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The Impact of an Intronic Dopamine Receptor Type-2 Single Nucleotide Polymorphism on Growth, Reproduction and Prolactin Gene Expression in Beef Bulls

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THE IMPACT OF AN INTRONIC DOPAMINE RECEPTOR TYPE-2
SINGLE NUCLEOTIDE POLYMORPHISM ON GROWTH, REPRODUCTION
AND PROLACTIN GENE EXPRESSION IN BEEF BULLS

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ABSTRACT

Prolactin (PRL) is a ~22 kDa peptide hormone reported to regulate over 300 distinct biological functions in vertebrates. Tonic inhibition of prolactin (PRL) by dopamine binding to dopamine receptor-type 2 (DRD2) is well established and changes in PRL serum concentration resulting in detrimental effects to male reproductive physiology have been observed. Release and synthesis of PRL may be altered due to a single nucleotide polymorphism (SNPs) in the DRD2 gene. The objective of these studies were to evaluate associations of a previously identified DRD2 SNP to PRL serum concentrations, growth traits, semen quality, and PRL gene expression in beef bulls. Semen quality, growth traits, and serum PRL concentrations were recorded from bulls exposed to a dopamine agonist over a 4-year study. Testis and epididymis were collected from bulls in Year 1 at the end of a 126 d study. Genotyping was performed on genomic DNA from semen samples by restriction fragment length polymorphism (RFLP) and Taqman custom SNP genotyping assays. Genotype was not associated with semen quality, serum PRL concentrations, or growth traits; however, treatment of a dopamine agonist lowered serum PRL concentrations. Immunohistochemistry revealed the presence of DRD2 in testis, epididymis and sperm cells. Prolactin protein expression, assessed by western blotting, indicated presence of PRL protein in only anterior pituitary. Pituitaries were collected from a local abattoir with selection of male posterior pituitaries performed by duplex PCR and Southern blotting methods. Genotyping was performed by RFLP for the DRD2 SNP. Slot blot and densitometry analysis for PRL protein expression was performed on a subset of male pituitaries (n = 92). No association between the DRD2 SNP and PRL protein

expression in bovine anterior pituitary was observed. Taken together, these data indicate no DRD2 SNP genotypic effect on growth or semen quality. Further, PRL protein expression was similar across genotype in the anterior pituitary, the main source of PRL synthesis and secretion.

<u>DEDICATION</u>

I would like to dedicate this dissertation to my other half, my twin, Samantha DeCarlo. We started every journey in life together. I watched your every move growing up, wanting to not only look alike, but to also do everything you did. I began college, only because you did. Through all our accomplishments and all our struggle in school together, I found an unexpected love of learning. On a journey I only started in pursuit of likeness to you, I found myself and my passion. It is you that I attribute so much of my interest in learning and it is you that instilled in me a need to go further. I hope someday Kota will look back and be so proud and inspired. To my mom and dad, Aggie and Nick DeCarlo. So much of what I am today is because of you both and all the sacrifices you both had to make along the way. Your encouragement and support over the years has kept me driven to make you proud. A special thank you to my mom, who spent countless late nights on the phone, and countless months traveling and visiting Clemson to support me. To my husband, Jonathan. Graduate school took so much of our beginning married. You took on an impossible burden to let me pursue my dreams, putting yourself last. I only hope to get the chance to give you what you gave me all these years, the opportunity to dream, to pursue, and to succeed. I look forward to our new beginning, hopeful, as we start this next chapter of life.

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CHAPTER ONE

LITERATURE REVIEW

Prolactin

Structure

Prolactin is a member of the GH/PRL gene family (Anthony et al., 1995). Prolactin cDNA has been characterized across a number of species including cattle, human, rat, and mouse (Wallis, 1974; Miller et al., 1981; Cooke et al., 1980; Linzer and Talamantes, 1985). to rat and humans, respectively (Miller et al., 1981). The PRL gene is made up of 5 exons separated by 4 introns (Miller et al., 1981; Truong et al., 1984). The PRL amino acid sequence characterized from cDNA for the coding region of the PRL gene, excluding pre-sequences, share 60.5% identity between cattle and rats and 74% identity between cattle and humans (Miller et al., 1981). The cDNA characterized for the PRL gene coding region in cattle pituitary encoded for a protein of ~227 amino acids (~26 kDa) with a 28-nucleotide long signal peptide. Once the signal peptide is cleaved the mature PRL protein is ~199 amino acids in length with a molecular weight of ~22 kDa (Wallis, 1974; Miller et al., 1981). The sequence does not possess a traditional AAUAAA poly (A) region; however, Sasavage and others (1982) reported three poly (A) sites and suggested the possibility of multiple processing mechanisms through alternative polyadenylation, therefore producing multiple transcripts from the single gene (Sasavage et al., 1982). Camper and others (1984) determined PRL gene number by genomic titration of bovine pituitary DNA against a standard curve of genomic equivalents produced from a PRL cDNA clone. Genomic DNA

was blotted to a membrane in increasing concentrations and hybridized with a PRL-specific ^{32}P-labeled probe. Detection of signal was carried out by a scintillation counter. The curve was linear with 10 μg of pituitary DNA plotted at 1 genomic equivalent indicating a single copy gene per haploid genome (Camper et al., 1984). According to PRL cDNAs characterized across mammals all encode for a protein consisting of six conserved cysteine residues predicted to form 3 disulfide bonds making up a large central loop, and small N- and C-terminal loops (Wallis, 1974; Cooke et al., 1980; Miller et al., 1981; Linzer and Talamantes, 1985).

Variants of PRL form by proteolytic cleavage or upon reduction of disulfide bonds. Posttranslational processing results in cleaved PRL isoforms, 14 and 16 kDa in size (Freeman et al., 2000). The 16 kDa isoform has been reported in rat pituitary cells. Cleavage of the large central loop occurs at a Tyr145 to produce the 16 kDa isoform (Andries et al., 1992) possibly by proteases that are present in the anterior pituitary (DeVito et al., 1992). Cole and colleagues (1991) purified monkey and baboon PRL identifying a 16 kDa PRL protein. They proposed a cleavage site at Ile133 that would theoretically result in a 14 kDa fragment. The 14 kDa fragment was confirmed as a posttranslational product of the native 23 kDa PRL, as RT-PCR and Southern analysis only revealed the 23 kDa PRL product, indicating the 14 kDa variant would actually be independent from the 16 kDa fragment. fragment was not a result of alternative splicing (Clapp et al., 1994). A year later Torner and colleagues (1995) confirmed the 14 kDa variant was the dominant form in rat hypothalamo-neurohypophyseal system (Torner et al., 1995).

Phosphorylation is the main post-translational modification of PRL proteins, with upwards of 80% of all PRL in the bovine pituitary phosphorylated (Kim and Brooks, 1993). Phosphorylation of PRL occurs predominantly at serine 90 residue but has also been shown to occur at two minor sites, ser26 and ser34. In Nb2 cell culture a PRL mixture of both nonphosphorylated and phosphorylated ^{125}I bovine PRL was set up to compete for binding with either nonphosphorylated or phosphorylated bovine PRL. Phosphorylated PRL was not able to compete for binding of the intermediate PRL receptor present in Nb2 cell culture (Kelly et al., 1992) at concentrations up to 65 nM, indicating phosphorylation produces a less biologically active form of PRL (Wicks and Brooks, 1995).

Glycosylated PRL (N-linked glycosylation) makes up as little as 15% of PRL in the pituitary of sheep and humans but upwards of 40% in swine (Sinha, 1992). Prolactin proteins are glycosylated through attachment of a carbohydrate at asparagine residue 31 in sheep and humans at a consensus sequence of Asn-X-Ser. Bovine pituitary possesses only a small portion of glycosylated PRL, approximately 1% due to the lack of a glycosylation sequence with an Asp in place of the Asn at residue 31, therefore glycosylated PRL is not prevalent in cattle at as low as 1% in the pituitary (Lewis et al., 1984; Sinha et al., 1995). Further, a cell-free protein synthesis system was utilized with isolated bovine pituitary microsomes to identify post-translational modification of bovine and ovine PRL. Protein extracts from bovine pituitaries did not contain glycosylated forms of PRL (Strickland and Pierce, 1985). Glycosylated PRL has been shown to elicit greater lactogenic but not mitogenic activity than non-glycosylated forms in swine (Young et al., 1990) and has been shown to impact metabolic clearance rate of PRL (Sinha et al., 1991). It is plausible that

cattle lack the N-linked glycosylation sites for regulation of PRL function through conformation, since addition of a carbohydrate moiety would alter conformation and therefore binding properties. Conformational changes as a result of glycosylation could play a role in dictating specificity for ligand receptor binding across species. An example of this species specificity is with human growth hormone receptors, which will only bind growth hormone from primate origin, but human growth hormone ligand will bind to other species' growth hormone receptors (Li et al., 1957). Human glycosylated PRL exhibited weak competitive binding, comparable to bovine PRL in human kidney cells transfected with a hybrid human prolactin receptor (PRLR) containing a 5'rat untranslated sequence. (Lochnan et al., 1995). Therefore, the lack of N-glycosylation consensus sequence for post-translational modification by glycosylation may be specific for bovine PRL to permit high affinity binding to bovine PRLR but not the human PRLR.

Larger PRL moieties of PRL can be formed by covalent and non-covalent binding of PRL monomers with other PRL monomers, binding proteins, and immunoglobulins. A PRL binding protein in human serum has been observed to bind to human PRL and growth hormone, producing larger protein aggregates of ~30-60 kDa (Kline and Clevenger, 2001). Immunoglobulins can also form complexes with PRL (Cavaco et al., 1995). These high molecular weight forms are often referred to as "big" or "big-big" PRL and can range from 45 to over 100 kDa, respectively (Sinha, 1992). Research on larger PRL moieties has been extensively studied in human medicine in relation to a condition termed "macroprolactinemia," as a form of hyperprolactinemia (Cavaco et al., 1995). Macroprolactin has been shown to be bioactive *in vitro* but not *in vivo*, indicating that larger

PRL forms cannot gain access to receptors as they may not be able to pass through the pituitary capillary bed (Andersen et al., 1982). Big PRL makes up ~15-30% of serum PRL while big-big forms range from 0-10% in humans (Suh and Frantz, 1974).

Synthesis and secretion of prolactin

The pituitary gland is made up of two main lobes, anterior and posterior. An intermediate lobe exists in most species, but is not present as a defined structure in humans (Larkin and Ansorge, 2017). The anterior lobe contains specialized cells called lactotrophs, that synthesize and secrete of PRL. The pituitary is formed with the expansion of Rathke's pouch (Brinkmeier et al., 2009) at ~20 d of gestation in the rat (Fisher et al.,1977) and ~4 wks in humans (Larkin and Ansorge, 2017). In cattle, development of the pituitary must occur prior to 60 d of gestation, as secretion of GH was observed at ~60 d of gestation while secretion of PRL was observed at 98 d of gestation (Kineman et al., 1992).

Earlier identification of pituitary cell types within the anterior lobe were named according to their staining properties by aldehyde-fuchsin which will stain lactotrophs (acidophils) orange, basophils will stain blue or green and chromophobes will not take up stain as readily as either acidophils or basophils (Halmi, 1950). With immune-histochemistry and more advanced microscopy, populations of cell types were named according to what hormone each produce and secrete as well as their target organs (Kurosumi, 1968). Corticotropes, thyroropes, somatotropes, gonadotropes and lactotropes produce and secrete, adrenocorticotropin (ACTH), thyroid stimulating hormone (TSH), growth hormone (GH), leutinizing hormone (LH), follicle stimulating hormone (FSH), and PRL, respectively. However, there are exceptions for somatotrophs, with cell morphology

able to change under hormonal influence; an example being the change in somatotroph secretion during pregnancy (Goluboff et al., 1969). In the presence of estrogen, somatotropes are able to switch secretion from GH to PRL (Stratmann et al., 1974).

Prolactin undergoes packaging in granules prior to release. Granules are formed in the Golgi apparatus, within the lamellae (Farquhar and Wellings 1957) and range in size from approximately 200 nm to the largest at around 500 nm cattle (Tesar et al., 1969). Granule size varies in response to varying physiological status and need. Granules reach maximum size when storage of hormones is high and they become more localized at the cell membrane in anticipation for exocytosis. When in a state of higher synthesis, the granules are localized in the Golgi (Farquhar and Rinehart, 1954). Experiments to assess dynamic state of lactotrophs in relation to PRL heterogeneity observed that hyperstimulated lactotrophs bypass packaging and polymerization of PRL when the need for exocytosis was greater, as in the presence of a secretogogue, whereas interruption of the stimulation caused lactotrophs to build up secretory granules due to lack of exocytosis (Torres and Aoki, 1985).

There are four types of granules categorized by size and positioning within the lactotroph. Studied in rats, granules 100-200 nm in size are considered type I granules, found within the Golgi. Slightly larger granules, type II, are also within the golgi or associated with the Golgi but form aggregates with other granules. Type III and Type IV granules are much larger and more irregular shaped then the smaller Type I round granules. Type IV granules can get to approximately 900 nm in size (Farquhar, Reid and Daniell, 1978). Through a pulse-chase experiment, in which pituitary cells were "pulsed" with [^{3}H]

leucine to label PRL proteins followed by "chased" with unlabeled medium it was concluded that the path PRL takes through intracellular processing and then onto packaging within secretory granules is through the endoplasmic reticulum (ER) and then the Golgi in which immature granules form and then mature granules move out to the cytoplasm for exocytosis. Localization of granules in the ER occurred immediately after cells were pulsed. Within 5 minutes all labeled granules were associated with the Golgi. These labels then showed a progression from Type I all the way to Type IV granules for packaging within 15 minutes of transport from the Golgi (Farquhar, Reid and Daniell, 1978). Monomeric PRL was observed localized to organelles while big PRL moieties were observed aggregated and stored in granules (Torres and Aoki, 1987).

Secretion of PRL has been shown to be dependent on calcium influx (Zorec et al., 1991; Ratovondrahona et al., 1998). The activation of phospholipase C (PLC) is followed by hydrolysis of membrane bound phosphatidylinositol 4,5-bisphosphate (PIP$_2$) for formation of inositol phosphates and diacylglycerol (DAG). Protein kinase C (PKC) is recruited and activated by DAG. Inositol phosphate 3 binds its receptor in the endoplasmic reticulum (ER) to increase mobilization of calcium from the ER. Phospholipase 3 phosphorylates voltage-gated calcium channels to facilitate increased influx of intracellular calcium for exocytosis of PRL (Fomina and Levitan, 1995).

There is evidence that secretion of PRL varies in response to physiological status. The most classic example originates from the early pigeon crop assays. Prolactin, facilitates brooding in pigeons, further an increase in mitotic activity (Lahr and Riddle, 1938) and differentiation (Dumont, 1965) of crop epithelial cells is observed for the production of

crop milk to feed young (Lahr and Riddle, 1938). Heightened serum PRL concentrations are also observed due to longer photoperiod (Pelletier, 1973; Bourne and Tucker, 1975; Peters and Tucker, 1978; Kennaway et al., 1983; Griffith and Minton, 1992). For example, rodents experience a decrease in synthesis and secretion of PRL during testicular regression, occurring as a result of a shorter photoperiod (Filippa and Mohamed, 2010). Increased stress has also been shown to increase levels of prolactin (Angelier and Chastel, 2005; Dorshkind and Horseman, 2000).

Other sources of prolactin

Aside from the pituitary, PRL is synthesized in other tissues. Most research on extra-pituitary sources of PRL is in females, as reviewed extensively by Ben-Jonathan (1996). Most recently, PRL has been reported in seminal fluid of humans and cattle (Sheth et al., 1975; Pratt et al., 2014). Further research to determine synthesis of PRL in male reproductive tissues is needed to uncover the mechanism of impact of PRL on male reproductive physiology. Prolactin's presence in seminal fluid implicates involvement in semen quality for possibly autocrine and paracrine actions of PRL. Further, the possibility also exists that endocrine effects could be exerted by PRL from the pituitary on male reproductive tissues such as the testis and accessory glands, impacting semen quality or fertility. Investigation of the presence of PRLR in these male reproductive tissues, and further, assays to asses binding of PRLR locally could help to elucidate how PRL exerts effects on male reproductive physiology.

Regulation

Gene expression of PRL is regulated through a proximal promoter region spanning 40 to 250 bp upstream of a canonical TATA box in the proximal region of the PRL gene (Peers et al., 1990). Extra-pituitary sources of PRL are regulated through another distal regulatory element 5.8 kilobases upstream of Exon 1a (Berwaer et al., 1994). This distal promoter controls extra-pituitary prolactin expression in primates, but is not present in rodents (Christensen et al., 2013). Further modulation occurs by regulatory elements located within the proximal promoter (Peers et al., 1990), containing pit-1 binding sites, a transcription factor known to control prolactin transcription (Elsholtz et al., 1991; Lew et al., 1994). Moreover, two more regulatory elements are located even more distally at approximately 1750 bp and another at approximately 5000 bp upstream of the promoter region (Peers et al., 1990).

Pituitary PRL is under tonic inhibition by hypothalamic sources of dopamine (Ben-Jonathan and Hnasko, 2001). Dopamine suppresses PRL synthesis through binding to the dopamine Type-2 receptor (DRD2), a G-protein coupled receptor (Baertschi et al., 1992; Lledo et al., 1992). Binding initiates a conformational change that will prompt an exchange of GDP for GTP on the alpha subunit of the coupled G-inhibitory protein, resulting in dissociation of the alpha subunit from the beta and gamma subunits. The dissociation allows for the subunits to interact with other proteins, receptors, and ion channels to facilitate inhibition of PRL synthesis and secretion. In the case of interaction with ion channels, coupling is facilitated by the GTP-binding protein beta and gamma subunits interaction with gated inward-rectifying potassium channels, increasing potassium conductance and causing calcium channels to close resulting in decreased intracellular

calcium concentration (Pillai et al., 1998). The coupled G-inhibitory protein alpha subunit can also interact with and inhibit adenylyl cyclase, preventing the conversion of ATP to second messenger cAMP needed for activation of protein kinase A (PKA) (Ben-Jonathan and Hnasko, 2001; Lledo et al., 1992; Enjalbert and Bockaert, 1983) (Figure 1.1). Without activation, PKA remains in a deactivated state and does not phosphorylate cAMP response element binding proteins (CREB). As a result, CREB is unable to bind to cAMP response element (CRE) domains and subsequently bind cAMP binding protein. Consequently, binding of CBP to facilitate activation of pit-1 for stimulation of PRL transcription will not occur (Kapiloff et al., 1991). The alpha subunit can also interact with and inhibit PLC, inhibiting the subsequent hydrolysis of PIP_2, formation of inositol phosphates and DAG proteins. Activation of PKC does not occur and Inositol phosphate 3 cannot bind its receptor in the ER for mobilization of calcium necessary for exocytosis of PRL (Figure 1.2) (Fomina and Levitan, 1995).

Dopamine supply to lactotrophs for regulation of PRL originates from neurons located in the arcuate nucleus that extend to the median eminence where dopamine is transported through the portal blood system terminating in the anterior pituitary (Bjorklund et al., 1974; Fuxe, 1964). The nuclei are classified according to Andén et al. (1966) and are indicated by A11-A15. The perikarya responsible for dopamine transport, specifically to the anterior pituitary, are within the A12 region of the arcuate nucleus, called tuberoinfundibular neurons (TIDA). Axons project into the median eminence where released dopamine is picked up by longer portal vessels that carry the supply of dopamine to the capillaries of the hypophysial portal vessels and directly to the anterior pituitary (Fuxe, 1963). Prolactin

receptors are located on dopaminergic neurons which allow prolactin to regulate its own secretion through a short feedback loop. Prolactin is proposed to feedback in this manner to effect mRNA abundance of TH, and therefore, the production of dopamine (Arbogast and Voogt, 1991).

Less defined methods of dopaminergic inhibitory effects on PRL are through the Ras-Raf-MEK-ERK pathway. Suppression of ERK was diminished following mutation of DRD2 coupled G-inhibitory proteins. Specifically, $G_{\alpha o,}$ but not $G_{\alpha i2}$ coupled to dopamine receptors suppresses ERK1 and ERK2 activation (Liu et al., 2002). This is inconsistent with research in glioma cells, indicating a stimulatory role for dopamine on ERK (Luo et al., 1997). Differences may be attributed to cell type as the suppression of ERK by dopamine seems to be unique to pituitary cells. Moreover, abundance of isoform, long or short DRD2, may differ between cell types. Transgenic mice produced with overexpression of the short DRD2 isoform in lactotrophs, increases induction of the Ras-Raf-MEK-ERK pathway and consequently, pituitary hypoplasia and decreased serum PRL concentrations. Overexpression of DRD2 long isoform does not induce activation of the ERK pathway and therefore, PRL serum concentrations are higher in DRD2 long isoform overexpressed mice (Laccarino et al., 2002).

Function in male reproduction

The role of PRL is not well understood in male reproductive physiology. Most of the research regarding PRL and its effects on male physiology were performed in knockout mouse models. However, research is conflicting and has not yet provided a clear and consistent role. Prolactin ablation experiments showed fertile PRL deficient males when

they were mated with heterozygous females (Horseman et al., 1997) while experiments utilizing ablation of the PRLR in mice report none or only a slight decrease in fertility (Binart et al., 2003; Ormandy C.J. et al., 1997). Moreover, most knockout experiments were conducted under optimal conditions with few females and adequate feed available, which does not compare to conditions that would occur naturally or in an agricultural setting. Further, differences in species exist with PRL not found in rodent testis (Christensen H.R. et al., 2013).

Consumption of a forage containing a naturally ocurring dopamine agonist, ergot alkaloids, decreases serum PRL concentrations and negativley impacts fertility in cattle (Schuenemann at al., 2005). In human males, fluctuations in PRL concentrations have also been observed to affect male reproductive function with hyperprolactinemia patients associated with a high instance of infertility (Segal et al., 1976). Findings are contradictory with the ablation of the PRL gene in mice resulting in no differences in fertility measured by number of live pups per litter and rate of pregnancy in male PRL knockout mice mated to normal female mice (Horseman et al., 1997; Stegar et al., 1998). Studies from our laboratory, performed across three years, utilized fresh extended semen from bulls exposed to a dopamine agonist or not for artificial insemination to primi- and multiparous cows. Serum PRL concentrations were determined in the second study by radioimmunoassay. Results indicated lower serum PRL concentrations due to exposure to a dopamine agonist; however, pregnancy rates 35 d post-timed artificial insemination in the second study did not differ in cows artificially inseminated with fresh extended semen from bulls with decreased PRL serum concentrations. (Burnett et al., 2018).

Prolactin has been implicated in a role for stimulation of testicular responsiveness. The mechanism in which PRL exerts affects are not well understood (Bartke et al., 1977; Sanford and Baker, 2010). Hamsters treated with a PRL anterior pituitary transplant more rapidly experience testicular recrudescence in the presence of human chorionic gonadotropin, while hamsters treated with a dopamine agonist, bromocriptine, displayed delayed testicular recrudescence in the Spring (Bartke et al., 1980). Effects of PRL could be exerted by effects on steroidogenesis, as PRL-deficient mice treated with exogenous ovine PRL or an ectopic homograft exhibit elevated testosterone in response to treatment of human chorionic gonadotropin *in vitro* (Bartke et al., 1977). However, changes in testosterone due to serum PRL concentrations has not been reported across species with studies indicating no change in testosterone levels in cattle (Smith et al., 1973; Pratt et al., 2015). Leydig cells do possess high affinity binding sites for PRL (Ka = 8.7 nM) (Barkey et al., 1987), but in cell culture performed with Percoll gradient isolated rat Leydig cells in a serum-free medium, a dose-dependent response to treatment of ovine PRL was observed to exacerbate inhibitory effects of PRL on testosterone secretion in 3 d culture (Barkey et al., 1987). Testosterone secretion by Leydig cells in response to treatment with human chorionic gonadotropin was inhibited maximally at 100 ng/mL and minimally at the highest does of 500 ng/mL. This same inhibition was not observed short term in culture for 4 h. (Barkey et al., 1987).

Effects on steroidogenesis by PRL may also be due to effects on LH receptors. Evidence for maintenance of LH receptors on Leydig cells by PRL is observed in rams treated with a dopamine agonist, bromocriptine (Sanford and Baker, 2010). A tendency

was observed for 25-30 % decreased LH receptor abundance in testis during testicular regression in rams treated with a dopamine agonist. In response to treatment with LH, rams treated with bromocriptine displayed a 20 % decrease in testosterone secreted by the testis (Sanford and Baker, 2010). If PRL does play a role in augmentation of testosterone, there is likely more involved than solely maintenance of LH receptors as the effects observed in rams. The reduction in LH receptors in the testis were observed during testicular regression, when testosterone is much lower to begin with (Sanford and baker, 2010). In contrast to rams, LH receptor availability on Leydig cells is increased with treatment of exogenous PRL in hypophysectomized rats but did not alter binding affinity (Purvis et al., 1979). Prolactin was observed to act synergistically with LH to stimulate steroidogenesis in the testis as measured by the dose response of isolated Leydig cells to human chorionic gonadotropin in rats; however, like in rams, testosterone levels were unaltered by treatment of exogenous PRL (Purvis et al., 1979). Taken together, findings on PRL role in steroidogenesis is unclear and warrants further investigation.

Overabundance of PRL, or hyperprolactinemia, is associated with hyperplasia of the prostate (Wennbo et al., 1997). Moreover, PRL has been implicated in cancer of the prostate (Mee et al., 1984; Costello and Franklin, 1994) attributed to PRL enhancement of testosterone levels, as testosterone is the main regulator of the prostate (George and Peterson, 1988). Prolactin has been shown, in conjunction with testosterone implants in mice, to increase the level of androgen receptor availability in the prostate, specifically in the lateral lobe, allowing for increased responsiveness to testosterone (Prins, 1987). However, later studies in human prostate cancer cells, confirmed both PRL and

testosterone function to enhance zinc uptake, with increasing zinc corresponding to prostate malignancy (Costello et al., 1999). Results in both testis and prostate confirm a contradictory role for PRL on testosterone levels. However, research findings in the prostate indicate a synergistic role of both PRL and testosterone in trophic effects (Costello et al., 1999). However, experiments to elucidate the precise role or roles of PRL on steroidogenesis is warranted.

Prolactin has been identified in seminal fluid of cattle and humans (Pratt et al., 2014; Sheth et al., 1975). Prolactin's role in semen quality is very unclear as there are differences in findings within the literature. In cattle consuming a forage containing a dopamine agonist, in which serum PRL concentrations are lowered, semen motility and morphology are negatively affected (Looper et al., 2009; Pratt et al., 2014). Moreover, hypoprolactinemia is highly associated with semen quality issues leading to infertility in human males (Gonzalez et al., 1989). Interestingly, semen from bulls exposed to a dopamine agonist exhibit decreased semen freezing potential. Post-thaw, semen exhibits decreased motility as assessed by sperm-quality analysis. Sperm cells from semen collected from these bulls exhibited an overall lower percentage of normal morphology compared to those bulls not exposed to a dopamine agonist suggesting a role for PRL in sperm structure for why motility is so affected post-thaw. Semen utilized to test freezing was above acceptable sperm morphology percentage (75 %) at the start of freezing and total sperm number was unchanged post-thaw indicating the effect was not due to concentration or initial quality pre-freezing (Pratt et al., 2015). Others have reported no change in semen

quality as a result of decreased serum PRL concentrations (Schuenemann et al., 2005; Stowe et al., 2013).

There is evidence that PRL may indirectly play a role in spermatogenesis by increasing lipids needed for the spermatogenic processes (Gunasekar et al., 1991). Monkeys, immature and mature were injected with 1mg/kg body weight of either ovine PRL or bromocriptine (a dopamine agonist) twice a day for 10 days. Total cellular lipids increased in germ cells, ~15 mg/g to 25 mg/g in mature monkeys and ~12 mg/g to ~20m mg/g in immature monkeys (Gunasekar et al., 1991). A decrease in mg/g lipids was observed in Leydig cells of mature monkeys but not in immature monkeys treated with PRL at 40 mg/g in untreated monkeys compared to 30 mg/g in PRL treated monkeys (Gunasekar et al., 1991). Prolactin treatment showed the same effect on cholesterol levels in Leydig cells, with a decrease in cholcterol in Leydig cells of PRL treated mature monkeys compared to untreated controls (1 mg/g; 5 mg/g). Taken together, age could play a role in the effect of PRL on lipid accumulation in Leydig cells and suggests that there could be further effecs on steroidogenesis through Leydig cell production of testosterone (Gunasekar et al., 1991).

Prolactin's role in semen quality is further supported by a restriction in maturation of spermatocytes to spermatids in rats following a decrease in PRL concentration (Nag et al., 1981). However, exposure to a dopamine agonist had no effect on semen quality in yearling bulls (Burnett et al., 2018). Further, when bulls were monitored through two full spermatogenic cycles, bulls grazing a dopaminergic agonist exhibited no differences in semen quality as assessed by computer assisted semen analysis (CASA), a computer

program that utilizes an algorithm to track motility parameters of sperm cells as they move on recorded short video clips (Stowe et al., 2013). Given all the conflicting findings, at present, PRL's role in semen quality has not been clearly defined.

<div align="center">**Prolactin Receptor**</div>

Structure

The Class 1 cytokine receptor family includes receptors for PRL and growth hormone (GH) (Goffin and Kelly, 1997). The general structure of PRLR protein consists of a single chain protein with a single transmembrane domain, a cytoplasmic domain, and an extracellular region. The receptors bind in a 2:1 ratio, with 2 receptors binding to one PRL protein (Bole-Feysot et al., 1998). The extracellular domain of PRLR consists of two regions, the amino-terminal and membrane-proximal regions. The receptors contain two sets of conserved disulfide-linked cysteines located in the amino-terminal portion (Boutin et al., 1988, 1989). Within the membrane proximal region, there is a conserved region assigned the 'WS motif' (WSXWS) that facilitates ligand binding (Rozakis-Adcock and Kelly, 1992). Radiolabeled ligand binding assays performed with a WSXWS motif mutated PRLR (all five residues mutated with alanine) resulted in decreased binding affinity as measured by Kd, 0.29 ± 0.04 nM for wild type unmutated control, and 6.76 ± 1.4 nM for the mutated receptor, (Rozakis-Adcock and Kelly, 1992). Similar findings have been implicated in other cytokine receptor types finding the motif functions for assistance in folding of secondary protein structure in erythropoietin receptors (Hilton et al., 1996). A decrease or complete loss of erythropoietin receptor availability at the cell membrane has been observed due to reduced migration from the endoplasmic reticulum consequence of

mutation of the serine and tryptophan residues in the WS motif effects on secondary structure (Hilton et al., 1996). Similar results have been reported with mutation of the tryptophan residues in the same receptor resulting in impaired or abolished ligand internalization and binding (Quelle et al., 1992). The importance of tryptophan residues adjacent to the serine residues in the WSXWS motif may be due to polarity of the sequence and the ability of the tryptophan residues to provide the necessary scaffolding for proper support of the serine polar residues and therefore proper hydrogen binding to beta sheets (Quelle et al., 1992). This would agree with observations by Bazan (1990) who proposed the WSXWS motif was located on a loop connecting the amino-terminal and membrane proximal regions of the extracellular domain in which proper folding would not be permitted with substitutions of the outer tryptophan residues do to its location on a hinged region.

The intracellular portion of PRLR contains two regions, deemed Box 1 and 2, that are conserved across all isoforms of the PRLR with the exception of Box 2 in some isoforms of the receptor with shorter cytoplasmic lengths (Goffin et al., 1997). Box 1 is a proline rich region that has been shown to be essential for association of JAK2 with PRLR and further, activation (Tanner et al., 1995). Experiments in human kidney fibroblasts transfected with a pRc/CMV expression vector containing the PRLR cDNA encoding various mutated prolines within Box 1 indicate that association of Janus Kinase 2 (JAK2) to the PRLR requires the last proline in Box 1 (residue 250). When this proline is mutated, JAK2 does not associate with the PRLR and subsequent phosphorylation of signal transducer and activators of transcription 5 (STAT5) cannot occur for facilitation of PRL

transcriptional activity (Pezet et al., 1997). It is not surprising that Box 1 plays such an important role in PRLR signaling since the sequence is conserved across both short and long PRLR across species; however, Box 1 alone is not sufficient to permit PRL signal transduction as two other regions, one between Box 1 and 2 and the other in the carboxyl terminal have been shown as necessary for transcriptional activity of the β casein promoter, a promoter who's activation is dependent on PRL (Lebrun et al., 1995).

The PRLR gene is located on Chromosome 2 in rats, Chromosome 5 in humans (Arden et al., 1990), Chromosome 13 in mice (Jackson et al., 1988), and Chromosome 20 in cattle (Hayes et al., 1996). Complimentary DNA sequences have been characterized in a number of species and encode for PRLR proteins of varying cytoplasmic domain length (Bignon et al., 1997; Anthony et al., 1995; Shirota et al., 1990). Characterization of a cDNA clone performed in rat liver encoded for a short PRLR protein predicted to be 291 amino acids in length with an average mass of ~40 kDa (Boutin et al., 1988). The cDNA encoded a predicted transmembrane domain of 25 amino acids in length and the extracellular domain was ~210 amino acids in length. A ~55 amino acid cytoplasmic domain was observed for the short PRLR protein in rat liver (Boutin et al.,1988). A longer PRLR protein was characterized from a cDNA clone derived from rat ovary that encoded for a PRLR protein with a cytoplasmic domain ~300 bp longer than the previously identified short PRLR isoform in rat liver with a molecular mass of 80 kDa (Shirota et al., 1990). An intermediate isoform has been identified in the rat lymphoma Nb2 cell line and in a human breast cancer cell line (Ali et al., 1991; Kline et al., 1999). The intermediate isoform lacks ~200 amino acids in the cytoplasmic domain with a molecular mass just shy

of the long receptor at 60 kDa in rats (Ali et al., 1991). In humans the intermediate isoform is homologous to the long PRLR up to nucleotide position 1582 in which a 573-nucleotide deletion was found, causing a frame shift that resulted in an early stop codon in the carboxyl terminal (Kline et al., 1999). Similarly, multiple PRLR isoforms have been identified in mice, however mice possess three short and one long isoform, identified by characterizing cDNA clones generated from the liver (Davis and Linzer, 1989). In humans, a long PRLR isoform has also been cloned from the liver (Boutin et al., 1989), an intermediate form from a breast cancer cell line (Kline et al., 1999), multiple short forms, and a soluble binding protein (lacking the entire cytoplasmic portion of the receptor) have been identified (Hu et al., 2001; Trott et al., 2003). The intermediate isoform has similar binding affinity and signaling properties to long PRLR (Kline et al., 1999).

One long and one short PRLR have been identified in bovine fetal and placental tissues (Scott et al., 1992; Schuler et al., 1997). The long receptor was characterized from endometrial cDNA and the preprotein was predicted to be 557 amino acids in length (Scott et al., 1992). The long bovine PRLR differs in length from human and rats due to an extra stop codon in the 3' region encoding the cytoplasmic domain (Scott et al., 1992). Messenger RNA expression of short variants of the mouse PRLR have been observed to vary throughout pregnancy in the ovary. One short PRLR variant was differentially expressed in atretic follicles, corpora lutea and interstitial cells suggesting a role in follicular atresia (Clarke and Linzer, 1993). Findings for a specialized role of short PRLR in the mouse placed emphasis on possibilities of these PRLR isoforms in other species. Shortly after, one short and one long PRLR were isolated and characterized in ovine fetal

liver and adult ovaries (Anthony et al., 1995). Characterization of the second sequence revealed an almost identical cDNA sequence up until nucleotide 420 in which an extra 39 base pairs were inserted. The cDNA encoded for a protein lacking the conserved box 2 sequence due to a truncation in the cytoplasmic domain, encoding for the short ovine PRLR (Anthony et al., 1995). The primer sequences used to identify PRLR in sheep (Anthony et al., 1995) were then used for the identification of a short bovine PRLR variant that encoded a protein similar to that reported in sheep, with a truncated receptor at 227 amino acids in length containing no Box 2 sequence (Schuler et al., 1997).

In ruminants, the short isoform of the PRLR is produced as a result of an extra 39 bp sequence at residue 420, containing two alternative 3' stop codons. Ruminants utilize an intron retention mechanism of alternative splicing that spans a single 5' or two 3' alternative sites on the extra 39 bp insert to produce a short or long receptor. Splicing patterns for PRLR are different in rodents which utilize excision or inclusion of three exons in the 3' region to produce long and short PRLR, respectively. However, the end resulting protein is extremely similar across ruminants and rodents regardless of their splicing method (Bignon et al., 1997).

Short isoforms of the PRLR serve as compensatory signaling if the loss of long PRLR occurs. This effect has been demonstrated by restoration of normal mammary gland development in mice through overexpression of short PRLR in mice heterozygous for the long PRLR (Binart et al., 2003). However, this is not what is observed in cattle. The long, but not short PRLR was able to promote gene expression when transfected into a bovine

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endometrial stromal cell line indicating the specialized functionality of short PRLR in rodents is not the same in cattle (Schuler et al., 1997).

Differences in functionality may be due to splicing out of tyrosine residues within the cytoplasmic domain. Shorter PRLR isoforms lack some if not all of these essential tyrosine residues depending on where splicing occurs (Schuler et al., 1997). In cattle, the long PRLR is slightly truncated due to an extra stop codon, resulting in the loss of a tyrosine residue thought to be important for STAT5 docking in rats; however, the slightly truncated receptor was still able to stimulate transcription (Schuler et al., 1997). The receptor's ability to stimulate transcription indicates it may use other tyrosines for STAT docking and initiation of transcriptional activity. Importance of tyrosine residues have been evaluated with mutation experiments. Particularly tyrosine 580, results in depletion of STAT5 activation and downstream gene expression. However, tyrosine 479 and 473 allow for retention of ~20 % activation when tyrosine 580 is mutated. (Pezet et al., 1997). Explanation of differences across species in ability to initiate signaling through short PRLR could also be due to heterodimerization. Short PRLR isoforms inhibit long PRLR thought heterodimerization. When the two receptors were co-expressed in human embryonic kidney fibroblast cells, activation by phosphorylation of JAK2 and the PRLR does not occur, therefore transcriptional activation is prevented (Perrot-Applanat et al., 1997). Such heterodimerization has also been reported in humans (Qazi et al., 2006).

Expression and Function in the male

Prolactin receptor mRNA has been reported in rat interstitial cells, Leydig and Sertoli cells, and sperm cells (Hondo et al., 1995). *In situ* hybridization in the rat indicates

signal for PRLR in Leydig and endothelial cells within interstitial space in the testis as well as staining in the epithelial layer of the seminiferous tubules (Hondo et al., 1995). Similar localization was confirmed in rams and red deer with *in situ* hybridization revealing signal for PRLR in the seminiferous tubules, specifically Leydig and developing sperm cells, interstitial space, and the epithelial layer of the epididymis of the red deer. (Jabbour and Lincoln, 1999). More recently, PRLR gene expression has been reported in bull and human testis (Hair et al., 2002; Pratt et al 2014). Immunohistochemistry confirmed localization of PRLR in Leydig cells and the interstitial space in the seminiferous lumen in the testis in humans and bulls, respectively (Hair et al., 2002; Pratt et al., 2014).

Prolactin receptor gene expression in the interstitial space and in Leydig cells suggests a possible role for PRL on steroidogenesis, as Leydig cells produce testosterone. *In vitro* studies in the rat testis indicate that circulating serum PRL concentrations impact LH receptors (Aragona et al., 1977). However, *in vivo* experiments in hamsters indicate PRL alone does not modulate steroidogenesis, as other factors such as FSH and LH are needed to gain full enhancement of steroidogenesis in the testis (Klemcke et al., 1990).

Prolactin receptor expression in developing sperm cells suggests a direct role for PRL in spermatogenesis. Evidence for effects on germ cells has been reported. Hypophysectomized rats treated with exogenous PRL display an increase in number of primary spermatocytes as well as overall number of germ cells (Dombrowicz et al.,1992); however, sperm concentration does not seem to be altered in patients with hyperprolactinemia (Merino et al., 1997) but increased serum PRL has been associated with reduced semen motility (Gonzales et al., 1989). Studies using PRLR ablation in mice

indicate that PRL does not play a direct role in spermatogenesis or steroidogenesis, as histology of the testis was unaffected and there was no change in testosterone levels (Binart et al., 2003). Effects may differ due to species with rodents possessing possible compensatory mechanisms.

Mouse PRLR ablation experiments report that the loss of PRLR slightly impacts male fertility (Ormandy et al., 1997). However, later experiments utilizing the same PRLR knockout model, found that there was no change in male fertility due to PRLR knockout (Binart et al., 2003). Male fertility was assessed by mating a male mouse with complete knockout of the PRLR (PRLR$^{(-/-)}$) with a heterozygous female PRLR$^{(-/+)}$. Males were considered fully fertile if they produced pregnancies after the first vaginal plug observed, and considered partially or completely infertile if they took several times to produce pregnancies after the observation of a vaginal plug or they never produced pregnancies, respectively (Binart et al., 2003). Knockout mouse models therefore predict that there is no association of PRL on overall fertility in males. However, across species this differs with hyperprolactinemic human males reported as infertile (Segal et al., 1976) and cattle with decreased serum PRL concentrations displaying detrimental effects to semen motility and morphology (Looper et al., 2009; Pratt et al., 2014).

Overall, research in PRLR$^{(-/-)}$ models differ in observations within and across laboratories on the role of PRL in spermatogenesis and steroidogenesis. Results also vary in *in vitro* models versus *in vivo* animal studies on the role of PRL in steroidogenesis. Given the severity of differences, further exploration of the PRLR and PRL impact on male reproductive physiology are intriguing.

JAK/STAT Pathway

The JAK/STAT pathway is activated by cytokines including PRL (Ihle and Kerr, 1995) (Figure 1.3). Prolactin binds to its receptors possessing two binding sites in which each single chian protein receptor binds to one of the two sites on the PRL protein. Dimerization and ligand binding prompt a conformational change to occur in the cytoplasmic region of the receptor (Ihle and Kerr, 1995; Gertler et al., 1996). The conformational change activates the the constitutively associated JAK2 to be transphosphorylated (Rui H. et al., 1992). Activation of JAK2 results in phosphorylation of tyrosine residues on the PRLR allowing STAT5 molecules to bind to these tyrosine sites and undergo phosphorylation by JAK2 (Gouilleux et al., 1994; Kisseleva et al., 2002). The STAT5 molecules then disassociate from the receptor and dimerize, followed by translocation to the nucleus to effect transcriptional activity by binding to enhancer regions, such as gamma interferon activated sites (GAS) (Ihle, 1996).

Four different JAKs have been identified, JAK 1, JAK 2, JAK 3, and TYK 2 with all except JAK 3 expressed ubiquitously (Kisseleva et al., 2002). Janus kinases are 120-130 kDa proteins, ~1150 amino acids in length and are structurally categorized by 7 domains (JH1-JH7) (Kisseleva et al., 2002). The tyrosine kinase domain (JH1) contains all the conserved tyrosine residues that are responsible for the conformational change to the PRLR proceeding phosphorylation (Hubbard, 2018). Phosphorylation of JAK2 occurs in tyrosine residues Tyr1007 and 1008 (Hubbard, 2018) located in the activation loop to facilitate activation of JAK2. Phosphorylation of Tyr 813 has been shown to be a main site

for phosphorylation to facilitate binding the Src homology 2 (SH2) domain in STAT5 to JAK2 (Kurzer et al., 2004). The N-terminal domain (JH3-JH7) has been implicated in binding specificity for cytokine receptors (Zhao et al., 1995) and is the least conserved in family members of the JAKs (Kisseleva et al., 2002). However, not much else is known about its function. The pseudokinase domain (JH2) lacks catalytic activity (Velazquez et al., 1995). Until recently, not much was known about the JH2 domain or its function. The JH2 domain functions to regulate JAK activation as observed by deletion of the JH2 domain resulting in an increase in tyrosine phosphorylation of both JAK2 and STAT5 (Saharinen et al., 2003). Further, two residues have been implicated in this negative regulation through mutation of the pseudokinase domain, (Ser 523 and Tyr 570) (Ungureanu et al., 2011). Phosphorylation of Ser 523 in the JH2 domain is the initial step of autophosphorylation of JAK2, followed by Tyr 570 (Ungureanu et al., 2011).

There are seven identified STATs, approximately 750-850 amino acids in length at approximately 95 kDa. All STAT proteins share six conserved domains with the amino-terminal portion of STATs has been shown to facilitate DNA-binding (Vinkemeier et al., 1998). Structural characterization studies indicate that the N-terminal domain is made up of a folded dimerized structure in which the conformation permits binding to DNA elements (Vinkemeier et al., 1996). The first ~100 amino acids of the amino-terminal specifically, have also been shown to facilitate translocation of STAT dimers to the nucleus and after translocation, deactivation (Strehlow and Schindler, 1998). These first 100 amino acids are highly conserved among the STAT family, however, when chimeric STAT proteins were produced, the dimer was unable to translocate and deactivate, indicating that

the N-terminal domain is involved in more than was originally thought (Horvath et al., 1995). The DNA binding domain favors a TTC(T/C)N(G/A)GAA GAS sequence corresponding to three conserved amino acids phenylalanine, glutamine, and glutamic acid for binding of STAT homodimers to DNA (Soldaini et al., 2000). The specific site of interaction occurs through the DNA-binding domain, specifically residues 470-474, that are inserted into the major groove of DNA (Soldaini et al., 2000) The coiled-coil domain directly proceeding the N-terminal domain, has been shown to facilitate nuclear translocation of STATs, sequestration to the receptor, as well as dimerization (Zhang et al., 2000). These functions are all tyrosine phosphorylation dependent and deletions of 5' coiled coil domain areas result in a loss of these functions due to a loss of phosphorylation when assessed in STAT3 function (Zhang et al., 2000). The SH2 domain plays a role in several functions, essential for STAT recruitment to the receptor by docking on phosphorylated tyrosine residues, dimerization that occurs prior to translocation to the nucleus, and associations with JAK for subsequent activation (Kisseleva et al., 2002). Ability of the SH2 domain to facilitate these actions occurs through recognition of specific sequences. The SH2 domain of STAT5 specifically recognizes Tyr, Leu, Asp, Pro, Thr motif for PRL receptor signaling (Lebrun et al., 1995).

Ras/Raf/MEK/ERK signaling pathway

Aside from activation of the JAK-STAT pathway, mitogen-activated protein kinase (MAPK) pathways can also be activated through PRLR signaling. Adaptor proteins, src homology 2 domain containing protein (Shc), growth factor receptor-bound protein 2 (Grb2), and son of sevenless (SOS) anchor to the phosphorylated tyrosine residue on the

PRLR to bridge the JAK-STAT signaling pathway to the Ras/Raf/MEK/ERK cascade (Das and Vonderhaar, 1996). Son of sevenless is a guanine nucleotide exchange factor that activates Ras, a small GTPase, through exchange of coupled GDP for GTP. Ras then phosphorylates Raf, a protein kinase related to an oncogene, followed by phosphorylation of MEK1/2 and ERK1/2 (extracellular signal regulated kinase). ERK1/2 then translocate to the nucleus to activate transcription factors such as (E26 transformation-specific) ETS factors (Bole-Feysot C. et al., 1998; Booth and Gutierrez-Hartman 2015; Clevenger et al., 1998) (Figure 1.3). A Thr38 residue in ETS-1 is essential for the activation of ETS-1 by Ras (Yang et al., 1996). Transformation-specific factor E-26 and pit-1, the necessary homeodomain for the transcriptional activation of the prolactin gene (Ryan and Rosenfeld, 1997), interact on the DNA to increase PRL promoter activity (Wasylyk B. et al., 1998).

Dopamine

Biosynthesis, storage, and exocytosis

Dopamine synthesis occurs in the brain and in the kidneys (Soares Da Silva et al., 1992; Ben-Jonathan and Hnasko, 2001). In the brain there are four major neuronal systems dopamine can be produced by; the mesocortical, tuberoinfundibular, mesolimbic, and nigro-striatal systems. Dopamine in hypophyseal blood was originally detected in rats by collection of portal blood from female rats in various stages of cyclicity, and in male rats either castrated or intact; detection of dopamine by radioenzymatic assay (Ben-Jonathan et al., 1977; Plotsky et al., 1978). The source of dopamine to hypophyseal blood was proposed to come from tuberoinfundibular (TIDA) neurons, with axons originating in the arcuate nucleus and terminating in the median eminence, releasing dopamine at the hypophysial

capillary bed (Smelik and Van Maanen, 1968). The capillary loops are connected to long portal vessels that carry released dopamine to the anterior pituitary (Smelik and Van Maanen, 1968). Tuberohypophyseal (THDA) neurons were later suggested as contributors of dopamine to the anterior pituitary when experiments utilizing posterior lobectomy resulted in elevation in serum PRL. The THDA neurons project axons from the periventricular nucleus to the posterior pituitary to supply dopamine to the anterior pituitary by transport through the small vascular networks connecting the posterior pituitary to the anterior lobe (Murai et al.,1986).

Dopamine is in the class of neurotransmitters called catecholamines. Dopamine, as with all catecholamines, have structurally defining features such as a catechol group, consisting of a benzol ring with two hydroxyl side chains and a single amine group (Nagatsu et al., 1964). Like all catecholamines, the synthesis of dopamine starts with tyrosine (Nagatsu et al., 1964). Tyrosine is readily available in the diet but can be produced by the hydroxylation of phenylalanine by phenylalanine hydroxylase in the liver (Moss and Schoenheimer, 1940). In dopaminergic neurons, tyrosine is hydroxylated to produce dihydroxyphenylalanine (DOPA) by the enzyme tyrosine hydroxylase (TH), with TH serving as the rate limiting step in dopamine synthesis. Further, DOPA is converted to dopamine by the enzyme, L-amino acid decarboxylase (Nagatsu et al., 1964).

Tyrosine hydroxylase is structurally unique from other aromatic acid hydroxylases, as it contains an extra four serine residues in its R-domain (Campbell et al., 1986). Dopamine exerts negative feedback on TH activity, dependent on the need for neurotransmitter synthesis (Daubner et al., 2011). Phosphorylation of Ser40 primarily by

PKA, results in activation of TH (Lovenberg et al., 1975) due to a conformational change that decreases affinity of dopamine for TH (Flatmark and Stevens, 1999). Inhibition of TH activation by dopamine is facilitated by competitive binding of dopamine and TH cofactor, tetrahydrobiopterin, for binding of the active site in the N-terminal region (Gordon et al., 2008). Phosphorylation of Ser40 results in a 300-fold reduction in dopamine affinity for binding TH (Daubner et al., 1992; Ramsey et al., 1998). Deactivation of TH occurs with dephosphorylation by phosphatase PP2A (Saraf et al., 2010). Prolactin and TH participate in a short feedback loop for regulation of TH transcriptional activity. Increased signaling of PRL through the PRLR promotes increased TH mRNA, specifically in the hypothalamus regions in rats resulting in increased conversion of TH to L-DOPA (Arbogast and Voogt, 1991). Tuberoinfundibular neurons were later discovered to possess PRLR (Arbogast and Voogt, 1997).

Dopamine is taken up into secretory vesicles by vesicular monoamine transporters (VMATs). After packaging into secretory vesicles, the vesicles are moved to the synaptic region for exocytosis (Nirenberg et al., 1996). Monoamine transporter cDNA has been characterized and is present in bovine adrenal medulla. The cDNA encodes for a protein 517 amino acids in length with a molecular weight of ~56 kDa (Howell et al., 1994). Vesicular monoamine transporters are found in two isoforms, VMAT 1 and VMAT 2. The VMAT 2 serves as a specific marker for monoamine containing neurons in the brain (Nirenberg et al., 1996). Transport and packaging are dependent on an electrochemical gradient and pH. Hydrogen ions are released, two for each dopamine molecule taken up by

the transporter and low acidic pH allows for the packaging of dopamine into storage vesicles (Njus et al., 1986).

Further regulation of dopamine is through post-translational regulation of VMAT2. Phosphorylation occurs at both the N-terminal and C-terminal ends of VMAT2, both eliciting a different response. Phosphorylation at the N-terminal is likely performed by PKC on serine residues 15 and 18 and is implicated in regulation of efflux away from the synapse. A reduction of monoamine sequestration to vesicles and efflux from the synapse in response to treatment with methamphetamine (to permit a high-efflux state) has been observed (Torres and Ruoho, 2014). N-linked glycosylation occurs on the C-terminal and between transmembrane 1 and 2 (Yao and Hersh, 2007). Both must be phosphorylated to permit VMAT2 vesicle packaging (Yao and Hersh, 2007)

Release and Reuptake

Exocytosis of dopamine at the synapse is controlled through a flickering fusion pore or "kiss and run" exocytosis. Dopamine is released in multiple flickering events per single exocytotic event. It is the number of flickering events that control overall release of dopamine as each flicker releases ~25% of vesicle stored dopamine allowing controlled release of only partial vesicle content for use of vesicles multiple times (Staal et al., 2004). Trigger of exocytotic events is controlled by calcium influx through sodium and calcium exchange at the synapse (Taglialatela et al., 1990). Rate of diffusion and distance to binding target of dopamine have also been observed to influence number of flickers occurring and how much dopamine is therefore released (Cragg et al., 2004).

Dopamine transporters (DATs) participate in reuptake of dopamine that is released from the synapse but does not bind to its target receptor thereafter (Giros and Caron, 1993). Dopamine transporter cDNA has been characterized and is present in bovine substantia nigra. The reported cDNA sequence was 2340 nucleotides in length and encoded for a predicted protein 693 amino acids in length with an approximate molecular weight of 70 kDa; 80 kDa when glycosylated. The protein consists of 12 transmembrane domains and three N-linked glycosylation sites located in the larger extracellular loop (Usdin et al., 1991).

Due to TIDA neuron termination in the median eminence and transportation of dopamine through perivascular space, TIDA neurons were thought not to form true synapses and were agreed to lack a functional DAT reuptake system (Demarest and Moore, 1979). Original experiments were performed using radioenzymatic assays to determine the ratio of total dopamine concentration to dopamine accumulation in the presence or absence of a reuptake inhibitor. Findings indicated that not much reuptake occurs in the median eminence where TIDA neurons release dopamine (Demarest and Moore, 1979). Immunohistochemical experiments allowed for the visualization of DAT transporter immunoreactivity, observed localized in the median eminence where TIDA neurons release dopamine (Revay et al., 1996) and further, found in the pituitary stalk by immunocytochemistry (Demaria et al., 2000). Suppression of PRL gene expression was observed in the anterior pituitary of rats treated with a DAT blocker for 7 d indicating that without the reuptake mechanism in the median eminence, increased levels of dopamine were being transported from the median eminence through portal blood, to the anterior

pituitary to suppress PRL expression (Demaria et al., 2000). The DAT transporters have been localized in both perisynaptic and extra-synaptic regions (Nirenberg et al., 1996). Extra-synaptic localization of DAT suggests a role in regulating already released dopamine. Even though TIDA neurons release dopamine as a neurohormone, regulation by DAT is still possible after dopamine diffuses away from the synaptic region. After reuptake by DAT, dopamine is degraded through oxidation by monoamine oxidase or repackaged into a secretory vesicle (Nirenberg et al., 1996).

Dopamine receptors largely regulate DAT function through increasing or decreasing abundance of DAT on the plasma membrane. Dopamine transporter and DRD2 co-expressed in oocytes, treated with a DRD2 agonist, exhibited upregulated expression of DAT on the plasma membrane. Treatment with pertussis toxin blocked the effect, further implicating DRD2 (Mayfield and Zahniser, 2001), as the DRD2 receptor is coupled to G-inhibitory proteins $G\alpha_i$ and $G\alpha_o$ (Ben-Jonathan and Hnasko, 2001). Interestingly, DAT is also able to directly bind to the third intracellular loop of DRD2 resulting in upregulation of DAT at the plasma membrane (lee et al., 2007).

Dopamine transporter protein expression and availability are regulated by post-translational modification. The DAT transporter plasma membrane expression has been observed to be PKC dependent (Grånäs et al., 2003). Truncation of the first 22 residues of the N-terminal results in a loss of phosphorylation, however, no changes in internalization (Grånäs et al., 2003). Internalization regulation seems to be mediated on the C-terminal, specifically residues 587-590 (Boudanova et al., 2008). The predicted mechanism for regulation of internalization of DAT includes an endocytic "brake." The proposed brake

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closely associates with DAT residues 587-590 to decrease binding to an adaptor protein necessary for internalization. Once PKC is activated the brake dissociates from DAT and the resulting residues are free to associate with the adaptor protein to facilitate endocytosis (Boudanova et al., 2008).

Dopamine transporters can be recycled back to the plasma membrane or marked for lysosomal degradation by ubiquitination (Miranda et al., 2007). Experiments to induce mutations in ubiquitination proteins such as a protein ubiquitination E3 ligase protein (Parkin) have been observed to increase degradation of misfolded DAT (Jiang and Feng, 2004). A mechanism for ubiquitination has been proposed with another implicated ubiquitin, neural precursor cell expressed developmentally down-regulated protein 4 (NEDD4-2) or through PKC mediated phosphorylation (Miranda et al., 2007). After activation of NEDD4-2 or by phosphorylation, ubiquitinated DAT is transported to a clathrin-coated pit by binding to Eps15 subsequently could to AP-2 on the clathrin coated pit. The ubiquitinated DAT is endocytosed and degraded by lysosomes as endosomes mature. Non-ubiquitinated DAT is recycled back to the plasma membrane through extensions of multi-vesicular recycling endosomes (Miranda et al., 2007). Glycosylation of DAT transporters occurs within asparagine binding sites between transmembrane domains 3 and 4 (Nirenberg et al., 1996). Site directed mutation experiments revealed that mutation of all three N-linked glycosylation sites of DAT transporters strengthened the inhibitory action of cocaine on dopamine uptake (Li et al., 2004).

Function in male reproduction

Lack of or increased dopamine is associated with many mental health issues such as schizophrenia, Parkinson's disease, and depression, all of which have been reviewed extensively (Meltzer and Stahl, 1976; Lotharius and Brundin, 2002; Dunlop and Nemeroff, 2007). These conditions are treated with dopamine antagonistic and agonistic drugs that are known to cause a variety of side effects in male patients. Symptoms range in severity from sexual dysfunction (Segal et al., 1979) to infertility (Segal et al., 1976). These drugs are classified as neuroleptics and are known to cause hyper- or hypoprolactinemia depending on whether they are dopamine antagonistic or agonistic drugs, respectively (Dickson and Glazer, 1999). Specifically, hyperprolactinemia is associated with high instance of male infertility (Segal et al., 1976) and impotence (Segal et al., 1979). Hypoprolactinemia has been associated with reduced semen quality (Gonzales et al., 1989) and decreased libido (Svare et al., 1979).

Cattle consuming a forage containing a dopaminergic agonist experience lowered serum PRL concentrations and exhibit similar repercussions on reproductive physiology including decreased cleavage rates of oocytes fertilized with semen from bulls grazing fescue infected with a dopamine agonist and decreased semen quality and freeze-thaw survivability (Schuenemann et al., 2005; Pratt et al., 2015). Others report no change in semen quality (Schuenemann et al., 2005; Stowe et al., 2013). Dopamine type-2 receptors are present in spermatozoa of cattle, rats, mice, humans (Otth et al., 2007) and boar (Ramírez et al., 2009). Receptors are also present in mouse Leydig cells (Gonzalez et al., 2015). The presence of DRD2 receptors in Leydig cells and sperm cells suggests a direct role for dopamine signaling in steroidogenesis and spermatogenesis. Report of a role for

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dopamine in capacitation *in vitro* in pig sperm confirms functionality of dopamine signaling in male germ cells (Ramírez et al., 2009). Indeed, lower cleavage rates were observed in oocytes fertilized with semen from bulls grazing fescue infected with a dopamine agonist (Schuenemann et al., 2005). However, the majority of the literature does not support a direct effect of dopamine to DRD2 receptors on sperm cells, and instead research has focused on an indirect mechanism, as most of the work performed has focused on dopaminergic agonist and antagonists and their effects on the PRL signaling pathway.

Dopamine Receptors

Structure

Dopamine receptors are G-coupled, seven-transmembrane receptors (Missale et al., 1998). The receptors are classified as either D1-like, including dopamine type-1 (DRD1) and dopamine type-5 (DRD5) receptors or D2-like, including dopamine type-2, 3, and 4 receptors (DRD2, DRD3, DRD4; respectively). The DRD2 cDNA has been characterized and identified in human and cow pituitary (Senogles et al.,1988; Grandy et al.,1989), and in the rat brain (Bunzow et al., 1988). Human, rat, and cattle DRD2 cDNA share over 90% sequence identity among their amino acid sequence and ~85-90% shared identity in nucleotide sequence predicted through cDNA characterization (Grandy et al., 1989; Chio et al.,1990). The cattle DRD2 cDNA sequence is longer than the rat mRNA sequence and results in a protein product of 444 amino acids (Chio et al.,1990) compared to the rat sequence at 415 amino acids (Bunzow et al., 1988). Unlike the D1-like receptor genes that are intronless, the D2-like receptor gene contains 6 introns and 7 exons and encodes for a predicted protein of ~50 kDa in size (Civelli et al., 1993). Alternative splicing of an extra

exon encoding the additional 29 amino acids between introns 4 and 5 occurs in humans to produce different gene products, designated DRD2$_L$ and DRD2$_S$ for the long and short isoforms of the DRD2 receptor, respectively (Dal Toso et al., 1989). Isolation and characterization of DRD2 cDNA in cattle and rat brain encode for an extra 87 bp insert. The cDNA sequence was predicated to encode an extra 29 amino acids from this insert, located in the third intracytoplasmic loop (Chio et al., 1990). The sequence was homologous to the earlier identified human sequence confirming cattle also undergo alternative splicing to produce DRD2$_S$ and DRD2$_L$ (Chio et al., 1990).

The dopamine receptor type-3 cDNA was isolated and characterized in rat brain (Sokoloff et al., 1990). The DRD3 gene consists of five introns and six exons, 446 amino acids in length. The amino acid sequences of DRD2 and DRD3 share high identity among their transmembrane domains at ~75%; however, given the entire amino acid sequences, the two receptors only share ~50% sequence identity (Sokoloff et al., 1990). The DRD4 cDNA has been characterized in humans (Van Tol et al., 1991), rats (Sun et al., 1998), and the mouse (Suzuki et al., 1995). The gene contains five exons and encodes for a predicted protein of 387 amino acids in length (Van Tol et al., 1991). Like DRD3, the human DRD4 protein also shares high amino acid sequence identity to DRD2 when considering transmembrane domains alone; however, DRD4 shares the least homology to all D2-like receptors overall, when considering the entire amino acid sequence (Van Tol et al., 1991). Unique to the human DRD4 amino acid sequence are transposable element repeats that have been identified in the coding region (Lichter et al., 1993). Each variant is designated DRD4.3, DRD4.6, and DRD4.9 with the end digit corresponding to the

number of repeats. Repeats have been found in various locations throughout the DRD4 gene, Specifically within the third cytoplasmic loop. Repeats within DRD4 have been hypothesized to effect RNA stability and expression (Schoots and Van Tol, 2003) but are likely not involved in G-protein interactions or enhancement of functional activity for binding of dopamine agonistic drugs (Asghari et al., 1995; Jovanovic et al., 1999).

Dopamine type-1 receptor has been characterized and is present in human retina (Dearry et al.,1990), rat striatum (Monsma et al., 1990), and in cattle lung, kidney, and uterus (Haegeman et al., 1999). The cDNA encoded for a protein 446 amino acids in length with a molecular weight of 49 kDa. Similarly, DRD5 also has been characterized in cattle and is present in the lung and kidney, encoding for a protein 477 amino acids in length. Dopamine type-5 receptor shares 50% amino acid sequence identity with its counterpart, the DRD1 receptor (Sunahara et al.,1990). A conserved cysteine residue is located at the end of the D1-like receptor carboxyl terminal and at the beginning of D2-like receptor carboxyl terminal (Missale et al., 1998). Two conserved cysteines are also located in the extracellular loops 2 and 3. The cysteine residues have been implicated in other G-coupled receptor types to form a disulfide bridge for facilitation of ligand binding (Dohlman et al., 1989). There are two N-glycosylation sites in D1-like receptors and up to 4 in D2-like receptors (Civelli et al., 1993). Mutation experiments reveal that receptor activation and signal transduction is dependent on a conserved aspartate residue 80 (Neve et al., 1991). When asp80 was substituted for an adenine or guanine the DRD2 receptor lost its ability to inhibit adenylyl cyclase, as is necessary for the negative feedback on PRL (Neve et al., 1991).

Signaling pathways

Dopamine receptors have been categorized based on their ability to inhibit or stimulate adenylyl cyclase activity. In general, D1-like receptors stimulate adenylyl cyclase activity (Monsma et al., 1990). Experiments have demonstrated DRD1 receptor's ability to stimulate cAMP production by transfection of COS-7 cell lines with the DRD1 transcript followed by treatment with DRD1 agonists (Monsma et al., 1990). Further, activation of adenylyl cyclase was measured by conversion of ATP to cAMP and was associated with expression of GTP-binding proteins, G_{olf} rather than G_s detected by immunoblotting in the striatum of rats (Hervé et al., 1993). Dopamine type-1 receptor is bound by dopamine prompting a conformational change. The conformational change prompts dissociation of the alpha subunit of the GTP-binding proteins, $G_{s/olf}$. Once activated, adenylyl cyclase initiates the production of cAMP and subsequent activation of PKA. Activated PKA phosphorylates CREB that binds to CRE domains and subsequently binds cAMP binding proteins. Consequently, binding of cAMP binding protein facilitates activation of regulatory elements on the DNA (Nishi et al., 2011).

Dopamine type-2 like receptors work the opposite of D1-like receptors in that they inhibit adenylyl cyclase (De Camili et al., 1979). Primary examples of its inhibition are through the actions of dopamine binding DRD2 on lactotrophs for suppression of PRL synthesis and secretion (Ben-Jonathan and Hnasko, 2001). The inhibition of adenylyl cyclase is facilitated through coupling of the DRD2 receptor with adenylyl cyclase by GTP-binding proteins $G\alpha_i$ and $G\alpha_o$ (Enjalbert et al., 1988). Binding of dopamine to DRD2 induces a conformational change resulting in dissociation of the alpha subunit of the GTP-

binding protein trimer. The alpha subunit couples to and inhibits adenylyl cyclase, preventing activation of PKA, phosphorylation of CREB, and further, downstream transcriptional activation (Figure 1.4) (Ben-Jonathan and Hnasko, 2001; Lledo et al., 1992).

D2-like receptors can also mediate inhibition of PRL signaling through inhibition of the Ras-Raf-MEK-ERK pathway. Dopamine binds to DRD2 and a conformational change takes place that prompts $G\alpha_o$ GTP-binding protein subunit to dissociate from the G-protein trimer. Coupling of $G\alpha_o$ GTP-binding protein subunit inhibits the activation of ERK1 and ERK2 in GH4ZR7 cells (Liu et al., 2002). The mechanism of action is not known yet, although it is hypothesized that the mechanism may be through coupling to adenylyl cyclase or ion channels. Utilizing transgenic mice for either the DRD2 long or DRD2 short receptor types, induction of the Ras-Raf-MEK-ERK pathway has been measured in which overexpression of the short isoform resulted in increased phosphorylation of ERKs and pituitary hypoplasia and reduced serum PRL compared to the long isoform which decreased phosphorylation of ERKs when overexpressed in the pituitary and increased PRL (Laccarino et al., 2002). These results indicate that differential expression of particular DRD2 isoforms adds yet another layer of control over PRL protein expression and cell proliferation by induction of the Ras-Raf-MEK-ERK pathway (Figure 1.4).

Both D1- and D2-like receptors have the ability to regulate ion channels in order to elicit effects at the cellular level. Inositol phosphate accumulation has been observed in the rat striatum (Undie and Friedman, 1990). Slices of rat brain subjected to treatment with 2-

[^{3}H]inositol (for incorporation into inositol phosphates) and treatment with DRD1 agonists, indicated stimulation of inositol phosphates through DRD1 signaling (Undie and Friedman, 1990). A dose effect was observed with peak inositol phosphate accumulation at 100μM concentration of a dopamine agonist was required to elicit calcium mobilization through D1-like receptor signaling with the effect absent at lower doses in rats. Dopamine like-2 receptors function to inhibit calcium influx through interaction of GTP-binding proteins with ion gated channels (Lledo et al., 1992). Much of the research on hyperpolarization claims inhibition of PRL also occurs by coupling of DRD2 to activate potassium channels (Einhorn, Gregerson, and Oxford, 1991). Coupling is facilitated by the GTP-binding protein beta and gamma subunits interaction with gated inward-rectifying potassium channels, increasing potassium conductance and causing calcium channels to close resulting in decreased intracellular calcium (Pillai et al., 1998). Contrary to D2-like receptors, D1-like agonists were shown to inhibit activation of potassium channels (Surmeier and Kital, 1993).

Synthesis and export trafficking

Synthesis and folding of the G-coupled receptor occurs within the endoplasmic reticulum and the newly synthesized receptor must be transported to the plasma membrane (Rothman, 1994). The receptors are packaged into vesicles known as coat protein two (COPII) vesicles, or the coated protein vesicular transport system. The G-coupled receptor is taken up by the vesicles due to interactions between the receptor and COPII proteins (Kunduri et al., 2014). The receptors are not yet mature and must make their way from the endoplasmic reticulum, through the Golgi apparatus and the trans-Golgi network

(Rothman, 1994). As they make their way through the Golgi, receptor proteins undergo post translational modifications until they are sequestered to the plasma membrane. Aside from transport of the newly synthesized receptor, proteins meant to assist in folding and post-translational modification travel with the receptor to its destination. These chaperone proteins often act as quality control for the transported G-coupled receptor. This has been established between calnexin and a vasopressin receptor in a pulse chase experiment with mutated and wild type forms of the receptor (Morello et al., 2001). The vasopressin receptor with a nonsense mutation induced in the proximal portion of the carboxyl tail was maintained in the endoplasmic reticulum in association with calnexin longer than wild-type receptors, hypothesized to be unrecognizable by the chaperone proteins needed for transport to the plasma membrane (Morello et al., 2001). The dopamine receptor exits the endoplasmic reticulum by interaction of the COPII protein with chaperone protein motifs, or in the case of the DRD1 receptor, interaction with the DRiP78 protein (Bermak et al., 2001). In particular, the interaction with DRiP78 protein occurs at the FxxxFxxxF motif which has been identified in the DRD1 receptor C-terminal (Bermak et al., 2001). Trafficking mechanisms of DRD2 are not yet fully understood; however, Leucine-rich repeat kinase 2 (LRRK2) has been associated with decreased trafficking to the membrane, as DRD2 was localized to the Golgi when LRRK2 was overexpressed in a neuroblastoma cell line (Rassu et al., 2017).

Once the G-coupled protein receptor exits in the Golgi, export to the plasma membrane is facilitated by Rab 1 as shown in other G-coupled receptor types (Wu et al., 2003). Although the mechanism is not fully understood, Rab1 mutation and distribution

studies with adrenergic and angiotensin receptors show that mutation of Rab1 results in much less cell surface receptors (Wu et al., 2003). Internalization of DRD2 receptors at the membrane is performed for recycling of receptors. In the case of D2 receptors, a GTPase (dynamin), a Ras related protein (Rab5a) and G-protein coupled receptor kinase 2 (GRK2) facilitate internalization. Specifically, GRK2 is co-expressed with DRD2 upon internalization. Dynamin has been hypothesized to phosphorylate DRD2 and close the vesicles for internalization whereas Rab5a facilitates binding to the clatharin coated pit where internalization occurs (Iwata et al., 1999). In some cases, degradation is triggered by lysosomal degradation facilitated by Rab7, as Rab7 was found localized to the cell membrane of late endosomes by immunofluorescence (Chavrier et al., 1990).

Single Nucleotide Polymorphisms

Single nucleotide polymorphisms within the DRD2 gene

Single nucleotide polymorphisms are defined as single base changes within the DNA across individuals in a given population. These substitutions must occur > 1 % in any given population to be considered a SNP and can occur within coding and non-coding regions of the DNA to alter gene expression as well as protein structure or function (Kwok and Gu, 1999). The PRL signaling pathway has been implicated in effects on reproductive efficiency of several species and may provide a route for indirect effects on mammalian reproduction (Gonzales at al., 1989; Schuenemann et al., 2005). Some of these indirect effects may be explained by the presence of SNPs reported to be present in the bovine DRD2 gene (Campbell et al., 2014). Evidence for impact on male physiology due to dopaminergic SNPs exist.

An insertion/deletion SNP occurring in the promoter region of the DRD2 gene at position 141 across two cytosines in tandem, was highly associated with sexual dysfunction in men treated with DRD2 antagonist drugs. The three resulting genotypes are insertion/insertion, insertion/deletion, and deletion/deletion. Men with the cytosine deletions, whether they are homozygous or heterozygous for cytosine deletion, had overall lower blood PRL levels (Zhang et al., 2011). The polymorphism is located in the SP1 binding site of DRD2 (Arinami et al., 1997). The DRD2 lacks a TATA promoter sequence and relies on SP1 binding sites for promoter activity (Minowa et al., 1992; Yajima et al., 1998). The deletion genotype has been associated with higher density of DRD2 in the striatum in humans (Jönsson et al., 1999) suggesting this polymorphism may alter promoter activity and therefore DRD2 gene expression. Subsequently, PRL protein expression may be altered in deletion genotype patients due to the increased repression of PRL secretion by elevated DRD2 availability and signaling.

Another polymorphism resulting in a base change between cytosine and thymine in exon 7 at position 3420 of the human DRD2 gene (encoding the cytoplasmic portion of DRD2) has been reported to be associated with hyperprolactinemia (Hansen et al., 2005). The cytoplasmic portion of DRD2 functions in docking of inhibitory G-proteins (Missale et al., 1998) that facilitate inhibition of PRL (Ben-Jonathan and Hnasko, 2001). The polymorphism is silent and, therefore, does not change the amino acid sequence. Patients with the cytosine to thymine substitution resulting in a homozygous genotype were associated with increased occurrence of hyperprolactinemia (Hansen et al., 2005),

suggesting the base change may impact gene expression of the PRL pathway by altering G-coupled protein interactions.

Recently, a SNP was reported in cattle indicating an association to serum PRL concentrations in steers grazing a forage containing a dopamine agonist (Campbell et al., 2014). The DRD2 SNP is located within the third intron at position 404365, on Chromosome 15, inferring a base substitution between adenine and guanine (Campbell et al., 2014). Steers grazing the dopaminergic agonist in April and May, but not in June months, that possessed a genotype homozygous for an 'A' allele, were associated with higher serum PRL concentrations compared to those homozygous for a 'G' allele. Association of decreased PRL in GG steers was also observed to have increased hair coat scores. Homozygous 'A' heifers were also observed to have fewer days to first calf (Campbell et al., 2014). The same base substitution between adenosine and guanine on the DRD2 gene tended to be associated with hair coat score but had no effect on calving rates in Angus crossbred cows (Meyer et al., 2016). Elevated circulating PRL concentrations have been observed to cause reduced hair coat shedding in the summer months (Porter and Thompson, 1992); however, serum PRL concentrations were not measured, therefore associations of genotype to serum PRL concentrations were not explored.

Contrary to DRD2 SNPs in humans, the bovine DRD2 SNP is located within a non-coding region, and therefore would not affect protein structure/function however, its effects on PRL release and synthesis could be due to effects on gene expression within the PRL signaling pathway. An example could be the SNP alters mRNA transcript stability. Other DRD2 intronic SNPs have been reported to affect transcript abundance, and have also been

associated with increased binding of transcriptional repressors that decrease DRD2 mRNA expression in human striatum (Rogaeva et al., 2007). Conflicting research on male reproductive function due to exposure to a dopamine agonist warrants further investigation. Single nucleotide polymorphisms in the DRD2 gene have been associated to serum PRL concentrations (Campbell et al., 2014) which would provide an indirect mechanism in which binding of these agonists to DRD2 affect PRL, and therefore male reproductive function. Contrary to the prior findings, preliminary data from our lab indicate that DRD2 genotype (Campbell et al., 2014) does not impact male physiology, having little or no effect on semen quality parameters or prolactin serum concentrations in bulls grazing a dopamine agonist. Investigation of DRD2 genotype association with PRL protein and mRNA expression would be beneficial to perform directly at the source of PRL production, in the bovine pituitary to clarify the intronic SNP's association with decreased serum PRL concentrations in cattle.

Single nucleotide polymorphisms in genes within the PRL signaling pathway

Aside from SNPs in the DRD2 gene, other variants located within the PRL (Brym et al., 2005a; Looper et al., 2010), PRLR (Brym et al., 2005b), and STAT5 (Brym et al., 2004) genes have been reported. These SNPs have been implicated in association to productivity traits in cattle and may also provide more routes in which PRL synthesis and release may be affected that may subsequently impact bull reproduction.

A cytosine to thymine substitution at position 1286 of the PRL gene, within the promoter region was identified in cattle (Looper et al., 2010). Cattle grazing a forage containing a dopamine agonist possessing a homozygous thymine genotype had reduced

calving rates than those heterozygous or homozygous cytosine cows (Looper et al., 2010). Further, a second SNP was identified in the promoter region, an adenine to guanine substitution at position 1167. Cow possessing a lower BCS containing a 'G' allele had longer time to calving (Looper et al., 2010). An adenine to guanine substitution in position 8398, in exon 4 of the PRL gene, was associated with milk yield in cows (Brym et al., 2005a). Dairy cows with a heterozygous genotype had higher milk yields than homozygous individuals of either 'A' or 'G' alleles and homozygous 'G' cows yielded milk higher in fat (Brym et al., 2005a). Being located in the promoter region suggests that the SNPs could impact initiation of transcription directly.

Similar to the intronic DRD2 SNP reported in cattle (Campbell et al., 2014), intronic SNPs have been reported in the bovine STAT5 gene in intron 9, position 9501, including an adenine to guanine substitution (Brym et al., 2004). Jersey cows, but now black and white dairy cows possessing a genotype with an 'A' allele, either heterozygous or homozygous, were associated with higher protein content in milk while cows homozygous for a 'G' allele had overall higher milk yield (Brym et al., 2004). An intronic SNP has also been reported within the PRLR gene within intron 9 at position 205 (Brym et al., 2005b). The intronic SNP includes a substitution for adenine to cytosine. Jersey cows homozygous for a 'C' were associated with milk yielding higher protein content (Brym et al., 2005b). The SNPs located in a non-coding region are not likely to effect protein structure but could affect gene expression of STAT5. The possibility exists that downstream effects for activation of transcription by binding of STAT5 dimers to GAS sequences necessary for the activation of transcription of casein genes may also be effected.

The presence of SNPs in genes involved in the PRL signaling pathway, already associated with productivity traits in cattle, could serve as potential targets of future studies on associations of these SNPs to PRL gene expression and further, reproductive and growth traits in bulls. To the best of our knowledge, SNPs within the Ras Raf MEK ERK pathway have not been reported in association with reproduction traits or PRL serum concentrations in cattle and could also be investigated.

Conclusions

Prolactin (PRL) is a ~22 kDa peptide involved in over 300 diverse biological functions (Freeman et al., 2000). This peptide is produced by lactotrophs in the anterior pituitary, and it's release and synthesis is negatively regulated via tonic inhibition by dopamine through the dopamine type-2 receptor (DRD2) (Ben-Jonathan and Hnasko, 2001). Once released, PRL in circulation binds to its ubiquitously expressed receptor, activating the JAK-STAT pathway (Campbell et al., 1994). Janus 2 kinases bind to the SH2 domain on the cytoplasmic portion of the receptor and are phosphorylated upon PRL binding. This phosphorylation of JAK triggers the association and phosphorylation of STAT5 (Ihle, 1996). STATs dimerize and translocate to the nucleus to stimulate gene transcription by binding to defined nucleic acid sequences within promoter regions of genes (Ihle, 1996). Dopamine receptors are G-coupled protein receptors. Coupling of DRD2 to G-inhibitory proteins functions to inhibit PRL gene expression by suppression of adenylyl cyclase and therefore cAMP production (Ben-Jonathan and Hnasko, 2001). More immediately, upon binding of dopamine to DRD2 on lactotrophs, a rapid decrease in

intracellular calcium occurs suppressing the release of PRL from secretory granules (Ben-Jonathan and Hnasko, 2001).

Cattle consuming forage containing a naturally occurring dopamine agonist, exhibit reduced circulating serum concentrations of PRL (Schillo et al., 1987; Strickland et al., 2011; Pratt et al., 2014). Consumption of dopamine agonists have been reported to decrease sperm motility and morphology in bulls (Looper et al., 2009; Pratt et al., 2015). Receptors for dopamine and PRL have been reported to be present in male reproductive tissues (Hair et al., 2002; Otth et al., 2007; Pratt et al., 2014), therefore, either hormone may have an effect on male reproduction. Antipsychotic drugs used in human medicine also antagonistically bind dopamine receptors (Seeman et al., 1975) and patients taking these dopamine antagonists experience erectile dysfunction as well as increased latency in time to ejaculate (Segraves, 1989). In humans, hyperprolactinemia is associated with infertility (Segal et al., 1979) and impotence (Segal et al., 1976) while hypoprolactinemia is associated with reduced semen motility in male patients (Gonzales et al., 1989). Moreover, early PRL and PRL receptor ablation experiments in mice resulted in infertile males (Ormandy et al., 1997). The mechanism in which male reproductive physiology is impacted could be due to either dopamine through binding of the DRD2 receptor at the level of the testis and accessory glands or due to lowered serum PRL concentrations.

The PRL signaling pathway has been implicated in effects on reproductive efficiency of several species and may provide a route for indirect effects on mammalian reproduction (Flisikowski et al., 2003; Hermanns and Hafez, 1981; Ormandy et al., 1997; Segal et al., 1976). Some of these effects may be explained by the presence of single

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nucleotide polymorphisms. The SNPs could impact signaling through DRD2 or PRL signaling and therefore could affect mammalian reproductive physiology. Evidence for impact on male physiology due to dopaminergic SNPs exist. A polymorphism in DRD2 exon 7 in which homozygosity is prevalent results in a high association with male infertility and impotence (Hansen et al., 2005). Further, a cytosine insertion in the DRD2 gene has been shown to impact PRL secretion as measured by increasing blood serum concentrations leading to erectile dysfunction (Zhang et al., 2011). A SNP has been identified that exists within the bovine DRD2 gene, within an intron, consisting of a base substitution between adenine and guanine (Campbell et al., 2014). Similarly, to humans, this SNP is associated with decreased serum PRL concentrations in cattle consuming dopamine agonists (Campbell et al., 2014). Other SNPs within genes involving the PRL signaling pathway have been reported within PRL (Looper et al., 2010), PRLR (Ormandy et al., 1997), and STAT5 (Brym et al., 2004) genes. These SNPs have also been implicated in association to productivity traits in cattle and may also alter PRL synthesis and release to subsequently impact bull reproductive physiology.
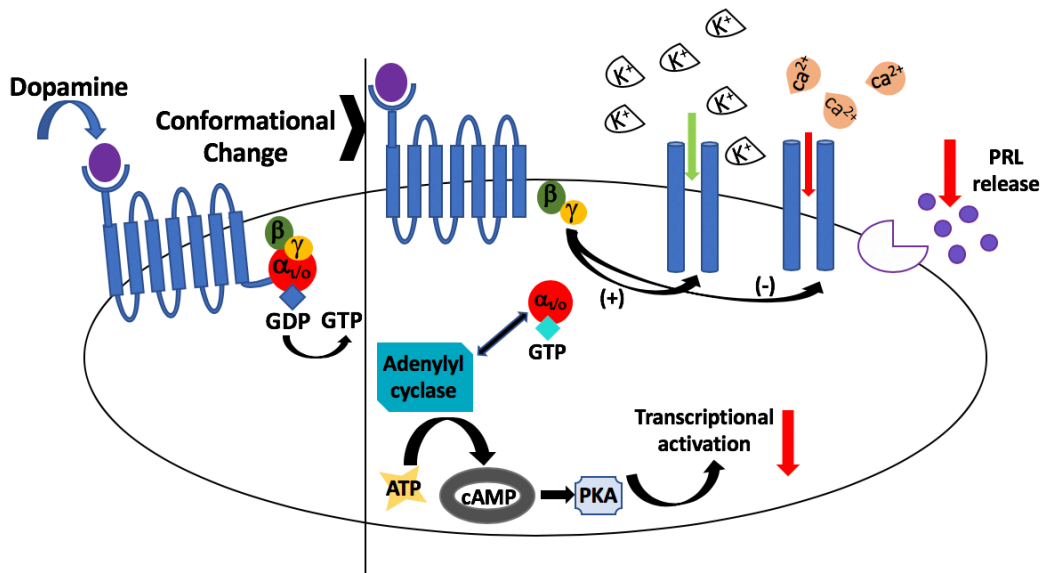
Figure 1.1 Dopamine type-2 receptor mediated inhibition of PRL synthesis and release by $G_{i/o}$ inhibition of adenylyl cyclase.

Dopamine binds to DRD2 coupled with G-inhibitory proteins alpha ($\propto$), Beta (β), and Gamma (γ). Ligand binding initiates a conformational change of the receptor. An exchange of GDP for GTP on the alpha subunit of the coupled G-inhibitory protein results in activation and dissociation of the alpha subunit from the beta and gamma subunits. **(1)** GTP-binding protein β and γ subunits interact with gated inward-rectifying potassium channels that stimulate potassium conductance. As a result, calcium channels close resulting in decreased intracellular calcium and hyperpolarization of the cellular membrane, decreasing secretion of PRL. **(2)** Interaction of alpha subunit $G_{i/o}$ with adenylyl cyclase suppresses production of cAMP, activation of protein kinase A (PKA), and therefore, downstream PRL gene expression. Interactions of the alpha subunit are marked by double-pointed black arrows. Inhibition is marked by red decreasing arrows and increases in expression or abundance are marked by upward facing green arrows.
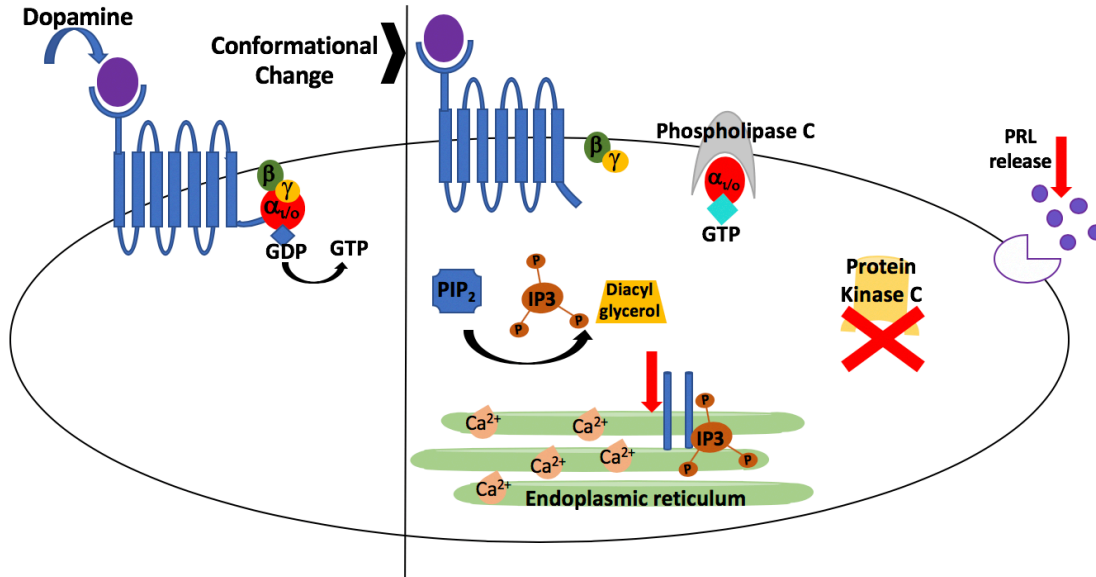
Figure 1.2 Dopamine type-2 receptor mediated inhibition of PRL synthesis and release by $G_{i/o}$ inhibition of phospholipase C (PLC) and protein kinase C (PKC).

Dopamine binds G-coupled DRD2. A conformational change occurs due to ligand binding and GDP is exchanged for GTP. The alpha (α) subunit disassociates from the beta (β) and gamma (γ) subunits and is able to associate with other proteins to facilitate inhibition. Inhibition of phospholipase C (PLC) prevents subsequent hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) and production of intermediates Inositol triphosphate (IP3) and Diacyl glycerol (DAG). Normally inositol triphosphate functions to bind to IP3 receptors for activation of voltage gated channels in the endoplasmic reticulum to allow for calcium influx needed for secretion of prolactin while DAG and calcium activate PKC. Inhibition of PLC prevents intermediate formation and activation of PKC needed for phosphorylation of transcription factors necessary for transcriptional activation of the PRL gene. Inhibition is marked by red decreasing arrows.
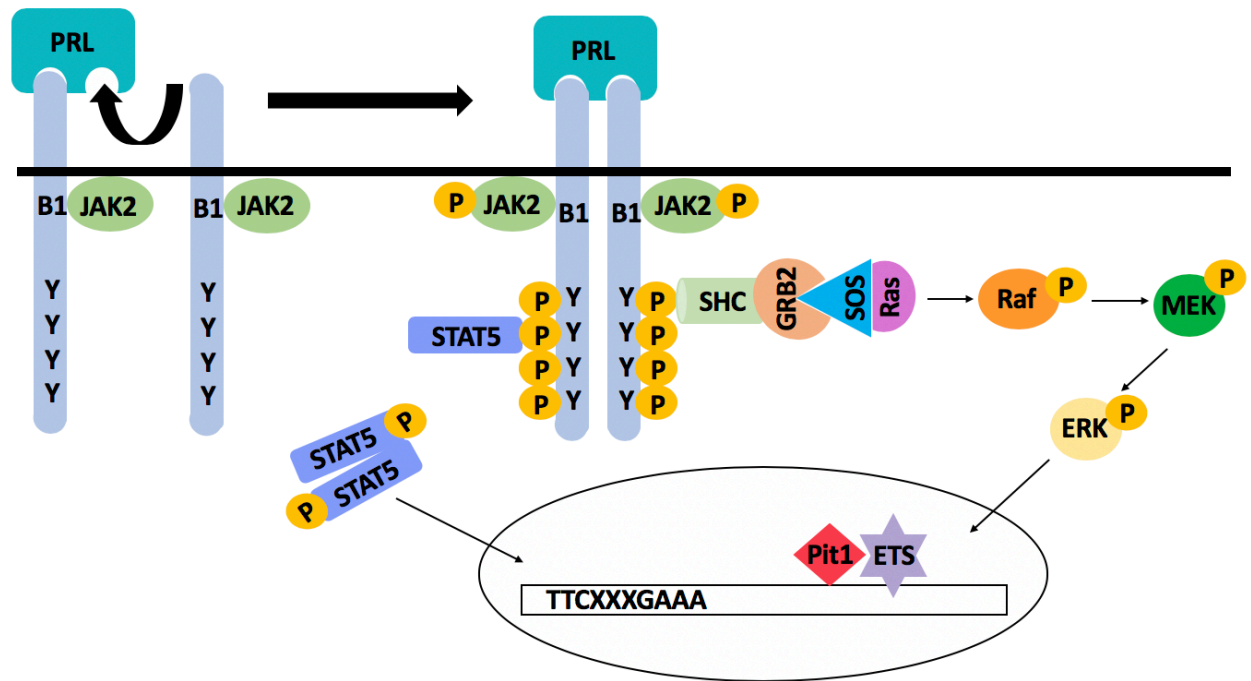
Figure 1.3 Prolactin stimulation of Janus kinase 2 (JAK2) and signal transducers and activators of transcription 5 (STAT5).

Ligand binding induces dimerization and a conformational change occurs in the cytoplasmic region of the PRLR. The constituativley active JAK2 is activated following auto phosphorylation of tyrosine residues on the receptor providing docking sites for STAT5 molecule. STAT5, in close proximity to JAK2 is also phosphorylated. The STAT5 molecules then disassociate from the receptor, dimerize, translocate to the nucleus to bind to CIS elements on the DNA. Activation of a MAPK pathway, the Ras/Raf/MEK/ERK cascade is also possible through bridging of the two signaling pathways by adaptor proteins SHC, GrB2, and SOS. Phosphorylated tyrosine residues on the PRLR serve as docking sites for the adapter proteins to bind. Activation of Ras, Raf, and MEK1/2 permit the

activation of ERK1/2 that translocate to the nucleus to modulate transcription factors such as ETS factors.
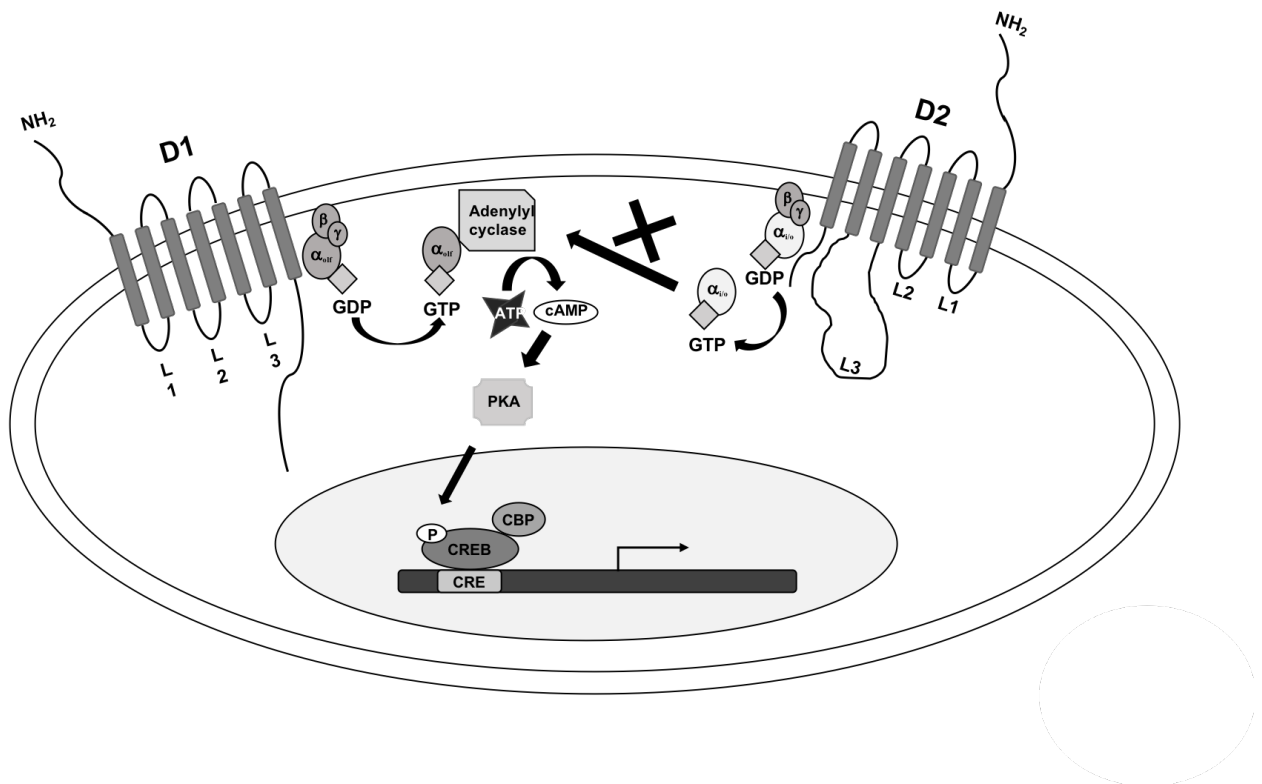
Figure 1.4 Antagonistic effects of dopamine receptor type-1 (DRD1) and dopamine receptor type-2 (DRD2).

Dopamine type-1 **(D1)** receptor is associated with GTP-binding proteins, $G_{s/olf}$ and Dopamine type-2 receptors **(D2)** are associated with signaling by GTP-binding proteins, $G_{i/o}$. Once bound by ligand, a conformational change of the receptors occurs prompting the dissociation of GTP-binding protein α subunits. GTP-binding proteins, $G_{s/olf}$ associates with and prompts stimulation of adenylyl cyclase activity followed by production of cAMP and subsequent activation of PKA. Activated PKA phosphorylates CREB that binds to CRE domains and subsequently binds cAMP binding proteins for stimulation of transcription. Dopamine type-2 receptor GTP-binding proteins $G_{i/o}$ bind to and inhibit

adenylyl cyclase therefore preventing the signaling cascade for activation of PKA, phosphorylation of CREB, and further, downstream transcriptional activation.

CHAPTER TWO

BOVINE DOPAMINE TYPE-2 RECEPTOR SNP HAS NO EFFECT ON GROWTH, SEMEN CHARACTERISTICS AND PROLACTIN CONCENTRATIONS IN BEEF BULLS EXPOSED TO A DOPAMINE AGONIST

Abstract

A dopamine type-2 receptor (DRD2) SNP, previously found to be correlated with serum prolactin (PRL) concentrations in cattle, was evaluated for impact on growth traits, serum prolactin concentration, and semen quality. Over a four-year period, yearling beef bulls were allowed diets containing or lacking ergot alkaloids (EA). Every 21 or 28 d semen was collected for semen motility and morphology assessment and blood samples were collected to measure serum PRL concentrations. In addition, body condition score and scrotal circumference were evaluated. Serum PRL concentrations were assessed using a radioimmunoassay. In the first year, all bulls were sacrificed at the end of a 126-day study. Testicles and epididymis were collected at the end of the study or 60 days after removal from treatment. Immunohistochemistry was performed on testis, epididymis, and sperm cells, incubated with or without a primary antibody for DRD2 and counterstained with DAPI. Isolation of DNA was performed on sperm pellets using DNAzol (Thermo Fisher Scientific, Waltham, MA, USA) methods. Polymerase chain reaction was performed to

amplify the region of the DRD2 gene containing the SNP of interest. The products were subjected to restriction fragment length polymorphism analysis. Further, all samples were subjected to genotyping using a custom Taqman genotyping assay (Applied Biosystems, Foster city, CA, USA). The presence of DRD2 was detected in the testis, epididymis, and sperm cells. The DRD2 genotype was not associated with semen quality, serum PRL, or growth traits. Consumption of EA resulted in lesser PRL serum concentrations but had no effect on values for other variable examined.

Introduction

Prolactin (PRL) is an ~ 22 kDa peptide produced primarily by pituitary lactotrophs and is regulated by dopamine through tonic inhibition (Ben-Jonathan et al., 2001). Prolactin is involved in over 300 biological functions across species (Bole-Feysot et al., 1998; Freeman et al., 2000). Hyperprolactinemia promotes erectile dysfunction as well as infertility in men which improves through treatment with a dopamine agonist (Segal et al., 1979). Dopamine is the main regulator of PRL via tonic inhibition (Ben-Jonathan et al., 2001) and the bovine dopamine type-2 receptor (DRD2) gene possesses several single nucleotide polymorphisms (SNP), with one SNP reported to be correlated with serum PRL concentrations (Campbell et al., 2014). This SNP is located within intron 3 and therefore does not likely affect protein structure or function and currently there is little information available on the mechanism in which this SNP located in a non-coding portion of the gene (Campbell et al., 2014) acts. Currently all information is associative and little direct evaluation of the DRD2 SNP on PRL expression is available as well as little to no evaluation has been reported on this SNP's impact on bull growth and reproduction.

Dopamine type-2 receptor genotype impact on PRL serum concentrations and hair coat scores was evaluated in steers consuming or not consuming forage containing ergot alkaloids (EA) (Campbell et al., 2014). The DRD2 SNP located within intron 3 at position nt 404365 on Chromosome 15, includes a base substitution between adenine and guanine at position 534 (Campbell et al., 2014). Dopamine type-2 receptor genotype was shown to be correlated with serum PRL concentrations, in which homozygous AA steers were associated with higher serum PRL concentrations compared to GG steers in April and May but not June months. While the DRD2 SNP (Campbell et al., 2014) is located within a non-coding region and is not likely involved in protein folding or structure, the SNP may be involved in altering gene expression within the PRL pathway. Interestingly, the SNP does not appear to represent an alternate acceptor site to generate alternative splice variants of the DRD2 mRNA, nor has splice variants been reported for the bovine DRD2. Alternate splicing of DRD2 does occur to form long and short forms of the receptor to facilitate coupling of G-proteins and has been reported in the rat and human pituitary and brain (Dal Toso et al., 1989). Both, long and short forms of DRD2 are able to suppress PRL transcription (McChesney et al., 1991). However, this alternative splicing of DRD2 has not yet been reported in cattle. Evidence for effects on the PRL pathway due to dopaminergic SNPs exists with some continued differences in severity of effects in male physiology. Homozygosity of the polymorphism located on exon 7 of DRD2, was found to be associated with hyperprolactinemia in humans (Hansen et al., 2005). Moreover, an insertion-deletion located within the DRD2 promoter region was associated with sexual dysfunction and lower serum prolactin concentrations in human male patients (Zhang et

al., 2011). However, the aforementioned polymorphisms were not located within a non-coding region such as the SNP of interest in this study.

Cattle grazing forage producing EA, a naturally occurring dopamine agonist, possess decreased serum PRL concentrations (Schillo et al., 1988; Strickland et al., 2011). Ergot alkaloids can bind many neurotransmitter receptors (Larson et al., 1995; Mizinga et al., 1993; Schoning et al., 2001; Wang et al., 2009) to elicit physiological responses in cattle that consume them. Ergot alkaloids have been shown to act agonistically with DRD2, inhibiting the synthesis and secretion of prolactin (Caron et al., 1978; Paterson et al., 1995; Sibley et al., 1983; Thompson et al., 1993). Ergot alkaloid consumption may negatively affect semen motility and morphology (Looper et al., 2009; Pratt et al., 2015), as well as impact bull fertility (Schuenemann et al., 2005). However, there are contradictions between and within laboratories. Some report no change in semen quality (Schuenemann et al., 2005; Stowe et al., 2013) or *in vivo* fertility in PRL knockout mice (Horseman et al., 1997). It is possible that EA may have an impact on male physiology by binding to DRD2 receptors located in male reproductive tissues. Moreover, DRD2 is localized in the acrosome and tail regions of spermatazoa in bovine, rats, and mice, and is also present in spermatocytes and spermtids within the testes of rats. Further, dopamine type-2 receptors are also present in human spermatazoa, with the exception of the acrosome (Otth et al., 2007). This is consistent across species with DRD2 receptors present in male reproductive tissues (Otth et al., 2007; Ramírez et al., 2009; Gonzalez et al., 2015).

Results to date have demonstrated negative effects on growth traits in cattle exposed to a dopamine agonist. Earlier studies report a clear decrease in average daily gain

(ADG) in cattle grazing EA (Hoveland et al., 1984; Crawford et al., 1989) while other studies report no difference in BW or BCS between treatments of bulls with and without a dopamine agonist (Stowe et al., 2013). The decreased growth in animals grazing EA may be attributed to lower concentrations of growth hormone (GH). However, GH levels have been reported to increase across treatment of EA (Thompson et al., 1987) or remain unchanged (Paterson et al., 1995).

With much variation in reports on growth and semen characteristics in cattle, clarification of the role of DRD2 genotypic effects is warranted. The objective of this study is to assess the presence of DRD2 in bovine testis, epididymis, and sperm and to assess if the DRD2 SNP (Campbell et al., 2014) has any impact on growth, semen quality, or serum PRL concentrations in bulls exposed to a naturally occurring dopaminergic agonist, EA.

Materials and Methods

Animals

All animal research was approved by the Clemson University Institutional Animal Care and Use Committee (AUP #2010-068 and AUP # 2014-60).

Experimental Design and Treatments

Over four years, 2011 *(n = 14)*, 2012 *(n = 21)*, 2014 *(n = 25)* and 2015 *(n = 29)*, yearling beef bulls were fed a ration that included or excluded ergot alkaloids. In 2011, bulls were fed a concentrate ration which contained or lacked tall fescue seeds containing or lacking EA at 0.8 µg/g dry matter (DM) (Stowe et al., 2013). In all other years bulls were subjected to grazing forage that produced or lacked EA. Grazing treatments were performed according to Burnett et al. (2017). Bulls were subjected to electroejaculation

every 21 d for all animals in year 2011 with a total grazing period of 126 d. For all other years, electroejaculation was performed every 28 d with a total of 155 d for 2012 and 168 d for years 2014 and 2015. Breeding soundness exams (BSE) were performed on all bulls to assess semen motility and semen morphology, scrotal circumference (SC), body condition (BCS) and structural soundness. Bulls passing the BSE were allotted to treatment and blocked according to body weight (BW) and BCS. Semen samples collected were centrifuged, separated into cell and fluid portions, and each individually stored at - 80° C until used for DNA isolation. Caudal venipuncture was performed to collect blood which was processed into serum. Blood was allowed to clot overnight at 4° C and serum was collected by whole blood centrifugation at 2000 x g for 15 min at 4° C and serum was stored at - 20° C. All bulls were sacrificed at the end of a 126-day study and testicles and epididymis collected either immediately at the end of the study (group A with 5 and 3 bulls on E- and E+ diet, respectively) or 60 days after removal from treatment (group B with 3 bulls from each treatment) for year 2011 only. Data for growth, SC and semen quality from all animals in all years were assessed across periods 2 (0 d) through 5 (84 d).

Radioimmunoassay

Serum PRL concentrations were determined through a previously validated radioimmunoassay (RIA) performed by the F. Neal Schrick laboratory (Bernard et al., 1993). The intra-assay coefficient of variation was 9.7 % and the inter-assay coefficient of variation was 6.0 % (Burnett et al., 2017).

Immunohistochemistry and western Blotting

Formalin-fixed samples were processed and IHC performed as described by

Calcatera et al. (2011). Slides were washed in phosphate buffered saline (PBS) 3X, and incubated in blocking solution of 10 % goat serum/PBS for 15 min. Blocking serum was removed and sections were incubated with primary antibody for 1 hr at RT. A primary mouse monoclonal Ab for DRD2 (Sc-5303; Santa Cruz Biotechnology Inc., Dallas, Texas, USA) was diluted in blocking solution to a concentration of 4 µg/mL. Negative controls were treated with blocking solution in place of primary antibody. Following incubation in primary Ab, slides were washed in blocking solution followed by a final 1-hr incubation using secondary antibody, Alexafluor 594-conjugated goat anti-mouse IgG (A11032; Invitrogen, Carlsbad, CA, USA), diluted in blocking solution (10 µg/mL). After incubation, secondary antibody was removed and slides were washed in PBS. Nuclei staining was done by DAPI (0.33 µg/mL; Sigma, St Louis, MO, USA) and was followed by PBS wash as above. Slides were mounted with PBS, coverslipped, and immediately examined.

Protein extracts were produced from cattle testis, epididymis and pituitary tissues by homogenization in a 1X RIPA buffer (Alfa Aesar, Ward Hill, MA) with addition of proteinase and phosphatase inhibitors at 10µl/mL (HALT; Thermo Scientific, Waltham, MA, USA). Concentrations of extracts were obtained using the DC microplate assay (Bio-Rad; Hercules, CA, USA). Samples were processed by boiling 5 min in a 2X laemeli buffer with 1/8th volume beta mercaptoethanol. Denatured samples were centrifuged briefly and then loaded at 45µg total mass per well on a 12 % mini protean TGX precast gel (Bio-Rad; Hercules, CA, USA). Proteins were blotted onto a 0.2 µm nitrocellulose membrane (Bio-Rad; Hercules, CA, USA). The membrane was blocked in 2.5 % non-fat dry milk Tris

buffered saline with added 0.1 % Tween 20 solution (TBST). Immunodetection was carried out using primary antibody against DRD2 (B-10) at 1:20 dilution (5303; Santa cruz biotechnology, Dallas, TX, USA) overnight at 4° C followed by three washes in TBST and an incubation with a goat anti-mouse IgG HRP conjugated secondary antibody (Sc-2031; Santa cruz biotechnology, Dallas, TX, USA) for 1 hr at RT. The membrane was washed again 3X in TBST followed by incubation with a clarity ECL western substrate (Bio-Rad; Hercules, CA, USA) for 5 min at RT. Chemiluminescent detection was performed in a Fluor Chem FC2 imager (Alpha Innotech; San Leandro, CA, USA) for 10 min.

RNA isolation and RT-PCR

Total RNA was isolated from testis and epididymis using the mirVana mRNA isolation kit (Ambion, Austin, Texas, USA). Purity of RNA was assessed by the nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) to obtain A260:A280 ratio. Quality was assessed using the Agilent 2100 bioanlayzer (Agilent Technologies Inc, Santa Clara, California, USA) ribonucleic acid integrity number (RIN), utilizing samples with an 8.0 or greater for analysis. Complimentary DNA was generated by reverse transcription reaction using Superscript II Frist-Strand kit (Invitrogen, Carlsbad, Ca, USA). Primers for DRD$_2$ were generated through IDT PrimerQuest Tool (San Jose, CA, USA) for use in polymerase chain reaction (PCR), specific for bovine DRD2 (NM_174043.2). corresponding to nucleotide positions 510-531 forward primer and 576-597 reverse primer of the mRNA DRD2 sequence. End-point PCR was performed using GoTaq green (Promega Madison, WI, USA) and the resulting amplified cDNA was subjected to slab gel electrophoresis on 1.5 % agarose gels. In addition to size analysis PCR

products were ligated into pDrive cloning vectors and used to transform competent E. coli (Qiagen, Valencia, CA). Transformations were plated and subsequent colonies selected, propagated, and plasmid DNA isolated and subjected to dideoxysequencing.

DNA isolation

Sperm pellets were suspended in PBS to approximately $1X10^6$ cells, as assessed by sperm quality analyzer (SQA) (Advanced Agricultural Technologies) or semen samples were aliquoted to 100µl for use in DNA isolation. Samples were centrifuged at 10,000 x *g* for 1 min, supernatant removed, and the sperm pellet washed in 100 µl of 1X PBS. For cell lysis, 1mL DNAzol (Thermo Fisher Scientific, Waltham, MA, USA) was added to each 100µl semen sample with 1/8th volume of beta mercaptoethanol (BME). Cells were lysed by gentle pipetting. DNA was precipitated by addition of 500 µl of 100 % ethanol to each lysed sample. Samples were inverted until spooling of DNA was visible with a white precipitate. DNA was sedimented by centrifugation at 4,000 x *g* for 1 min. The supernatant was discarded and the pellet washed in 80 % ethanol (500 µl), centrifuged at 4,000 x *g* for 1 min for a total of two washes. After the last wash, the DNA pellet was allowed to dry on the benchtop for 1 min, then resusupended in 100 µl 8mM NaOH. Samples were stored up to 1 week at 4° C until complete solubilization of the DNA was achieved. Samples were subjected to Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) to determine A260:A280 ratio to assess purity.

Genotyping

Genotypes for each bull were obtained through restriction fragment length polymorphism (RFLP) analysis (Campbell et al., 2014) and Taqman SNP genotyping

assay. In brief, polymerase chain reactions were generated for a previously identified dopamine SNP region (Campbell et al., 2014). The subsequent products were purified using the wizard SV gel and PCR clean-up system (PROMEGA, Durham, NC, USA) and were subjected to slab gel electrophoresis for amplification of the dopamine SNP region (Campbell et al., 2014) to ensure amplification was successful. Purified products (200 ng) were digested using a Pfel (Tfil) restriction enzyme (New England Biolabs, Ipswich, MA, USA), allowed to digest at 37° C for 30 min. Slab gel electrophoresis was performed with each sample, digested and undigested. Digested samples yielded three genotypes with different size products; AA (532 and 261bp), AG (793, 532 and 261bp), and GG (793bp). A custom Taqman SNP genotyping assay (Applied Biosystems, Foster city, CA, USA) specific to the DRD2 SNP region (Campbell et al., 2014) was performed using 10 ng of the purified PCR products. Samples were run in 96-well format and run on the CFX Real-Time System (BIO-RAD Hercules, CA, USA) for a single step at 95° C for 10 min followed by 40 cycles of denaturation at 92° C for 15 sec and annealing and extension at 60° C for 1 min. Allelic discrimination analysis was performed using CFX Maestro software (BIO-RAD Hercules, CA, USA) to determine genotype. Sample of known genotype for each of the three genotypes were run on each plate as positive controls as well as a no template control (NTC). These results were compared to the RFLP analysis to ensure efficiency in genotype determination.

Statistical analysis

For serum PRL concentration, BW, BCS and semen characteristics, Analysis of Variance, followed by pairwise student's t-tests among LSMeans was used to assess the

impact of genotype and EA treatment on semen quality and growth traits. The model for the Analysis of Variance included fixed effects for treatment, genotype, time on treatment and their interactions; and year as a random effect. The time on treatment effect was included in the model since animals in each year were assessed across periods 2 (0 d) through 5 (84 d). Equality of variances were examined by Levene's test ($P \ll 0.05$). JMP software (SAS Institute Inc.) was used for all statistical calculations.

Results

DRD2 Expression in bull reproductive tissues

The DRD2 receptor was identified to be present in the bovine testis, epididymis, and sperm using IHC (Figure 2.1); however, attempts using the same antibody in western blotting did not yield definitive results (data not shown). To further confirm the presences of DRD2 gene products, end point (RT-PCR) and dideoxysequencing of the amplified product (Figure 2.1) were conducted and the expected product size and sequence were produced. The receptor was present in all samples examined regardless of treatment or bull genotype.

Genotype frequencies

Genotypic frequency was determined to be 18 % AA, 61 % AG, and 21 % GG using RFLP and 21 % AA, 59 % AG, 20 % GG by the custom Taqman genotyping methods across all years. Comparison of RFLP and Taqman DRD2 genotyping methods are shown (Figure 2.3). Genotypes were identical for all bulls with the exception of 3 animals across all years in the study. A representative sample is shown for each method RFLP and Taqman (Figure 2.4).

Growth, semen characteristics, and PRL serum concentrations

Prolactin concentrations were not significantly changed across DRD2 genotype but showed a treatment effect, with higher concentrations of serum prolactin in bulls not consuming EA compared to bulls consuming EA (144.02 ng/mL ± 49.3; 53.10 ng/mL ± 49.3, respectively ($P < 0.05$) (Figure 2.2). A year by treatment by genotype random effect was observed for PRL concentrations at a 37.6 % of total variance ($P = 0.02$). As expected, period had a significant effect ($P < 0.05$) on BCS and BW with animals exhibiting a higher BCS and BW earlier in treatment. Period also effected scrotal circumference, with bulls at a lower scrotal circumference (35.4 ± 0.41; $P < 0.05$) at the start of the study, increasing up to 84 d (36.3 ± 0.44; $P < 0.05$) (Table 2.1). A random interaction including year, treatment, genotype, and period accounted for 9.7 % of the total variance for BCS *($P =$ 0.03)* and 0 % for BW and SC ($P < 0.0001$). There were no significant differences in semen concentration due to treatment, period, genotype or their interactions. There were no differences in motility, progressive motility, number of motile sperm cells or number of progressively motile sperm cells due to treatment, period, genotype or their interactions. However, period effected velocity with bulls longer on study presenting elevated semen velocity ($P < 0.05$). Sperm cell morphology as well as the total number of sperm cells per ejaculate showed no significant differences. A random effect for cell morphology including treatment, year, genotype, and period accounted for none of the percent total variance ($P = 0.009$). Further, no significance was seen for total motile sperm or total progressive motile sperm cells per ejaculate in relation to genotype, treatment, period or their interactions (Table 2.2).

Discussion

There is contradiction between observations in the body of literature surrounding the topic of dopamine agonist effects on male reproduction and physiology. The reason for the variability may be attributed to genetic makeup of animals utilized in the studies. This study evaluated the presence of DRD2 expression in bovine male reproductive tissues and assessed genotypic differences in animals for the DRD2 gene in an attempt to determine any possible differences in bovine male physiology due to a particular DRD2 SNP (Campbell et al., 2014) on bull serum PRL concentration, growth traits, and semen quality in bulls grazing a dopamine agonist. Using IHC techniques, the DRD2 receptor was found to be present in bull testis, epididymis and sperm. This is similar to observations in other species showing the presence of DRD2 receptor throughout the male reproductive system, including sperm cells (Gonzalez et al., 2015; Otth et al., 2007; Ramírez et al., 2009). To further verify the expression of DRD2 receptor in male reproductive tissues, end-point RT-PCR identified products of the correct size and sequence. The primers utilized in RT-PCR flank the intronic region the SNP of interest is located. A single product was identified indicating no splice variants present due to genotype; however, this does not negate the possibility of alternative spliced forms of DRD2 present in cattle. Alternate splicing of DRD2 has been reported in rats and humans, resulting in long and short forms of the receptor; however, both forms are functionally coupled to G-proteins and can suppress PRL expression (Dal Toso et al., 1989; McChesney et al., 1991).

These data show that EA consumption could impact male reproductive physiology by binding to DRD2 receptor in the testis and accessory glands could impact reproduction.

This binding could alter spermatogenesis, sperm maturation or the makeup of seminal fluid impacting sperm physiology and subsequent fertility. Further, if the toxin is present in seminal fluid, sperm physiology could be altered through binding to the DRD2 receptor or other receptor types which may be present and are capable of binding EA. Dopamine receptors have been well established in male physiology in both rats and humans. Dopamine type-2 receptor agonists and antagonists have been heavily studied linking DRD2 to initiation of ejaculation (Ferrari et al., 1994; Peeters et al., 2008; Stafford et al., 2006). However, this retrospective study would indicate little if any effect on bovine male growth and semen quality at time of collection due to EA consumption. In contrast, two previous studies show an impact of EA consumption on the ability of bovine semen to survive normal extension and freezing procedures (Burnett et al., 2017; Pratt et al., 2015), supporting the observation of Gallagher and Senger (1989), showing that incubation of sperm with dopamine agonists decrease sperm freezing potential.

Genotyping was originally attempted by nested PCR. In brief, end-point PCR was performed for the F1-R793 DRD2 region (Campbell et al., 2014). The resulting products were subjected to nested PCR with primers flanking the SNP site (Campbell et al., 2014) for genotype (AA, AG, GG) on either the forward or reverse primer. The specific forward and reverse primers were run with primers flanking either the end of the sequence at 793 bp or the beginning of the sequence, 1bp, respectivley. The resulting products should have amplified only for the specific genotype, but when run with known genotyped animals, results were inconclusive. The RFLP method was then conducted and the resulting products were subjected to dideoxysequencing along with subcloned samples of known

DRD2 genotype to assess issues with specific amplification of the prior methods. Results were again inconclusive, with clones of known genotype and resulting products of RFLP unable to read 'G' in all samples sequenced, suggesting the polymerase may be unable to read through the 'G' 'C' rich region of this particular site. The custom Taqman geontyping assay was utilized to compare to RFLP methods. Both produced nearly identical results in known genotyped animals, with 3 animals differing between methods, accounting for a 3.4 % error rate among calls. These three samples may have differed due to possible contamination issues, with genotypes all resulting in AA in the taqman genotyping assay. These samples were limiting and we were unable to genotype them again. An attempt to isolate DNA from urine samples from these same animals was made but was unsuccessful. The DNA isolated from urine never resulted in quality template due to contaminants and sediment in the urine. However, the error in genotypic calls is extremely low and does not directly impact the overall distribution within the study. This comparison of Taqman methods to RFLP conclude both methods' reliability for use in further studies for genotyping.

Body weight and BCS in relation to period are in agreeance with our lab's previous work in which BW and BCS were unaffected by treatment alone, with BCS effected by day (Stowe et al., 2013). When examining a subset of bulls in 2011, it was found that SC was decreased at the end of a 126 d study when bulls were consuming a high concentration of EA; however, considering data across all four years SC were not affected by treatment. A lack of effect on SC may be due to the duration of the periods analyzed, as this current study only looked out to 84 days whereas Stowe et al. (2013) extended their analysis to

71

126 days. A change in SC was observed across all bulls due to period, with prolonged time on study resulting in a higher SC. However, these two studies differ in treatment delivery, with the current study grazing, with the exception of year 2011, and the previous study by Stowe et al. (2013) fed a concentrate diet throughout. Moreover, the current observations on SC are consistent with conclusions by Looper et al. (2009) and Schuenemann et al. (2005) who also report no change in SC due to EA exposure.

The current observations agree with the consensus of the literature to date in which animals grazing a dopamine agonist, experience a decrease in circulating serum PRL concentrations (Porter et al., 1992; Pratt et al., 2015; Schillo et al., 1988). However, the conclusions on serum PRL concentration are contradictory to findings by Campbell et al. (2014) who observed differences in serum PRL concentrations according to DRD2 genotype. In the current study, PRL levels remained consistent across DRD2 genotypes. Campbell et al. (2014) observed a correlation with serum PRL concentrations with GG genotypes experiencing decrease in PRL and steers with the AA genotype experiencing higher PRL concentration when grazing E+ in May. However, they did not observe this same effect consistently across all months that animals were on treatment. Their results for the month of June, although attributed to heat overriding genotypic advantage, more closely align with our current findings in which genotype had no effect on PRL concentrations. Moreover, our study assessed effect of period on cattle grazing E+ and E- spanning from April to August or February to August. Period was found to have no significant effect on PRL serum concentrations.

Previous data are contradictory on semen quality regarding bull exposure to EA. Ergot alkaloids have been shown to impact semen quality, with previous studies reporting decreased semen concentration (Pratt et al., 2015), motility and morphology (Looper et al., 2009; Pratt et al., 2015) while others have reported no effects (Schuenemann et al., 2005; Stowe et al., 2013). Our current findings support these later observations, with no effect on motility, concentration or morphology due to treatment or period. Further, our results conclude that DRD2 genotype has no effect on semen quality. Interestingly, velocity was impacted by period, with bulls longer on study exhibiting an increase in semen velocity. This has been observed in our previous findings for bulls grazing a dopamine agonist in which velocity was effected by day (Burnett et al., 2017) and is most likely attributed to increasing bull age and/or seasonal change from winter to spring temperatures. These results are contradictory to what is seen in hyperprolactinemia patients, in which velocity is increased with hightened circulating serum PRL concetrations (Panidis et al., 1997).

Summary

Results from this research indicate that DRD2 SNP genotype (Campbell et al., 2014) does not play a role in male physiology pertaining to semen quality, growth, or circulating levels of serum PRL. These data are however, in agreement with a treatment effect on serum PRL concentrations in bulls grazing EA. However, these results are contradictory to previous findings on this SNP that did find an association of serum PRL concentrations to SNP genotype in steers (Campbell et al., 2014). The treatment effect observed with serum PRL concentrations decreased in bulls exposed to a dopamine agonist indicated that the SNP did not alter structure or function of DRD2. No differences in growth

or semen quality due to SNP genotype also indicate that the SNP has no systemic effects. The SNP is located within a non-coding region of the DRD2 gene, so the current observations presented here are plausible.
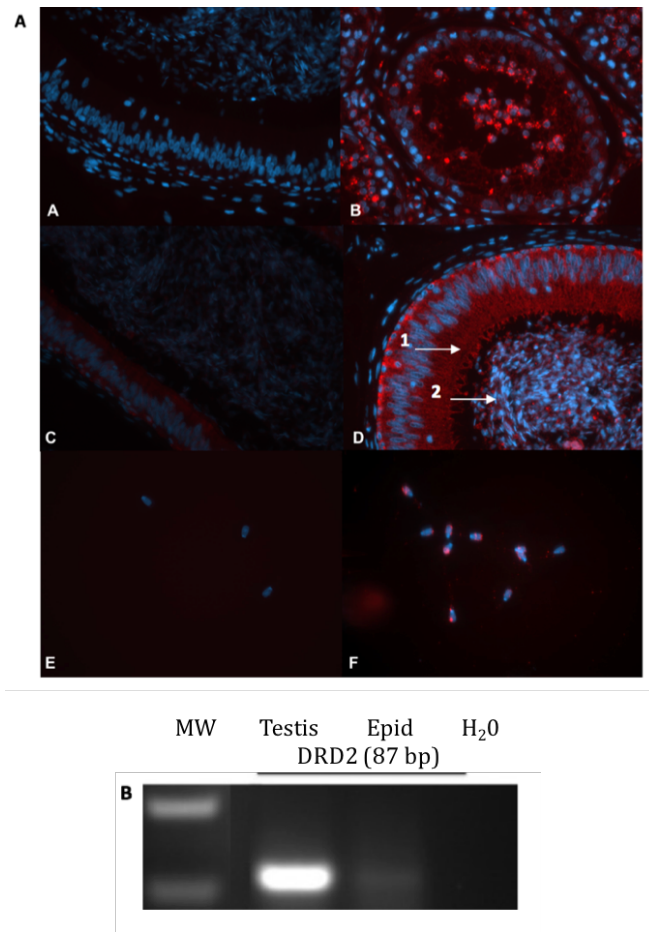
Figure 2.1 Immunohistochemical detection and end-point PCR of DRD2 in testis and epididymis.

Panels are labeled at the top left in bold. Panel (A) Immunohistochemistry of DRD2: Panels (A), (C) and (E) are negative controls for the primary Ab and panels (B), (D) and (F) were incubated with anti-DRD2 Ab. Dopamine receptor type-2 staining is shown in red. Slides were counterstained with DAPI (blue) for visualization of the nuclei. Both stains are indicated by white arrows in panel (D) with staining for DRD2 and DAPI indicated by arrows labeled 1 and 2, respectively. Tissue types are as follows: panels (A) and (B) are testis, panels (C) and (D) are epididymis, panels (E) and (F) are sperm cells. All images were taken at 40x magnification. Ab, antibody; DRD2, Dopamine type-2

receptor. Panel (B) End-point PCR: An 87 bp amplicon for DRD2 mRNA expression is shown in testis and epididymis. MW, molecular weight ladder; DRD2, Dopamine type-2 receptor.
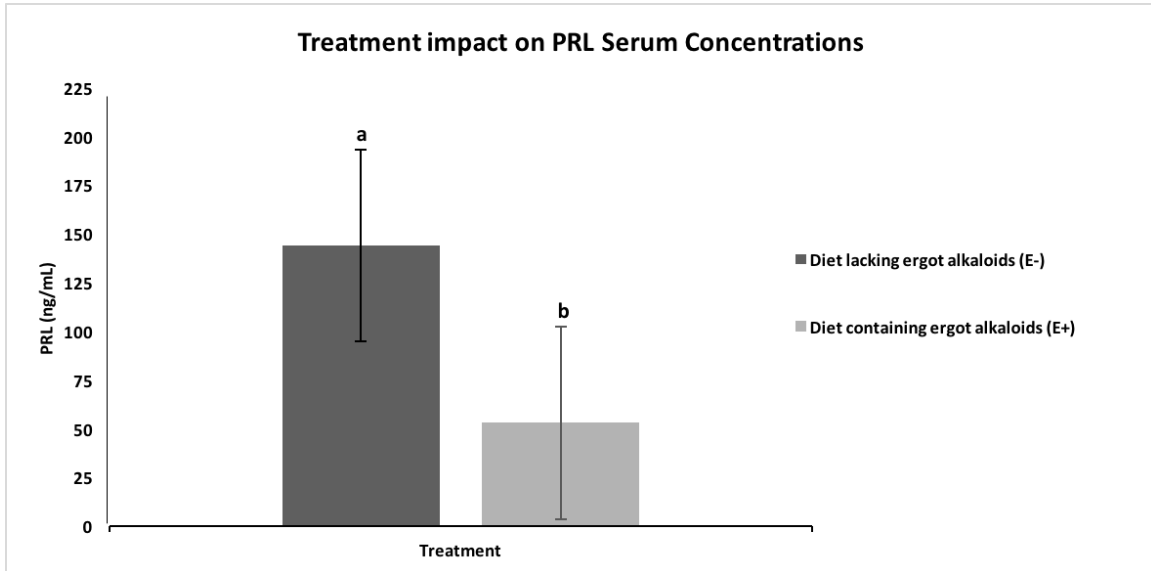
Figure 2.2 Treatment effect on prolactin serum concentrations of bulls grazing a forage containing (E+) or lacking (E-) a dopamine agonist.

Bulls on treatment showed overall lower serum concentrations of PRL than E-bulls. Different letters indicate significance ($P = 0.02$) due to treatment.
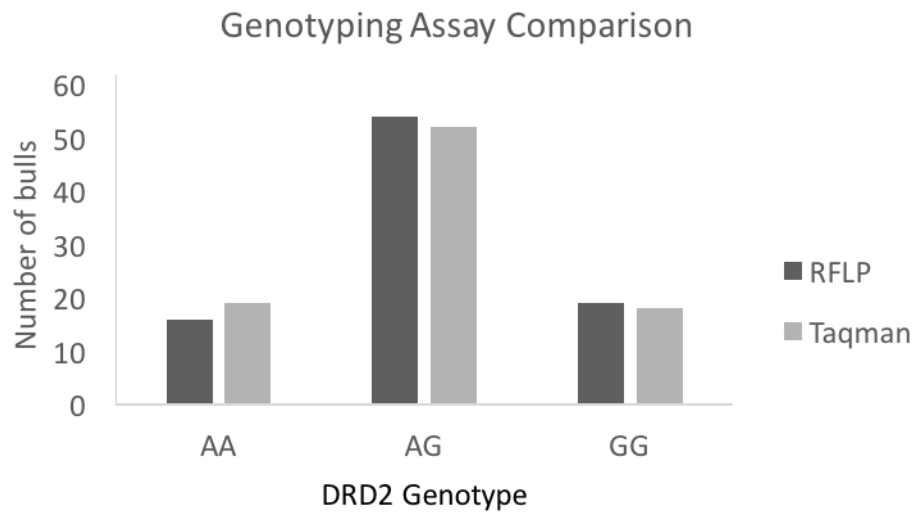
Figure 2.3 Comparison in genotype calls for RFLP and the custom Taqman genotyping assays.

Genotyping of the DRD2 SNP by Taqman and restriction fragment length polymorphism methods resulted in 96.6% identical calls between both methods.
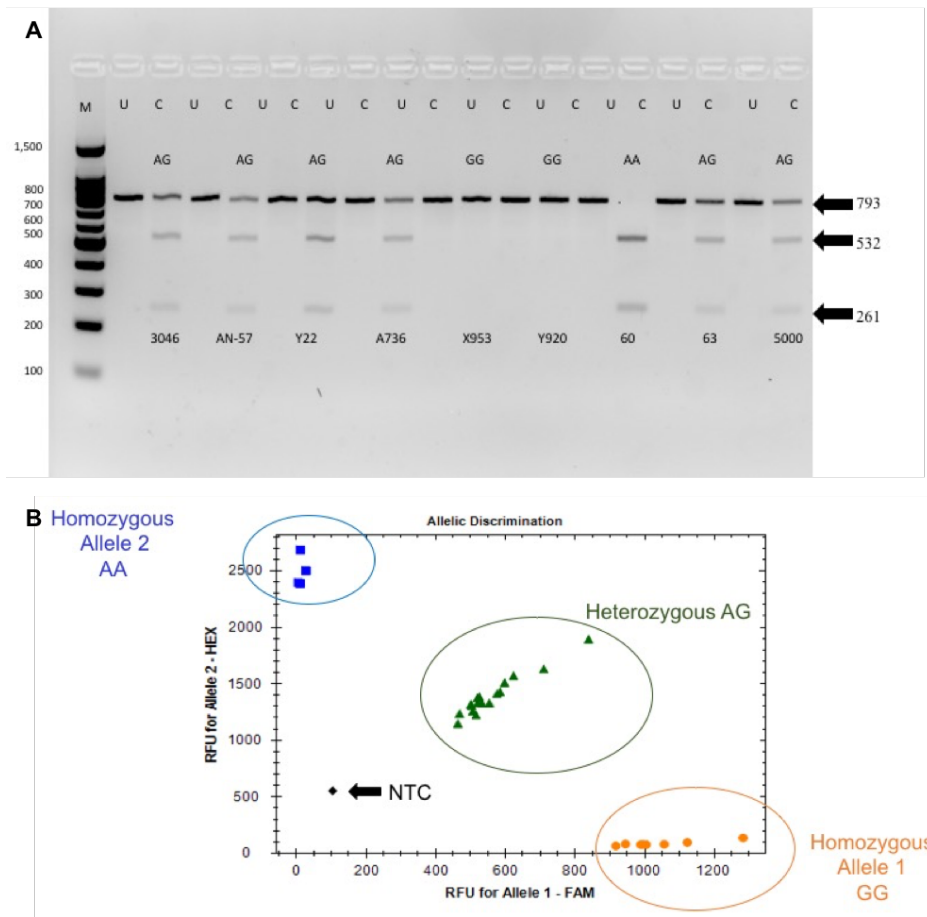
Figure 2.4 A representative sample of bulls analyzed by RFLP and Taqman analysis for DRD2 genotypes.

Panels are denoted on the top left in bold. Panel (A) RFLP analysis: molecular weight marker is on the left and product sizes are on the right. Bull IDs are below each lane. All products were incubated in the absence (U) or presence (C) of TfiI restriction enzyme. A single band present at 793 bp indicates a lack of the TfiI restriction site and is genotyped as GG. The presence of two bands at 532 and 261 indicate homozygous for the TFiI site and genotyped as AA. The presence of all three bands are genotyped as AG. Panel (B) Taqman analysis: The allelic discrimination plot from the custom Taqman genotyping assay for the DRD2 SNP region contains a shape, each representative of a

single animal with each cluster represented by a different color, representing a genotype. Plots are represented as relative fluorescence units (RFU) for each allele. Allele 1 representing RFU for 'G' on the horizontal axis and Allele 2 representing 'A' on the vertical axis. Genotypes are represented as the following: Orange circles, GG; Green triangles, AG; Blue squares, AA; Black dot, non-template control (NTC).

Table 2.1. Mean body weight, body condition and scrotal circumference of bulls grazing ergot alkaloids (E+) or a diet lacking ergot alkaloids (E-) up to 84 days.

Parameter	d 0	d 21-28	d 42-56	d 84	P-Value
Body weight (BW) (kg)	508.56 ± 11.8[c]	520.90 ± 11.8[bc]	534.6 ± 11.8[b]	551.8 ± 12.3[a]	< 0.001
Scrotal Circumference (SC)	35.4 ± 0.41[c]	35.8 ± 0.41[bc]	36.5 ± 0.41[a]	36.3 ± 0.44[ab]	0.0053
Body Condition (BCS)	5.9 ± 0.21[a]	5.5 ± 0.21[b]	5.5 ± 0.21[b]	5.6 ± 0.22[b]	0.0009

[a-c] **Means within the same row possessing a different superscript lowercase letter differ due to day**.

Table 2.2. Mean semen characteristics of bulls grazing ergot alkaloids (E+) or a diet lacking ergot alkaloids (E-) up to 84 days.

Parameter	d 0	d 21-28	d 42-56	d 84	P-Value
Concentration (million/mL)	383.2 ± 54.9^a	377 ± 54.6^a	435.3 ± 56.5^a	448.5 ± 59.7^a	0.42
PROG Motility (%)	66.5 ± 2.96^a	71.5 ± 2.95^a	71.6 ± 3.13^a	72.9 ± 3.49^a	0.35
Motility (%)	76.5 ± 2.49^a	82.1 ± 2.46^a	82.3 ± 2.74^a	84.2 ± 3.13^a	0.27
Normal Morphology (%)	85.6 ± 4.91^a	84.9 ± 4.73^a	84.2 ± 5.00^a	92.2 ± 5.42^a	0.55
Motile sperm cells (x $10^{\wedge 6}$)	304.1 ± 34.9^a	298.0 ± 34.7^a	351.2 ± 36.2^a	345.7 ± 38.8^a	0.31
Velocity (∞/sec)	51.5 ± 3.77^b	56.2 ± 3.74^{ab}	62.8 ± 3.96^a	63.6 ± 4.39^a	0.04
Total number sperm cells ($10^{\wedge 6}$)	2.08 ± 0.639^a	2.65 ± 0.637^a	3.11 ± 0.654^a	2.47 ± 0.682^a	0.24
Total Motile Sperm (million/mL)	1.68 ± 0.423^a	2.04 ± 0.421^a	2.42 ± 0.432^a	$1.90 \pm .452^a$	0.17
Total Progressive sperm cells (million/mL)	1.49 ± 0.348^a	1.80 ± 0.346^a	2.15 ± 0.356^a	1.64 ± 0.376^a	0.16

[a-b] **Means within the same row possessing a different superscript lowercase letter differ due to day.**

CHAPTER THREE

EVALUATION OF AN INTRONIC BOVINE DRD2 SNP

ASSOCIATION TO PROLACTIN PROTEIN EXPRESSION

IN BOVINE REPRODUCTIVE AND ANTERIOR PITUITARY TISSUES

Abstract

Detrimental effects to male reproductive physiology have been observed due to changes in prolactin (PRL) serum concentration. Regulation of PRL by dopamine binding to the dopamine type-2 receptor (DRD2) is well defined and associations between male physiology and single nucleotide polymorphisms (SNPs) within the DRD2 gene have been observed. Association of a bovine DRD2 SNP with serum PRL concentrations has been previously reported in steers. In contrast, the DRD2 SNP was not associated with PRL serum concentration, growth traits, or semen quality in bulls, warranting further investigation on the SNP genotypic effects on PRL production and secretion in bulls. The objective of the current study was to evaluate association of a DRD2 SNP to PRL protein expression in bull reproductive and anterior pituitary tissues. Testis and epididymis were collected from bulls grazing a forage containing or lacking a dopamine agonist at the end of a 126 d study ($n = 14$) and bovine pituitaries ($n = 587$) were collected from a local abattoir. Prolactin protein expression was assessed in all tissues by western blotting; however, PRL protein was below detection range in reproductive tissues but was present in pituitary, therefore experiments continued in pituitary. Sex determination of pituitaries was performed by duplex PCR and Southern blotting methods and genotyping was

performed by restriction fragment length polymorphism (RFLP). The DRD2 SNP region was amplified followed by digestion with a Tfil enzyme, producing 3,2, or 1 band (AG, AA, GG, respectively). Pituitary genotype population was 17.4% AA, 63% AG, and 19.6% GG. Protein expression was assessed by slot blot and densitometry analysis, normalized to GAPDH expression ($n = 92$). Prolactin protein expression in the pituitary was similar across genotype ($P = 0.23$) and agrees with previous observations on this SNP indicating no genotypic effect on growth or semen quality traits in bulls.

Introduction

Prolactin (PRL) is a 22kDa peptide involved in over 300 diverse biological functions (Freeman et al., 2000). Prolactin is produced by lactotrophs in the anterior pituitary, and it's release and synthesis is negatively regulated via tonic inhibition by binding of dopamine to the dopamine type-2 receptor (DRD2) (Ben-Jonathan et al., 2001). Once released, PRL binds to its receptor, activating the Janus Kinase-Signal transducers and activators of transcription (JAK-STAT) pathway (Campbell et al., 1994). Phosphorylation of JAK2 triggers the association and phosphorylation of STAT5 (Ihle et al., 1996). Phosphorylation of STAT1, STAT3, and STAT 5 have been observed in PRL signaling through PRLR; however, the predominant transcription factor involved is STAT5 (Gouilleux et al., 1994; Lebrun et al., 1995). Transcription factor STATs form heterodimers and translocate to the nucleus to stimulate gene transcription by binding to defined nucleic acid sequences within promoter regions of genes (Ihle et al., 1996). The main source of inhibition of PRL is by suppression of adenylyl cyclase to inhibit PRL production. Cells treated with a dopamine agonist exhibit reduced PRL transcription which can be rescued

by treatment with cyclic AMP (Maurer, 1981). More immediately, upon binding of dopamine to DRD2 on lactotrophs, a rapid decrease in intracellular calcium occurs suppressing the release of PRL from secretory granules (Ben-Jonathan et al., 2001).

Cattle consuming dopamine agonists such as ergot alkaloids, which bind to DRD2 receptors, exhibiting reduced circulating serum concentrations of PRL (Schillo et al., 1988; Strickland et al., 2011; Pratt et al., 2015). Further, decreased semen motility and morphology (Looper et al., 2009; Pratt et al., 2014) have also been reported in these bulls, supporting observation in humans where hypoprolactenemia is associated with semen motility (Gonzales, et al., 1989). In contrast, hyperprolactinemia in human males is associated with infertility (Segal et al., 1976) and impotence (Segal et al., 1979). Further supporting effects of PRL on male reproductive physiology, early PRLR ablation experiments in mice resulted in infertile males (Ormandy et al., 1997). Unfortunately, later data using knockout models did not support these observations and mechanisms in which reproductive male physiology is impacted by PRL, whether through a direct or indirect mechanism, remain unclear.

The PRL signaling pathway has been implicated in effects on reproductive efficiency of several species and may provide a route for indirect effects on mammalian reproduction (Segal et al., 1979; Gonzales et al., 1989; Ormandy et al., 1997). Some of these indirect effects may be explained by the presence of single nucleotide polymorphisms reported to be present in the bovine DRD2 (Campbell et al., 2014), PRL (Looper et al., 2010), PRLR (Ormandy et al., 1997), and STAT5 (Brym et al., 2004; Flisikowski et al., 2003) genes. The SNPs reported for these four genes result in over 2,000 possible genetic

combinations that could impact PRL signaling and therefore indirectly affect mammalian reproductive physiology. Evidence for impact on male physiology due to dopaminergic SNPs exist. A polymorphism in DRD2 exon 7 in which a cytosine to thymine substitution at nucleotide 3420 results in homozygous genotype is highly association with hyperprolactinemia in male patients (Hansen et al., 2005). Further, a cytosine insertion in the DRD2 gene has been shown to impact PRL secretion as measured by increasing blood serum concentrations leading to erectile dysfunction (Zhang et al., 2011). Recently in cattle, a SNP has been identified that exists within the DRD2 gene located on bovine Chromosome 15 at position 404365, on the third intron. The SNP consists of a base substitution between adenine and guanine (Campbell et al., 2014). The bovine DRD2 SNP yields three genotypes when digested with Tfi I, AA (532 and 261bp), AG (793, 532 and 261bp), and GG (793bp) (Campbell et al., 2014). Similarly, to humans, the DRD2 SNP genotype homozygous 'GG' genotype is associated with decreased serum prolactin concentrations in cattle exposed to a dopamine agonist (Campbell et al., 2014). The bovine DRD2 SNP is located within a non-coding region, and therefore would not affect protein structure/function however, its effects on PRL release and synthesis could be due to effects on gene expression. Contrary to the prior findings, recent research indicates that genotype does not impact male physiology, having little or no effect on semen quality parameters or prolactin serum concentrations in bulls grazing a dopamine agonist (DeCarlo et al., 2019). This research aims to elucidate the role of the DRD2 SNP (Campbell et al., 2014) on PRL protein expression in the pituitary.

Materials and Methods

Treatment and tissue collection

Treatment of the yearling beef bulls in which testis and epididymis tissues were collected are described by Stowe et al. (2013). In brief, bulls were fed a ration that included or excluded ergot alkaloids at 0.8μg/g dry matter, then allowed to graze for a total period of 126 d. Testes and epididymides were collected 60 days after removal from ergot alkaloids.

Bovine pituitaries ($n = 587$) were collected at local abattoir, the anterior and posterior lobes were carefully excised, placed into a cryotube, flash frozen in liquid N_2 and stored at -80 °C until analysis. Bovine ovaries ($n = 5$) were also collected from the abattoir, transported on ice to the laboratory, and frozen at -80°C until used for further analysis.

RNA isolation and RT-PCR

Total RNA was isolated from anterior pituitary tissues using the TriZol isolation methods (Invitrogen, Carlsbad, CA, USA) and isolated from testis and epididymis tissue using the mirVana mRNA isolation kit (Ambion, Austin, Texas, USA). Samples were subjected to nanodrop 1000 spectrophotometry (Thermo Fisher Scientific, Waltham, MA, USA) to assess concentration and purity by A260:A280 ratio. Quality of RNA samples were confirmed through Agilent 2100 nano chip assay (Agilent Technologies, Santa Clara, CA, USA) to obtain a RIN value. Only samples that possessed a RIN of ≥ 7 were utilized for analysis.

Complimentary DNA was generated using qScript cDNA Supermix (Quantabio, Beverly, MA, USA). Primers for PRL (NM_173953.2) flanking nucleotide positions 146-

431, TH (NM_173884.2) (an indirect measurement of dopamine synthesis) flanking nucleotide positions 1263-1381, DRD2 (NM_174043.2) flanking nucleotide positions 510-597 of the mRNA DRD2 sequence, and PRLR (L02549) flanking nucleotide positions 1107-1216 were generated through IDT primer quest tool (Integrated DNA technologies, Coralville, IA) for use in polymerase chain reaction (PCR) (Table 3.1). End-point PCR was performed utilizing the following reaction conditions: denaturation at 95° C for 2 min followed by 35 cycles at 94° C for 30 sec, annealing 30 sec (PRL, 60° C; DRD2, 52° C; TH, 52° C; PRLR, 55° C) and 72° C extension for 30 sec followed by 72° C for 5 min and a 4° C hold. Slab gel electrophoresis was performed and the resulting products were visualized on a 1.5% agarose gel.

To further validate identity of the products, cDNA from testis and epididymis were ligated into a pDrive cloning vector overnight at 4° C (Qiagen PCR Plus Cloning kit, Hilden, Germany). Ligations were used to transform competent E. coli (EZ competent; Qiagen, Hilden, Germany). Resulting transformations were plated on LB agar plates containing 100 μg/ml Ampicillin and IPTG/Xgal and allowed to incubate overnight at 37° C for blue/white screening. Subsequent colonies were selected from each plate for each respective insert, and were propagated through inoculation of LB broth, incubated overnight, shaking to aerate at 250 rpm at 37° C. Plasmid DNA was purified using Wizard mini-prep plus (Promega, Madison, WI), digested with EcoR1 (Promega, Madison, WI, USA) and products of the expected sizes were subjected to dideoxysequencing.

DNA Isolation

Isolations of genomic DNA were performed according to Current Protocols in Molecular Biology (Wiley and Sons, Inc., 1998). Posterior pituitary lobe tissues (200 mg) and ovary (50 mg) were homogenized directly in DNA lysis buffer (100 mM NaCl, 10 mM TrisCl pH 8, 25 mM EDTA pH8, 0.5 % SDS, 0.1 mg/ml proteinase K) (n = 587). Phenol:chloroform:isoamyl alcohol (25:24:1; pH 8) were added to the homogenized sample, equal to the volume of the lysate. Samples were vortexed for 1 min and centrifuged at 1,700 x g for 10 min at RT. The aqueous layer was carefully aspirated and put into a sterile tube, followed by addition of 0.5 volume of 7.5 M ammonium acetate and 2 vol of 100 % ethanol relative to the aqueous layer volume. Resulting precipitated DNA was pelleted at 1700 x g for 2 min at RT and DNA pellet saved and the supernatant decanted. The DNA pellet was washed in 70 % ethanol and allowed to dry for 15 min followed by resuspension in 100 μl of nuclease free water. Samples were heated at 65° C for 30 min and then stored at 4° C until further analysis of sex and genotype. Isolated DNA was evaluated for concentration and nucleic acid to protein ratio (260/280) by spectrophotometry (Nanodrop 1000; Thermofisher).

Sex Determination

Sex was determined (n = 587) utilizing a duplex PCR method described by Curry et al. (2008) and Southern blotting techniques. In short, 2 ng of DNA was amplified in a 25 μl PCR reaction (GoTaq Green; Promega, Madison, WI, USA) including 10 μl of Go-taq, 1 μl of each β-actin primer and 2 μl of each SRY primer. Controls, including a known male sample (testis) and a known female sample (ovary) were also subjected to duplex PCR amplification. Single products of β-actin and SRY generated from 2ng of

purified plasmid DNA products, verified by dideoxysequencing served as additional controls on each blot. Reaction conditions consisted of 95° C for 2 min, 94° C for 30 sec, annealing at 60° C for 30 sec and extension at 72° C for 30 sec for a total of 35 cycles, followed by 72° C for 5 min and a 4° C hold. Reactions were treated according to Curry and others (2008). In brief, samples were denatured by addition of NaOH (0.1 vol) and heated at 37° C for 5 min followed by addition of SSPE (3 M NaCl, 0.2 M NaH_2PO_4, 0.02 M EDTA) at a final 6X concentration. Reactions were blotted using a dot blot manifold. Nylon membrane (Biobond-Plus Nylon Membrane; Sigma, St. Louis, MO, USA) was soaked in nuclease free water for 10 min prior to assembling the blot manifold. The manifold was set up with the pre-soaked nylon membrane placed on top of two Whattman filter papers. Each well was washed initially with 300 μl nuclease-free water followed by blotting of products. After addition of samples to the membrane, the wells were washed with 300 μl 0.4 M NaOH. The manifold was disassembled and the resulting blot was rinsed briefly in 2X SSC (20X solution; 0.3 M sodium citrate, 3 M NaCl, pH 7.0) and allowed to dry completely before crosslinking at 120 cm^2/j for 1 min (XL-1000 UV crosslinker).

Amplification of single, β-actin and SRY products were generated using 2 ng genomic DNA (GoTaq Green; Promega, Madison, WI, USA). Reaction conditions are as described above. Purified products were ligated into pDrive cloning vector (Qiagen PCR Plus Cloning kit, Hilden, Germany), subcloned, and resulting transformations plated. Vectors containing the inserts of interest were propagated and plasmids isolated. Inserts were amplified using end-point PCR for β-actin and SRY (Genewiz, Plainfield, NJ, USA).

Resulting PCR products were purified (Wizard SV Gel and PCR Clean-Up System, Promega, Madison, WI, USA) and subjected to spectrophotometry to assess concentration and quality by 260/280 nucleic acid to protein ratio. Samples with a ratio of 1.8 - 2.0 were utilized for generation of probes. Labeling was then carried out by random prime labeling (North2South Biotin Random Prime DNA Labeling Kit, Thermo Fisher Scientific, Waltham, MA, USA). Probes were generated using 200 ng of purified PCR products in a final volume of 24 μl with nuclease-free water followed by addition of 10 μl heptanucleotide mix. Samples were denatured by boiling for 5 min and quickly placed in a methanol dry ice bath. The samples were thawed on ice followed by the addition of 10 μl of 5X dNTP mix, 5 μl of 10X reaction buffer, and 1 μl Klenow fragment for a final volume of 50 μl. The samples were then incubated at 37° C for 1 hr following inactivation with addition of 2 μl 500 nM EDTA (pH 8.0). Unincorporated nucleotides were removed by size exclusion chromatography. Sephadex G50 (1 g) was allowed to swell in approximately 10 mL phosphate buffered saline (PBS) (1X) at RT overnight. A column was produced using a 1 mL syringe with steel wool packed into the bottom measuring to approximately 0.1 mL on the syringe. The swelled sephadex mixture was loaded into the syringe barrel to the top and allowed to drip for 1 hr at RT followed by centrifuging at 2000 x g for 1 min at RT to pack the column. The column was placed into a 15 mL conical tube to catch flow-through and was initially washed with PBS (100 μl) by slow addition to the top of the column and centrifugation at 2000 x g for 1 min at RT. The flow through was discarded and the column was placed into a new sterile tube. The full volume of the labeled probe was then loaded onto the column followed by centrifugation, 2000 x g for 1 min. The flow-

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through was saved and the column was washed once more with 100 μl PBS utilizing the same flow-through tube. The resulting flow through was transferred to a new sterile tube and subjected to denaturation by boiling for 10 min followed by 5 min incubation on ice.

Pre-hybridization, hybridization, and chemiluminescent detection were performed using the North2South Chemiluminescent Hybridization and Detection Kit (Thermo Fisher Scientific, Waltham, MA, USA). Kit contents were allowed to come to RT or incubated at 37° C until no precipitate existed before use. Pre-hybridization of blots were performed in two separate hybridization tubes in a hybridization oven at 55° C (Problot Hybridization Oven, Labnet, International) in 10 mL of pre-hybridization buffer for 30 min followed by addition of probe, either β-actin or SRY, directly to the buffer for each blot (hybridization buffer). Hybridization was carried out overnight at 55° C, rotating.

The hybridization buffer was discarded and each blot washed 3X in stringency wash buffer at 55° C for 15 min each wash, rotating. The membranes were then blocked for 15 min at RT with gentle agitation. Streptavidin was diluted 1:300 in blocking buffer and then added to the membrane to incubate again for 15 min at RT with gentle agitation. The solution was then decanted and the blots were incubated in substrate equilibration buffer for 5 min at RT. The blots were then covered in substrate for 5 min at RT then exposed to chemiluminescent detection for 2 min (FluorChem FC2, Alpha Innotech, San Leandro, CA, USA). Confirmation of males was performed by densitometry. Samples were considered male if they met or exceeded the density of the testis control on each blot. Samples that came up inconclusive, in which signal density was just below or between the

threshold of background versus male control sample, were blotted an additional time to assure accuracy in calls.

Genotyping

Genotyping of male pituitaries (*n* = 259) were performed according to the restriction fragment length polymorphism method described by DeCarlo et al. (2019), with modifications. In brief, amplification of the DRD2 region flanking a SNP (A534G) was performed (Campbell et al., 2014) with 10 ng starting template genomic DNA (GoTaq Green Master Mix, Promega, Madison, WI, USA). Resulting products were digested with Tfil enzyme overnight at 37° C (ThermoFisher Scientific). Digested products were visualized on a 1.0 % agarose gel.

SDS-PAGE and western blotting

Protein extracts were produced from testis, epididymis, ovary, and anterior pituitary tissues (100 mg starting material). Samples were homogenized in an ice-cold lysis buffer (1X RIPA with 10 μl/mL Protease inhibitor HALT). Protein concentrations were determined using the DC protein assay with each sample performed in duplicate (Bio-Rad; Hercules, CA). Samples were stored at -20° C until used for further protein analysis.

Protein extracts of testis, epididymis, ovary, and anterior pituitary were diluted in molecular grade water and 2X laemmali buffer with addition of 1/8th volume of beta mercaptoethanol to a total mass of 30 μg and 60 μg, for detection of PRL and GAPDH, respectively. Samples were denatured for 5 min by boiling and directly chilled on ice for 5 min. Denatured samples were centrifuged at 13,000 x *g* for 10 min and loaded onto a 12 % mini protean TGX precast polyacrylamide gel at a total volume of 20 μl per well (Bio-Rad;

Hercules, CA). Gel electrophoresis was performed at 75 volts for 1 hr and 15 min and 200 volts for 20 min for GAPDH and PRL, respectively. Proteins were blotted onto a 0.2 μm nitrocellulose membrane (Bio-Rad, Hercules, CA, USA) at 100 volts for 1 hr. Transfer of proteins was assessed by staining with Ponceau S for 5 min and the polyacrylamide gel stained with coimmasse blue overnight. The membrane was allowed to dry overnight at RT, protected from light.

For the detection of PRL, blotted proteins (30 μg) were blocked in 3.5 % non-fat dry milk in a tris-buffered saline buffer with 0.1 % Tween added (TBST) for 1 hr at RT with gentle rocking. The membrane was briefly washed in TBST followed by the addition of PRL primary antibody diluted to 1:1000 in 1 % non-fat dry milk/ TBST (sc-69874; Santa Cruz Biotechnology, Dallas, TX, USA) and allowed to incubate overnight at 4° C. The blot was then washed in TBST 3X, 5 min each wash. Secondary antibody was diluted 1:1000 in TBST (610-703-124; Rockland, Limerick, PA, USA) was added and allowed to incubate at RT for 1 hr followed by three washes in TBST, 5 min each. The membrane was laid on an acetate sheet and covered in substrate (Clarity Western ECL Substrate; Bio-Rad Hercules, CA, USA) for 5 min at RT. The substrate was drained from the blot, covered by another piece of acetate, and exposed to chemiluminescent detection for 15 min (Alpha Innotech; San Leandro, CA, USA).

For the detection of GAPDH, blotted proteins (60 μg) were blocked in 5 % non-fat dry milk in TBST for 1 hr at RT with gentle rocking. The membrane was briefly washed in TBST followed by the addition of GAPDH primary antibody diluted to 1:1000 in 5 % BSA/TBST solution (2118; Cell Signaling Technology, Danvers, MA, USA) and allowed

to incubate overnight at 4° C. The membrane was then washed in TBST 3X, 5 min each wash. Secondary antibody was diluted 1:1000 in TBST (7074; Cell Signaling Technology, Danvers, MA, USA) was added and allowed to incubate at RT for 1 hr followed by three washes in TBST, 5 min each. Substrate was added to the blot and after 5 min exposed to chemiluminescent detection for 15 min.

Evaluation of PRL protein expression in bovine pituitary

Linear range of detection was determined by blotting increasing mass of protein by slot blot manifold followed by exposures at varying times by chemiluminescent detection. Blotting by slot blot manifold was performed using 6 size No. 1 Whattman filter papers topped with a 0.2 μm nitrocellulose membrane. The manifold was sealed and each well washed with 200 μl of nuclease-free water followed by application of vacuum. Anterior pituitary protein (AA $n = 16$; AG $n = 58$; GG $n = 18$) (10 μg) was then blotted followed by an additional wash with deionized water. Blots were then stained with Ponceau S (Sigma-Aldrich, St. Louis, MO, USA) to assess transfer, rinsed with TBST briefly and treated with hydrogen peroxide (3 %) at 37° C for 10 min with gentle agitation to remove peroxidase activity. Blots were then placed in non-fat dry milk blocking solution and immune-detection carried out as described above for GAPDH. Chemiluminescent detection was performed using an Fluorchem FC2 (alpha innotech, San Leandro, CA, USA) for a 15 min exposure. Blots were then washed in TBST for 5 min and stripped using two washes of glycine (400nm, pH 2.5) for 5 min each at RT, followed by two PBS washes and a TBST wash for 5 min each. Immuno-detection for PRL was then carried out according to the protocol outlined above. Membranes were again exposed at 15 min for chemiluminescent

detection. Total protein on each blot was also evaluated by densitometry utilizing Ponceau S stain. Inter- and intra-assay coefficients were 7.4 % and 6.9 %, respectively.

Statistical analysis

Densitometry data for PRL protein expression was normalized to a GAPDH housekeeping protein density as follows. Background subtraction was applied to each GAPDH and PRL sample densities for all samples in duplicate. Blot normalization factors were calculated for each blot (highest GAPDH expression value on blot/ GAPDH signal of each individual sample). The density (after background subtraction) of each sample for PRL signal was then divided by the blot normalization factor to obtain relative protein expression. Analysis of Variance was performed for all densitometry data to assess DRD2 genotype impact on pituitary PRL protein expression. The model for the Analysis of Variance included genotype as a fixed effect. Significance was set at (P < 0.05) and all statistical analysis was performed using JMP software (SAS Institute Inc.).

<div align="center">

Results

</div>

Messenger RNA and protein expression

The messages for PRL and TH were present in testis and epididymis (figure 3.1). Previously published work also confirms the presence of DRD2 in testis and epididymis (DeCarlo et al., 2019). Further work to assess protein expression of PRL in testis and epididymis by western blotting was unsuccessful as the protein concentrations may be below detection range in these tissues. Prolactin (~22kDa) was present in pituitary as expected and therefore, experiments continued in bovine anterior pituitary (Figure 3.2).

Amplifications of PRL, DRD2, PRLR, and TH were performed in anterior pituitary with the message for all present (Figure 3.3).

Sexing and genotypic distribution

Sexing by PCR and Southern blot revealed 55.9% females and 44.1% males. An example of dot blots probed with SRY and β-actin are shown in Figure 3.4. Genotyping was performed by RFLP procedures. Genotypic distribution was determined to be AA 17.4%, AG 63%, and GG 19.6%.

Prolactin protein expression in anterior pituitaries

Prolactin protein expression was evaluated across genotype by slot blot and immunodetection procedures. Densitometry analysis revealed no differences in PRL protein expression due to DRD2 genotype ($P = 0.23$) (Figure 3.5).

Discussion

Initial goals of these experiments were to assess if the message for PRL and its regulatory system (DRD2 and its rate-limiting enzyme tyrosine hydroxylase) were present in male reproductive tissues. Our findings indicate that the message for PRL and TH are present in bovine testis and epididymis. These findings agree with other species in which PRL has been identified in semen, male accessory glands (Nevalainen et al., 1997; Sanford and Baker, 2010) and germ cells (Pratt et al. 2014; Sheth et al., 1975). Dopamine type-2 receptor has also been identified in male germ cells (Otth et al., 2007; Ramirez et al., 2009) as well as in the testes of mice (Gonzalez et al., 2015) and most recently in cattle testes, epididymides, and germ cells (DeCarlo et al., 2019).

We were not able to detect PRL in any testis, epididymis or ovary by western blotting. Given that the message for PRL is present in testis and epididymis, it is possible that PRL protein expression was just below the detection range for western blotting. Bovine anterior pituitary, the main site of production of PRL, did reveal ~22 kDa band as expected. without possible detection of PRL protein in testis and epididymis, experiments continued in the pituitary to examine possible genotypic effects of the DRD2 SNP previously associated with decreased serum PRL concentrations.

Slot blotting of anterior pituitary protein was performed to assess PRL protein expression across DRD2 SNP genotype. Original experiments to assess antibody specificity revealed evidence of high peroxidase activity in bovine anterior pituitary protein. To combat this issue, membranes with slot blotted anterior pituitary protein were treated with a 3% hydrogen peroxide solution prior to immunodetection procedures (Figure A.1). Experiments to determine optimal time, temperature, and hydrogen peroxide concentration to eliminate peroxidase activity were performed (Appendix A).

Sex determination of pituitaries were performed by duplex PCR and Southern blotting techniques. Original DNA isolations were performed using only 50 mg of posterior pituitary tissue starting material and a simple phenol chloroform extraction method that resulted in genomic DNA isolations containing large quantities of RNA. Increased specificity of Southern blotting utilizing hybridization to combat this issue. Isolations were then performed with a much higher, 200 mg of tissue for genotyping. The RFLP genotyping protocol was adapted from the previously used protocol (DeCarlo et al., 2019) in order to eliminate the need for the PCR purification step prior to digestion. Experiments

were performed with known genotyped samples to ensure enzyme digestion accuracy (data not shown) and an overnight digestion was able to allow for immediate digestion after amplification of the SNP sequence, without PCR clean-up.

The DRD2 SNP (A534G) is located in a non-coding region and therefore is not expected to affect protein folding or structure but possibly could affect gene expression within the PRL pathway. These data indicate no association of DRD2 SNP genotype to PRL protein expression in the bovine anterior pituitary. Previous studies on this DRD2 SNP showed no changes in serum PRL concentrations observed due to SNP genotype (DeCarlo et al., 2019). These two observations would indicate that the reported bovine DRD2 SNP does not affect the production or release of PRL. The genotypic distribution presented here is nearly identical to previous reported distributions for this DRD2 SNP (Meyer et al., 2016; DeCarlo et al., 2019) but are contradictory to Campbell et al. (2014) who observed a genotypic effect on PRL serum concentrations. The same study also reported a genotypic effect associated with hair coat score, which has been linked to PRL levels in cattle (Campbell et al., 2014); however, Meyer and others (2016) only observed a tendency for this association, although they did not directly measure serum PRL concentrations. Observations from this current study agree with findings by Meyer and others (2016) in which no change in PRL protein was observed according to genotype. Further, genotyping methods used in this study have been validated by a second genotyping method previously (DeCarlo et al., 2019), while no validation was used for Campbell and others (2014). The DRD2 SNP examined here is located in a non-coding region and therefore it is not surprising there was no change in PRL protein expression due to

genotype. Many SNPs within other genes affecting the PRL signaling pathway exist (Brym et al., 2005; Looper et al., 2010) and may provide more informative avenues to explore when assessing SNP genotypic effects on PRL synthesis and secretion.

Summary

In summary, the message for PRL, TH, and DRD2 are present in bovine testis, epididymis, and pituitary. The message for DRD2 has previously been reported in testis and epididymis (DeCarlo et al., 2019). The presence of PRL protein was observed in pituitary protein extracts and was not detectable in testis, epididymis or ovary samples. These data indicate that PRL protein expression may be lower than what is possible to detect by western blotting in male reproductive tissues. Experiments continued at the source of PRL production, the pituitary.

Genotyping ratios were extremely similar to what has previously been reported for this SNP (Meyer et al., 2016; DeCarlo et al., 2019) and for what is closest to a normal, random distribution (25:50:25). Further investigation of relative protein expression across genotype in the pituitary revealed no differences due to DRD2 genotype. These data agree with previous findings from this laboratory in which this DRD2 SNP had no effect on PRL serum concentrations and furthermore, no effects on growth, reproduction, and semen characteristics (DeCarlo et al., 2019). These data are contradictory to what has been previously reported on this DRD2 SNP, which indicate a genotype association to PRL serum concentrations (Campbell et al., 2014). This DRD2 SNP is located within an intron and does not likely affect protein structure but could affect gene expression; however, these data suggest no association between protein expression to the intronic DRD2 SNP.

Figure 3.1 Polymerase chain reactions were performed to assess the presence of PRL (A) and TH (B) in bovine male reproductive tissues.

Products revealed appropriate size bands at 285 bp and 118 bp for PRL and TH, respectively. Lanes are as follows: 100bp ladder (L), no template control (H_2O), PRL products in testis (T) and in Epididymis (E). (B) Bovine PRL and TH cDNA clones produced from the testis and epididymis were subjected to dideoxysequencing and aligned to the Bos Taurus PRL mRNA (NM_173953.2) and TH (NM_ 173884.2) sequence. Sequence alignments are shown to the right.
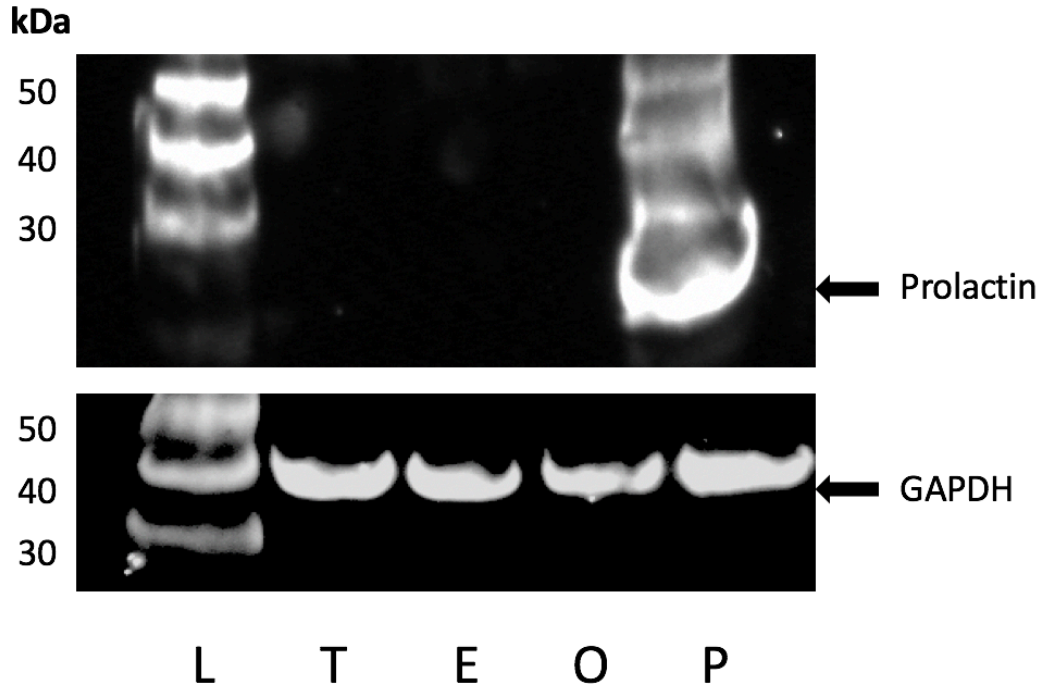
Figure 3.2 Western blotting analysis of Prolactin (PRL).

A band at ~22kDa is present in anterior pituitary (P) but no bands were present in testis (T), epididymis (E), or ovary (O). Loading control GAPDH is shown below at ~37 kDa. Biotinylated ladder is indicated by (L) with band sizes for the ladder shown on the left (kDa). Total protein loaded for each PRL and GAPDH was at 30μg and 60μg, respectively.
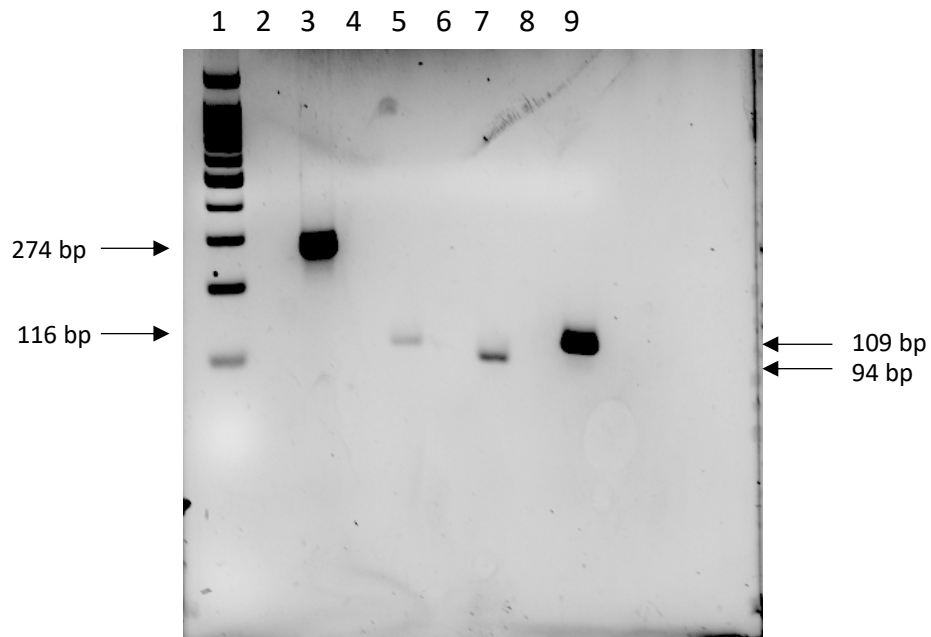
Figure 3.3 Polymerase chain reaction for detection of prolactin (PRL), prolactin receptor (PRLR), tyrosine hydroxylase (TH) and dopamine receptor type-2 (DRD2) was performed on pituitary cDNA.

Ladder (100bp; Promega) is shown in lane 1. Products are as follows: PRL (lane 3), DRD2 (lane 5), TH (lane 7), and PRLR (lane 9). Lanes 2,4,6, and 8 are no template controls for each reaction. Sizes are indicated for PRL (274bp) and DRD2 (116 bp) on the left and sizes for PRLR (109 bp) and TH (94 bp) are indicated on the right.
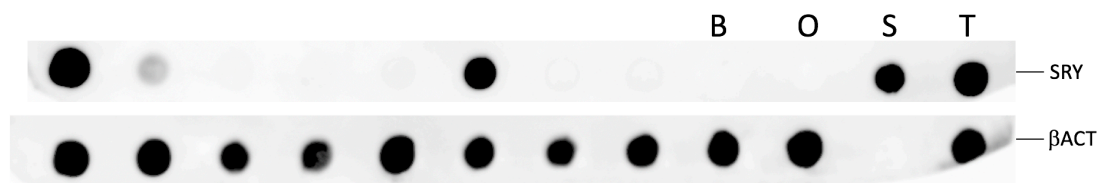
Figure 3.4 Southern blotting performed to determine sex of each pituitary sample.

Each dot represents a single sample. Probes for each blot are indicated to the right, the top blot (SRY) and the bottom blot (βACT). Control samples are noted at the top: purified PCR products for βACT (B) and SRY (S) to assess probe specificity; positive control female ovary (O) and positive control male testis (T) samples to assess density for sex. All samples to the left of controls are pituitary samples of unknown sex. All densitometry for unknown samples were compared to testis sample density. Samples were considered male if they met or exceeded the density of the testis control.
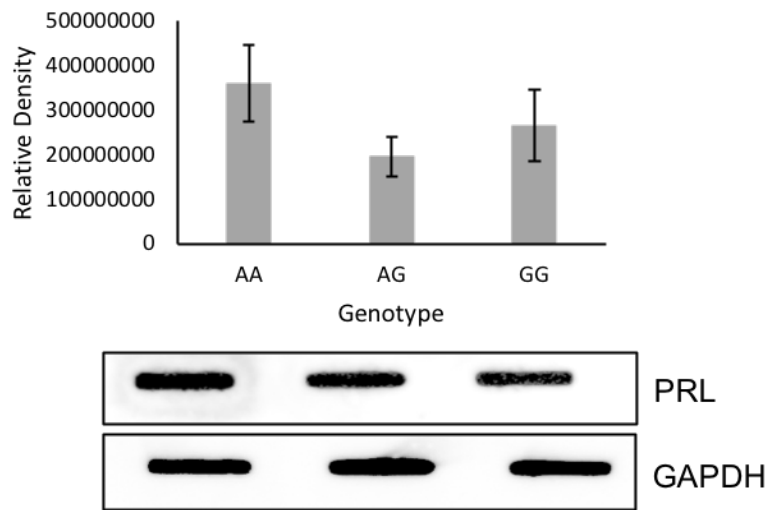
Figure 3.5 Relative PRL protein expression across DRD2 genotype in bovine pituitaries.

Relative density of PRL protein expression is shown across DRD2 genotype with error bars representative of the standard error of the mean. Slot blots are shown below the bar graph for PRL and GAPDH loading control for the respective genotypes indicated above. No differences in relative PRL protein expression were observed across DRD2 genotype.

Table 3.1 Primer sets utilized for qPCR. Primer sequences, efficiencies, and product sizes

Gene (accession no.)	Primer set ID	Forward Primer (5' to 3')	Reverse Primer (5' to 3')	Efficiency (%)	Product size (bp)
Glyceraldehyde-3-dehydrogenase (NM_001034034.2)	GAPDH5	GAGATCAAGAAGGTGGTGAAG	GCATCGAAGGTAGAAGAGTG	85.03%	122
peptidylprolyl isomerase A (AY911355.1)	Cyclo	CACCGTGTTCTTCGACATCG	ACAGCTCAAAAGAGACGCGG	96.84%	62
Actin Beta (AY141970)	BACT	CTCTTCCAGCCTTCCTTCCT	GGGCAGTGATCTCTTTCTGC	88.49%	178
Prolactin Receptor (L02549)	PRLR ICD2	ATGCACCTGGATCTTGACAG	GAGTATGGAACTTGGAGGGATG	105%	109
Dopamine Receptor D2 (NM_174043.2)	DRD2-3	CTGAACCTGTCCTGGTATGATG	GTGAGCAGCATGGCATAGTA	96.71%	116
Tyrosine Hydroxylase (NM_173884.2)	TH5	AGAGCAAAGTCCTCTGGTT	TGATCCAAGTCCAGGTCAG	93.96%	94
Prolactin (NM_173953.2)	PRL2	TTGGGTTGCTGCGCTCCTGG	CGCAGGCAGTGGAGCAGGTT	90.78%	274

OVERALL CONCLUSIONS

The goals of these experiments were to evaluate an intronic DRD2 SNP, previously found to be associated with decreased bovine serum PRL concentrations. Associations assessed included DRD2 genotype association with growth traits, semen quality, PRL serum concentrations, mRNA expression in male reproductive tissues, and PRL protein expression in bovine pituitaries.

Evaluation of DRD2 genotype association with growth traits, semen quality, and PRL serum concentrations was performed on bulls grazing (E+) or not grazing (E-) a diet containing a dopamine agonist. The presence of DRD2 was observed in testis and epididymis by immunohistochemistry and polymerase chain reaction.

Genomic DNA was isolated from semen and used for genotyping by restriction fragment length polymorphism and TaqMan techniques. Both techniques were almost identical with genotypic frequencies at 18 % AA, 61 % AG, and 21 % GG using RFLP and 21 % AA, 59 % AG, 20 % GG by the custom Taqman genotyping methods. Results on association of DRD2 genotype on serum PRL concentrations is contradictory to what has been previously found, in which there was no association found. However, this is consistent with a treatment effect in which E+ bulls experience a decrease in PRL serum concentrations. No differences were observed in growth traits and semen quality parameters due to genotype.

The message for DRD2, PRL, and TH was present in the testis, epididymis, and pituitary. Further, DRD2 was also present in sperm cells and the message for PRLR was also identified in bovine pituitary. Protein expression was evaluated in testis, epididymis,

ovary, and pituitary tissues. Western blotting and immunodetection revealed PRL expression in the pituitary as expected, but we were unable to detect PRL in reproductive tissues, as they may be below detection range. Experiments continued in the pituitary to evaluate PRL protein concentrations across genotype.

Bovine pituitaries were collected at a local abattoir. A duplex PCR and southern blotting technique were utilized to probe for SRY and Beta actin for sexing of samples. Subsequent male samples were genotyped by restriction fragment length polymorphism method for the DRD2 SNP. Genotypic frequencies were almost identical to our previous report at AA 17.4 %, AG 63 %, and GG 19.6 %. Blotting of anterior pituitary protein and immunodetection for PRL was performed. No differences in PRL protein expression were observed across genotype. These data indicate that the DRD2 SNP previously found to be associated with serum PRL concentrations is not associated with PRL protein expression, directly at the source of PRL synthesis and secretion, the pituitary.

Both studies taken together indicate that we cannot yet negate where effects on reproduction are originating. The first study indicates decreased serum PRL concentrations in bulls exposed to a dopamine agonist, indicating that DRD2 is functional. Further, genotype did not impact PRL serum concentrations indicating that the DRD2 SNP does not alter structure or function of DRD2. Dopamine receptors, DRD2 located within reproductive tissues and sperm cells and imply that they can be bound by a dopamine agonist to elicit effects directly in these tissues to alter processes such as spermatogenesis, concentration of semen or maturation; however, the possibility also remains that detrimental effects could also be due to overall decreases in serum PRL concentrations.

APPENDICES

Appendix A

Treatment for removal of endogenous peroxidase activity

Prior to immunodetection, membranes were treated with hydrogen peroxide to remove peroxidase activity for all western blot experiments assessing pituitary protein expression across genotype (Sennepin et al., 2009). To validate the procedure for removal of endogenous peroxidase activity, bovine pituitary proteins were blotted onto a 0.2 μm nitrocellulose membrane (Bio-Rad, Hercules, CA, USA) by slot blot manifold in increasing mass from 1.2 μg to 40 μg. Membranes were cut into individual strips, pre-wet in TBST for 5 min followed by individual treatment of each strip as follows: (1) treatment with hydrogen peroxide (3%) for 10 min at 37 °C (2) treatment with hydrogen peroxide (3%) for 10 min at 37 °C followed by incubation with secondary antibody only (7074; Cell Signaling Technology, Danvers, MA, USA) to assess cross reactivity (H_2O_2; Secondary only) (3) treatment with hydrogen peroxide (3%) for 10 min at 37 °C followed by GAPDH immunodetection (H_2O_2; GAPDH) (4) untreated followed by GAPDH immunodetection (Untreated; GAPDH) (2118; Cell Signaling Technology, Danvers, MA, USA) (5) Substrate only (Clarity Western ECL Substrate; Bio-Rad Hercules, CA, USA) (Substrate only). Blots not treated with hydrogen peroxide were incubated in TBST at 37 °C with gentle shaking instead. The hydrogen peroxide or TBST wash were decanted and the membranes were washed in TBST for 5 min followed by immunodetection procedures for GAPDH outlined in chapter III materials and methods.

Incubation with only ECL substrate resulted in signal at all pituitary masses, indicating endogenous peroxidase activity. Without treatment of hydrogen peroxide,

signal of GAPDH is much stronger than with treatment. To validate removal of endogenous peroxidase activity, blots were treated with the 3% hydrogen peroxide treatment followed by incubation in secondary antibody only. The secondary only blot resulted in no signal, indicating that there is no secondary antibody cross reactivity and endogenous peroxidase activity was quenched, as incubation in ECL substrate resulted in absence of signal.
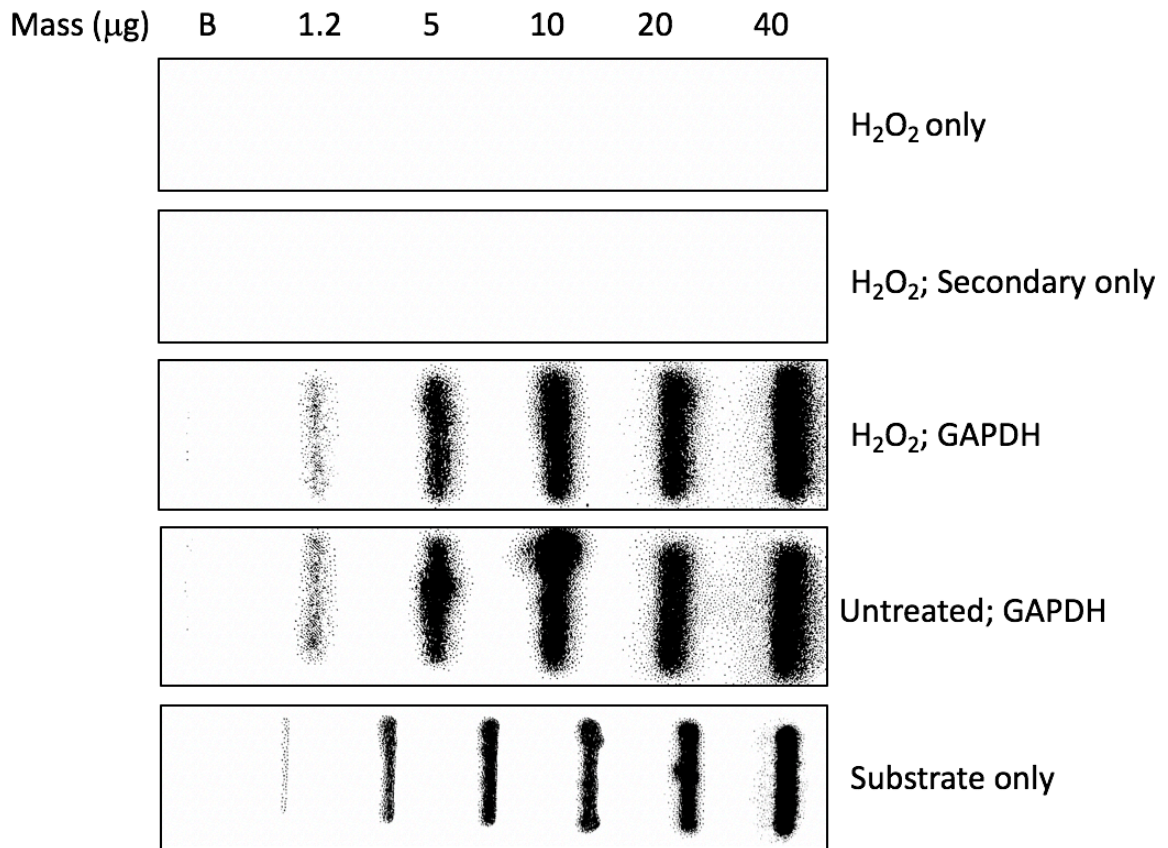
Figure A.1 Treatment of hydrogen peroxide for removal of endogenous peroxidase activity in bovine anterior pituitary.

Bovine pituitary protein blotted in increasing mass (indicated at the top of the figure) (B) indicates a blank slot with no protein. Treatments are indicated on the right side of the figure: (from top to bottom) (1) treatment with hydrogen peroxide (3%) for 10 min at 37 °C (2) treatment with hydrogen peroxide (3%) for 10 min at 37 °C followed by incubation with secondary antibody only (7074; Cell Signaling Technology, Danvers, MA, USA) to assess cross reactivity (H$_2$O$_2$; Secondary only) (3) treatment with hydrogen

peroxide (3%) for 10 min at 37 °C followed by GAPDH immunodetection (H_2O_2; GAPDH)

(4) untreated followed by GAPDH immunodetection (Untreated; GAPDH) (5) Substrate

only.

Appendix B

Methods for detection of single nucleotide polymorphisms

Identification of SNPs can be carried out through a variety of methods including, array-based hybridization, sequencing, and PCR-based methods. Hybridization techniques are utilized in array technology with two of the most popular platforms called Affymetrix and Illumina. Affymetrix chips are hybridized with oligonucleotide sequences complimentary to the SNP region of interest, with fluorescent probes for each possible allele outcome. Fragments of DNA containing the SNP of interest will bind if they are similar or an exact match to the hybridized probes on the chip. Signals are obtained from these bound fragments, in which those that are completely complimentary will bind with higher affinity and therefore obtain a stronger signal then those that bind with decreased affinity if they are not completely complimentary. It is also typical to have probes on the array that do not contain a complementary sequence that matches the SNP site in order to measure background. (LaFramboise, 2009). Illumina utilizes sequences complimentary to the adjacent sequence to the SNP of interest and has an extension from that sequence containing each possible allele outcome. The oligonucleotides, approximately 50 nt in length, are attached to an Illumina bead. The DNA will then bind to its complimentary allele and a red or green signal is obtained depending on the allele, with each separate allele labeled a specific color. Both chip technologies report over 99.5% accuracy in genotyping (LaFramboise, 2009). Either platform can be performed for targeted genotyping across many individuals as described above or whole-genome genotyping can be performed to discover new variations in DNA of individuals across treatments or phenotype. Software

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such as SNPtest can be used to determine SNPs of interest pertaining to their association with phenotype (Agler et al., 2019). Association of the mutation to phenotype is not always based on the smallest P-value, but rather, the abundance in which the mutation occurs in a large population that shares a given phenotype (Xu and Taylor, 2009).

Next generation sequencing (NGS) is performed through multiple steps and relies heavily on preparation of a library (Head et al., 2014). Genomic DNA is digested into fragments and adaptors are added to the 3' or 5' ends for recognition by the primers during sequencing. The library is amplified by PCR and sequencing performed by either pyrosequencing or sequencing by ligation (SOLiD) (Hurd and Nelson, 2009). Pyrosequencing is based on complementary strand synthesis through detection of pyrophosphate release after addition of nucleotides to the DNA. Pyrophosphate will only be released when the correct base is added onto the template strand and is then converted into ATP for the conversion of luciferin to oxyluciferin to produce signal. Light intensity corresponds to how many of that correct base is added in succession (Fakruddin et al., 2013). Sequencing by ligation is performed through emulsion PCR in which a ssDNA adaptor is added onto the 3' end of the sequence that is to be sequenced and all are attached to a bead. The beaded sequences are subjected to PCR utilizing primers specific for the adaptor sequence and probes that contain fluorescent dyes at the 5' end of different colors corresponding to each base they will recognize. Once the primers bind to the adaptor sequence, the probe will bind and DNA ligase with enable joining of the probe to the primer. The fluorescent signal is given off by this joining as it will release the fluorescent dye that was bound to the probe at the 5' end. With each round of sequencing, the extension

is melted off and the process begins again except with a primer that is 1 less base in length each time until the entire sequence is finished (Albrecht et al., 2000). Reads from these methods of sequencing can be used to identify variants between groups of samples. There are software programs available to assist in variant identification and genotyping of NGS reads. Microarrays pose issues with SNP genotyping due to nonspecific binding of similar sequences. Signal to noise ratios also become an issue when multiple sequences reach either high or low threshold making it difficult to distinguish between groups of those highly or lowly expressed SNPs. Moreover, NGS allows for SNP detection through genome wide sequencing without a reference genome, whereas microarrays are limited to prior knowledge of the genome. Due to preparation of a library and multiples steps for performance of NGS, the chance of contamination from one step to another is higher (Hurd and Nelson 2009).

Another form of hybridization detection of SNPs includes a technology called fluorescence resonance energy transfer (FRET). This technology utilizes fluorescently labeled primers that correspond to each possible allele. When the DNA sequence is complimentary to the primer, extension incorporates the labeled probe into the sequence and fluorescence is emitted. (Sobrino et al., 2005). This FRET technology has been used in conjunction with the exonuclease activity of the Taq polymerase. Taqman assays for SNP genotyping require one set of primers that flank the region containing the SNP of interest. A set of probes, each corresponding to each allele for the SNP of interest and each of a specific fluorophore color, will then hybridize to the DNA if it is the correct base match. The fluorophore is attached to the 5' end and a quencher is attached to the 3' end.

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When the both are in close proximity to one another the signal remains quenched; however, when Taq polymerase reads through and cleaves the 5' fluorophore, the fluorescent dye is relieved of its close proximity to the quencher and is able to give off a signal that can be measured in real time (Schleinitz et al., 2011) (Figure 1.5)

Kompetitive allele specific PCR (KASP) genotyping requires multiple forward primers with the base at the 5' end specific for each possible outcome of the SNP and a universal reverse primer. The forward primers will bind the SNP site but will only be able to elongate if they bind with the correct complimentary base to the SNP. A probe, complimentary to the SNP sequence is then incorporated, a separate fluorescent dye for each SNP outcome, and the sequence is amplified, and with it the signal intensifies and can be quantified (Ayalew et al., 2019).

RNase H2 enzyme-based amplification (rHAMP) also utilizes allele specific primers with a 3' blocking modification consisting of an RNA base. The assay also utilizes a set of universal primers that flank the SNP region and universal probes. The allele specific primer will bind to the complimentary SNP sequence and RNase H2 will cleave the RNA 3' modification to allow for extension by Taq polymerase. A tail sequence is incorporated in subsequent cycles and a universal probe recognizes the tail sequence to bind, and through extension, a signal is generated (Ayalew et al., 2019).

Overall, Taqman SNP genotyping allows only up to 6 bp sequences to be genotyped offering less flexibility in assay design compared to KASP or rHAMP. The Taqman and KASP techniques lack the modified 3' end primers that rHAMP technology utilizes to reduce non-specific binding and unwanted dimerization, therefore, allelic discrimination is

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more accurate and defined in rHAMP techniques compared to KASP and Taqman (Ayalew et al., 2019). Taqman is the most expensive of the three genotyping assays at ~$250 for 2,000 reactions compared to KASP and rHAMP at less than $100 for 5,000 reactions including primers and probes. Master mix is sold separately and costs ~$550 for 2,000 reactions for Taqman assays and ~$1000 for KASP and rHAMP for 10,000 reactions. Lastly, all assays utilize minimal DNA input with Taqman and KASP at a required 5 ng DNA, and rHAMP at 20 ng (Ayalew et al., 2019).

Other methods that include PCR are single strand conformation polymorphism analysis (SSCP) and restriction fragment length polymorphism (RFLP) analysis. The SSCP analysis can be used to identify polymorphism at any position (Orita et al., 1989; Rajatileka et al., 2014). Methods include isolating DNA, amplification of the sequence of interest and denaturation of products in a formamide solution. When the products are denatured, they will reveal different conformational shapes due to polymorphisms they contain that will affect how they migrate through a non-denaturing gel. These patterns can then be used to identify mutations in the DNA (Orita et al., 1989). Cost is relatively low for this method of genotyping however the use of formamide poses some extra risk in performing this procedure.

The RFLP analysis takes advantage of a mutation that may or may not abolish a restriction site. Polymerase chain reaction is used to amplify the region containing the SNP of interest. The product is purified and digested with the restriction enzyme that will cut at the restriction site where the SNP is located, resulting in multiple fragments that vary in size. The band patterns that result are used for SNP genotyping (Maeda et al., 1990).

Considerations for this method include sample number and cost, as some of the enzymes will not tolerate the taq polymerase in the original PCR reaction and cost is incurred for PCR cleanup for each reaction prior to digestion. Since there are two reactions, PCR and digestion, there is also concern of contamination from sample to sample.

Sanger sequencing is also a powerful tool to identify SNPs. This method works through production of fragments that contain dideoxynucleotides at the 3' end. These fragments are separated by capillary electrophoresis which will separate each base by molecular weight. Each is tagged with a different colored fluorescent dye at the 3' end for identification by base calls. Each base will be identified by a different color fluorescence as they pass through capillary electrophoresis (Imelfort et al., 2009). The base calls resemble peaks of fluorescence that can be used to identify SNPs by varying color and intensity of the peak. Single nucleotide polymorphisms will show overlap of signal or base calls for heterozygotes and a single signal for homozygotes. Although Sanger sequencing was once considered and may still be largely considered the hallmark of this technology, NGS has largely replaced this technique, as larger data sets can be collected for a fraction of the price, $0.25 per SNP compared to $2.95 for Sanger sequencing. Aside from price, platforms such as Illumina are also only able to perform short reads while Sanger sequencing can perform at over 500 nt (Imelfort et al., 2009).

Overall, when choosing a method for genotyping the main considerations are time, cost, sample abundance, and abundance of SNPs being studied. The NGS and microarray options are much more expensive but provide high throughput data allowing for detection of thousands of SNPs in many samples within a short time frame (Goodwin, McPherson,

and McCombie, 2016). The PCR based assays are also capable of processing thousands of samples as most assays are sold as sets of 2,000-5,000 reactions and are performed in plate format; however, the region of interest needs to be known prior unlike high throughput methods that allow for screening of the entire genome for SNPs. The cost for FRET-based qPCR assays are higher than the cost of SSCP or RFLP; however, the FRET based assays are much less labor intensive (Chen and Sullivan, 2003). Other considerations include cost of and access to equipment for high throughput processing and cost of sending samples out of the laboratory for processing, which can take some time. This extra time and cost can be worth the vast amount of data generated by high throughput screening for SNPs if the goal is to identify large quantities of SNPs across a given population or SNPs across the entire genome.
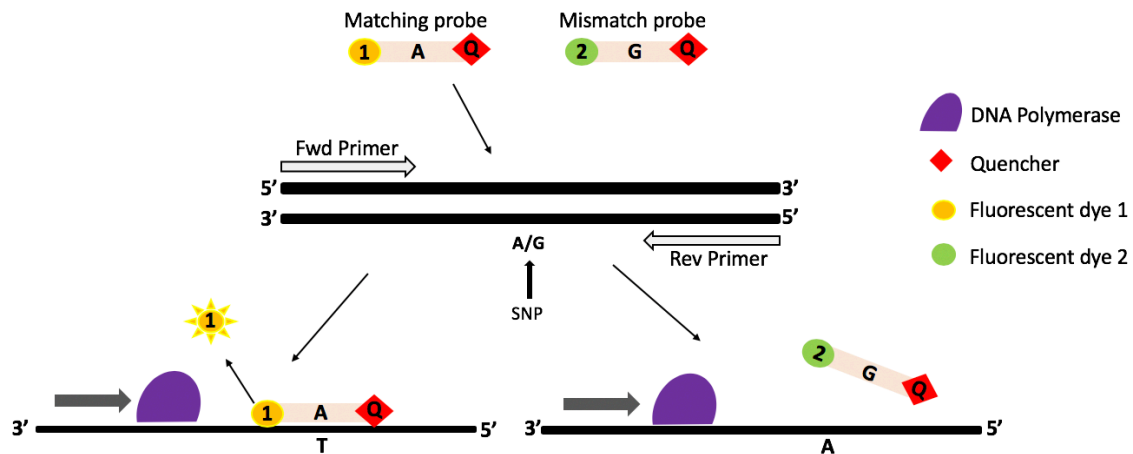
Figure B.1 Genotyping using fluorescence resonance energy transfer (FRET) technology (Taqman custom SNP genotyping assay).

(A) Primers are utilized to amplify a region containing a single nucleotide polymorphism (SNP) of interest. Custom probes that are complimentary to the SNP sequence, each contain a possible allele outcome for the SNP site with a fluorophore attached to the 5' end and a quencher attached to the 3' end of each probe. When the fluorophore is in close proximity to one another the signal remains quenched. (B) When a probe is complimentary to the SNP allele, Taq polymerase reads through and incorporates the probe while the 5' fluorophore is cleaved, displacing the fluorescent dye molecule and giving off a signal. (C) If the probe hybridizes only partially to the DNA due to a mismatch to the SNP allele, the intact labeled probe will become displaced and no signal is obtained.

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