on the standard curve. The lower limit of detection was 2 pg/well.
2.10 Statistical Analysis

Pup survival for the post-partum day 0 through post-partum day 1 and the post-partum day 0 through day 6 experiments, was analyzed using a Chi-square test to assess differences between genotypes and a paired t-test for differences within a genotype. A two-sample t-test was used to assess differences in pup body weights on post-partum day 0 through post-partum day 1. Body weight changes before and after suckling were determined using an ANOVA statistical analysis. The presence or absence of pup milk bands before and after suckling was compared between genotypes using a Fisher’s exact test. The pup body weights on post-partum day 0 through day 6, maternal behaviors, cross-fostered pup survival data, western blot, RIA, and Nb 2 lymphoma assay data were analyzed using two sample t-test, assuming unequal variance (Microsoft Excel).

3. Results

3.1 Pup Survival – Post-Partum Day 0 through Post-Partum Day 1

There was no difference in the total number of pups/litter delivered, nor the number of pups/litter that were alive on the day of delivery (day 0) between litters born to knock-out or wild-type dams (Figure 1A). However on post-partum day 1, the number of live pups/litter was significantly less in litters born to knock-out mothers than on the day of birth (day 0) (p<0.01) and significantly lower than the number of live pups (day 1) in the litters born to wild-type dams (Figure 1A) (p<0.01). Additionally, by day 1 post-partum, the body weight of pups born to knock-out mothers was significantly lower (p<0.01) than those of the pups born to wild-type dams (Figure 1B). Absence or presence of milk bands in the pups was determined as an indication of milk in the pup’s stomach. On post-partum day 0, only 14 of 40 (35.0%) litters born to knock-out females had at least 50% of their pups with milk bands, while 22 of 31 (71.0%) litters born to wild-type females had at least 50% of their pups with milk bands. By day 1 post-partum, there was no difference between genotypes in the number of litters in which more than 50% of their pups contained milk bands (68.4% of the knock-outs litters and 80.7% of the wild-types litters) (Table 2). No morphological differences were observed in the mammary glands or in the number of uterine implantation sites between the knock-out and wild-type mothers on post-partum day 1 (data not shown).
Pituitary PRL content was quantified in wild-type and knock-out mothers to determine if sufficient PRL was available for secretion during the post-partum period. On post-partum day 1, pituitary prolactin content was significantly greater in knock-out dams than wild-type dams (Figures 2A and B) (p<0.04). However, due to the high rate of offspring mortality in litters born to knock-out mothers, none of these knock-out dams had any living pups. On post-partum day 1, plasma prolactin levels were significantly less in the knock-out dams that had lost all their offspring (p<0.02) as well as in knock-out dams that still had at least 50% of their pups alive (p<0.04) compared to wild-type dams (Figure 2C).

3.2 Maternal Behavior – Post-Partum Day 1

To determine if OFQ/N gene deletion had deleterious effects on maternal behavior, which would contribute to decreased survival of offspring born to knock-out mothers, several indicators of maternal behavior were measured. The behaviors were: nursing, pup licking, pup retrieval, nest building, time spent crouching over the nest to provide warmth, and time spent not in contact with their pups (Lucas et al., 1998; Chiang et al., 2002). No significant differences were observed between genotypes in any of the maternal behaviors recorded (Figure 3).

3.3 Pup Survival - Post-Partum Day 0 through Day 6

Although there was no difference between the number of live pups born to knock-out and wild-type mothers, by post-partum day 1, only 13.3% (2 of 15 total) of litters born to knock-out mothers had 100% survival. In contrast, 75.0% (6 of 8 total) of the litters born to wild-type mothers had 100% survival. The difference in the number of live pups in knock-out litters on post-partum days 1 (p<0.01) and 6 (p<0.01) was significant compared to wild-type litters. Furthermore, the number of pups remaining on day 6 in the knock-out litters was significantly less than the number of pups born (day 0)(p<0.01)(Figure 4).

Circulating PRL levels were determined in both wild-type and knock-out mothers between days 2 and 6 post-partum. Prolactin levels were significantly lower (p<0.02) in the knock-out dams compared to the wild-type dams (Figure 5).
3.4 Cross-fostering Experiment

Due to the high infant morality in offspring of knock-out mothers, pups were cross-fostered (Table 1) to ensure that the genotype of the pups did not affect their ability to survive. Fewer pups housed with knock-out mothers reached weaning age (day 21) compared to those housed with wild-type mothers (Figure 6) (p<0.02). Pup genotype did not affect survival (data not shown).

3.5 Suckling Experiment

Knock-out pups had significantly lower body weights compared to the wild-type pups both before being reunited with their mother and after suckling (p<0.01) (Figure 7). Prior to the observation period, only 6 of the 17 (35.3%) knock-out females had litters in which more than 50% of their pups had milk bands compared to the pups with wild-type females in which 14 of 18 (77.8%) litters had more than 50% of their pups with milk bands. Regardless of the amount of time being suckled, the knock-out females still had significantly fewer litters (p<0.01) in which more than 50% of their pups had milk bands (Table 3).

Knock-out females had more prolactin in the anterior pituitary gland compared to the wild-type females when suckling was not observed (44.6% more than wild-type dams) and after 5-10 of suckling (31.8% more than wild-type dams) (Figure 8A and 8B). No difference in the circulating levels of prolactin was detected at any time period following suckling (Figure 8C).

Litters were grouped according to the amount of time they spent suckling. In one group (designated 0 minutes), the pups did not suckle. In the second group, the pups spent at least 1 minute, but less than 5 minutes suckling (designated 1-5 minutes). Pups in the third group spent at least 5 minutes, but less than 10 minutes suckling (designated 5-10 minutes).

3.6 Estrogen Receptor-α Expression (ERα)

Pituitary estrogen receptor-α expression levels did not differ between genotype on post-partum day 1 (Figure 9A and 9B).
Discussion

The results of this study demonstrate that maternal OFQ/N is necessary for pup survival during the post-partum period. In the absence of OFQ/N, milk band accumulation and body weight gain in the pups were reduced, however maternal mammary gland development, maternal behavior, and pituitary ERα expression were not affected. OFQ/N is also necessary for prolactin release from the anterior pituitary into the circulation on post-partum day 1, regardless of the presence or absence of pups. Following separation, OFQ/N is necessary for prolactin release from the anterior pituitary in the absence of suckling (0 minutes) and following at least 5 minutes (5-10 minutes) of suckling.

Within 24 hrs of parturition, the inability of knock-out dams to sustain their litter as effectively as wild-type mothers was evident. Without OFQ/N present, anterior pituitary prolactin content was higher than in wild-type mice, but circulating prolactin levels were lower. These results suggest that the pituitary prolactin is being synthesized, but not released. Although there were no morphological changes in the mammary glands (data not shown), the reduced body weight and the lower incidence of milk bands in knock-out pups indicate that prolactin is not being secreted normally. Mammary gland development may have been normal due to the actions of hormones during pregnancy; estrogen, progesterone, and placental lactogens stimulate mammary gland growth and development (reviewed in Goffin, et al., 2002). However, when pups suckle, TIDA neuronal activity must be inhibited to allow prolactin levels to increase and stimulate milk synthesis and secretion. Previous results from our laboratory indicate that OFQ/N is critical in the suckling-induced prolactin release and that OFQ/N administration does inhibit TIDA neurons (Chesterfield, et al., In Review). If OFQ/N is necessary for TIDA neuronal inhibition in mice, one possible mechanism for reduced circulating prolactin levels is that OFQ/N is not available to inhibit these neurons.

The results from the cross-fostering experiment demonstrated that the offspring, regardless of genotype, are capable of suckling because, no significant loss in pups occurred between post-partum day 0 and post-partum day 21 when pups were housed with a wild-type mother. A significant loss in the number of live pups occurred between post-partum day 0 and post-partum day 21 when pups were housed with a knock-out dam. In the cross-fostering experiment, only dams with at least 6 viable pups on post-partum day 0 were used. Because
there is a significant decline in pup survival within the first 24 hours after parturition when pups are reared by knock-out dams, there were a number of knock-out dams that were unable to be used in this cross-fostering experiment due to poor pup survival on post-partum day 0. Therefore, the loss in the number of live pups per litter is not as great in the cross-fostering experiment as the loss observed when pups are housed with their knock-out birth mother (pup survival- post-partum day 0 through day 1 and pup survival – post-partum day 0 through day 6). Taken together, these results indicate that knock-out females were capable of delivering viable pups but were not able to sustain them due, at least in part, to the reduced level of prolactin secretion. Even when knock-out mothers had at least 50% of their litters alive, the circulating prolactin levels were still significantly lower than the wild-type dams.

OFQ/N appears to be necessary for anterior pituitary prolactin release during lactation. To measure the suckling-induced prolactin response, mothers were separated from their pups for 2 hours. After being returned to their pups, animals were observed and suckling time was determined. Anterior pituitary prolactin levels were greater in knock-out dams than in wild-type dams when neither were suckled (0 minutes). Furthermore, even when pups suckled for more than 5 minutes (5-10 minutes), pituitary prolactin levels remained higher in knock-out females. Therefore, it appears that prolactin is not being released in the OFQ/N knock-out dams in the presence of pups when suckling does not occur (0 minutes) and with prolonged suckling (5-10 minutes). When dams were suckled for 1-5 minutes, knock-out females did not have more pituitary prolactin compared to the wild-type dams. Therefore, prolactin releasing factors such as oxytocin, EOP, and TRH may be stimulating prolactin release during short bouts of suckling (1-5 minutes) however this release is not is present when the pups are not suckling (0 minutes) and the release is not sustained in the absence of OFQ/N with prolonged suckling (5-10 minutes).

Prolactin regulation during lactation is complex and involves numerous factors (reviewed in Freeman et al., 2000) however the results of this study demonstrate that OFQ/N is also an important factor in prolactin regulation in the post-partum period.

One likely mechanism of action of OFQ/N is that it inhibits TIDA neuronal activity. This has been demonstrated in rats (Chesterfield, et al., In review). Alternatively, OFQ/N may influence the activity of the other endogenous opiates or prolactin releasing factors, e.g. serotonin and/or TRH, although other studies from our laboratory indicate OFQ/N does not significantly influence serotonin activity in virgin female rats (Kraska, et al., 2005). OFQ/N may
also be involved in regulating oxytocin release, thus OFQ/N may be necessary for milk ejection. Nevertheless, when OFQ/N is absent, anterior pituitary prolactin levels were increased, circulating prolactin levels were decreased, and pup survival was reduced.

In conclusion, OFQ/N is necessary for pup survival during the post-partum period because it regulates prolactin release from the anterior pituitary which is critical during lactation. When reared by a female without OFQ/N, pups obtained less milk, gained weight more slowly, and had an increased mortality rate compared to offspring housed with wild-type dams. OFQ/N does not appear to be necessary for proper mammary gland development or the onset of maternal behavior. A likely mechanism of action for OFQ/N is that it contributes to the suckling-induced TIDA neuronal inhibition; OFQ/N does inhibit TIDA neurons in intact, virgin female rats, and is necessary for the suckling-induced prolactin increase in post-partum rats (Chesterfield, et al., In review). This study demonstrates that OFQ/N is responsible, at least in part, for the release of prolactin from the anterior pituitary and is essential for pup survival during the post-partum period.
Table 1: Cross-fostering experimental procedure. On post-partum day 0, all litters were culled to 6, 3 pups were removed from their birth mother and replaced with 3 pups from a litter born to a dam with the opposite genotype.
<table>
<thead>
<tr>
<th></th>
<th>WT mother</th>
<th>KO mother</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pups culled to 6</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Pups removed</td>
<td>-3</td>
<td>-3</td>
</tr>
<tr>
<td>Pups added</td>
<td>+3 KO</td>
<td>+3 WT</td>
</tr>
<tr>
<td>Total pups fostered</td>
<td>6</td>
<td>6</td>
</tr>
</tbody>
</table>
**Figure 1:** Pup survival (A) and mean pup body weight (B) on post-partum day 0 and day 1. In litters born to knock-out dams, there was a significant loss in the number of live pups on post-partum day 1 compared to day 0 (p<0.01) and compared to the number of live pups in the wild-type litters (p<0.01) (A). Pups in the knock-out litters had significantly lower (p<0.01) body weights on post-partum day 1 compared to the wild-type pups (B). The number of litters is indicated in parenthesis. Values are means +/- SEM.

* Significantly different from wild-type.

† Significantly different from day 0.
1A)  
Number of Live Pups/Litter

- Wild-type (n=27)
- Knock-out (n=33)

Total Delivered  Day 0  Day 1

1B)  
Pup Body Weight (g)

- Wild-type (n=30)
- Knock-out (n=37)

Days Post-Partum

0  1
Table 2: Presence of milk bands in pups on post-partum day 0 and day 1. On post-partum day 0, only 35.0% of the knock-out females had litters in which more than 50% of their pups had milk bands compared to 71.0% of litters born to wild-type females.
<table>
<thead>
<tr>
<th>Days Post-Partum</th>
<th>Genotype</th>
<th>Litters with &lt; 50% of the Pups Containing Milk Bands</th>
<th>Litters with = 50% of the Pups Containing Milk Bands</th>
<th>Percent of Litters with &lt;50% of the Pups Containing Milk Bands</th>
<th>Percent of Litters with = 50% of the Pups Containing Milk Bands</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 0</td>
<td>KO n=40</td>
<td>26</td>
<td>14</td>
<td>65.0%</td>
<td>35.0%</td>
</tr>
<tr>
<td></td>
<td>WT n=31</td>
<td>9</td>
<td>22</td>
<td>29.0%</td>
<td>71.0%</td>
</tr>
<tr>
<td>Day 1</td>
<td>KO n=38</td>
<td>14</td>
<td>26</td>
<td>36.8%</td>
<td>68.4%</td>
</tr>
<tr>
<td></td>
<td>WT n=31</td>
<td>6</td>
<td>25</td>
<td>19.3%</td>
<td>80.7%</td>
</tr>
</tbody>
</table>
Figure 2: Prolactin content in the anterior pituitary (A and B) and in the circulation (C) on post-partum day 1. The anterior pituitary prolactin content was quantified using western blot analysis. Due to the high rate of offspring mortality in litters born to knock-out mothers, these data represent anterior pituitary prolactin levels in knock-out dams without any living pups compared to that of wild-type dams that had 100% of their pups surviving on post-partum day 1. The knock-out dams had significantly more prolactin in the anterior pituitary compared to the wild-type dams (p<0.04) (A). A representative western blot is shown (B). Lanes 1-3 and 7-9 are knock-out females with 5, 10, and 20 µg of protein. Lanes 4-6 and 10-12 are wild-types containing 5, 10, and 20 µg of protein. Actin was used as an internal control (43 kDa) and the prolactin (23 kDa) content was normalized to actin (B). Knock-out dams had significantly less prolactin in their circulation when no live pups were present (p<0.02) and when more than 50% of their litter was still alive (p<0.04) on post-partum day 1, compared to the wild-type dams (C). Values are means +/- SEM.

* Significantly different from wild-type.
2C)

- Wild-types - 100% of litter (n=9)
- Knock-outs -> 50% of litter (n=7)
- Knock-outs - 0% of litter (n=7)

Prolactin (ng/mL) vs Genotype
Figure 3: Maternal behaviors on post-partum day 1. Dams were separated from their pups for 2 hours. Upon return, dams were allowed a 5 minute acclimation period. Maternal behaviors were monitored for 10 minutes. These behaviors were: nursing, pup licking, pup retrieval, nest building, time spent crouching over the nest to provide warmth, and time spent not in contact with their pups. No significant difference was observed between genotypes in the amount of time (seconds) the dams spent engaging in any of the maternal behaviors measured. Values are means +/- SEM.
**Figure 4:** Pup survival on post-partum day 0 through post-partum day 6. There was a significant loss in the number of live pups in the knock-out litters on post-partum days 1 (p<0.01) and 6 (p<0.01) compared to the wild-types. On post-partum day 6, the number of pups still alive in the knock-out litters was significantly less than the number of pups born (day 0) (p<0.01). Values are means +/- SEM.

* Significantly different from wild-type.
† Significantly different from post-partum day 0.
Figure 5: Circulating prolactin levels on post-partum day 2 through day 6. Prolactin levels were quantified by radioimmunoassay. Knock-out dams had significantly less prolactin in the circulation compared to the wild-type dams (p<0.02). Values are means +/- SEM.

* Significantly less than wild-type.
**Figure 6:** Cross-fostered pup survival. All litters were cross-fostered on post-partum day 0. Each knock-out mother and each wild-type mother had 6 pups; each cross-fostered litter included 3 wild-type and 3 knock-out offspring. By weaning age (day 21), knock-out dams had significantly fewer pups survive than the wild-type dams (p<0.02). Values are means +/- SEM. * Significantly different from wild-types.
Figure 7: Pup body weights before and after suckling. Pups in the knock-out litters had significantly lower body weights before and after suckling compared to pups in the wild-type litters (p<0.01). The number of litters is indicated in parenthesis. Values are means +/- SEM.
* Significantly different from wild-type litters.
**Table 3**: Presence of milk bands in the pups before and after suckling. Prior to the observation period, only 6 of the 17 (35.3%) knock-out females had litters in which more than 50% of their pups contained milk bands compared to the pups from wild-type females in which 14 of 18 (77.8%) litters had more than 50% of the pups with milk bands. After the 10 minute observation period, the knock-out mothers still had significantly fewer litters (23.5%) in which more than 50% of their pups contained milk bands compared to the wild-types (81.2%)(p<0.01). Values are means +/- SEM.

* Significantly different from wild-type litters.
<table>
<thead>
<tr>
<th>Genotype</th>
<th>Litters with &lt; 50% of the Pups Containing Milk Bands</th>
<th>Litters with = 50% of the Pups Containing Milk Bands</th>
<th>Percent of Litters with &lt;50% of the Pups Containing Milk Bands</th>
<th>Percent of Litters with = 50% of the Pups Containing Milk Bands</th>
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</thead>
<tbody>
<tr>
<td>Before Suckling KO n=17</td>
<td>11</td>
<td>6</td>
<td>64.7%</td>
<td>35.3%</td>
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<tr>
<td>Before Suckling WT n=18</td>
<td>4</td>
<td>14</td>
<td>22.2%</td>
<td>77.8%</td>
</tr>
<tr>
<td>After Suckling KO n=17</td>
<td>13</td>
<td>4</td>
<td>76.5%</td>
<td>23.5% *</td>
</tr>
<tr>
<td>After Suckling WT n=16</td>
<td>3</td>
<td>13</td>
<td>18.8%</td>
<td>81.3%</td>
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</tbody>
</table>
**Figure 8:** Prolactin levels in females with pups that did not suckle (0), or following suckling; at least 1-5 minutes (1-5), or 5-10 minutes (5-10). Knock-out mothers with litters that didn’t suckle (0) and those that suckled at least 5 minutes (5-10) of suckling had higher pituitary prolactin levels than the wild-type mothers in the same groups (44.6 % and 31.8%, respectively)(A). A representative western blot from animals in which no suckling was observed (0 min) is shown. Lanes 1-3 and 7-9 are levels in knock-out dams when 5, 10, or 20 µg of protein was loaded. Lanes 4-6 and 10-12 are levels in wild-type dams in samples containing 5, 10, or 20 µg of protein. Actin was used as an internal control (43 kDa) and the prolactin (23 kDa) content was normalized to actin (B). Circulating prolactin levels (C) were determined using the Nb2 lymphoma assay. There was no difference between genotypes at any time. The number of litters is indicated in parenthesis. Values are means +/- SEM.

* Significantly different from wild-type litters.
8A)

![Bar graph showing PRL/Actin Content % of WM-types over minutes suckled](image)

8B)

<table>
<thead>
<tr>
<th>Lanes:</th>
<th>KO</th>
<th>WT</th>
<th>KO</th>
<th>WT</th>
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<tr>
<td></td>
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<td></td>
<td>9</td>
<td>10</td>
<td>11</td>
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</table>

- Actin 43 kDa
- PRL 23 kDa
Figure 9: Estrogen Receptor-α (ERα) anterior pituitary expression. No significant difference in pituitary ERα expression was detected between genotypes on post-partum day 1 (A). A representative western blot is shown. Lanes 1-3 and 7-9 are ERα levels in knock-out females when 5, 10, or 20 µg of protein was loaded. Lanes 4-6 and 10-12 are levels in wild-type females containing 5, 10, or 20 µg of protein. Actin was used as an internal control (43 kDa) and the ERα (66 kDa) content was normalized to actin (B). Values are means +/- SEM.
9A) ER α/Actin Content % of WT-types

Genotype

Wild-type (n=15)
Knock-out (n=15)

9B) Lanes:

<table>
<thead>
<tr>
<th></th>
<th>KO</th>
<th>WT</th>
<th>KO</th>
<th>WT</th>
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<tbody>
<tr>
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<td>7-9</td>
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<tr>
<td>ERα 66 kDa</td>
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<tr>
<td>Actin 43 kDa</td>
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Literature Cited


