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Elevated utero/placental GR/NR3C1 is not required for the induction of parturition in the dog

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Abstract

The endocrine mechanisms that lead to initiation of parturition in dogs are still not fully understood. The prepartum luteolysis is associated with increased prostaglandin (PG) F2α secretion; however, there is no pregnancy- or parturition-related increase in estrogens. Moreover, unlike in other mammalian species, in the dog, increased peripartum levels of cortisol measured sporadically in maternal peripheral blood are not mandatory for normal parturition. Nevertheless, auto/paracrine effects of cortisol at the placental feto-maternal level cannot be excluded. Therefore, the aim of this study was to investigate the expression and localization of glucocorticoid receptor (GR/NR3C1) in canine utero/placental (Ut/Pl) units and uterine interplacental sites at selected time points during pregnancy (pre-implantation, post-implantation and mid-gestation), and at normal and antigestagen-induced parturition. The Ut/Pl expression of *GR/NR3C1* did not change significantly from pre-implantation until mid-gestation; however, it was strongly induced during the prepartum luteolysis. Within the interplacental samples, expression of *GR/NR3C1*-mRNA was greater post-implantation than pre-implantation and did not change afterward, i.e. toward mid-gestation. Compartmentalization studies within the Ut/Pl units, involving placenta, endometrium and myometrium separately, performed at the prepartum luteolysis revealed the highest *GR/NR3C1*-mRNA levels in placenta compared with endometrium and myometrium. Interestingly, in antigestagen-treated mid-pregnancy dogs, Ut/Pl and interplacental *GR/NR3C1*-mRNA expression remained unaffected. At the cellular level, placental GR/NR3C1 was clearly detectable in *placenta fetalis*, i.e. in trophoblast cells. In conclusion, increased expression of GR/NR3C1 during normal parturition, but not following antigestagen-treatment, suggest that it is not required for initiating the signaling cascade of PG synthesis leading to the induction of parturition in the dog.

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Introduction

Cortisol is a steroid hormone produced mainly by the adrenal gland; its production increases in response to stress. Acting via its nuclear receptor subfamily 3, group C, member 1 (NR3C1 or glucocorticoid receptor; GR), cortisol regulates many reproductive processes including fetal and placental development as well as initiation of parturition (Chida *et al*. 2011, Li *et al*. 2014, Wyrwoll 2014). Fetal plasma concentrations of cortisol increase at the end of gestation in several mammalian species, e.g. cattle, sheep and goat, due to maturation of the fetal hypothalamic–pituitary–adrenal axis (Smith *et al*. 1973, Challis *et al*. 1977, Flint *et al*. 1978, Magyar *et al*. 1980). As also shown in sheep (Mason *et al*. 1989), glucocorticoids and presumably also increased fetal cortisol levels stimulate the activity of placental cytochrome P450 17α hydroxylase (CYP17A) and cytochrome P450 (P450arom). This results in a switch of placental steroid production in favor of estrogen, i.e. redirection of steroid synthesis from the delta(Δ)4 to Δ5 pathways (Mason *et al*. 1989). Similar to the sheep, bovine placental CYP17A activity is strongly upregulated at parturition (Schuler *et al*. 1994, Schuler *et al*. 2006). Additionally, the role of locally acting steroid sulfatase (STS) in placental production of free, active estrogens from biologically inactive sulfoconjugated estrogens during the prepartal increase in estrogens has been postulated in cattle (Janszen *et al*. 1995, Greven *et al*. 2007). The decreased progesterone (P4)/estradiol-17β (E_2) ratio causes elevated uterine and myometrial oxytocin receptor (OTR) expression (Larcher *et al*. 1995, Wu *et al*. 1996, Helmer *et al*. 1998, Robinson *et al*. 2001), which results in increased sensitivity to oxytocin. Oxytocin is capable of inducing uterine synthesis of luteolytic and contractile prostaglandins (PGs) as shown in cattle (Fuchs *et al*. 1999) and sheep (Meier *et al*. 1995).

Interestingly, in addition to the above-described roles of cortisol and GR/NR3C1 during initiation of parturition in large animal species, a parallel observation in humans showed that GR/NR3C1 may act as a local placental antigestagen, allowing parturition in women to take place in spite of relatively high circulating P4 levels (Karalis *et al*. 1996). Furthermore, peripartum cortisol is important for fetal organ development, especially

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maturation of the lung (Bolt *et al*. 2001, Chida *et al*. 2011). In mice, GR-knockout results in perinatal death of fetuses, possibly due to lack of surfactant production that is necessary to decrease surface tension at the air–liquid interface of the alveoli in the lung (Bolt *et al*. 2001, Chida *et al*. 2011).

As for the dog, increased levels of cortisol measured sporadically in maternal peripheral blood peripartum are not mandatory for normal parturition, which generally occurs 63 days after mating, and have been attributed by some authors to maternal stress (Concannon *et al*. 1978, Hoffmann *et al*. 1994). In line with this, application of the antigestagen aglepristone to early- and mid-pregnancy dogs did not affect circulating levels of cortisol (Fieni *et al*. 2001). An important feature of canine reproductive physiology is the lack of placental steroidogenic activity (Hoffmann *et al*. 1994, Nishiyama *et al*. 1999). Therefore, the maintenance of pregnancy in this species entirely depends on luteal secretion of P4 (Concannon *et al*. 1989). The prepartum drop in P4 concentration is, however, associated with increased placental PGF2α output (Nohr *et al*. 1993, Kowalewski *et al*. 2010). Importantly, in contrast to other domestic animal species, e.g. cattle, in dogs, there is no pregnancy- or parturition-associated increase in E_2 concentration (Nohr *et al*. 1993, Hoffmann & Schuler 2002). This excludes any possible roles of cortisol and GR/NR3C1 as trigger factors in the placental 'switching mechanism' between the Δ4 and Δ5 steroidogenic pathways in dogs. The source of the prepartum PGF2α seems to be predominantly within the utero/placental compartment (Ut/Pl), specifically in the fetal trophoblast cells, in which all prostaglandin system members are present (Kowalewski *et al*. 2010, Gram *et al*. 2013, Gram *et al*. 2014*b*). P4 has a signaling function in the prepartum PGF2α release, acting at the feto-maternal interface between the trophoblast cells and the maternal stroma-derived decidual cells. The decidual cells are the only cells of the canine placenta expressing progesterone receptor (PGR) (Vermeirsch *et al*. 2000, Kowalewski 2012) and OTR (Gram *et al*. 2014*a*). Interfering with PGR function in these cells, e.g. by applying a receptor-specific blocker (antigestagen), leads to changes in utero-placental PG synthesis similar to those observed during normal prepartum luteolysis, and results in elevated PGF2α output (Nohr *et al*. 1993, Kowalewski *et al*. 2010, Gram *et al*. 2014*a*).

Apart from the variable plasma cortisol concentrations found in bitches during spontaneous whelping, no information is available concerning the localization and expression levels of its receptor GR/NR3C1 in canine reproductive tissues. Therefore, possible endocrine effects of cortisol at the feto-maternal level, and their involvement in the process of parturition in dogs, cannot be excluded. Consequently, this study aimed to better understand underlying endocrine mechanisms by investigating the expression and localization of GR/NR3C1 in canine Ut/Pl units and interplacental uterine sites during selected time points of pregnancy, as well as during natural and induced parturition.

Materials and methods

Animals, tissue collection and preservation

Tissues were obtained from healthy, cross-breed pregnant bitches (2–8 years of age) by ovariohysterectomy (OHE) at selected stages of gestation, as follows: pre-implantation (days 8–12, $n=5$), post-implantation (days 18–25, $n=5$), mid-gestation (days 35–40, $n=5$) and prepartum luteolysis ($n=3$). Additionally, mid-pregnancy dogs (days 40–45 of pregnancy; $n=10$) were treated twice with the antigestagen aglepristone in order to selectively block the function of PGR (Alizine, Virbac, Bad Oldesloe, Germany; 10 mg/kg bw; 2×/24 h apart); then, tissue samples were collected 24 h ($n=5$) and 72 h ($n=5$) after the second treatment.

Dogs were mated 2–3 days after ovulation (time needed for oocyte maturation and completion of the first meiotic division), which was determined by vaginal cytology and P4 measurements (>5 ng/mL in peripheral blood). The day of mating was then designated as Day 0 of gestation. The pre-implantation stage of gestation was determined by finding free-floating embryos in uterine flushings. The prepartum luteolysis (prepartum progesterone decrease) was assessed by performing regular measurements of circulating P4 concentrations by well-established in-house radioimmunoassay (RIA) after sample extraction with hexane (Hoffmann *et al*. 1973) every 6 h, starting from Day 58 of pregnancy. Once a steep P4 decline was observed over three consecutive measurements, OHE was performed. The canine placenta, which is classified as endotheliochorial, zonaria (Amoroso 1952, Kehrer 1973), starts to form immediately after implantation, around day 17–18 of embryonal life. Besides the formation of decidua, the strong invasion by trophoblast is associated with the development of marginal hematomas, and formation of the so-called glandular chambers, which are highly enlarged superficial endometrial glands. These establish a thick border layer between the placental labyrinth and the connective tissue covering the deep endometrial glands. Their development continues until around Day 40, when fully developed Ut/Pl units are established (Amoroso 1952). Accordingly, samples collected between days 35 and 45 of gestation were referred to as mid-gestation samples in our experiments.

All tissue samples were used for our previous studies (Kowalewski *et al*. 2010, Gram *et al*. 2013). Experimental procedures utilizing dogs were performed according to the guidelines of animal welfare legislation and approved by the respective authorities of Justus-Liebig University, Giessen (permit no. II 25.3-19c20-15c GI 18/14 and VIG3-19c-20/15c GI 18,14), Giessen, Germany, and the University of Ankara (permit no. Ankara 2006/06), Ankara, Turkey. Respective mean P4 concentrations were previously reported (Kowalewski *et al*. 2009): 35.71 ± 7.9 ng/mL during the pre-implantation period, 29.73 ± 13.23 ng/mL post-implantation, 13.32 ± 8.66 ng/mL at mid-gestation and 2.07 ± 0.99 ng/mL during the prepartum luteolysis. After Alizine treatment, P4 concentrations were as follows (Kowalewski *et al*. 2009): 15.11 ± 6.7 ng/mL before the

first application; 13.61 ± 8.2 ng/mL at the second treatment; 5.1 ± 2.7 ng/mL 24 h later; 2.33 ± 1.44 ng/mL 48 h later and 1.2 ± 0.6 ng/mL 72 h later. No additional serum samples were available for this study.

Following OHE, uterine samples (following implantation, these tissues were referred to as interplacental sites) and utero/placental compartments (the whole thickness of the uterine wall, i.e. uterus with adjacent placenta, comprising *placenta fetalis* and *placenta materna*; Ut/Pl) were rinsed with phosphate-buffered saline (PBS), trimmed of surrounding connective tissue and preserved for further experiments. The Ut/Pl units were collected from the middle part of the placental girdle, avoiding marginal hematomas. No interplacental samples collected during prepartum luteolysis were available for the study.

For immunohistochemistry (IHC), tissue samples were fixed by immersion in 10% neutral phosphate-buffered formalin for 24 h at +4°C and subsequently washed for 1 week with PBS and embedded in paraffin. For total RNA retrieval, samples were incubated in RNAlater (Ambion Biotechnology GmbH) for 24 h at +4°C and stored at −80°C until further use.

Total RNA isolation and real-time (TaqMan) PCR

Total RNA was extracted using the standard chloroform-isopropanol method with TRIzol reagent (Invitrogen). The total RNA amount and purity in each sample were assessed using a NanoDrop 2000C spectrophotometer (Thermo Fisher Scientific AG). In the next step, DNAse treatment with RQ1 RNase-free DNase (Promega) of total RNA from each tissue was performed according to the supplier's instructions. Reverse transcription (RT) was set up with random hexamers as primers and other reagents from Applied Biosystems by Thermo Fisher Scientific as reported previously (Kowalewski *et al*. 2006*b*, Kowalewski *et al*. 2011). Semiquantitation of canine *GR/NR3C1* was performed by real-time (TaqMan) PCR using an automated fluorometer ABI PRISM 7500 Sequence Detection System (Applied Biosystems by Thermo Fisher Scientific). A commercially available gene- and canine species-specific TaqMan system of canine *GR/NR3C1* was used, which was purchased from Applied Biosystems (Prod. No. Cf02627498_m1). In order to exclude any potential genomic DNA contamination, PCR reactions were performed with samples that were not reverse-transcribed (the so-called 'minus-RT control'). For an additional control, water was used instead of cDNA. The reaction mixture consisted of 200 nM TaqMan Probe, 300 nM of each primer, 12.5 µL Fast Start Universal Probe Master (ROX) (Roche Diagnostics) and 5 µL cDNA corresponding to 100 ng total RNA per sample. The thermal cycler setups were as follows: denaturation at 95°C for 10 min, 40 cycles at 95°C for 15 s and 1 cycle at 60°C for 60 s. The comparative delta CT threshold method (ΔΔCT) was used for quantitation of target gene according to the manufacturer's protocols for the ABI PRISM 7500 Sequence Detection System (Applied Biosystems) and as described previously (Kowalewski *et al*. 2006*b*, Kowalewski *et al*. 2011). The sample with the lowest concentration of the target gene served as the calibrator in the ΔΔCT method. *GAPDH*, *18SrRNA* and *CYCLOPHILIN A* were used as reference genes for normalization of relative gene expression. The efficiency of all TaqMan assays was tested by the CT slope method with a range of 10-fold serial dilutions of the targeted PCR product and using samples from different stages of pregnancy (in order to test the efficiency at different quantities of the targeted RNA expected at different stages of pregnancy) according to the instructions of the manufacturer of the ABI PRISM 7500 Sequence Detector, and was set up to achieve approximately 100% efficiency. Primer Express software version 2.0 (Applied Biosystems by Thermo Fisher Scientific) was used to design the *GAPDH* and *18SrRNA* primers and probes. The following TaqMan systems including 6-carboxyfluorescein (6-FAM) and 6-carboxytetramethylrhodamine (TAMRA)-labeled TaqMan probes were purchased from Microsynth AG, Balgach, Switzerland: *GAPDH* forward 5′-GCT GCC AAA TAT GAC GAC ATC A-3′, reverse: 5′-GTA GCC CAG GAT GCC TTT GAG-3′, TaqMan probe 5′-TCC CTC CGA TGC CTG CTT CAC TAC CTT-3′ (GenBank: AB028142; product length: 75 bp); *18SrRNA* forward 5′-GTC GCT CGC TCC TCT CCT ACT-3′, reverse 5′-GGC TGA CCG GGT TGG TTT-3′, TaqMan probe 5′-ACA TGC CGA CGG GCG CTG AC-3′ (GenBank: FJ797658; product length: 125 bp). For *GR/NR3C1*, as well as for *CYCLOPHILIN A*, a commercially available gene-specific TaqMan system was purchased from Applied Biosystems (Prod. No. Cf03986523_gH).

Compartmentalization of utero/placental GR/NR3C1-mRNA expression during prepartum luteolysis

Under sterile conditions, two tissue sections (5 µm each) of formalin-fixed, paraffin-embedded Ut/Pl units from the prepartum luteolysis group were cut with a microtome and placed on Arcturus PEN Membrane Glass slides (Applied Biosystems). Sections were dried overnight at 37°C. Thereafter, slides were dewaxed in xylene, rehydrated in graded ethanol and stained with hematoxylin to distinguish each tissue layer (i.e. placenta, endometrium and myometrium) for dissection. Due to the structural characteristics of the strongly invasive endotheliochorial placenta of the dog, the dissected placental compartments comprised both maternal and fetal parts of the organ. Following air-drying, dissection was performed using a sterile scalpel blade under a stereomicroscope at 50× magnification. The separated tissue fragments were then transferred into sterile Eppendorf tubes, and total RNA was immediately extracted using the RNeasy FFPE kit (Qiagen) following the manufacturer's instructions and as described previously (Gram *et al*. 2014*a*).

Immunohistochemical staining

Immunohistochemical (IHC) staining was performed using our previously described method (Kowalewski *et al*. 2006*a*,*b*). Briefly, formalin-fixed tissue samples were embedded in paraffin, cut into 2–3 µm thick slices, mounted on Super Frost microscope slides (Menzel-Glaeser, Braunschweig, Germany), dewaxed in xylene and rehydrated through graded ethanol solutions to water. Afterward, sections were immersed in 10 mM citrate buffer pH 6.0 and heated in a microwave oven at 560W for 15 min to retrieve antigens. Endogenous peroxidase activity was quenched by treating slides with 0.3% hydrogen peroxide in methanol for 30 min on a shaker at

ambient temperature. Afterward, in order to avoid background staining, slides were incubated with 10% normal serum from the same species in which the secondary antibody was made.

The following primary antibodies were used: monoclonal mouse anti-rat GR/NR3C1 IgG2, dilution 1:300 (LSBio Inc, Seattle, WA, USA); monoclonal mouse anti-VIMENTIN IgG2a (M7020; Clone 3B4), dilution 1:100 (Dako Schweiz AG); affinity-purified polyclonal rabbit anti-CYTOKERATIN, wide spectrum screening, dilution 1:300 (Dako Schweiz). The primary antibodies were diluted in IHC buffer/0.3% Triton X, pH 7.2–7.4 (0.8 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 2.68 mM KCl, 137 mM NaCl) and incubated overnight at 4°C. For isotype controls, instead of the primary antibody, sections were overlaid with irrelevant, nonimmune antibodies at the same protein concentration as for primary antibodies: mouse IgG2a (Dako Schweiz) for GR/NR3C1 and VIMENTIN, or rabbit IgG for CYTOKERATIN (I-1000; Vector Laboratories, Burlingame, CA, USA). Slides were subsequently rinsed with IHC buffer/0.3% Triton X buffer in order to remove unbound primary antibody and incubated with biotinylated secondary antibodies: horse anti-mouse IgG BA2000 or goat anti-rabbit IgG BA1000, both at 1:100 dilution (Vector Laboratories, Burlingame, CA, USA). After rinsing, a streptavidin-peroxidase Vectastain ABC-peroxidase kit was applied to the biotinylated secondary antibody, according to the manufacturer's instructions (Vector Laboratories, Burlingame, CA, USA). Color development was achieved with a Liquid DAB+ substrate kit (Dako Schweiz AG). Then, slides were washed in running tap water, counter-stained in hematoxylin, dehydrated in a graded ethanol series and xylene and finally mounted in Histokit (Assistant, Osterode, Germany).

Statistics

Statistical analysis was performed for the evaluation of real-time (TaqMan) PCR data. One-way ANOVA was performed followed by the Tukey–Kramer multiple comparisons post-test in order to determine the effect of pregnancy stage on *GR/NR3C1* expression at selected time points during pregnancy. Dunnett's multiple comparison test was applied to reveal significance among levels of *GR/NR3C1* expression after aglepristone-induced luteolysis; the results show the *n*-fold change in target gene expression compared with its expression at mid-gestation.

The relative levels of *GR/NR3C1*-mRNA expression in the different dissected tissue compartments (i.e. placenta, endometrium and myometrium) of the utero/placental units during prepartum luteolysis were assessed by pairwise, unpaired, two-tailed Student's *t*-test. The statistical software program GraphPad 3.06 (GraphPad Software) was used for all tests. Numerical data are presented as the mean ± S.D.

Results

Expression of GR/NR3C1-mRNA in canine utero/placental units and interplacental uterine sites during pregnancy, and during natural prepartum luteolysis and aglepristone-induced abortion

Expression of *GR/NR3C1*-mRNA was detectable in Ut/Pl units and interplacental samples at all pregnancy

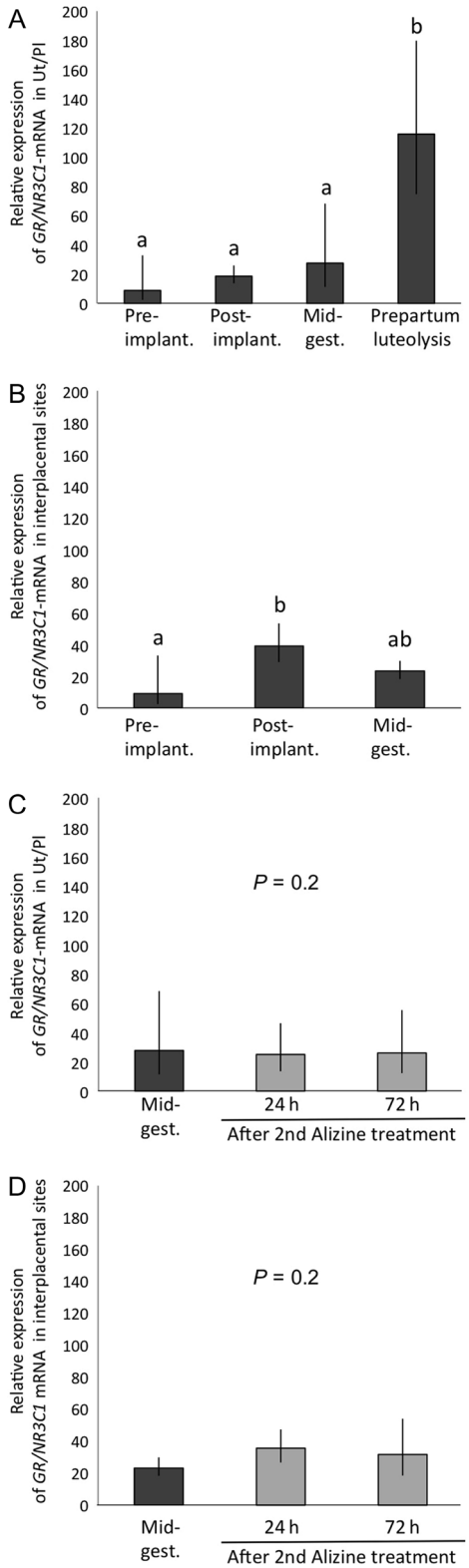

Figure 1 Expression of *GR/NR3C1*-mRNA as determined by real-time (TaqMan) PCR (mean ± S.D.) in canine Ut/Pl compartments (A) and interplacental uterine sites (B) during pregnancy and aglepristone-induced parturition (C and D). Bars with different letters differ at $P<0.01$ in (A) or at $P<0.05$ in (B). ((C) and (D) Compared with the mid-pregnancy group as a nontreated control).

stages examined (Fig. 1A). The Ut/Pl mRNA expression was low from the pre-implantation stage of pregnancy until mid-gestation (Fig. 1A). Afterward, before parturition, i.e. during the prepartum luteolysis, a significant increase ($P<0.01$) was detected (Fig. 1A). Within the interplacental tissues, expression of *GR/NR3C1*-mRNA increased significantly ($P<0.05$) from the pre-implantation to post-implantation stage and did not change significantly ($P>0.05$) during mid-gestation (Fig. 1B).

For experiments investigating the expression of *GR/NR3C1* during antigestagen-induced parturition/abortion on days 40–45 of gestation, samples collected from mid-pregnancy dogs were used in the Dunnett's multiple comparison test as nontreated controls. However, the expression of *GR/NR3C1* remained unaffected ($P=0.2$) in response to aglepristone treatment in both kinds of tissues, i.e. in the Ut/Pl units and at interplacental sites (Fig. 1C and D).

Compartmentalization of utero/placental GR/NR3C1-mRNA expression during prepartum luteolysis

The expression of *GR/NR3C1*-mRNA within the three dissected tissue layers (i.e. placenta, endometrium and myometrium) of canine Ut/Pl units during prepartum luteolysis (Fig. 2) showed highest levels in the placental layer, compared with myometrium ($P=0.02$) and endometrium ($P=0.03$). No statistically significant difference was noted between myometrial and endometrial *GR/NR3C1*-mRNA levels ($P=0.62$) (Fig. 2).

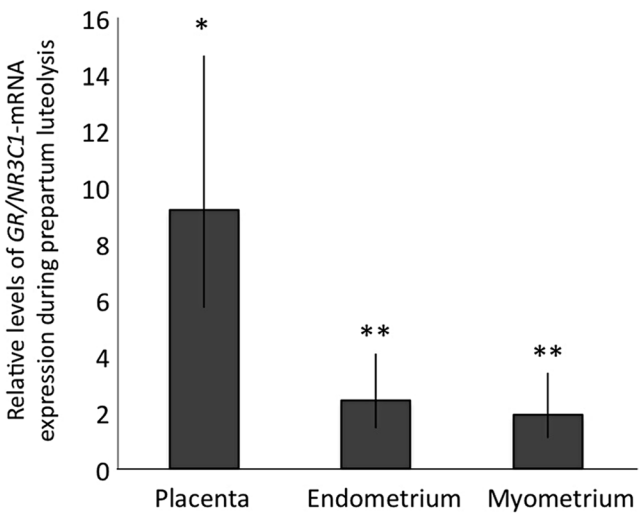

Figure 2 Compartmentalization of *GR/NR3C1*-mRNA expression in the canine utero/placental compartment during prepartum luteolysis as determined by real-time (TaqMan) PCR (mean ± S.D.). Bars with different asterisks differ at $P=0.03$ between placenta and endometrium, and at $P=0.02$ between placenta and myometrium.

Immunolocalization of GR/NR3C1 in canine utero/placental units

As determined by IHC, the expression of GR/NR3C1 protein was clearly localized to the endometrial luminal/surface epithelial cells and superficial glands (Fig. 3A), including the so-called glandular chambers at placentation sites, and to myometrium (Fig. 3B, C and D). The endometrial signals appeared weaker in deep uterine glands (Fig. 3B). These expression patterns of endometrial and myometrial GR/NR3C1 did not change throughout gestation both in Ut/Pl units and at the interplacental sites. Within the placental labyrinth, GR/NR3C1 was clearly detectable in fetal trophoblast cells throughout the placentation period as well as during prepartum luteolysis (Fig. 3E and 3H). Sporadically, only weak or no signals were detected in maternal stroma-derived decidual cells (Fig. 3E and H). For easier differentiation of cells within the canine placenta (i.e. endothelial, trophoblast and decidual cells), epithelial cell-specific anti-CYTOKERATIN (wide spectrum, pan-cytokeratin; Fig. 3F and I) and mesenchyme cell-specific anti-VIMENTIN (Fig. 3G and J) staining was performed on two consecutive sections following those used for GR/NR3C1 detection.

Discussion

In the dog, the prepartum PGF2α release is only sporadically associated with increased cortisol levels in maternal peripheral blood (Concannon *et al.* 1978, Hoffmann *et al.* 1994). Possible local, i.e. utero-placental effects, cannot, however, be ruled out. Therefore, aiming to determine possible autocrine/paracrine effects of glucocorticoids during canine gestation, in this study, we investigated the time-dependent expression and localization of GR/NR3C1 in tissues of canine uterus and placenta collected at selected stages of pregnancy. Tissue samples were examined spanning the time-frame from pre-implantation until mid-gestation, as well as during normal and antigestagen-induced luteolysis. Following more or less constant expression until mid-gestation, utero-placental levels of *GR/NR3C1* increased greatly during the prepartum luteolysis. This increase must originate predominantly in the fetal compartments of the placenta, i.e. in the trophoblast cells, as revealed in our experiments investigating both the compartmentalization of mRNA expression and IHC staining.

These findings corroborate observations made in other species, e.g. in sheep and humans, in which similar spatio-temporal expression patterns of GR/NR3C1 were seen before parturition (Driver *et al.* 2001, Gupta *et al.* 2003, Mparmpakas *et al.* 2014). Together with the increased levels of cortisol observed in the fetal and maternal plasma of these species,

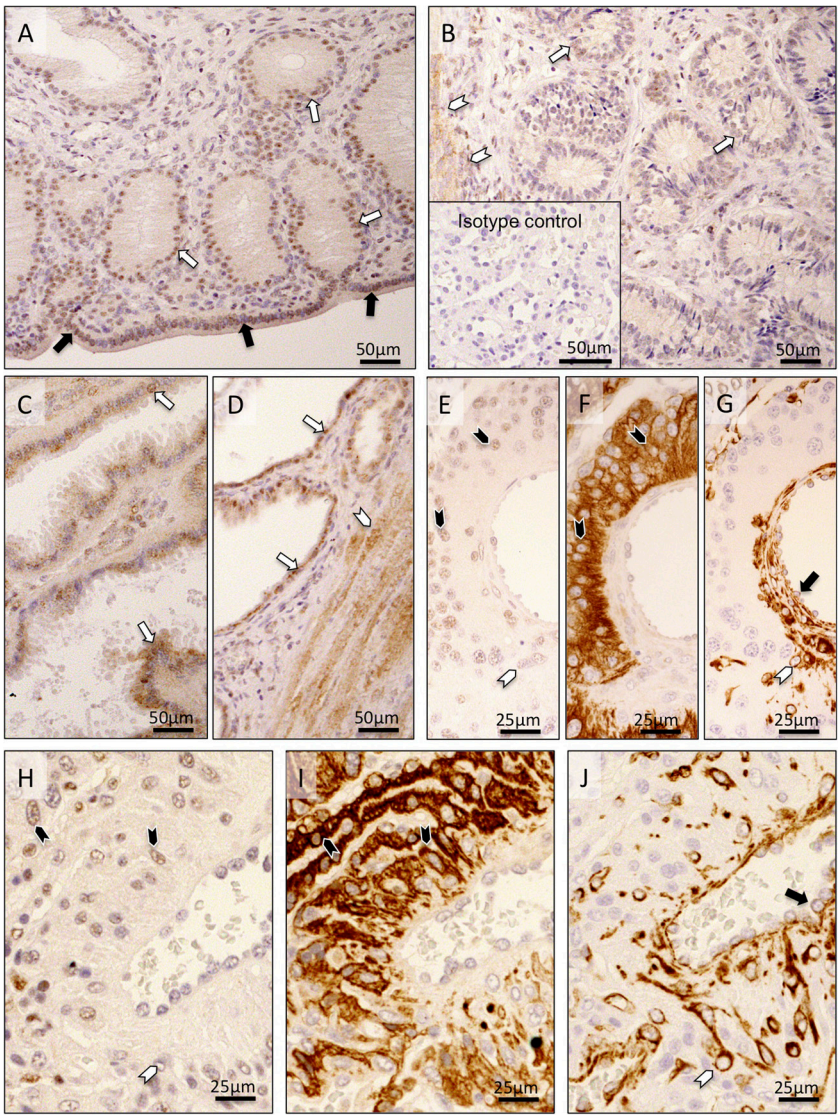

Figure 3 Immunohistochemical (IHC) localization of glucocorticoid receptor (GR/NR3C1) in the canine uterine and utero/placental (Ut/Pl) compartments at selected time points during pregnancy. At the pre-implantation stage (A and B), in the Ut/Pl units during mid-gestation (C, D and E) and in the Ut/Pl compartments at prepartum luteolysis (H). (A and B) At pre-implantation, nuclear staining of GR/NR3C1 is predominantly localized to the endometrial luminal (surface) epithelial cells (solid arrows in A), glandular epithelial cells of the superficial uterine glands (open arrows in A) and myocytes (open arrowheads in B). The signals in deep uterine glands appear weaker than in superficial glands (open arrows in B). During mid-gestation, within the Ut/Pl units, endometrial GR/NR3C1 expression is detected in the superficial glands (the so-called glandular chambers) and deep uterine glands (open arrows in C and D, respectively), and myometrium (open arrowhead in D). In the placental labyrinth during mid-gestation, signals are localized in fetal trophoblast cells (solid arrowheads in E). At prepartum luteolysis, fetal trophoblast cells stain strongly (solid arrowheads in H). No, or sporadically only weak, signals can be identified in maternal decidual cells (open arrowhead in E and H). In order to distinguish between fetal and maternal cell types within the canine placenta, i.e. endothelial, trophoblast and decidual cells, (pan)CYTOKERATIN (wide spectrum) and VIMENTIN staining was performed in consecutive sections following those used for GR/NR3C1. Trophoblast cells stain positively for CYTOKERATIN (solid arrowheads in F and I), whereas endothelial cells (solid arrows in G and J) and decidual cells (open arrowheads in G and J) stain positively for VIMENTIN. There is no background staining in the isotype control (inserted in B).

GR/NR3C1 plays an important role in modulating utero-placental steroid synthesis in favor of estrogens, e.g. in sheep and cattle (Mason *et al*. 1989, Schuler *et al*. 1994, 2006). This applies also to the human placenta, which, albeit lacking 17αHSD activity, is equipped with other endocrine mechanisms facilitating upregulation of estrogen production close to and at term (reviewed in (Kota *et al*. 2013)). Due to the lack of placental steroids and prepartum estrogen increase, no such causality can, however, exist for the dog. Importantly, the prepartum luteolysis, besides being characterized by steeply decreasing P4 concentrations, is also associated with diminishing levels of circulating E_2, further indicating the luteal origin of both steroids (Hoffmann *et al*. 1994, Onclin *et al*. 2002). In this respect, in dogs, like in most other mammalian species, e.g. rabbits, mice, rats, goats, pigs or cattle, P4 withdrawal is required for initiation of parturition (Ash & Heap 1975, Sugimoto *et al*. 1997, Brown *et al*. 2004, Zakar & Hertelendy 2007, Ratajczak & Muglia 2008). This strongly contrasts with the endocrine milieu observed during parturition in guinea pigs, humans and most other primates that maintain high P4 plasma levels during parturition (Heap & Deanesly 1966, Karalis *et al*. 1996, Nnamani *et al*. 2013). Interestingly, however, as shown *in vitro* in the human term placenta model, GR/NR3C1 might antagonize the action of P4 in the placenta by acting as a local antigestagen at the end of pregnancy and thereby facilitating the prepartum endocrine cascade (Karalis *et al*. 1996). GR/NR3C1 and PGR can bind to the same recognition site in promoters of target genes (von der Ahe *et al*. 1985). However, while P4 binds to GR/NR3C1 with 25–50% of the affinity of cortisol (Philibert *et al*. 1991) and 12–42% of the affinity of synthetic glucocorticoid dexamethasone (Ojasoo *et al*. 1988), cortisol at physiological concentrations does not bind to PGR (Ojasoo *et al*. 1988). Taking into account the increased expression of *GR/NR3C1* in the canine placenta during prepartum luteolysis, and

the lack of placental steroids, functional competition between PGR and GR/NR3C1 for P4 binding could also apply in the dog, especially since no other local withdrawal mechanisms of P4 are known in this species. At the cellular level, as presented herein, this putative endocrine mechanism would involve competition between fetal trophoblast, where the GR/NR3C1 is localized, and maternal decidual cells expressing PGR. This hypothesis, while it still needs to be validated, might explain the observation of variable, lower progesterone concentrations at the end of canine gestation during the initiation of parturition (Concannon *et al*. 1975, 1989, Concannon & Hansel 1977). Noteworthy, regrettably, no additional serum samples were available to this study that would allow determination and correlation of *GR/NR3C1* expression levels with circulating peripheral cortisol levels, especially at the time of prepartum luteolysis.

As shown in our previous studies, inhibition of PGR function by the antigestagen aglepristone in mid-pregnancy dogs results in activation of the placental PG system and, unequivocally, leads to preterm luteolysis/abortion (Kowalewski *et al*. 2010, Kowalewski 2014, Gram *et al*. 2014*a*). This further emphasizes the important signaling role of P4 in the endocrine cascade leading to initiation of parturition in the dog. Locally, aglepristone treatment results in suppression of uterine but not placental PGR expression, whereas both uterine and placental OTR levels become strongly upregulated (Kowalewski *et al*. 2010, Kowalewski 2014, Gram *et al*. 2014*a*). The spatio-temporal expression pattern of OTR within the canine uterus and placenta clearly indicates its involvement in the prepartum signaling cascade in this species.

In this context, one of the most important observations from this study is the unaltered uterine and placental expression of *GR/NR3C1* following application of an antigestagen. Notably, an aglepristone-related antigestagen, mifepristone, was shown to act as an agonist/antagonists of GR/NR3C1 (Zhang *et al*. 2007). The degree of its glucocorticoid agonist activity depends on the concentration of GR/NR3C1 in the cell, and increases with the presence of the receptor (Zhang *et al*. 2007). Even though mifepristone has a very similar molecular structure to aglepristone (Hoffmann & Schuler 2002), there is no information currently available on aglepristone-binding capacity of GR/NR3C1.

Regardless, however, the unaltered expression of *GR/NR3C1* in antigestagen-treated dogs suggests that its upregulated expression and presumably increased functionality are not needed to initiate the prepartum luteolytic cascade in dogs.

In conclusion, for the first time, our study reveals the uterine and placental expression of GR/NR3C1 at selected time points during canine pregnancy and at normal and antigestagen-induced parturition. We introduce GR/NR3C1 as a new and possibly important player involved in the placental cross-talk between maternal decidual cells and fetal trophoblast in the canine term placenta. Importantly, especially when the unaltered expression of the receptor during induced luteolysis is considered, other roles of GR/NR3C1 in the prepartum endocrine cascade in the dog need to be taken into account, e.g. including those involved in local, i.e. placental, functional withdrawal of P4.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported. All authors read and approved the final version of the manuscript.

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