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In the ovine pituitary, CXCR4 is localized in gonadotropes and somatotropes and increases with elevated serum progesterone

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Abstract

The pituitary is the central endocrine regulator of reproduction and in addition to various hormones regulating its actions, other molecules, such as chemokines, influence pituitary physiology as well. Despite reports over two decades ago that chemokines regulate the pituitary, much of the basic biology discerning chemokine action in the pituitary is unclear. A small number of chemokines and their receptors have been localized to the pituitary, yet chemokine ligand 12 (CXCL12) and its receptor, CXCR4 have received the most attention as both are increased in human pituitary adenomas. This chemokine duo was also reported in normal human and rat pituitary; suggestive of a functional role and that this chemokine axis might function in pituitaries from other mammalian species. To date, reports of CXCL12 and CXCR4 in pituitary from livestock are lacking and research on pituitary during pregnancy in any mammalian species is limited. Moreover, progesterone regulates CXCR4 expression in a tissue dependent manner, but whether differing concentrations of progesterone reaching the pituitary modulate CXCL12 or CXCR4 is not known. To address these gaps, our first objective was to determine if CXCL12 and CXCR4 expression and protein abundance differ in sheep pituitary during early gestation (days 20, 25, and 30 of gestation) compared to non-pregnant ewes. The second objective was to determine if CXCL12 or CXCR4 production was altered in the ovine pituitary when circulating progesterone concentrations are elevated. Expression of CXCL12 mRNA decreased on day 20 of gestation compared to non-pregnant ewes; CXCL12 protein was similar across all days tested. In non-pregnant and pregnant ewes, CXCR4 was localized to somatotropes and gonadotropes on all days tested. Abundance of CXCR4 increased in pituitary tissue from pregnant ewes with elevated circulating progesterone compared to pregnant ewes with normal circulating progesterone concentrations (control). The current study details CXCL12 and CXCR4 in normal ovine pituitary and reveals gonadotropes and somatotropes may be regulated by CXCL12/CXCR4, underscoring this signaling axis as a potential new class of modulator in endocrine functions.

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Keywords

CXCL12; CXCR4; pituitary; gonadotrope; somatotrope; progesterone

1. Introduction

Functions of the pituitary gland are essential to reproductive biology and in addition to various hormones regulating pituitary actions, other molecules, such as chemokines, influence pituitary physiology as well [1]. Though reports of chemokines regulating the pituitary were proposed over 20 years ago [2], an understanding of physiological roles and basic biology of chemokines and their receptors in the pituitary is lacking. More than 50 chemokines and approximately 20 chemokine receptors have been identified [3,4], though only a few have been observed in hypothalamus or pituitary. With respect to pituitary physiology much of the research has focused on chemokine (C-X-C motif) receptor 4 (CXCR4) and its ligand CXCL12 as both proteins are increased in human pituitary tumors, suggesting this axis might promote adenoma development [5–7]. A paucity of information on potential roles for CXCL12 and CXCR4 in normal pituitary functions exists. Using immunohistochemistry in normal human pituitary, CXCR4 was localized to ~34% of anterior pituitary cells while CXCL12 was identified in ~12% of the cells [5]. The same study noted rat pituitaries expressed CXCR4, but less CXCL12. The fact that CXCL12 and CXCR4 are expressed in normal human and rat pituitary tissue would suggest a functional role and that this chemokine duo might function in pituitaries from other mammalian species.

Our group and others have demonstrated key roles for CXCL12/CXCR4 signaling during early gestation, specifically during implantation and placentation in humans [8,9], baboons [10], sheep [11–14] and mice [15]. Interestingly, in separate studies investigating the role of CXCL12/CXCR4 in the corpus luteum (CL) we observed both proteins also increased in CL during early pregnancy compared to non-pregnant (NP) ewes (unpublished data). It was curious similar expression patterns for CXCL12 and CXCR4 existed in different reproductive tissues (i.e. endometrium, fetal extraembryonic membranes, and CL) during the same time period of early gestation. Whether CXCL12 or CXCR4 are present in ovine pituitary and which cell types may express these proteins has not been described in any livestock species; also, whether they fluctuate with pregnancy, as observed in other tissues, or if elevated serum progesterone (P4) in vivo influences CXCL12 or CXCR4 in ovine pituitaries are unknown. To address these gaps, our first objective was to determine if CXCL12 and CXCR4 synthesis differed in sheep pituitary during early gestation (days 20, 25, and 30 of gestation) compared to NP sheep (day 10 of the estrous cycle). As circulating P4 concentrations are similar during luteal phase and early gestation in sheep we were able to evaluate CXCL12 and CXCR4 in the pituitary dependent on pregnancy and not P4. The second objective was to determine if CXCL12 or CXCR4 abundance in pituitary is altered when circulating P4 concentrations are elevated. In a previous study we administered human chorionic gonadotropin (hCG) to sheep on day 4 of gestation, which resulted in sustained elevated serum P4 concentrations [16]. Intriguingly, we observed increased CXCR4 in CL and endometrium when endogenous P4 concentrations were elevated compared to pregnant

ewes on the same day of gestation with normal physiological P4 concentrations. To determine if similar responses occurred in the pituitary, we elected to analyze CXCL12 and CXCR4 in pituitary tissue collected during this study. Because a major goal was to evaluate CXCL12 and CXCR4 in pituitary from pregnant sheep, this model not only permitted examination in pregnant ewes, but also allowed us to evaluate effects due to elevated circulating P4 in vivo as opposed to exogenous P4 administration. Our data provide novel information on CXCL12 and CXCR4 in normal sheep pituitary, underscoring this signaling axis as a potential new class of modulator in endocrine functions.

2. Materials and methods

2.1 Pituitary collection from ewes on days 20, 25, and 30 of gestation

The New Mexico State University Animal Care and Use Committee reviewed and approved all experimental procedures that used animals. Pituitary tissue used for the current study was collected from our previously published work [13]. To summarize, estrus was synchronized in twenty mixed-aged western whiteface ewes during the mid-to-late luteal phase with 2 injections of dinoprost tromethamine (5 mg i.m.; Lutalyse; Pfizer, New York, NY, USA) administered 4 h apart. On detection of estrus (day 0) by a vasectomized ram, ewes were placed in experimental groups and mated to an intact ram of known fertility. Pregnant ewes (n=5/d) were anesthetized with 20 mg/kg body weight of sodium pentobarbital (Vortech Pharmacy, Dearborn, MI, 48126) on day 20, 25, or 30 of gestation and on day 10 (n=5/d) of the estrous cycle (non-pregnant [NP] control ewes). Ewes were considered pregnant based on presence or absence of a conceptus. Following exsanguination, the pituitary was removed and half snap frozen in liquid nitrogen and stored at -80°C for subsequent RNA and protein isolation. The other half was fixed in 4% paraformaldehyde.

2.2 Treatment with hCG to increase progesterone synthesis

Pituitary tissue collected from our previously published research [16] was used for the current study. Briefly, nineteen mixed-aged western whiteface ewes were randomly assigned to one of two treatments: HiP4 (600 i.u. hCG i.m.; n=9) or control (4.8 mL saline i.m.; n=10). Ewes were treated 4 d post-mating. Within each treatment, ewes were randomly assigned to one of two groups where half the ewes were euthanized 13 d post mating (control n=4; HiP4 n=5) and the remaining ewes euthanized 25 d post mating (control n=6; HiP4 n=4). Days were selected to correspond to pre-attachment (d13) and post-attachment (d25) of the conceptus to endometrium. Ewes were anesthetized with 20 mg/kg body weight of sodium pentobarbital via i.v. administration. Ewes were euthanized by exsanguination and pituitary tissues were collected as described above. Our previous publication details the statistically higher concentrations of serum progesterone observed in ewes exposed to hCG [16].

2.3 RNA isolation and cDNA synthesis

Total cellular RNA was extracted from 100 mg of pituitary tissue in 1 mL of Tri Reagent BD (Molecular Research Center Inc., Cincinnati, OH), according to manufacturer's directions, eluted in nuclease-free water, and subsequently treated with DNase using the TURBO DNA-free kit (Ambion, Foster City, CA) to ensure samples were not contaminated with genomic

DNA. The quantity and purity of RNA was determined using a NanoDrop-2000 spectrophotometer (Thermo Scientific, Waltham, MA) and stored at -80°C until further analysis. Complementary DNA was synthesized from 1 µg RNA using the iScript cDNA Synthesis Kit (BioRad) according to the manufacturer's recommendations. The products were diluted to a final volume of 100 µL (10 ng/µL).

2.4 Quantitative real-time PCR (qPCR)

The qPCR analysis was performed using a CFX96 Touch Real-Time PCR Detection System and components of the iQ SYBR green supermix (BioRad Laboratories, Hercules, CA) as previously described [13]. Forward and reverse primers were used at a final concentration of 0.525 µM and 20 ng of cDNA for each sample was assayed. The following primers were used to amplify cDNA fragments: CXCL12 (accession number XM_012105583.1) sense 5'-CCTTGCCGATTCTTTGAGAG-3', antisense 5'-GGTCAATGCACACTTGCCTA-3'; CXCR4 (accession number NM_174301) sense 5'-AAGGCTATCAGAAGCGCAAG-3', antisense 5'-GAGTCGATGCTGATCCCAAT-3'; GAPDH (accession number NM_001190390) sense 5'-CGTTCTCTGCCTTGACTGTG-3', antisense 5'-TGACCCCTTCATTGACCTTC-3'. The efficiency of all primers was tested using varying amounts of cDNA (0.05–50 ng per reaction); all were within acceptable limits [17], and no primer-dimers were formed. The qPCR conditions were 95°C for 3 min followed by 40 cycles of 95°C (30 s), 55°C (30 s), 72°C (15 s) and then a melt curve was performed per manufacture's conditions. The C_q values were attained during logarithmic amplification phase of PCR cycling. The *GAPDH* amplicon did not change across days or pregnancy status and was used to normalize each target.

2.5 Protein isolation

Protein was isolated from ovine pituitary by homogenizing 100 mg of tissue in 1 mL of RIPA buffer (50 mM Tris (pH 7.4), 2 mM EDTA, 150 mM NaCl, 0.1% sodium dodecyl sulfate (SDS), 1.0% TritonX-100) supplemented with phosphatase and protease inhibitor cocktails (Roche Applied Science, Germany). Samples were placed on ice for 15 min then centrifuged at 12,000 × *g* for 10 min at 4°C. The supernatant was collected and stored at -80°C. Concentrations of protein were determined using the BCA protein assay (Pierce, Rockford, IL).

2.6 Western blot

Unless indicated, all reagents used for western blot analysis were purchased from Bio-Rad Laboratories Inc. (Hercules, CA). Equal protein amounts (50 µg) were combined with 6X dye (187 mM Tris (pH 6.8), 6% SDS, 30% glycerol, 440 mM beta-mercaptoethanol, 0.2% bromophenol blue, deionized H₂O) and denatured on a heat block at 100°C for 5 min, then cooled and subjected to SDS-PAGE. After electrophoresis, protein was transferred to polyvinyl difluoride (PVDF) membranes for immunoblotting. After blocking in 5% non-fat dry milk made in Tris-buffered saline plus tween (TBS-T; 68.4 mM Tris base, 10 mM NaCl, 0.10% tween 20, pH 7.6) for 1 h at room temperature, membranes were incubated with primary antibody in blocking solution over night at 4°C. Antibodies used for western blot analysis were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies specific to CXCR4 (sc-9046), or beta-actin (ACTB; sc-47778) were used to probe

membranes. Membranes were washed, and an appropriate secondary antibody (goat anti-rabbit IgG-HRP, sc-2004, or goat anti-mouse IgG-HRP, sc-2005, respectively) was added in blocking solution for 1 h at ambient temperature. The working concentrations for primary antibodies were 1:1000 (CXCR4) and 1:2000 (ACTB); secondary antibodies were used at a concentration of 1:5000. Proteins were visualized by Clarity Western ECL Substrate peroxide solution and Luminol/enhancer solution for 5 min and immediately detected using the ChemiDoc™ XRS and Image Lab Software Version 3 (BioRad Laboratories, Hercules, CA). If antibody removal was required in order to assess loading controls, membranes were stripped in stripping buffer (TBS-T, 143 μ M beta-mercaptoethanol, 2% SDS) heated to 65°C, then washed with TBS-T and blocked in 5% non-fat dry milk for 1 h at room temperature. Following the stripping procedure, membranes were assessed to assure lack of chemiluminescence in the absence of antibody before being probed for the loading control.

2.7 Immunofluorescence

Pituitary tissue was collected and fixed in 4% paraformaldehyde (Polysciences Inc., Warrington, PA) for 24 h and then transferred into 70% ethanol. After fixation, samples were paraffin embedded, sectioned at 5 μ m, mounted onto glass slides, and rehydrated with a histologic clearing agent (Histoclear; National Diagnostics, Atlanta, GA, USA) and series of ethanol washes (100%, 95%, 70%, 50% ethanol, respectively). Antigen retrieval was performed by microwaving samples for 7 min in sodium citrate buffer (10 mM trisodium citrate, 0.5% Tween-20, pH 6), and allowed to cool at room temperature for 30 min. Each slide was then rinsed 2 times in phosphate-buffered saline with Triton X-100 (PBS-TX; 10 mM Na₂HPO₄, 1.76 mM KH₂PO₄, 0.14 M NaCl, 2.68 mM KCl, 0.1% Triton X-100, pH 7.4). To block nonspecific binding of antibodies, each slide was treated for 20 min with blocking buffer (1% normal goat serum in PBS). Tissue sections were incubated with specific primary antibody for CXCL12 (monoclonal mouse, R&D Systems mab350) and CXCR4 (monoclonal mouse, R&D Systems mab172) at 1:50 dilution in PBS. Tissues were incubated with CXCL12 primary antibody overnight at 4°C, or with CXCR4 primary antibody for 30 min at 37°C. CXCL12 or CXCR4 was detected with a 1:200 dilution of fluorescent secondary dyes (anti-mouse, AlexaFluor 568 Invitrogen A11031, and anti-mouse, AlexaFluor 488 Invitrogen A11001 antibodies, respectively) in PBS protected from light for 1 h at room temperature. Each slide was mounted with Fluoromount Aqueous Mounting Medium (Sigma Aldrich, St. Louis, MO, USA) with 0.5 μ g/mL 4',6-diamidino-2-phenylindole (DAPI) to stain nuclei. Images were acquired from randomly chosen areas of pituitary tissues on Axio Scope.A1 with ZenPro 2012 software (Carl Zeiss Canada Ltd, Toronto, ON, Canada). Tissue sections stained with CXCR4 were co-stained with primary antibodies for GH (polyclonal rabbit, AbD Serotec 4750–0104; 1:100) and LH (polyclonal guinea pig, Biosensis GP-036–50; 1:200) and their respective fluorescent secondary antibodies (anti-rabbit, AlexaFluor 568 Invitrogen A11005; 1:500, and anti-guinea pig, AlexaFluor 633 Invitrogen A21106; 1:200). Control sections were incubated with normal serum in place of primary antibodies.

2.8 Statistical analysis

The experimental design was completely randomized. For qPCR the C_q value of the gene of interest was normalized to the C_q value of *GAPDH* for each sample and analysis completed

using the 2^{-Cq} values for HiP4 compared to control ewes, and Cq for NP compared to early days of gestation using GraphPad Prism (Version 6 from GraphPad Software, Inc.). The chemiluminescent signals for western blots were quantified using the mean value (intensity) using Image Lab software (Version 4.1). Each band of interest was normalized by dividing by mean value (intensity) for the protein of interest divided by mean value (intensity) of ACTB. Significant changes were determined at $P<0.05$ using one-way ANOVA for comparing different days of gestation. Significant changes were determined at $P<0.05$ using an unpaired, two-tailed student's t -test comparing control and HiP4 ewes. If the variance significantly differed, Welch's correction was used. Mean fluorescent intensity was measured using ImageJ 1.47v software. For comparison of CXCR4 protein in gonadotrope and somatotrope, the mean fluorescent intensity was measured from individual cells co-stained with LH or GH and intensity values within a cell type were averaged. Data was graphed using the mean value for each day and SEM in GraphPad Prism.

3. Results

3.1 Expression of CXCL12 mRNA decreases in ovine pituitaries on day 20 of gestation and CXCR4 mRNA is upregulated when circulating P4 concentrations are increased

Pituitary expression of *CXCL12* was diminished in pregnant sheep on day 20 of gestation compared to NP controls, while *CXCR4* was similar across days of gestation (Fig. 1A and 1B). Gene expression of *CXCL12* was similar between control and HiP4 ewes on days 13 and 25 (Fig. 1C). Expression of *CXCR4* mRNA on days 13 ($P<0.05$) and 25 ($P<0.01$) of gestation increased in HiP4 ewes compared to controls ewes with normal physiological concentrations of P4 (Fig. 1D). Circulating concentrations of P4 are greater and remain elevated in pregnant ewes treated with hCG on day 4 of gestation compared to control (Fig. 1E).

3.2 Ewes synthesizing greater concentrations of P4 have increased CXCR4 protein in ovine pituitary tissue

To ascertain if the noted increase in *CXCR4* mRNA in HiP4 ewes resulted in enhanced protein abundance, immunoblotting for CXCR4 protein was conducted. Abundance of pituitary CXCR4 was detected by western blot on days 13 and 25 of pregnancy in both control and HiP4 ewes (Fig. 2). Similar to mRNA expression, greater amounts of CXCR4 protein were observed in ewes with elevated serum P4 concentrations. On day 13 of gestation, CXCR4 tended ($P=0.09$) to increase in HiP4 compared to control ewes (Fig. 2A and 2B). On day 25, CXCR4 protein abundance significantly ($P<0.01$) greater in pituitary tissue from HiP4 ewes compared to controls (Fig. 2C and 2D).

3.3 Localization of CXCR4 protein in somatotropes and gonadotropes

We observed changes in CXCR4 protein with immunoblots conducted on whole-tissue isolations, but were unable to delineate which cell type was responsible for CXCR4 production. We sought to determine if CXCR4 protein was localized to gonadotropes and somatotropes. As gonadotropins and growth hormone (GH) play important roles during pregnancy [18–20], we performed immunofluorescent imaging for CXCR4, GH, and luteinizing hormone (LH) in fixed pituitary tissues collected during early pregnancy.

Sections of pituitary were probed with antibodies against CXCR4, GH and LH (Fig. 3). Immunoreactive CXCR4, GH, and LH were detected on days 20, 25, and 30 of gestation and in NP ewes (Fig. 3A). CXCR4 staining was perinuclear and evident in somatotropes and gonadotropes as shown in the merged panels of Figure 3. Other pituitary cells not specifically distinguished in this study were also positive for CXCR4. The abundance of CXCR4 was significantly ($P<0.01$) higher in somatotropes compared to gonadotropes (Fig. 3C). Akin to pituitary sections on different days of gestation and NP ewes, pituitary tissue from control or HiP4 ewes displayed abundant immunoreactivity for CXCR4, which was present in somatotropes, gonadotropes and other pituitary cell types (Fig. 4).

3.4 Less CXCL12 protein compared to CXCR4 in ovine pituitary

Gene expression of *CXCL12* was reduced on day 20 compared to NP (Fig. 1). To determine if CXCL12 protein abundance was influenced by day of gestation in the pituitary, immunofluorescent imaging was performed on pituitary sections to determine CXCL12 immunoreactivity. Immunopositive cells for CXCL12 were detected in pituitary sections from pregnant ewes on days 20, 25, and 30 as well as NP ewes (Fig. 5). The CXCL12 protein was not as robustly apparent as CXCR4 and appeared scattered throughout the pituitary. Likewise, control or HiP4 ewes revealed CXCL12 immunopositive cells in the pituitary gland (Fig. 6). The nature of the CXCL12 staining appears cytosolic or extracellular in many sections of the pituitary, which may represent secreted CXCL12.

4. Discussion

The pituitary is often termed the “master gland” because it is the central endocrine regulator of growth, reproduction, metabolism and responses to stress. Though reports of chemokines regulating the hypothalamic-pituitary axis were proposed over 20 years ago [2], an understanding of physiological roles and basic biology of chemokines and their receptors in the pituitary is lacking. Our laboratory has primarily focused on the chemokine-receptor pair CXCL12/CXCR4 and its role during early gestation at the fetal-maternal interface. However, our studies and others [9,11,13,14,21–24] demonstrate the CXCL12/CXCR4 signaling axis is a pivotal player in many biological functions and is regulated by steroid hormones [25–27]. To advance our understanding of CXCL12/CXCR4 in reproductive biology, we sought to determine if the ovine pituitary produces CXCL12 and CXCR4, if synthesis changes during early gestation, and if elevated concentrations of P4 production in vivo alter CXCL12 and CXCR4 abundance.

First, we evaluated mRNA expression of *CXCL12* and *CXCR4* in pituitary tissues collected from either NP ewes on day 10 of the estrous cycle or days 20, 25, or 30 of pregnancy. Transcripts for both ligand and receptor were observed across all days tested with only *CXCL12* displaying a significant decrease in expression on day 20 compared to NP, while *CXCR4* mRNA was similar on all days (Fig. 1). To decipher if changes in *CXCL12* mRNA correlated with protein abundance, we probed for CXCL12 in pituitary sections using immunofluorescence. Immunoreactive CXCL12 was observed in ovine pituitary tissue on all days tested (Fig. 5) with sparse distribution. Similar reports in humans highlight low amounts of CXCL12 protein in the pituitary with paltry immunoreactivity [5]. We also

attempted evaluating CXCL12 protein using western blot (data not shown), but despite detecting CXCL12 in placental tissue [13], we were unable to detect it using immunoblotting in ovine pituitary. Based on the low expression noted with immunofluorescent imaging and observations that CXCL12 is often secreted, it may be difficult to analyze CXCL12 in whole pituitary protein isolates. Further, the hypothalamus synthesizes CXCL12 [28,29] and it is possible CXCL12 travels from the hypothalamus or through systemic circulation from other sources to activate CXCR4 in the pituitary. Regardless of whether CXCL12 is inducible in the pituitary during early pregnancy or not, our data demonstrate ovine pituitary tissue produce CXCL12 both during gestation and without pregnancy.

Immunofluorescent imaging for CXCR4 in ovine pituitary sections revealed robust staining (Fig. 3 and 4) which is in agreement with previous studies in human pituitary tissue demonstrating greater amounts of CXCR4 compared to CXCL12 [5]. Interestingly, CXCR4 cellular localization appeared predominantly in the nuclear membrane and/or nucleus as opposed to plasma membrane. Others have noted similar nuclear expression and activation of CXCR4 in a variety of cells and tissue types [30,31]. The cellular localization of CXCR4 did not change across different days of pregnancy or from NP ewes (data not shown). Whether differential nuclear or plasma membrane localization for CXCR4 will dictate functional variances in receptor action in the pituitary remains to be determined. To define which cell types were CXCR4 positive, we employed colocalization studies probing for CXCR4 in conjunction with either antibodies recognizing GH or LH. Analogous to studies completed in rats and humans [5,32], we observed CXCR4 in ovine somatotropes (Fig. 3). However, in human and rat studies it is not clear if pituitary samples were from males or females, or, regarding the latter, if samples were at specific stages of the menstrual/estrous cycle or from pregnant individuals. Notably, in our study CXCR4 was analyzed only in female sheep and either from NP or on three different days of early gestation to provide a foundation for understanding potential normal functions of CXCL12/CXCR4 in the pituitary gland. Though expression patterns or cellular localization of CXCR4 did not appear to differ in pituitary tissue from NP ewes or on varying days gestation, the presence of CXCR4 in somatotropes may indicate another method by which GH secretion is regulated in sheep. Indeed, treatment of rat primary pituitary cells as well as the rat adenoma-derived cell line GH4C1 and somatotrope GH3 cell line with CXCL12 results in GH secretion [32–34]. Additional studies are needed to determine if similar actions of CXCL12 occur in ovine pituitary cells.

Release of gonadotropins from the anterior pituitary is paramount for normal reproductive functions. To investigate potential regulation through CXCL12/CXCR4 signaling in gonadotropes, we performed colocalization experiments of CXCR4 with LH in ovine pituitary tissue. Interestingly, we observed immunoreactive CXCR4 in gonadotropes (Fig. 3 and 4), suggestive of possible influence on gonadotrope functions by CXCL12. Though CXCR4 was found in LH producing cells, CXCR4 was more prominent in somatotropes compared to gonadotropes. Whether this is indicative of functional consequences warrants additional studies. CXCR4 was also evident in ovine cells not producing GH or LH. In human pituitary, in addition to GH, CXCR4 was observed in ACTH- and prolactin-producing cells as well as cells not synthesizing hormones, which may represent

undifferentiated/progenitor cells [5]. Similar colocalization of CXCR4 may exist in sheep, accounting for CXCR4 positive cells that did not co-stain with chosen markers. To our knowledge, this is the first report of CXCR4 in gonadotrope cells, which is exciting and may represent additional mechanisms influencing gonadotrope functions and thus overall reproductive physiology through the hypothalamic-pituitary-gonadal axis. Specifically, based on known roles of CXCL12/CXCR4 regulating cell migration, proliferation, and survival in other tissues, it's possible this chemokine duo performs similar functions in pituitary cells.

Steroid feedback to the hypothalamus and pituitary is central to controlling release of pituitary hormones. Given that P4 regulates CXCL12 and CXCR4 expression [26] and the presence of CXCR4 on gonadotropes in the current study, we evaluated if increasing P4 synthesis in vivo and thus the concentration of P4 reaching the pituitary alters CXCR4 production. As we previously reported, a single dose of hCG to sheep on day 4 of gestation results in significantly greater P4 production and serum P4 concentrations up to day 25 of gestation [16]. We analyzed CXCL12 and CXCR4 mRNA and protein in the pituitary from control and HiP4 ewes on days 13 and d25. Gene expression for *CXCL12* was similar across treatments and days, yet CXCR4 mRNA and protein significantly increased in pituitary tissue from sheep with greater P4 serum concentrations (Fig. 1 and 2). Because only one administration of hCG was given on day 4 of gestation, we propose the increase in pituitary CXCR4 on days 13 and 25 is due to higher concentration of P4 signaling in the pituitary, as hCG should not be present at these time points after hCG administration. However, we cannot rule out hCG as an inducer of CXCR4 as human first trimester decidua tissue treated with hCG results in elevated CXCR4 [8] and similar regulation was observed in baboons [10]. It is important to note, our study was conducted in vivo and demonstrates that pituitary abundance of CXCR4 is enhanced when P4 concentrations are elevated. We previously observed an analogous increase of CXCR4 in endometrium from these same sheep with elevated concentrations of P4 [16] and, as this agrees with reports in human mammary cells [26], we suggest CXCR4 is regulated by P4. This integration of steroid and chemokine signaling at the pituitary may represent a novel avenue that contributes to control of the pituitary-gonadal axis.

5. Conclusions

Dysregulation of CXCL12/CXCR4 signaling is involved in several malignant diseases and both are constitutively overexpressed in pituitary adenomas with resultant alterations in cell survival, proliferation, as well as hormone hypersecretion. However, little is known about normal physiological functions of CXCL12 and CXCR4 in the pituitary and to date nothing has been reported of this axis in sheep pituitary. Our study provides novel insights into possible roles of CXCL12/CXCR4 signaling in the ovine pituitary. Collectively, our data provide the first information on pituitary production of CXCL12 and CXCR4 in sheep and the presence of CXCR4 in gonadotropes, underscoring this signaling axis as a potential new modulator in endocrine functions.

The CXCL12/CXCR4 axis may exert autocrine/paracrine effects on pituitary cells as a means to maintain homeostasis or alert to pathological conditions. This axis may also be

involved with pituitary hormone secretion. In rat pituitary adenoma cells CXCL12 stimulates GH secretion [33], but if similar functions occur in livestock is not known. Based on our finding that CXCR4 is found in gonadotropes, future research will explore whether this chemokine axis regulates release of gonadotropins. While other chemokines might affect pituitary function, we suggest CXCL12/CXCR4 signaling serves to fine-tune and regulate the hypothalamic-pituitary axis. Our data provide a platform for future studies to delineate specific functions of CXCL12/CXCR4 on pituitary physiology and reveal this axis can be modulated in vivo by altering P4 concentrations.

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Abbreviations

CXCL12	chemokine (C-X-C motif) ligand 12
CXCR4	chemokine (C-X-C motif) receptor 4
hCG	human chorionic gonadotropin
NP	non-pregnant
P4	progesterone
GH	growth hormone
LH	luteinizing hormone

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Highlights

- The first study to demonstrate CXCL12 and CXCR4 in ovine pituitary.
- Ovine somatotropes and gonadotropes are CXCR4+
- The first report of CXCR4 present in gonadotropes in any mammalian species.
- Ewes with elevated serum levels of P4 exhibit greater levels of CXCR4.
- CXCL12/CXCR4 signaling may modulate endocrine functions.

Figure 1. Expression of *CXCL12* mRNA is altered in sheep pituitary during early gestation and elevated P4 serum concentrations correlate with increased *CXCR4* mRNA

Gene expression in the ovine pituitary was conducted using qPCR from non-pregnant (NP; day 10 of the estrous cycle) or pregnant ewes on days 20, 25, and 30 of gestation, or on days 13 and 25 after hyperstimulating P4 synthesis (HiP4). (A) *CXCL12* mRNA decreased on day 20 of gestation compared to NP, but was similar to NP on days 25 or 30 of gestation. (B) Expression of *CXCR4* mRNA in pituitary tissue was similar during early gestation and NP control. (C) Ewes with normal physiological concentrations of P4 (control) and HiP4 ewes express similar amounts of *CXCL12* mRNA on days 13 and 25 of gestation in the pituitary. (D) Expression of *CXCR4* mRNA increased in the pituitary from HiP4 ewes on days 13 and day 25 of pregnancy. (E) On day 4 of gestation, ewes that received hCG (HiP4) displayed

higher concentrations of serum P4 compared to ewes receiving saline alone (Control).
*P<0.05, **P<0.01.

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Figure 2. In the ovine pituitary CXCR4 abundance increases with elevated serum P4

Equal amounts of pituitary protein lysate were subjected to SDS-PAGE; immunoblotting was used for detection of CXCR4 on days 13 and 25 of gestation in ewes with normal physiological concentrations of P4 (control) or after hyperstimulating P4 synthesis (HiP4). The same protein samples were also probed for beta-actin (ACTB) to further verify equal loading of protein. Optical density of CXCR4 and ACTB were assessed, and values represent the mean $\pm$ SEM. (A) Representative blot for CXCR4 in pituitary tissue from control or HiP4 ewes on day 13 of gestation. (B) Compared to control, HiP4 ewes tended to synthesize more CXCR4 in the pituitary. (C) Representative blot for CXCR4 in the pituitary from control and HiP4 ewes on day 25 of gestation. (D) Compared to control, HiP4 ewes produce more CXCR4 in the pituitary. [^]P=0.09, ^{**}P<0.01.

Figure 3. Production of CXCR4 is evident in ovine somatotropes and gonadotropes with predominant nuclear localization

Pituitary sections from non-pregnant (NP; day 10 of the estrous cycle) or pregnant ewes on days 20, 25, and 30 of gestation were subjected to immunofluorescent imaging. (A) Representative immunofluorescent staining for CXCR4 (green), growth hormone (GH; magenta) marking somatotropes, or luteinizing hormone (LH; red) indicating gonadotropes in pituitary sections from NP or pregnant ewes on days 20, 25, and 30 of gestation. Immunolocalization of CXCR4 was predominantly perinuclear in the pituitary on all days tested and colocalized with GH and LH as shown in merged panels. (B) Greater CXCR4 protein production was observed in somatotropes (white arrowheads) compared to gonadotropes (yellow arrow). (C) Mean fluorescent intensity for CXCR4 in somatotropes compared to gonadotropes. (D) Pituitary sections were incubated with normal serum and secondary antibodies as negative controls; nuclei were counterstained with DAPI (blue). Original magnification, 40x. Scale bar: 5 μ m. ** $P < 0.01$.

Figure 4. The CXCR4 is found in somatotropes and gonadotropes with predominant nuclear localization in ewes with normal or elevated serum P4 concentrations

Pituitary sections from pregnant ewes on day 25 with normal physiological concentrations of P4 (control) or after hyperstimulating P4 synthesis (HiP4) were subjected to immunofluorescent imaging. Representative immunofluorescent staining for CXCR4 (green), growth hormone (GH; magenta) marking somatotropes, and luteinizing hormone (LH; red) indicating gonadotropes in pituitary sections is presented for control and HiP4 ewes. Staining for CXCR4 was perinuclear in the pituitary regardless of treatment and colocalized with GH and LH as shown in merged panels. Nuclei stained with DAPI (blue). Original magnification, 40x. Scale bar: 5 μ m.

Figure 5. In the ovine pituitary, CXCL12 protein is sparsely distributed during early gestation
 Pituitary sections from non-pregnant (NP; day 10 of the estrous cycle) or pregnant ewes on days 20, 25, and 30 of gestation were subjected to immunofluorescent imaging. Representative immunofluorescent staining for CXCL12 (yellow) in pituitary sections from NP or pregnant ewes on days 20, 25, and 30 of gestation is displayed. Immunoreactive CXCL12 (arrows) was cytoplasmic in pituitary of NP ewes and during early gestation. Nuclei stained with DAPI (blue). Original magnification, 40x. Scale bar: 5 μ m.

Figure 6. Ewes with normal or elevated serum P4 concentrations display sparsely distributed CXCL12 protein in the pituitary on day 25 of gestation

Pituitary sections from pregnant ewes on day 25 with normal physiological concentrations of P4 (control) or after hyperstimulating P4 synthesis (HiP4) were subjected to immunofluorescent imaging. A representative immunofluorescent image is shown for immunoreactive CXCL12 (yellow) in pituitary sections revealing cytoplasmic staining of CXCL12 (arrows). Nuclei stained with DAPI (blue). Original magnification, 40x. Scale bar: 5 μ m.