Regulatory mechanisms for natriuretic peptide (NP) signalling in sheep granulosa cells (GCs).

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ABSTRACT

Natriuretic peptides (NPs) have been reported in rodents to have critical roles in follicular development and oocyte maturation. This study aimed to extend our current understanding of NP-mediated signalling pathways and, mechanisms of action, in the follicles of a monovulatory species. Ovine GC and TC were cultured under conditions designed to allow gonadotrophin-stimulated cell-differentiation. Gene expression analysis was performed by qualitative (q)PCR for NPs and NPRs (between 16 and 96 hours of culture), and $VEGF_{120}$ and $VEGF_{164}$ (between 16 and 144 hours of culture). A qualitative analysis of the production of NP/NPR family members, and NP ligand/receptor associations was carried out utilising a highly sensitive immunological approach known as 'proximity ligation assay' (PLA). All NPRs were observed in GC, while NPRA was absent in TC. In GCs gene expression of NPPA, NPPB and NPPC was apparent but only active BNP and CNP, and not ANP, were detected. Also in GCs, ANP but not CNP was able to significantly ($P<0.05$) reduced oestradiol and increased ($P<0.05$) progesterone. Inhibition of $VEGF_{164}$ by ANP and CNP ($P<0.01$) after 48 hours of culture preceded up-regulation of $VEGF_{120}$ by ANP ($P<0.01$) after 144 hours, but not CNP. Taken together, these findings appear to demonstrate that NP responsiveness in the GC compartment of sheep follicles is multi-facilitated, utilising both autocrine and paracrine stimulation pathways.

INTRODUCTION

It is well established that members of the Natriuretic Peptide (NP) superfamily of ligands and their receptors (NPRs) are expressed in several locations within the female reproductive system of a number of poly-ovular mammals such as, rat (Gutkowska, et al. 1999), mice (Tsai, et al. 2005) and pigs (Zhang, et al. 2005). They have been reported in various compartments of the ovary including: follicular fluid, theca cells (TCs), granulosa cells (GCs), the oocyte and the corpus luteum, and this suggests they may have important roles in ovarian function. They have been linked to the regulation of follicular atresia, oocyte maturation and ovarian steroidogenesis (Dineva, et al. 2011). NP responsiveness in follicular somatic compartments continues to be an important area of research, especially in humans or a suitable model monovulatory species.

NPs interact with NPRs to mediate their actions through the activation of the second messenger cyclic guanosine monophosphate (cGMP). The family comprises a number of structurally related ligands (NPs) and their guanosine cyclase/receptors (NPRs). Currently receiving most research attention in several follicular locations (Mandich et al., 1991; Ivanova et al., 2003; Zhang et al., 2010) are, Atrial, Brain and C-type NPs (ANP, BNP and CNP respectively), and types A, B and C receptor (NPRA, NPRB and NPRC respectively). The NPs are first translated as stable but inactive, storage forms called natriuretic pro peptides (NPP)A, NPPB and NPPC, which are unable to bind to their receptors. In each case, protease-mediated cleavage releases a small active section from the C-terminal end of the protein (Yan et al., 2000; Wu et al., 2003; Pankow et al., 2007). Research progress has been hampered as antibody recognition alone cannot differentiate between the cleaved, active NPs and unmodified, intact pro-forms. In studies involving pig granulosa cells (Kim et al., 1992), where this problem was addressed, by the use of reverse-phase HPLC, a high molecular weight form of ANP, suggestive of the inactive storage form, was found to

predominate in GCs and follicular fluid. However, the need for a reliable routine laboratory protocol for the identification of specific active NPs, remains a research goal.

An area that has not been previously studied, in the context of NP responses in developing follicles, is the effect of reducing pericellular oxygen levels in the blood-isolated GC compartment due to the formation of multiple layers of cells. Ideally, such a study may be best performed under naturally occurring, as opposed to chemically-induced hypoxia and although the specialised culture systems used in this study have been designed (Campbell, et al. 1996; Campbell, et al. 1998) to provide the conditions necessary for gonadotrophin-stimulated steroidogenesis (GCs, oestradiol 17β and TCs, androstenedione), it has been shown that increasing seeding cell-density, accelerates the formation of multicellular 3-dimensional masses which undergo concomitant increases in naturally occurring pericellular hypoxia (Marsters, et al. 2014). Strong evidence was provided demonstrating that in these dense formations pericellular oxygen fell to hypoxic levels and expression of hypoxia-induced factor-1 (HIF-1) and levels of two key translation isoforms of the angiogenic factor group VEGFA (variant 120 and 164) were markedly increased. This culture system, therefore offers a promising approach to further investigate possible links between hypoxia and NP activity within the GCs of growing follicles. Putative relationships between NPs and VEGF isoforms in hypoxic GCs have previously not been investigated though, a number of groups have reported: that NPs have functions in the process of folliculogenesis (Noubani, et al. 2000; Zhang, et al. 2011; Zhang, et al. 2005). In addition, VEGFA variants are elevated in various compartments of growing follicles concomitant to induction of vascularisation (Robinson et al., 2007; Shimizu & Miyamoto, 2007); and more latter studies suggest the possibility of interactivity between NPs and VEGF variants (Bijsmans, et al. 2017; Kamai, et al. 2018; Spes, et al. 2019).

This study's over-riding objective was to extend the current knowledge of NP signalling in the GCs of a monovulatory species and investigate TC involvement in the process. As sheep ovaries are readily obtainable, and in size and function have many similarities to human ovaries, sheep were considered a good model, monovulatory species for this research. It was proposed as a first step to identify which NPRs and NPs were produced by each cell group. A recent report (de Cesaro, et al., 2018) provides good evidence that GCs taken from the dominant and subordinate follicles of cattle, an alternative monovulatory modal, may be NP-responsive as gene expression was detected for all three receptors. However, even though the same study also reported gene expression for all three NPPs it did not comment on levels of active NPs. As identification of the active forms have previously confounded research in this area, the present study aimed to utilise a novel ultra-sensitive immunological approach (PLA, Methods) for the detection of protein/protein associations in order to determine the local production of the active NP form from its interactions with specific NPRs on cultured cells.

As reduced oxygen conditions have been shown to be involved in the activation of NPs in various other cell-types (Chun, et al. 2003; Doi, et al. 1997), it was hypothesised that a similar hypoxia-mediated mechanism may be active in the GCs of large pre-antral and antral follicles. Thus a further aim of this present study was to gain a greater understanding of the role of hypoxia in NP signalling, utilising GCs grown under the cluster forming conditions, previously demonstrated by Marsters, et al. (2014) to produce a low pericellular oxygen environment as in the avascular, *in vivo* situation (Bianco, et al., 2005). It was aimed to investigate the relationship between NP stimulation and both steroidogenesis and expression of the two major VEGFA isoforms found in GCs ($VEGF_{120}$ and $VEGF_{164}$; ibid).

Materials and Methods

Unless otherwise indicated all reagents, including Duolink® proximity ligation assay (PLA) reagents and oligo-DNA subjugated secondary antibodies and Nunc® cell culture plates were purchased from Sigma-Aldrich™ Co. Ltd., Poole, Dorset, UK.

Tissue collection and cell preparation

Ovine ovaries were taken from freshly slaughtered, abattoir, animals and maintained at 37ºC in a collection/dissecting buffer of Dulbecco's modified eagle medium (DMEM) containing the ingredients fully described (Campbell, et al., 1996; Gutierrez et al., 1997). Granulosa cells (GCs) were obtained from cleanly dissected, morphologically healthy, small antral follicles (3mm or less in diameter) as previously described (ibid). Cells were then washed twice by flushing and centrifuging at 800g for 10 minutes in a GC culture medium of McCoys 5a containing the ingredients previously described (ibid). Intact theca cell (TC) 'shells' obtained after the GCs had been flushed from the hemisected follicles, were disrupted by incubation for approximately 20 minutes at 37ºC with a cocktail of proteases in dPBS as previously described (Campbell et al., 1998). This process also removed contaminant GCs. Following washing the TCs were resuspended in DMEM:Hams F12 culture medium containing ingredients as described previously (ibid).

Cell viability was determined by trypan blue exclusion and they were seeded, either in 8-well Nunc® Lab-Tek® chamber slides™ as per the Proximity Ligation Assay described later in Methods, or in the case of the NP and NPR expression, and the steroid hormone production under NP treatment studies, GCs were seeded in 24-well Nunclon® microtitre

plates, at the optimal seeding rate of 5 X 10^5 (Campbell et al., 1996) viable cells per well in 1 ml of culture medium. Both GCs and TCs were maintained in a humidified atmosphere of 95% air and 5% carbon dioxide, at 37ºC. Every 48 hours 80% of media was replaced with an equal volume of fresh media containing, treatments (below described). Spent media was reserved at -20ºC. To compare the expression of the VEGFA splice variants the GC were seeded at the either the high or low density (HD, LD) rates described by Marsters et al., (2014) of 10^6 viable cells or of 10^5 viable cells per well in 1 ml of medium. After 16, 24, 48 and 96 hours GC samples were retained at -20ºC, in 150 µl of RNeasy® RTL buffer (Qiagen®) containing 1% β-mercaptoethanol, for qPCR analysis. In both the VEGFA splice variants comparison, and the oestradiol 17β (E2) and progesterone (P4), studies the cells either had ANP or CNP (representative NP signallers via A- and B-type receptors respectively) added to the media to a concentration of 100 nM, or were untreated (NT). In serial dilution studies NPs were found to have similar effects in GC over a wide molar range (10 nM – 10 µM), with 100 nM having been previously reported (Zhang, et al., 2015) to illicit cellular responses.

Proximity ligation assay (PLA)

This assay was based on an *in situ* PLA, in which a pair of oligonucleotide-tagged secondary antibodies (PLA probes) produce a signal only when both are bound in close proximity (Fig. 1) either on the same target or two different targets (Soderberg et al., 2006). Ovine GCs or TCs were seeded, in their respective media, at 200 viable cells per well into 8-well Nunc® Lab-Tek® chamber slides™ and cultured. After 48 hours of culture the media was removed, and the cells washed, fixed and blocked according to the standard Duolink®

In situ fluorescence protocol (Sigma-Aldrich™), which varied according to whether there was a single or double target.

For the identification of individual natriuretic peptide receptors (single target), the initial step was to incubate with primary antibodies, specific for each of the NPRs, in concentrations as per Table 1, either for 1 hour at room temperature or overnight at 4ºC. Excess antibodies were removed by washing twice for 5 minutes. The *in situ* PLA was carried out as per the supplied manual. Briefly, when the DNA attached to pairs of antibodies hybridises, fluorescent oligonucleotides are incorporated, resulting in around 1000-fold amplification of signal. Finally, the wells were removed from the chamber slides as per the protocol and the slides mounted with a small amount of Duolink® in situ mounting medium with DAPI under a glass cover slide, and after a 15-minute incubation the fluorescent signals were visualised under a confocal microscope.

As only active NP forms interact with specific receptors, locally produced mature, active NPs were identified utilising the PLA approach for detecting interacting proteins (double target). Specific primary antibodies against NPs and NPRs, which had each been raised in a different species, were localized to their target proteins. It was also necessary to utilise secondary PLA plus and minus, antibodies which were species-specific, anti-primary IgG. All other steps followed the single target protocol.

Immunohistochemistry (IHC)

Ovine ovaries, collected from a local abattoir under the conditions earlier described, were

fixed in 4% PFA and embedded in paraffin wax. They were then serially sectioned into 10 μm slices and mounted onto SuperFrost Plus microscope slides (Menzel-Glaser, Brauncshweig, Germany), which were then and baked overnight at 45ºC. The slides were placed in a Bond Max Automated Immunohistochemistry Vision Biosystem (Leica Microsystems GmbH, Wetzlar, Germany) according to the following protocol. Slides underwent dewaxing in a descending alcohol series, followed by epitope retrieval by treatment with 0.1 M sodium citrate for 10 minutes. After being washed in PBS, blocking with a 3% hydrogen peroxide solution was carried out for 10 min, using the Bond Polymer Refine Detection Kit DC9800 (Leica Microsystems GmbH). In accordance with this protocol, slides were again washed and then incubated with the primary antibody (Anti-NPRA or Anti-NPRB, Table 1) for 30 min. Subsequently, the slides were incubated with poly-HRP-IgG conjugate for 10 min and developed with 3, 3'Diaminobenzidine tetrahydrochloride (DAB)-Chromogen for 10 minutes. Nuclei were counterstained with 0.02% haematoxylin.

qPCR Analysis

Total RNA was isolated from the GCs using the RNeasy® mini kit (Qiagen®) and protocol. First strand cDNA synthesis was performed using Revertaid™ H-minus reverse transcriptase (Thermo Scientific™) according to the prescribed protocol. Unless otherwise stated all qPCR, reagents were supplied by Applied Biosytems™ (Warrington, UK). All quantitative PCR (qPCR) was carried out on Applied Biosystem's 7500 FAST® Real-time thermocycler. Target genes $VEGF_{120}$, $VEGF_{164}$, NPPA, NPPB, NPPC, NPRA and NPRC, were amplified using gene- or transcript variant-specific primers (Table 2). Expression over

192 time profiles were produced after real-time qPCR. The VEGF variant analysis utilised a
193 common forward primer, oVEGFex3F and either oVEGF120R or oVEGF164R reverse
194 primers and the NPP and NPR analysis used Taqman® real-time primer/probe sets and
195 followed the prescribed Taqman® Universal Master Mix II protocol. Where possible all
196 qPCR primer sets were optimized to similar PCR parameters. Accordingly the conventional
197 reaction mixes containing 2X SYBR Green PCR mastermix (1X), specific primers (1pmol
198 each), and target cDNA (100 – 500ng) were heated to 94ºC for 10 minutes and cycled 35
199 times at 94ºC for 30 seconds, 57ºC for 30 seconds and 72ºC for 1 minute. Absence of non-
200 specific spurious products for each primer set was confirmed by melt-curve analysis and
201 product identity was confirmed by sequencing.
202
203 *ELISA Assays*
204 Oestradiol 17β (E2) and progesterone (P4) concentrations in the granulosa cell culture
205 media were assayed by competitive Enzyme-Linked Immuno Sorbant Assay (ELISA).
206 Microtitre plates (96-well) were either pre-coated with E2 antibody (ab 1025, Abcam®,
207 Cambridge, UK) or P4 antibody (R7044X, Scottish Antibody Production Unit®, Carluke,
208 UK) diluted in 0.05M carbonate coating buffer to a ratio of 1:64000 and 1:32000
209 respectively and left overnight at 4ºC. The plates were then washed three times in PBS
210 containing 0.005% Tween-20. Then wells were blocked in 3% BSA (in PBS) for 1 hour,
211 followed by three washes (as above), before addition of 100 µl of a 1 in 5 dilution of the
212 reserved spent media from the ANP and CNP stimulated, GC plates, and an equal volume
213 of either E2-HRP or P4-HRP conjugate (Abiox® Company, Portland, Oregon, USA). The
214 assay plates were then incubated for 2 hours on an orbital shaker at 170 rpm. This was

followed by five washes (as above) and addition of 50 µL of HRP substrate (3,3',5,5'-Tetramethylbenzidine, TMB). After about 15 minutes (or after the development of a blue colouration) the HRP enzymatic reaction was halted by addition of 50 µL of 'Stop' solution (1 N sulphuric acid) and the plate read at 450 nm. The sensitivities of the E2 and P4 assays were both 39 pg/ml (~90% of zero binding value) and the inter- and intra-assay coefficients of variation were both <10%.

Statistical analysis

Production of E2 and P4 were calculated as the concentration per 1 X 10^3 cells seeded in the cultures. The qPCR results were analyzed using a 'relative standard curve' method according to Applied Biosytems' analysis software package, version 2.0. Semi-quantitative comparisons between sample groups were made after the target gene expression had been normalised against an 18S endogenous control. The results shown are the means ± SEMs of at least three independent experiments with each experiment having been carried out in at least triplicate. 'Repeated measures ANOVA' was performed (SPSS software version16.0) to determine the level of significance between sample groups.

Results

Utilising a 'single target' proximity ligation assay (PLA, Methods) NP receptors A, B and C appeared abundant in cultured ovine GCs (Fig. 2). However only the C-type receptors appeared to be produced regardless of cell density or neighbour cell proximity, with the A- and B-type receptors seemingly only produced on clustered GCs. In cultured ovine TCs, NPRB and NPRC appeared similarly abundant, but in these cells cluster formation or cell-

cell proximity was not noted to be a prerequisite, and the A-type receptors were found to be absent or very poorly represented. These outcomes broadly matched the findings of the immuno-histochemical (IHC) study of NPRA and B in growing follicles (Fig. 3), which showed that though both could be immunolocalised on GCs throughout follicular development, only NPRB appeared on TCs and only on large antral follicles.

Utilising quantitative polymerase chain reaction (qPCR) it could be seen that, though both the A- and B-type receptor expression levels increased after 16 hours of culture (Fig. 4A), the expression of NPRA was markedly the more pronounced. After 48 hours of culture it was more than 60-fold ($P<0.05$) the level of NPRB. Over the following 48 hours of culture the expression level of NPRA decreased by around 85% ($P<0.05$) to a similar expression level as NPRB, which had increased almost 3-fold ($P<0.05$) over the same period. NPPA, NPPB and NPPC were all noted to have increased markedly after 16 hours of culture by around 90-fold ($P<0.05$) by their peak after 48 hours of culture (Fig. 4B). Over the following 48 hours of culture NP precursor gene expression decreased sharply by more than 60% ($P<0.05$) in all cases.

Using a ‘single target’ PLA approach, forms of ANP, BNP and CNP could all be detected in both cultured ovine GCs and TCs (Fig. 5) and all appeared markedly more abundant in the TC cultures. However, as the single target approach does not distinguish pro-forms (NPPs) from active forms a ‘double target’ PLA approach (Methods) was used. Under this highly sensitive scrutiny signals indicating active ANP in association with A-, B- or C-type receptor (Fig. 6) were not detected. A positive control was used which confirmed

exogenous ANP bound to NPRA in the GCs. Active endogenous BNP was detected associated with NPRA and NPRC in the cultured GCs but no signal was detected to show association with NPRB. Locally produced CNP was detected associated with NPRB and NPRC but not with NPRA.

In other ovine GC cultures, set up in 24-well plates (Methods) the production of the major steroid hormones, oestradiol 17β (E2) and progesterone (P4) were measured over time by ELISA (Methods) and the effects of 100 nM ANP or CNP were compared. In the untreated cultures E2 production over the 48 hours of culture preceding the 96 hours time-point was noted to more than double ($P<0.05$) the production over the first 48 hours of culture (Fig. 7A). However, in the 48 hours following the 96 hour time-point production was noted to fall back markedly ($P<0.05$) to similar levels to that produced in the first 48 hours of culture. While CNP was shown to have no effect on this profile, production was markedly ($P<0.05$) reduced it in the ANP-treated cells, over the 48 hours of culture preceding the 96 hour time-point. Over the same 144 hours of culture P4 production showed a trend of increasing production (Fig. 7B), with a 4-fold increase ($P<0.05$) noted after 144 hours of culture over the levels noted after the first 48 hours of culture. The addition of CNP to the culture media did not have a significant effects on the 'no treatment' profile.

In parallel studies the expression levels of the VEGFA ovine isoforms 120 and 164 were measured in GC cultured over time and the effects of 100 nM ANP or CNP were compared. These studies were carried out in cultures, designed (Methods) to either retard cluster formation (low-density seeded, LD), or to encourage the formation of multi-cellular

clusters (High-density seeded, HD) previously reported to induce naturally increasing levels of pericellular hypoxia (Marsters et al., 2014). In the LD cultures no significant effects of ANP or CNP treatment were noted, so those outcomes have not been included in Fig. 8. However, in the HD seeded GCs, though neither ANP nor CNP had a significant effect on the expression levels of $VEGF_{120}$ (Fig. 8A) over the first 48 hours of culture, that variant's expression increased by almost 6-fold ($P<0.05$) in the ANP-stimulated cells, over the next 48 hours to 96 hours of culture. In contrast CNP had no effect on the 120 variant's expression level over the whole 96 hours of culture. In the untreated HD-seeded GCs, the expression profile of $VEGF_{164}$ (Fig. 8B) was distinctly different than that of $VEGF_{120}$, which appeared to be expressed at a low level throughout the time course. Whereas, after 24 hours of culture the expression levels of $VEGF_{164}$ increased by over 5-fold ($P<0.05$) over the following 24 hours of culture, but fell away markedly ($P<0.05$) thereafter to starting levels by 96 hours of culture. This surge of expression in the untreated GCs, measured at 48 hours of culture, was not apparent in either of the ANP-stimulated or the CNP-stimulated cells.

Discussion

This study's over-riding objective was to expand the current understanding of natriuretic peptide (NP) responsiveness in the granulosa compartment of developing ovarian follicles in a monovulatory species. An important preliminary aim was to identify members of the NP family of ligands and receptors which may be active in the follicular, somatic cells. Utilising the specialised cell culture systems, which were designed to retard luteinisation and provide conditions for gonadotrophin-mediated cell differentiation in ovine GCs

(Campbell et al., 1996) and TCs (Campbell et al., 1998), the study found that the three key receptors NPRA, NPRB and NPRC, appeared abundantly on the cultured GCs and this highlights a possibly important difference to ovine TCs. In the cultured TCs only NPRB and NPRC were similarly in abundance with the A-type receptor appearing to be poorly produced. As these outcomes mirror the findings in follicles within sectioned ovine ovaries it seems likely that expression of A- and B-type receptors may be regulated differentially in the two cell types. This was evidenced in GCs with, both A- and B-type receptors observed only on multi-cellular clumps, indicating that in these cells they are more likely to be regulated by mechanisms associated with cluster formation, whereas, in TCs, A-type receptors seemed virtually absent and B-type receptors, though appearing antral follicle stage-specific, may not rely on cell clustering as *in vitro* their production also occurred on isolated TCs. As, ANP and BNP have been reported (Koller, et al., 1991; Suga, et al., 1992) to have only low binding affinity for the B-type receptor, it is likely that ovine TCs are only able to respond minimally to either. In contrast ovine GC appear to be equipped to respond to both A and B receptors via their high-affinity receptor, NPRA. As NPRC, the NP-clearance receptor was found to be highly represented in both cell-types it seems reasonable to conclude that both types can also curtail cellular responses to NPs. A further interesting observation was that A- and B-type receptors were absent on small clusters as well as on the dispersed GCs. Therefore, it seems possible that regulation of these receptors may rely on more than simply cell-cell communications being re-established. A feature of GCs cultured in the serum-free system is that they form 3-dimensional clusters which steadily enlarge, with the incorporated cells undergoing concomitant increases in pericellular hypoxia (Marsters et al., 2014). As the onset of NP receptor production in GCs coincides

with the development of a hypoxic environment it could suggest a connection between the two occurrences and even that NP-responsiveness may be part of a hypoxic adaptive response.

Further support for this contention has been provided by investigating the effect, in GCs, of NPs on the gene expression of suitable markers of hypoxia such as VEGFA variants (Levy, et al., 1995; Grasselli, et al., 2005; 2014; Ramanathan, et al., 2003). In the early stages of culture, when cell clusters were absent or sparse, neither ANP nor CNP induced changes in the consistently low expression levels of VEGFA variant 120 or 164, but under conditions known to elevate pericellular hypoxia, around 48 hours of culture, both ANP and CNP were able to abrogate the upsurge noted to peak in $VEGF_{164}$ expression in untreated cells. This profile appears to fit a scenario in which neither A- nor B-type receptor are produced while GCs are sparse and normoxic, but when naturally occurring hypoxia develops in larger cell clusters, signalling by ANP and CNP may be principally via their cognate receptors (NPRA and NPRB respectively). In marked contrast it appears that only NPRA is involved in up-regulating expression levels of $VEGF_{120}$ as ANP mediated an increase of around 6-fold that seen under no treatment, while CNP treatment did not elicit a statistically significant effect. It is also of interest that up-regulation of $VEGF_{120}$ expression increased in line with a reciprocal down-regulation of $VEGF_{164}$ suggesting the latter may be reliant on the former. However it is clear that more investigations are needed to fully elucidate these signalling pathways and their specificity to pericellular hypoxia.

The highly sensitive PLA process for double targets (Methods) provided a robust means to

354 determine which active NPs were endogenously produced by the cultured GCs, and which
355 specific NPRs they were able to complex with. To the authors' best knowledge the study's
356 detection, using this approach, of active BNP produced in ovine GC cultured *in vitro*, is the
357 first evidence of its occurrence in the follicular somatic cells of a monovulatory species.
358 Another noteworthy finding was that whereas, the BNP and CNP mature ligands were
359 produced in the *in vitro* GCs, activated ANP appeared not to be. As the pro-form was in
360 evidence, it appears, GCs may not produce the means by which cleavage of the active form
361 can be performed. Interestingly, previous studies have provided strong evidence showing
362 that human granulosa luteinised cells (GLCs) do express active ANP ligand (Dineva et al.,
363 2011; Ivanova et al., 2003), this therefore, could suggest that the mechanism for the
364 cleavage of NPPA may not occur in GCs until ovulation when luteinisation has occurred.
365 Although this has not been investigated here, it is tempting to speculate that, the presence
366 of NPRA on GCs, may mean that, in the *in vivo* scenario, neighbouring TCs are able to
367 induce signal transduction by the processing of active ANP, but this also remains to be
368 investigated.
369
370 A novel finding in the cultured GCs was that exogenous ANP formed detectable
371 associations with NPRA for relatively short periods. The optimal detection time point,
372 under the conditions utilised in this study, was around 15 to 20 minutes after treatment with
373 the signal being absent by 30 minutes. It is now well documented in other cell types
374 (Pandey et al., 2002; Pandey et al., 2005; Bonifacino and Traub, 2003) that NPs interacting
375 with NPRs transduce cellular responses which include ligand/receptor complex
376 internalisation and desensitisation. It seems plausible, therefore, that the loss of ANP-

NPRA signal in this study was most likely due to receptor-mediated endocytosis of the bound targets. As this phenomenon was not obvious in the cases of endogenously produced NPs (BNP-NPRA, CNP-NPRB and BNP- or CNP-NPRC) it could be speculated that the process of desensitisation is a further putative mechanism for the regulation of cellular responsiveness to unneeded NP-stimulation in these follicular somatic cells.

A number of studies (Campbell et al., 1996; Gutierrez et al., 1997; Campbell et al., 1998; Marsters et al., 2003) have confirmed that in GCs, taken from the small antral follicles of large ruminants and cultured under the optimal conditions described by Campbell et al., (1996), E2 production remains non-existent or at low levels for up to 48 hours of culture. Around this point cell differentiation occurs marked by E2 levels up-regulating, usually peaking after at least a further 48 hours of culture and this concurs with the outcomes of this present study showing E2 increased sharply between 48 and 96 hours of culture. It has been assumed that the subsequent marked decrease after that point is due to a limiting steroidogenesis precursor such as cholesterol (not included in the specialised culture medium). From earlier reports (ibid) and this current study it is tempting to hypothesise that E2 production may be initiated as a result of cells coming together and forming gap-junction-coupled clusters in which the pericellular environment undergoes steadily increasing hypoxia. However this may be quite transient as work reported by Marsters et al., (2014), along with findings reported here indicate that when numbers and sizes of cell clusters are well advanced the heightened density of large multi-laminar cell masses are concomitant to, and may induce, E2 inhibition. Utilising ANP to stimulate a NPRA-mediated response, the E2 ‘spike’ was noted to be markedly less than in the NT cultures.

Taken together these results suggest that stimulating NPRA with a high-affinity ligand (ANP), can abrogate the E2 surge in GCs. However, GC luteinisation may be unaffected under ANP stimulation as P4 production was not noted to deviate significantly from the 'steadily increasing' NT profile, though the resultant E2/P4 switch in dominance is suggestive of their juxtaposition in granulosa luteal cells, but this remains to be clarified.

This work has produced novel and compelling evidence that the regulation of NP-responsiveness in the GC and TC compartments of sheep is complex and multi-layered, and may involve ligand-mediated receptor desensitisation, receptor-mediated ligand clearance and cell-specific mechanisms for the production of receptors, and active ligand. Taken together these data suggest that this super family of ligands and receptors are highly active in the developing follicles of a monovulatory species and may have a number of important roles besides those already described in the literature.

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Tables

Table 1. Antibodies - Proximity Ligation Assay (PLA).

Antibody	Description	I.D.	Dilution
Anti-NPPA/proANP	mouse monoclonal (abcam™)	ab14442	1:50
Anti-NPPB/proBNP	mouse monoclonal (abcam™)	ab47699	1:50
Anti-NPPC/proCNP	rabbit polyclonal (Sigma™)	AB_2690054	1:50
Anti-NPRA	rabbit polyclonal (abcam™)	ab14356	1:50
Anti-NPRB	rabbit monoclonal (abcam™)	ab139188	1:50
Anti-NPRC	rabbit polyclonal (ThermoFisher™)	ER1914-07	1:50
PLA probe-anti-mouse plus/PLA probe-anti-mouse minus	Duolink® PLA Donkey anti-mouse IgG Probe (Sigma™)	DUO92001plus/DUO92004minus	1:5
PLA probe-anti-rabbit plus/PLA probe-anti-rabbit minus	Duolink® PLA Donkey anti-rabbit IgG Probe (Sigma™)	DUO92002plus/DUO92005minus	1:5

579

Table 2. PCR primers and probes.

Conventional PCR Primers

Primer Name	Primer Target site	Primer Sequence	Accession No.
oVEGFex3.F	Exon 3	F: 5' - ATTTTCAAGCCGTCCTGTGTGC - 3'	AF071015
oVEGF120.R	Exon 5/6a	R: 5' - TCGGCTTGTCACATTTTTCTTG - 3'	AF250375
oVEGF164.R	Exon 5/7a	R: 5' - CAAGGCCCACAGGGATTTTC - 3'	AF071015

Applied Biosystems Taqman Real-Time Primer/Probe Assays

Primer Name	Catalogue No.	Taqman Assay I.D. /(Probe Dye)	NCBI Ref. Seq.
oNPPA/ANP	4351372	Oa04657625_g1/(FAM/MGB)	NM_001160027.1
oNPPB/BNP	4351372	Oa04931155_g1/(FAM/MGB)	NM_001160027.2
oNPPC/CNP	4351372	Oa04931156_u1/(FAM/MGB)	NM_001009479.1
oNPR1/oNPRA	4351372	Oa04888681_g1/(FAM/MGB)	NM_027975457.1
oNPR2/oNPRB	4351372	Oa04829326_g1/(FAM/MGB)	NM_027964432.1
Eukaryotic 18S rRNA	4319413E	Hs99999901_s1/(VIC/MGB)	X03205.1

580

Figures

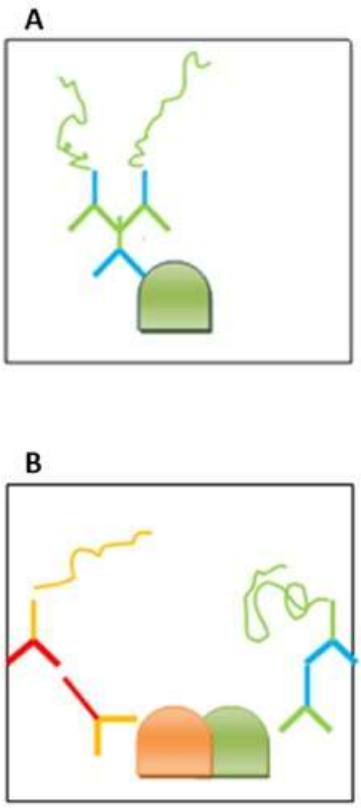

Figure 1. Proximity Ligation Assay (PLA). DNA strands attached to secondary Abs, which are primary Ab-specific, need to be in close contact in order for PCR amplification and detection-entity tagged nucleotide incorporation to occur (Methods). This was achieved using target-specific primary Abs and, in the case (A) single protein target, utilising equal amounts of sense (plus) and antisense (minus) DNA strands attached to anti-species-specific IgG, secondary Abs, and for (B) targeting proteins in close proximity (associating targets) primary Abs which have been raised in different host species are used which are specific for each of the tow target proteins and the plus and minus secondary Abs are each species-specific to target each of the primary Abs.

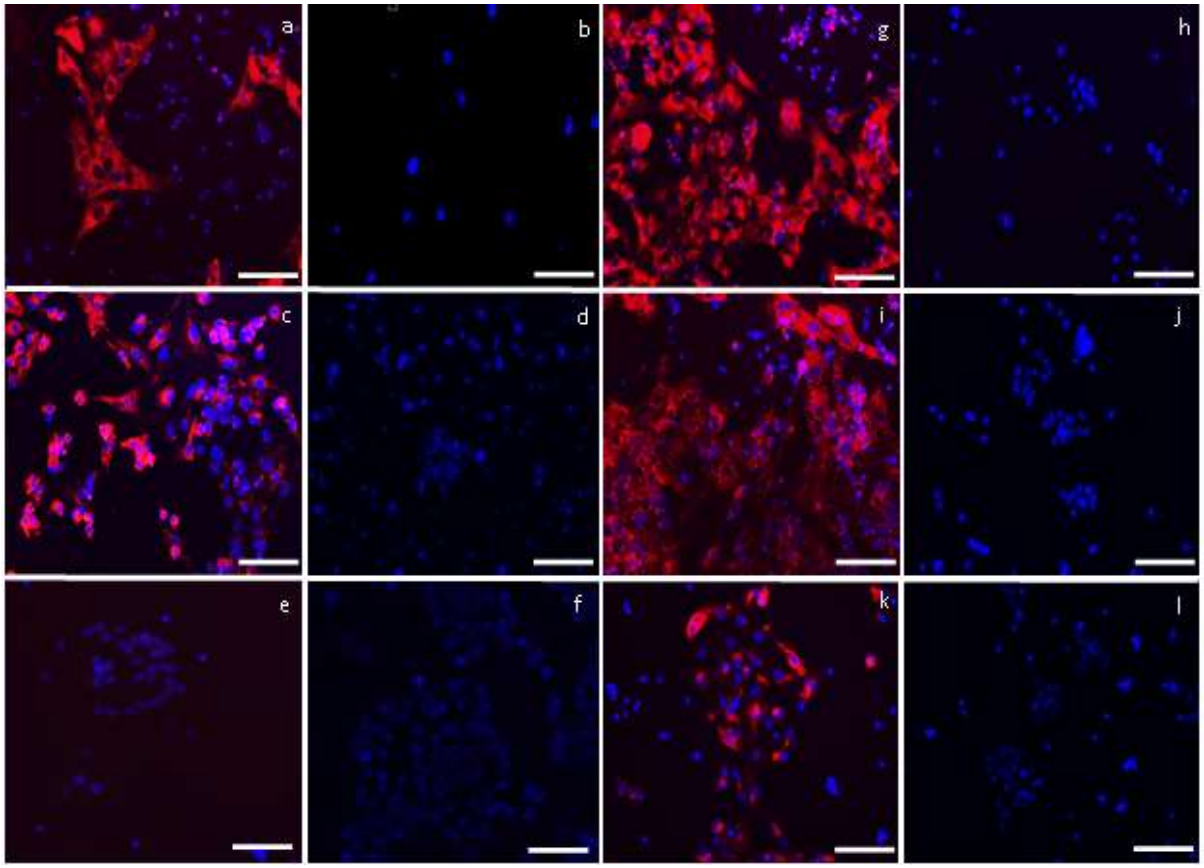

Figure 2. Cultured ovine TC and GC showing immunolocalisation (red fluorescence) of natriuretic peptide receptors; NPRA (a, TC) and (g, GC), NPRB (c, TC) and (i, GC), and NPRC (e, TC) and (k, GC). The respective, non-primary Ab negative controls (b, h, d, j, f and l), control against non-specific binding by secondary Abs. Cell nuclei were counter-stained blue using DAPI and bar = 100 µm

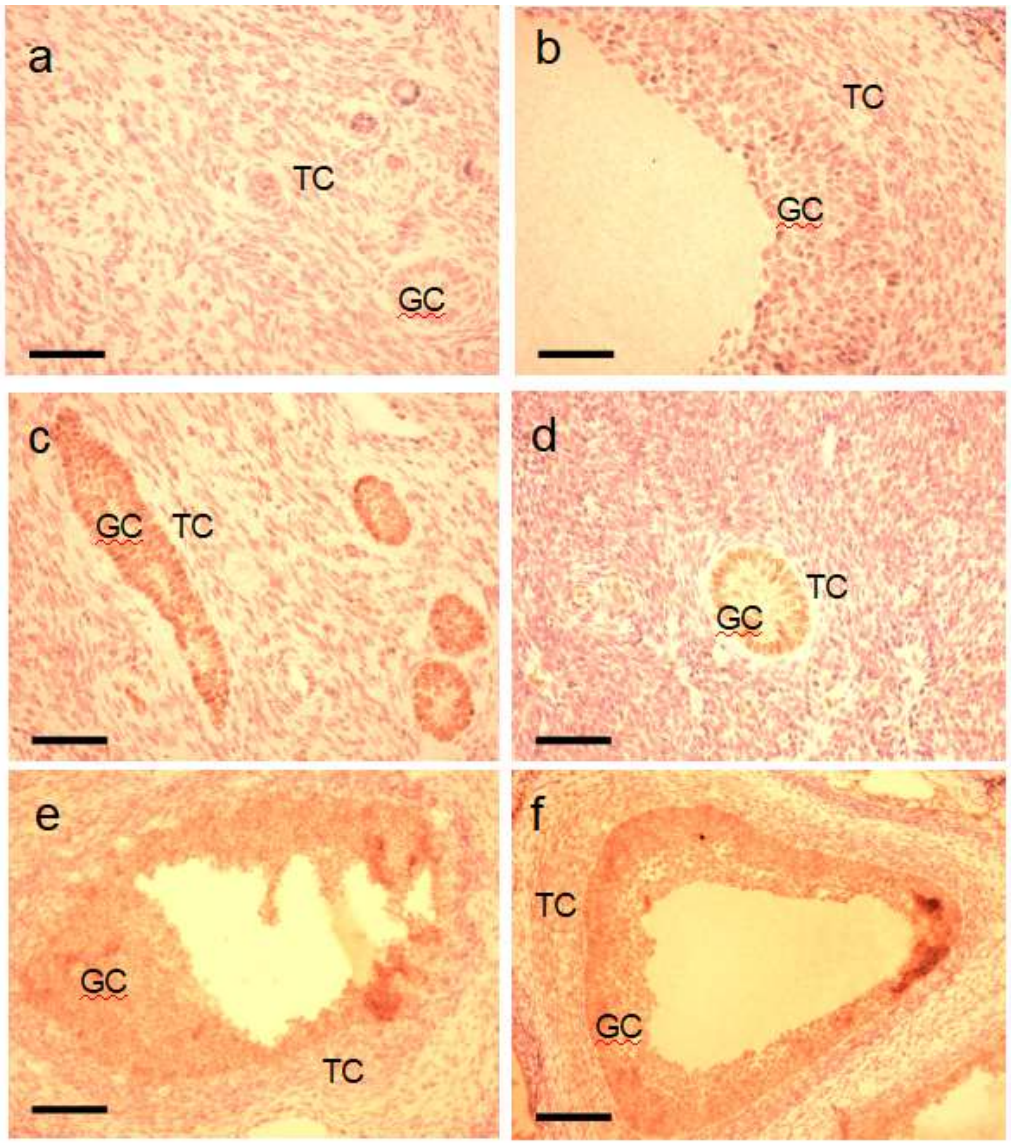

Figure 3. Immuno-histochemistry (IHC) analysis in ovine ovary sections localising anti- NPRA (c & e) and anti-NPRB (d & f) to various sized follicles Positive immune;ocalisations depicted in darker red. Primary Ab absent, negative controls (a & b) are included for NPRA and NPRB reseptivety. Theca and Granulosa cell compartments indicated by TC and GC respectively and bar =50µm

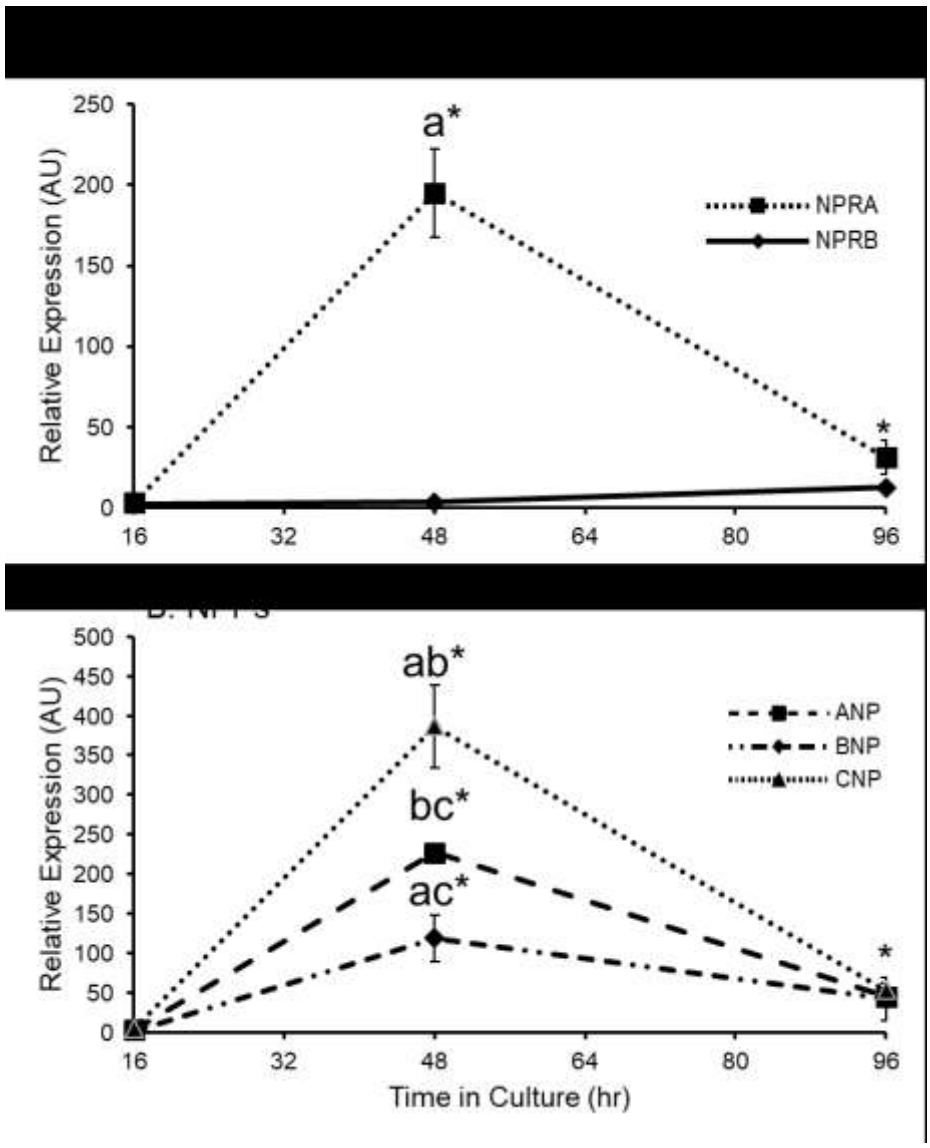

Figure 4. Expression in high or low density cultured cells (HD or LD respectively) of (A) NPRA (dotted line) and NPRB (filled line) and (B) NPPA (dashed line), NPPB (dot & dash line) and NPPC (dotted line) relative to an endogenous control (18S rRNA) in ovine GC cultured over time. The graphs represent the means, +/- the standard error of means (SEMs) of at least 3 separate experiments performed in duplicate. Statistically significant differences ($P<0.05$) in expression over the previous time point are denoted by asterisks (*) and NPRA over NPRB (a), NPPA over NPPB and C (bc), NPPB over NPPA and C (ac) and NPPC over NPPA and B (ab).

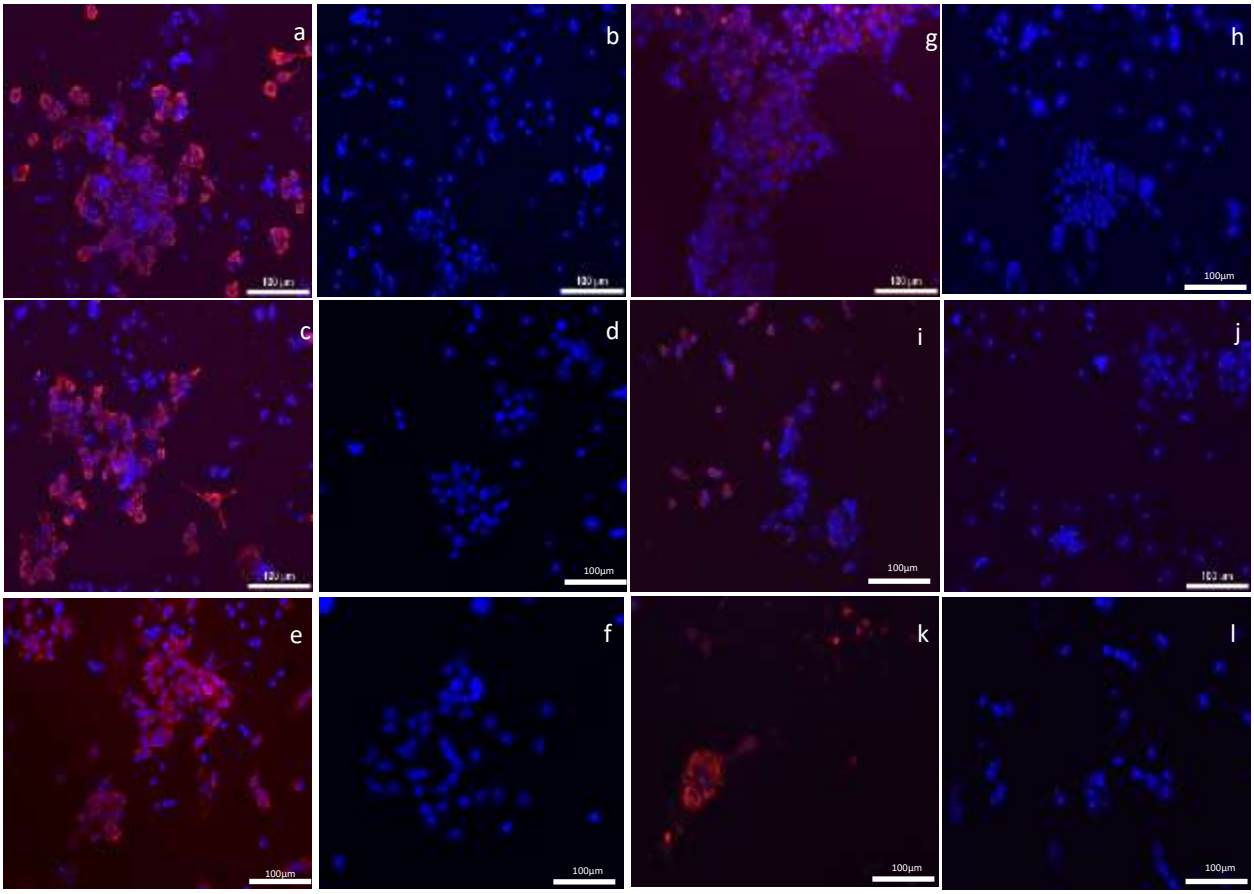

Figure 5. Cultured ovine TC and GC showing immunolocalisation (red fluorescence) of natriuretic peptide forms. ANP forms (a, TC) and (g, GC), BNP forms (c, TC) and (i, GC), and CNP forms (e, TC) and (k, GC). The respective, non-primary Ab negative controls (b,h,d,j,f and l), control against non-specific binding by secondary Abs. Cell nuclei were counter-stained blue using DAPI, and bar = 100 µm

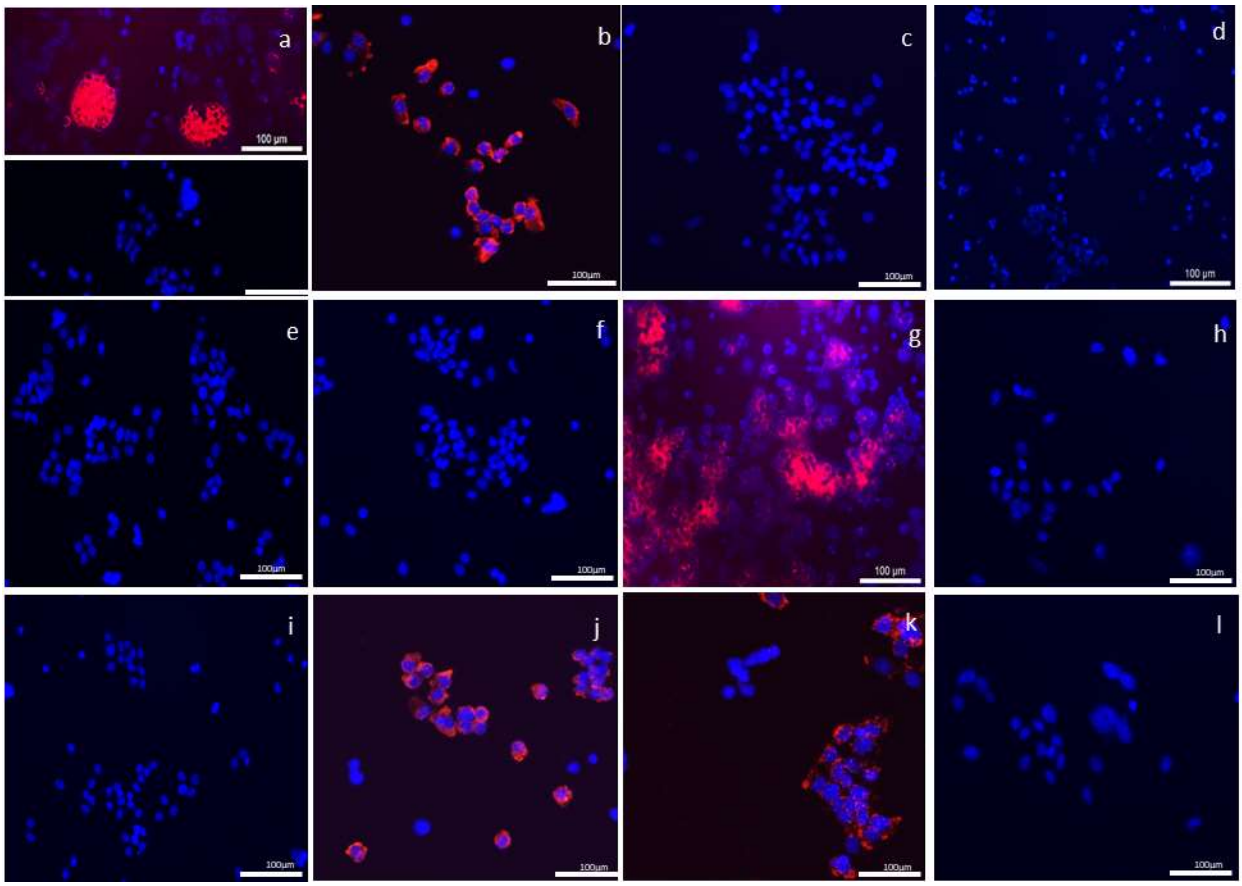

Figure 6. Cultured ovine TC and GC showing locally produced NP ligand associations with NPRs (red fluorescence) utilizing PLA detection and signal enhancement. Sets of target-specific primary antibodies were used which were specific for NPRA with ANP (a) with exogenous ANP in upper, BNP (b), and CNP (c); NPRB with ANP (e), BNP (f) and CNP (g); and NPRC with ANP (i), BNP (j) and CNP (k). The respective non-primary Ab negative controls (d, h and l), controls against non-specific binding by secondary Abs. Cell nuclei were counter-stained blue using DAPI and bar = 100 µm.

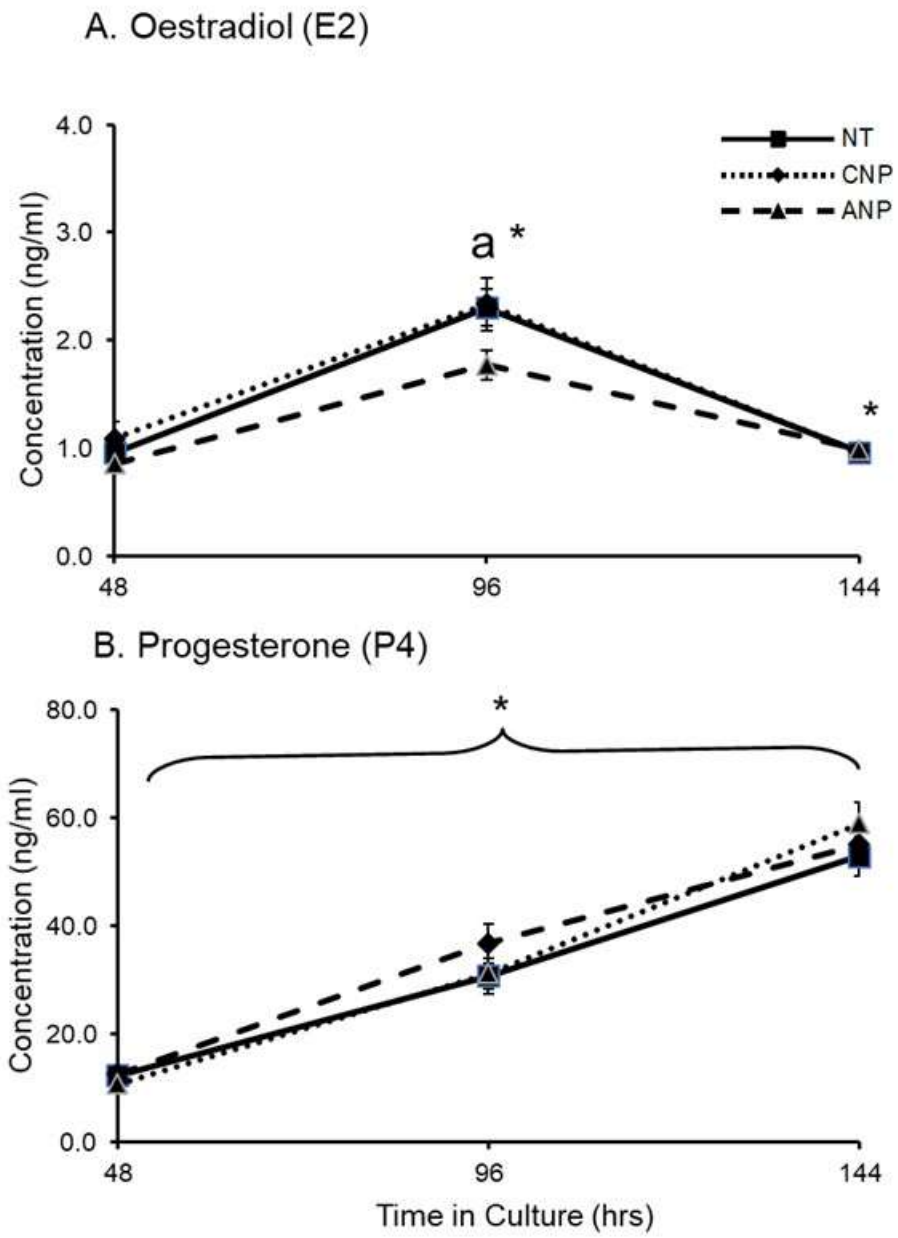

Figure 7. Oestradiol 17beta (E2, A) and progesterone (P4, B) produced by ovine GC cultured over time after treatment with either ANP (dotted line), CNP (dashed line) or no treatment (NT, filled line) and measured after simultaneous 48 hour periods. The graphs represent the mean concentrations, +/- the standard error of means (SEMs) of at least 3 separate experiments performed in duplicate. Statistically significant differences ($P<0.05$) of steroid concentrations over the previous time point are denoted by asterisks (*) and after treatment compared to untreated (NT) by the letter 'a'.

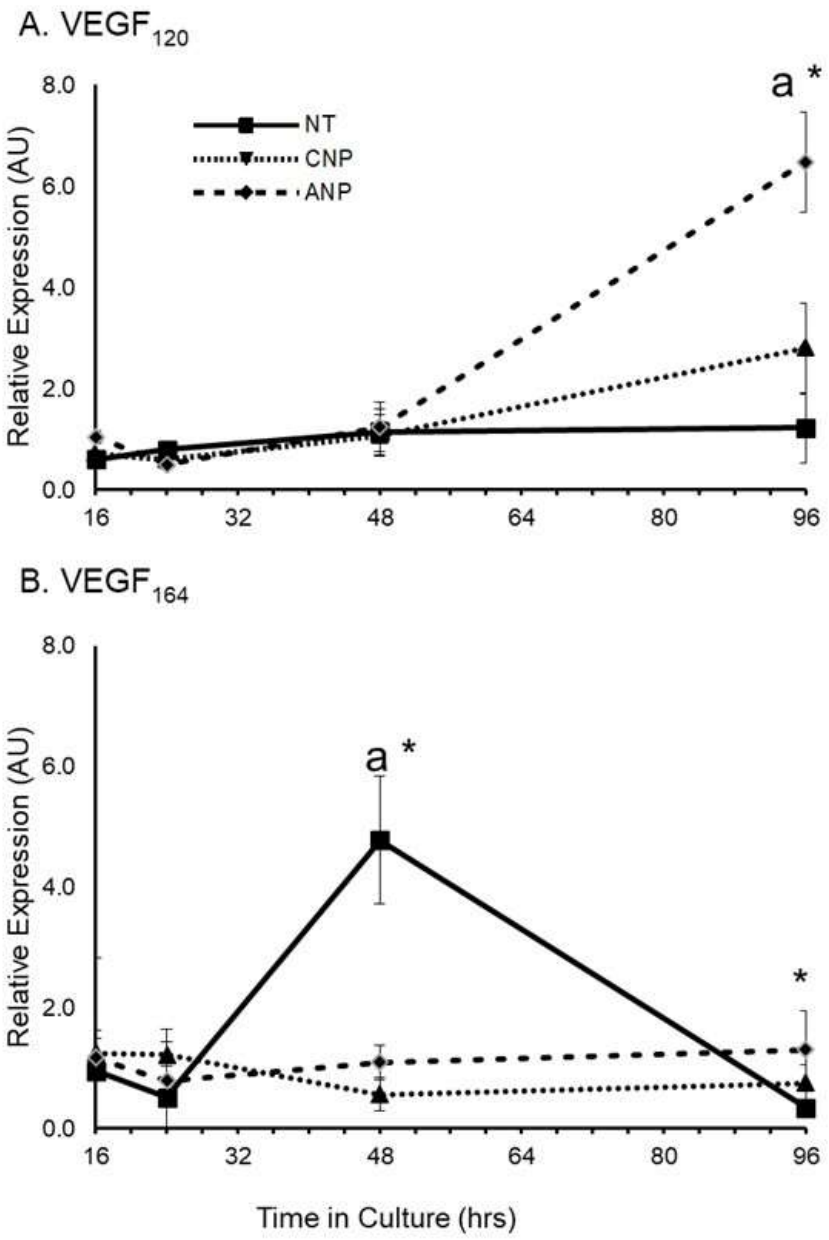

Figure 8. Expression of $VEGF_{120}$ (A) and $VEGF_{164}$ (B) relative to an endogenous control (18S rRNA) in ovine GC cultured over time in media treated with either ANP (dotted line), CNP (dashed line) and without treatment (NT, filled line). Graphs represent the means, +/- the standard error of means (SEMs) of at least 3 separate experiments performed in duplicate. Statistically significant differences ($P<0.05$) of gene expression over the previous time point are denoted by asterisks (*) and for, ANP or CNP compared to untreated (NT) by the letter 'a'.