

# Serotonin: A New Approach for Hypocalcemia

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## **Serotonin regulates mammary gland physiology during lactation**

Serotonin is synthesized in numerous tissues throughout the body and is incapable of crossing the blood-brain barrier. Serotonin is synthesized from the amino acid L-tryptophan in a two-step process. The first step is production of 5-hydroxytryptophan (5-HTP) via the rate-limiting enzyme, tryptophan hydroxylase (TPH). The second step is the conversion of 5-HTP to serotonin by aromatic amino acid decarboxylase (Wang et al., 2002). TPH1 is the rate-limiting enzyme for serotonin production in non-neuronal tissues, while TPH2 is used to produce serotonin in neuronal tissues. Our laboratory and others have shown that serotonin regulates milk protein gene expression, as well as the disassembly of tight junctions that occurs during the involution process (Matsuda et al., 2004; Stull et al., 2007; Hernandez et al., 2008; Pai and Horseman, 2008). Furthermore, we have shown that the mammary gland expresses a unique pattern of serotonin receptors in rodent, bovine, and human mammary epithelium (Hernandez et al., 2009; Pai et al., 2009). The bovine mammary gland epithelium expresses at least five serotonin receptor isoforms (5-HT1B, 2A, 2B, 4 and 7; Hernandez et al., 2009). Our lab determined that the 5-HT2B receptor subtype modulates serotonin's regulation of parathyroid hormone-related protein (PTHrP) production within the mammary gland in a rodent model (Hernandez et al., 2012; Laporta et al., 2013a; Laporta et al., 2014a,b). Furthermore, we showed that serotonin activates expression of various calcium (Ca) pumps and transporters in the mammary gland to stimulate transport of Ca from blood to milk during mouse lactation (Laporta et al., 2014a). Ca transport into the mammary gland is thought to occur through the Ca<sup>2+</sup> influx channel (ORAI1) and subsequent pumping into the milk by the apical plasma membrane Ca<sup>2+</sup> ATPase (PMCA2; Cross et al., 2014).

Current research in humans and rodents implicates PTHrP in the regulation of maternal Ca homeostasis during lactation. Our laboratory has demonstrated the necessity of serotonin for regulation of Ca transport in the mammary gland during lactation. We also showed that circulating serotonin concentrations post-partum are positively correlated with circulating Ca concentrations on the first day of lactation in dairy cows (Laporta et al., 2013b). Furthermore, we have demonstrated that serotonin is necessary for the production of mammary PTHrP during lactation. Mammary-derived PTHrP is critical for the mobilization of Ca from bone tissue to support lactation (Wysolmerski, 2010). Therefore, delineation of the mechanisms regulating the mammary gland serotonin-PTHrP axis in the dairy cow could lead to development of novel therapeutic interventions to reduce the incidence of subclinical hypocalcemia (SCH) and clinical hypocalcemia (CH) in the U.S. dairy cow population.

## Hypocalcemia and the Transition Period

The transition period (3 weeks pre-calving through 3 weeks post-calving) is an extremely critical time period in the life of the dairy cow. At this time, cows are highly susceptible to a variety of disorders that negatively impact the animal's health, and hence their overall production. Of particular concern during this time is the inability of the dairy cow to maintain adequate blood calcium concentrations due to increased demand for calcium at the onset of lactation by the mammary gland. This increase in calcium demand by the mammary gland results in decreased circulating calcium concentrations and can lead to the development of periparturient hypocalcemia (milk fever). Parturient paresis is one of the most common metabolic diseases of dairy cattle, with Jersey cows being more susceptible than Holsteins (Oetzel, 1988; NRC, 2001). Hypocalcemia is associated with numerous other health disorders during this time period (Oetzel, 1988). Due to inadequate blood calcium concentrations at the onset of lactation, animals experience a range of clinical symptoms, depending on the degree of hypocalcemia (Adams et al., 1996). Clinical hypocalcemia (CH), or milk fever, is clinically defined as a total blood calcium level of less than 1.4 mmol/L, and subclinical hypocalcemia (SCH) defined as total blood calcium of 1.4-2.0 mmol/L (DeGaris and Lean, 2008). Approximately 25% of heifers and 50% of older cows will succumb to SCH, and between 5 to 10% of all dairy cows will develop clinical hypocalcemia in the United States (Goff, 2008). Recent data suggest that as many as 25% of primiparous and 47% of multiparous lactating dairy cows are affected by SCH and are at increased risk of culling (Reinhardt et al., 2011). Cows that are afflicted with periparturient hypocalcemia exhibit a 14% decrease in milk production and are more susceptible to other transition disorders such as ketosis, retained placenta, displaced abomasum and muscle weakness, with the average cost of incidence of milk fever being \$334/animal (Oetzel, 1988). However, should a dairy cow be affected by an additional metabolic disorder or disease as a result of CH and/or SCH, costs increase substantially. SCH affects about 50% of second lactation and greater dairy cattle, and costs approximately \$125/animal to treat. Overall, prevalence of CH and SCH are more common in Jersey cattle, likely due to their higher milk production per unit body weight (Oetzel, 1988). Typically, in order to compensate for decreased blood calcium, increased intestinal calcium absorption and/or calcium resorption from the bone must occur. Calcium resorption from the bone is the primary mode used during this time frame. Dairy cows, in particular, exhibit a delay in calcium resorption from bone.

Adequate circulating calcium concentrations throughout the transition period are necessary for productive lactation, but large quantities of calcium are lost from maternal calcium pools into milk and colostrum. Cows undergoing SCH are at a greater risk of suffering other health disorders including dystocia, retained placentas, displaced abomasums, uterine disease, mastitis, and subclinical ketosis during the peripartum period (Chapinal et al., 2011; Chapinal et al., 2012; Martinez et al., 2012). To illustrate the negative impact of SCH on other disorders, previous data has estimated that completely eliminating SCH from a dairy herd could reduce the incidence of metritis and puerperal metritis by 66.6 and 91.3%, respectively (Martinez et al., 2012). Furthermore, as stated previously, SCH predisposes dairy cows to other metabolic disorders and diseases during the transition period (**Figure 1**; DeGaris and Lean, 2008; Chapinal et al., 2011; Chapinal et al., 2012).

It is important to prevent SCH and CH because the early symptoms of milk fever often go undetected. Because the early symptoms of SCH and CH are short-lived, they are difficult to detect and treat therapeutically. The economic impact of hypocalcemia is enormous: considering the 9.2 million cows in the U.S. dairy industry with a cost of \$125 and \$300 per case of subclinical and

clinical hypocalcemia, respectively, given treatments and lost milk yield, there is an estimated cost of \$900,000,000 annually. Translating these numbers to the 1.27 million cows in Wisconsin, the annual average cost of hypocalcemia to a WI farmer is approximately \$12,000 (Oetzel, 2013). While these estimates are purely economic, there are also animal welfare concerns, given that the cow may be unable to stand or walk until identified by the farmer. Potentially more troubling than the physical and economic ramifications of hypocalcemia is the fact that the subclinical form is nearly impossible to identify in a production setting, as cows do not display obvious clinical symptoms (Oetzel and Miller, 2012).

While there are prevention strategies currently utilized in the United States, they are often difficult to implement effectively. The primary target for prevention is through manipulation of the diet at the end of the dry period. The two major strategies are administration of low calcium diets (LCD) and adjustment of the dietary cation-anion difference (DCAD). Feeding of a LCD works by stimulating a transient hypocalcemia, inducing calcium resorption from the bone and increased absorption from the small intestine, in order to increase available calcium reserves (Horst et al., 1997). For the prevention of milk fever, a diet of 8 to 10 grams of calcium per day has been shown to have the greatest effect, but LCD with this little calcium are difficult to achieve mainly because the primary forage of alfalfa is quite high in calcium (Horst et al., 1997). Conversely, the strategy of DCAD manipulation is to increase availability of absorbable dietary anions and decrease the number of absorbable dietary cations through use of dietary anionic salts (Goff, 2008). While there is no doubt that this strategy aids in the prevention of milk fever (Charbonneau et al. 2006), there are two major concerns. The first is that the salts decrease palatability, reducing feed intake and predisposing the cow to other energy-related transition disorders. Importantly, new DCAD products are much more palatable than the original products developed. The second issue is that anionic salts are quite expensive, adding additional cost onto an already costly period in the cow's life. Additionally, the low DCAD diet is typically implemented during the 3 weeks immediately pre-partum, creating the need for two separate groups of cows in the dry pen. Further work has been done on vitamin D3 or oral calcium/metabolite administration, but these results are largely impractical and overly dependent on timing of administration (Martín-Tereso and Verstegen, 2011). Additional strategies are focused on administration of calcium boluses or gels post-calving as method to reduce CH and SCH. Recently, data has been presented demonstrating that using oral calcium boluses has differential effects on cows (Martinez et al., 2016a, b). Negative effects were seen in primiparous cows, and rebound effects were noted when oral boluses were only administered on d0 and d1 post-partum (Martinez et al., 2016a). However, SCH was reduced and pregnancies per AI were improved in multiparous cows receiving 4 boluses, and further reduced when given 7 boluses of oral calcium (Martinez et al., 2016a,b). Another recent study providing one subcutaneous calcium injection post-calving resulted in only elevating calcium concentrations for 24 h post-treatment and had no significant effects on risk of disease or culling, milk production, or reproductive performance (Miltenburg et al., 2016). Based on currently available strategies to manage hypocalcemia, there is room to improve the health of these animals. Improvement of these prevention strategies depends on a solid understanding of the physiological mechanisms that govern calcium homeostasis in the dairy cow. Our lab has shown that manipulation of a key regulator of calcium dynamics, serotonin, may have significant impact as a novel therapeutic target in the prevention of hypocalcemia.

### **New ideas about calcium and serotonin**

Our laboratory has demonstrated that serotonin is necessary for mammary PTHrP synthesis in

lactating rodents and mammary epithelial cells grown in lactogenic culture (Hernandez et al., 2012; Laporta et al., 2013a; Horseman and Hernandez, 2014). We also demonstrated that supplementation of a serotonin precursor, 5-HTP, to rats during the transition from pregnancy to lactation increased post-partum circulating serotonin, PTHrP, and Ca concentrations, and also increased total Ca content in milk (Laporta et al., 2013a). Furthermore, we observed increased osteocyte numbers in the femurs collected from rats supplemented with 5-HTP, indicating this response was due to bone Ca mobilization.

### **Mammary serotonin production is a significant source of maternal circulating serotonin concentrations during lactation in mice**

Using a mouse model in which we selectively deleted TPH1 in the mammary gland during lactation we revealed that the mammary gland is a substantial source of serotonin production during lactation. Serotonin is produced in many tissues in the body. Therefore, circulating serotonin concentrations are comprised of serotonin from numerous tissues. This allows serotonin to act in autocrine, paracrine and endocrine manners on the mammary gland during lactation. Dams lacking TPH1 in the mammary gland during lactation have an average circulating serotonin concentration on d10 of lactation of approximately 700 ng/ml, while the wild type counterparts average 1500 ng/ml (Weaver et al., In Review). Furthermore, when TPH1 is overexpressed in the mammary gland during lactation, maternal circulating serotonin concentrations increase to approximately 2000 ng/ml. These results support the hypothesis that serotonin production by the mammary gland is a significant contributor to circulating serotonin concentrations during lactation.

### **The onset of milk production drains Ca pools in dairy cows**

Colostrum and milk synthesis rapidly depletes Ca from the maternal circulation and therefore Ca must be mobilized from maternal bone to maintain adequate circulating concentrations. Circulating Ca concentrations are tightly regulated and controlled by several hormones including: Vitamin D, calcitonin, parathyroid hormone (PTH) and PTHrP. Liberation of Ca from bone stores can only be triggered when circulating Ca concentrations dip below the animal's minimal threshold for Ca, via a classic negative feedback loop. Dietary Ca is insufficient to maintain maternal Ca homeostasis during milk synthesis. This is demonstrated by the fact that a dairy cow will lose 9 to 13% of her bone mass during the first 30 days of lactation as part of the normal physiological response to low calcium levels. Bone loss during lactation is an evolutionary strategy of mammals used to support the cow and specifically the mammary gland demand for Ca for milk synthesis (Wysolmerski et al., 1995; Wysolmerski, 2010; Goff, 2014).

### **Our mouse studies revealed that serotonin is critical for the expression of key Ca sensors, pumps, and transporters in the mammary gland**

Utilizing a mouse model deficient in TPH1 we investigated the necessity of non-neuronal serotonin in maintenance of maternal Ca homeostasis. TPH1-deficient mice have little to no circulating serotonin. We demonstrated that intraperitoneal injections of 5-HTP to these mice restored and even elevated circulating serotonin concentrations compared to wild-type dams. Our results also demonstrated that total Ca concentrations are decreased in TPH1 null mice and that Ca concentrations can be restored with intraperitoneal injection of 5-HTP (Laporta et al., 2014a,b). RNA-sequencing analysis of mammary glands collected on d 10 of lactation from wild-type, TPH1-

deficient mice and TPH1-deficient mice injected with 5-HTP revealed that serotonin is critical for the cellular response to Ca (Laporta et al., 2015). Upon further analysis of the specific Ca pumps and transporters present in the mammary gland we observed that mRNA abundance of several Ca pumps and transporters was reduced in the TPH1-deficient mammary gland and was restored by exogenous 5-HTP (Laporta et al., 2014a). These results indicate that peripheral serotonin is critical for maintaining circulating Ca concentrations and mammary gland Ca transport during lactation.

### **The mammary gland functions as an “accessory parathyroid gland” during lactation**

The mammary gland produces the hormone PTHrP, which binds to receptors on bone to drive bone resorption and liberate Ca into the systemic circulation (Wysolmerski et al., 1995; Wysolmerski, 2010). Normal calcium physiology is unable to be maintained in the classical fashion by parathyroid hormone (PTH). PTHrP is produced by the mammary gland only during lactation, allowing the mammary gland to act as an accessory parathyroid gland to support the homeorhetic process of lactation. The Ca sensing receptor (CaSR) present in the mammary epithelium plays a crucial role in controlling maternal Ca concentrations during lactation. CaSR is highly expressed in the mammary gland during lactation, compared to virgin and pregnant time periods (VanHouten et al., 2003). Mammary PTHrP production is responsible for the mobilization of Ca from the bone during lactation, rather than the typical endocrine regulator of bone, PTH (Wysolmerski et al., 1995; VanHouten, 2005; Wysolmerski, 2010; Wysolmerski, 2012). Our lab made a novel discovery that serotonin is essential for the liberation of Ca from bone during lactation to sustain maternal Ca homeostasis in rodent models (**Figure 2**). This occurs through induction of PTHrP by the mammary gland (Hernandez et al., 2012; Laporta et al., 2014a, 2014b). Furthermore, we demonstrated that serotonin is critical for the expression of CaSR. This finding indicates that serotonin is crucial for mammary gland sensing of systemic Ca concentrations and subsequent endocrine response that liberates bone tissue.

### **Mammary gland coordination with the skeletal system liberates Ca during lactation**

The skeletal system maintains its structural and functional roles via communication between two cell types, osteoblasts (OB), which are responsible for bone formation, and osteoclasts (OC), which are responsible for bone resorption, and thus Ca mobilization. PTH regulates this mechanism under non-lactating conditions. Research in humans and rodents has suggested the PTH action on bone is uncoupled during lactation (Wysolmerski, 2010; VanHouten and Wysolmerski, 2013). PTHrP signals through the same G-protein coupled receptor (PTH1R) as PTH on the OB to decrease OB cell proliferation and up-regulate genes responsible for OC differentiation during lactation. In rodents and humans, the mammary gland is the main source of PTHrP found in the circulation (Thiede, 1994; Wysolmerski et al., 1995; Wysolmerski, 2010; VanHouten and Wysolmerski, 2013). Mammary-derived PTHrP, not PTH, is the critical hormone responsible for induction of bone Ca mobilization during lactation (Wysolmerski et al., 1995).

### **In order to evaluate the utility of the mammary serotonin-PTHrP axis in Holstein dairy cows, we performed several observational studies**

We have observed that serotonin concentrations are dynamic over the course of a given lactation, and decrease around the time of calving (d0 to d2 lactation), rebounding by approximately ten days into lactation (Moore et al., 2015). The overall average serotonin concentration in dairy cows is

approximately 1700 ng/ml. However, it should be noted that the concentrations fluctuate dependent on stage of pregnancy and lactation indicating that serotonin may have different physiological functions related to the physiological stage of the cow. These results combined with our rodent data support our hypothesis that serotonin and PTHrP are critical players in the regulation of Ca homeostasis in Holstein dairy cows. We have demonstrated in a small population of multiparous Holstein cows that serotonin and PTHrP concentrations are positively correlated with each other, and negatively correlated with total calcium concentrations (Laporta et al., 2013b).

### **Intravenous (IV) infusion of 5-HTP in late lactation, non-pregnant, multiparous Holstein dairy cows increases circulating serotonin concentrations and alters Ca dynamics**

In order to demonstrate the role of serotonin in Ca homeostasis in dairy cows, we performed a preliminary experiment in which we infused 5-HTP intravenously for one hour daily for four days in late-lactation dairy cows at varying doses (0, 0.5, 1.0, or 1.5 mg/kg) to determine an optimum dose of 5-HTP necessary to produce significant changes in Ca. All three doses of 5-HTP significantly increased circulating serotonin concentrations (Laporta et al., 2015) to a similar extent in the two hours after dosing, with concentrations returning to baseline concentrations observed in the saline controls by two hours after infusion. In addition to serotonin concentrations, we measured circulating total Ca concentrations following the same time course post infusion. While initially counter-intuitive, our data demonstrated that total Ca concentrations decreased in immediate response to 5-HTP treatments (Laporta et al., 2015). In order to determine where the circulating Ca was going after 5-HTP infusion, we measured urine and milk Ca concentrations prior to the start of infusion and two hours after the end of the infusion. Our results indicate that there was a decrease in urine Ca output with higher doses of 5-HTP treatment. This suggests that Ca is not being lost into the urine as a result of 5-HTP infusion. We then observed that the highest dose of 5-HTP increased total milk Ca concentrations. This supports the hypothesis that serotonin causes transient hypocalcemia by increased Ca transport into the mammary gland and subsequently into milk. Increased Ca transport into the mammary gland during lactation is critical for the stimulation of bone Ca mobilization by PTHrP because it decreases the circulating calcium concentrations sensed by the mammary gland, allowing for the production of PTHrP.

### **Use of 5-HTP before calving to prevent hypocalcemia: Does 5-HTP influence Ca during the cow transition period and does breed make a difference?**

In order to determine if elevating serotonin concentrations in pre-fresh dairy cows would alter post-calving Ca concentrations, we treated multiparous Holstein cows with daily IV infusions of 1.0 mg/kg of 5-HTP beginning 7 d before the estimated calving date until calving. Our data demonstrates that IV infusions of 5-HTP pre-calving increased post-calving total Ca concentrations compared to saline treated controls (Weaver et al., 2016). Collaborating researchers in Switzerland also showed that 5-HTP increased calcium concentrations in their system post-calving (Hernandez-Castellano et al., accepted). Furthermore, we measured deoxypyridinoline (DPD), a marker of OC activity and therefore bone resorption, in the urine of our cows. These data demonstrate that cows receiving 5-HTP before calving have increased bone resorption at calving, suggesting that 5-HTP treatment pre-calving may improve post-calving Ca concentrations by increasing bone Ca resorption. Furthermore, we tested the same hypothesis in multiparous Jersey cows. Interestingly, Jersey cows responded to 5-HTP differently than the Holstein cows. Jersey cows had decreased calcium concentrations prior to parturition, and then began to increase calcium concentrations at calving.

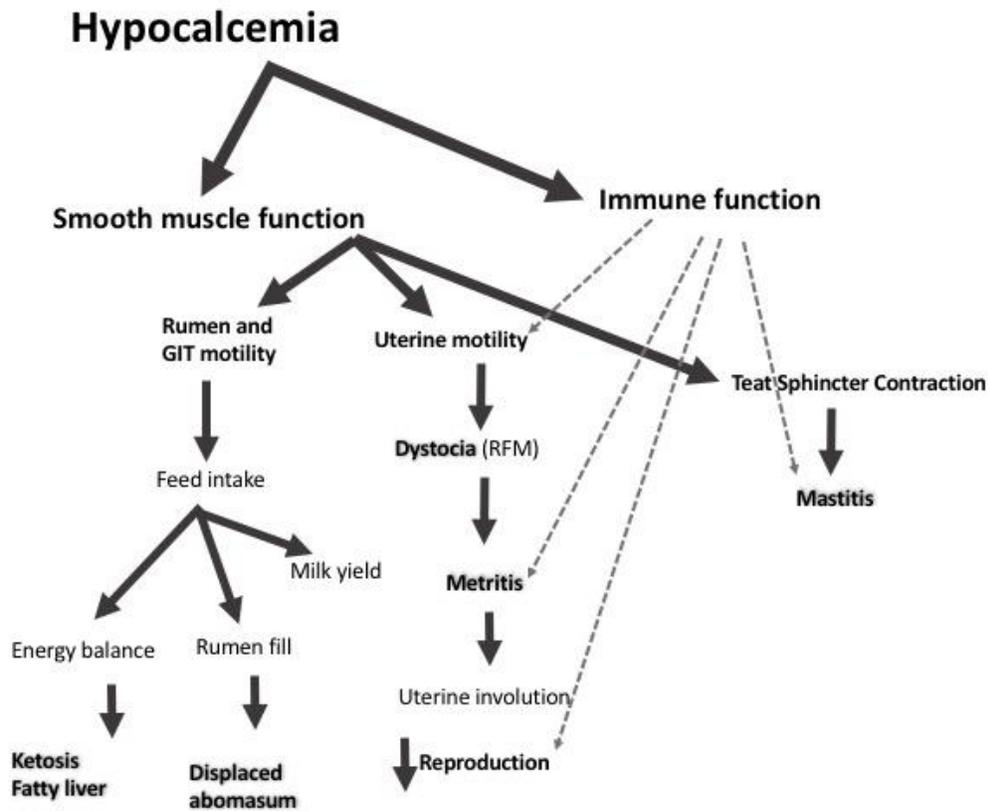
This was in contrast to the control Jersey cows who did not reach their total calcium concentration nadir until 1 day post-partum (Weaver et al., 2016). Furthermore, Jersey cows treated with 5-HTP had higher concentrations of calcium in their milk compared to the saline treated cows, which was opposite to what was seen in the Holstein cows. With the growing number of Jersey cows in the dairy cow population, efforts should be focused on understanding the physiological mechanisms these cows use to maintain homeostasis and homeorhesis during lactation. Our data suggest that Jerseys are vastly different than Holsteins in calcium metabolism, and potentially metabolism of other nutrients. These data indicate that serotonin positively impacts calcium homeostasis in both Holstein and Jersey cows, but the mechanisms underlying the serotonin-calcium axis appear to be different and should be further investigated.

### **Interrelationship of a negative DCAD and serotonin**

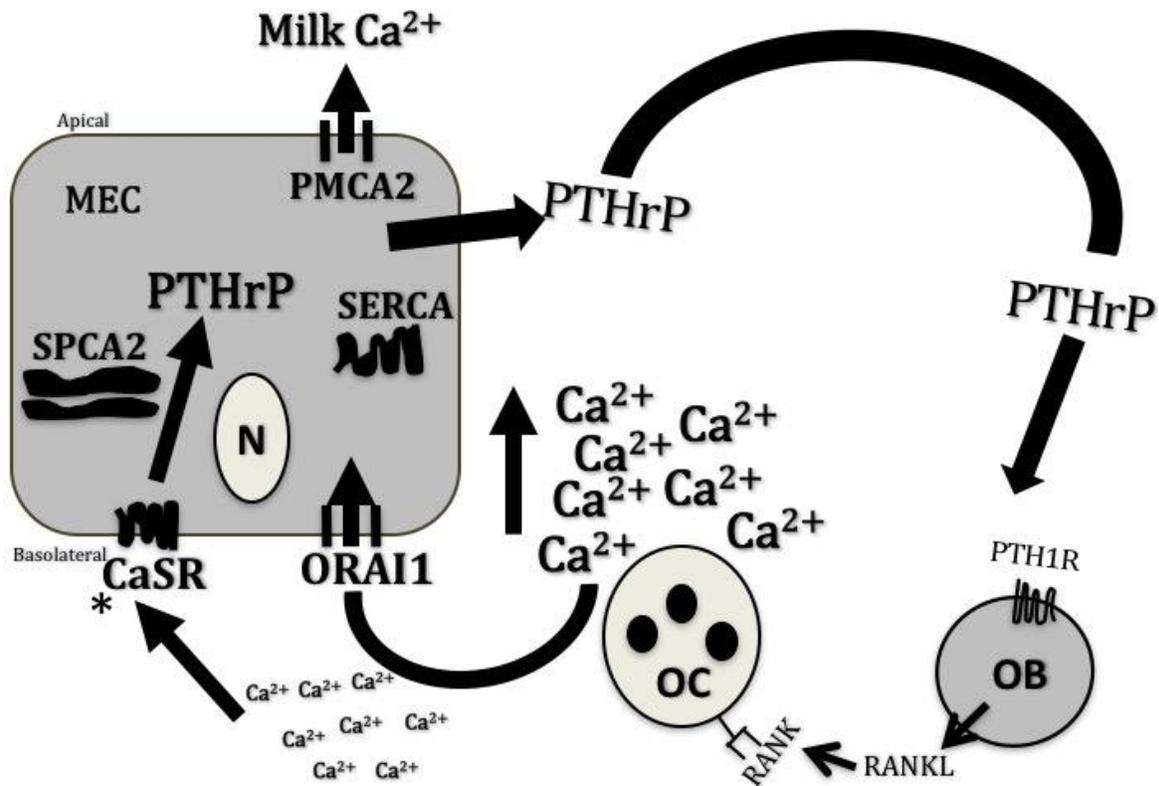
Given that 5-HTP treatment pre-calving was capable of increasing post-calving Ca concentrations, we wanted to determine if a common preventative treatment for SCH and CH, negative DCAD, controls Ca homeostasis via a serotonergic mechanism. To this end, we fed Holstein dairy cows a positive DCAD (+130 mEq/kg) diet for 21 days prior to calving or a negative DCAD (-130 mEq/kg) diet for 21 days prior to calving. Upon analysis of circulating serotonin concentrations from 9 days pre-calving through 6 days post-calving, we determined that a negative DCAD diet increased circulating serotonin concentrations pre-calving (Martinez et al., unpublished results). This suggests the resulting improvement in post-calving Ca concentrations in the cows receiving a negative DCAD diet pre-calving could be due to serotonin's control of Ca homeostasis. We have preliminary results from a study testing the hypothesis that 5-HTP and negative DCAD diets have a synergistic effect on post-calving calcium concentrations. Our preliminary results indicate that the combination of 5-HTP treatment combined with a negative DCAD diet results in the largest increase in post-calving ionized calcium concentrations (Slater et al., unpublished results).

### **Conclusion**

In conclusion, we have demonstrated that serotonin plays a critical role in regulation of maternal Ca transport, maternal Ca homeostasis, and mammary PTHrP production in the rodent. Additionally, our data demonstrate that mammary gland Ca transporter expression and induction of mammary-derived PTHrP during lactation are key regulators of maternal Ca homeostasis in rodent models. Furthermore, our rodent models indicate that the mammary gland is a significant source of serotonin during lactation. Our observational data in Holstein cows suggests that serotonin, PTHrP, and Ca are interrelated during the early post-partum period. Furthermore, our initial experiment exploring the effects of 5-HTP on maternal Ca homeostasis in late-lactation dairy cows supports the hypothesis that serotonin induces transient hypocalcemia by shuttling Ca into the mammary gland in order to stimulate bone Ca resorption. Treating pre-partum Holstein dairy cows with 5-HTP resulted in improvement of post-partum Ca concentrations. It also appears that Jersey cows respond differently to 5-HTP treatment and further research should be directed to understanding their physiology as compared to Holstein cows. Using a current therapeutic intervention for prevention of SCH and CH in the dairy industry, feeding of a negative DCAD diet pre-partum resulted in the increase of circulating serotonin concentrations. Our preliminary data examining the interaction of 5-HTP and negative DCAD suggests that two treatments together have a synergistic effect on improving post-calving calcium homeostasis.



*Figure 1. Hypocalcemia is a 'gateway' disease that leads to increased risks of other periparturient diseases. (DeGaris and Lean, 2008).*



**Figure 2.** Maternal Ca homeostasis is regulated by the mammary gland-bone axis. During lactation, the Ca sensing receptor (CaSR) on the basolateral side of the mammary epithelial cell (MEC) during lactation detects low blood Ca concentrations due to the increased transport of Ca into the MEC by Ca release-activated Ca channel protein 1 (ORAI1). Ca is either secreted into the milk through the apical plasma membrane Ca ATPase 2 (PMCA2) or sequestered in the Golgi apparatus by secretory pathways Ca ATPase 2 (SPCA2) or endoplasmic reticulum by the sarco(endo)plasmic reticulum Ca ATPase (SERCA). Detection of systemic decreased Ca by CaSR results in parathyroid hormone related-protein (PTHrP) production. PTHrP is secreted into the circulation and will bind its receptor PTH1R on the osteoblast (OB) cell in the bone increasing production of receptor activated nuclear factor kappa B (RANKL), which binds its receptor (RANK) on the osteoclast (OC) cell in the bone tissue, activating Ca liberation from bone.

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