

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

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1 **ABSTRACT**

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

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## 23 INTRODUCTION

24 It is well established that members of the Natriuretic Peptide (NP) superfamily of ligands  
25 and their receptors (NPRs) are expressed in several locations within the female  
26 reproductive system of a number of poly-ovular mammals such as, rat (Gutkowska, et al.  
27 1999), mice (Tsai, et al. 2005) and pigs (Zhang, et al. 2005). They have been reported in  
28 various compartments of the ovary including: follicular fluid, theca cells (TCs), granulosa  
29 cells (GCs), the oocyte and the corpus luteum, and this suggests they may have important  
30 roles in ovarian function. They have been linked to the regulation of follicular atresia,  
31 oocyte maturation and ovarian steroidogenesis (Dineva, et al. 2011). NP responsiveness in  
32 follicular somatic compartments continues to be an important area of research, especially  
33 in humans or a suitable model monovulatory species.

34

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

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

51

52 An area that has not been previously studied, in the context of NP responses in developing  
53 follicles, is the effect of reducing pericellular oxygen levels in the blood-isolated GC  
54 compartment due to the formation of multiple layers of cells. Ideally, such a study may be  
55 best performed under naturally occurring, as opposed to chemically-induced hypoxia and  
56 although the specialised culture systems used in this study have been designed (Campbell,  
57 et al. 1996; Campbell, et al. 1998) to provide the conditions necessary for gonadotrophin-  
58 stimulated steroidogenesis (GCs, oestradiol 17 $\beta$  and TCs, androstenedione), it has been  
59 shown that increasing seeding cell-density, accelerates the formation of multicellular 3-  
60 dimensional masses which undergo concomitant increases in naturally occurring  
61 pericellular hypoxia (Marsters, et al. 2014). Strong evidence was provided demonstrating  
62 that in these dense formations pericellular oxygen fell to hypoxic levels and expression of  
63 hypoxia-induced factor-1 (HIF-1) and levels of two key translation isoforms of the  
64 angiogenic factor group VEGFA (variant 120 and 164) were markedly increased. This  
65 culture system, therefore offers a promising approach to further investigate possible links  
66 between hypoxia and NP activity within the GCs of growing follicles. Putative  
67 relationships between NPs and VEGF isoforms in hypoxic GCs have previously not been  
68 investigated though, a number of groups have reported: that NPs have functions in the  
69 process of folliculogenesis (Noubani, et al. 2000; Zhang, et al. 2011; Zhang, et al. 2005).  
70 In addition, VEGFA variants are elevated in various compartments of growing follicles  
71 concomitant to induction of vascularisation (Robinson et al., 2007; Shimizu & Miyamoto,  
72 2007); and more latter studies suggest the possibility of interactivity between NPs and  
73 VEGF variants (Bijsmans, et al. 2017; Kamai, et al. 2018; Spes, et al. 2019).

74

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

89

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

99

100 **Materials and Methods**

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

104

105 *Tissue collection and cell preparation*

106 Ovine ovaries were taken from freshly slaughtered, abattoir, animals and maintained at  
107 37°C in a collection/dissecting buffer of Dulbecco's modified eagle medium (DMEM)  
108 containing the ingredients fully described (Campbell, et al., 1996; Gutierrez et al., 1997).  
109 Granulosa cells (GCs) were obtained from cleanly dissected, morphologically healthy,  
110 small antral follicles (3mm or less in diameter) as previously described (ibid). Cells were  
111 then washed twice by flushing and centrifuging at 800g for 10 minutes in a GC culture  
112 medium of McCoys 5a containing the ingredients previously described (ibid). Intact theca  
113 cell (TC) 'shells' obtained after the GCs had been flushed from the hemisected follicles,  
114 were disrupted by incubation for approximately 20 minutes at 37°C with a cocktail of  
115 proteases in dPBS as previously described (Campbell et al., 1998). This process also  
116 removed contaminant GCs. Following washing the TCs were resuspended in  
117 DMEM:Hams F12 culture medium containing ingredients as described previously (ibid).

118

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

123 plates, at the optimal seeding rate of  $5 \times 10^5$  (Campbell et al., 1996) viable cells per well  
124 in 1 ml of culture medium. Both GCs and TCs were maintained in a humidified atmosphere  
125 of 95% air and 5% carbon dioxide, at 37°C. Every 48 hours 80% of media was replaced  
126 with an equal volume of fresh media containing, treatments (below described). Spent media  
127 was reserved at -20°C. To compare the expression of the VEGFA splice variants the GC  
128 were seeded at the either the high or low density (HD, LD) rates described by Marsters et  
129 al., (2014) of  $10^6$  viable cells or of  $10^5$  viable cells per well in 1 ml of medium. After 16,  
130 24, 48 and 96 hours GC samples were retained at -20°C, in 150  $\mu$ l of RNeasy<sup>®</sup> RTL buffer  
131 (Qiagen<sup>®</sup>) containing 1%  $\beta$ -mercaptoethanol, for qPCR analysis. In both the VEGFA splice  
132 variants comparison, and the oestradiol 17 $\beta$  (E2) and progesterone (P4), studies the cells  
133 either had ANP or CNP (representative NP signallers via A- and B-type receptors  
134 respectively) added to the media to a concentration of 100 nM, or were untreated (NT). In  
135 serial dilution studies NPs were found to have similar effects in GC over a wide molar  
136 range (10 nM – 10  $\mu$ M), with 100 nM having been previously reported (Zhang, et al., 2015)  
137 to illicit cellular responses.

138

### 139 *Proximity ligation assay (PLA)*

140 This assay was based on an *in situ* PLA, in which a pair of oligonucleotide-tagged  
141 secondary antibodies (PLA probes) produce a signal only when both are bound in close  
142 proximity (Fig. 1) either on the same target or two different targets (Soderberg et al., 2006).  
143 Ovine GCs or TCs were seeded, in their respective media, at 200 viable cells per well into  
144 8-well Nunc<sup>®</sup> Lab-Tek<sup>®</sup> chamber slides<sup>™</sup> and cultured. After 48 hours of culture the media  
145 was removed, and the cells washed, fixed and blocked according to the standard Duolink<sup>®</sup>

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

148

149 For the identification of individual natriuretic peptide receptors (single target), the initial  
150 step was to incubate with primary antibodies, specific for each of the NPRs, in  
151 concentrations as per Table 1, either for 1 hour at room temperature or overnight at 4°C.  
152 Excess antibodies were removed by washing twice for 5 minutes. The *in situ* PLA was  
153 carried out as per the supplied manual. Briefly, when the DNA attached to pairs of  
154 antibodies hybridises, fluorescent oligonucleotides are incorporated, resulting in around  
155 1000-fold amplification of signal. Finally, the wells were removed from the chamber slides  
156 as per the protocol and the slides mounted with a small amount of Duolink® in situ  
157 mounting medium with DAPI under a glass cover slide, and after a 15-minute incubation  
158 the fluorescent signals were visualised under a confocal microscope.

159

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

166

167 *Immunohistochemistry (IHC)*

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



169 fixed in 4% PFA and embedded in paraffin wax. They were then serially sectioned into 10  
170  $\mu\text{m}$  slices and mounted onto SuperFrost Plus microscope slides (Menzel-Glaser,  
171 Braunschweig, Germany), which were then and baked overnight at 45°C. The slides were  
172 placed in a Bond Max Automated Immunohistochemistry Vision Biosystem (Leica  
173 Microsystems GmbH, Wetzlar, Germany) according to the following protocol. Slides  
174 underwent dewaxing in a descending alcohol series, followed by epitope retrieval by  
175 treatment with 0.1 M sodium citrate for 10 minutes. After being washed in PBS, blocking  
176 with a 3% hydrogen peroxide solution was carried out for 10 min, using the Bond Polymer  
177 Refine Detection Kit DC9800 (Leica Microsystems GmbH). In accordance with this  
178 protocol, slides were again washed and then incubated with the primary antibody (Anti-  
179 NPRA or Anti-NPRB, Table 1) for 30 min. Subsequently, the slides were incubated with  
180 poly-HRP-IgG conjugate for 10 min and developed with 3, 3'-Diaminobenzidine  
181 tetrahydrochloride (DAB)-Chromogen for 10 minutes. Nuclei were counterstained with  
182 0.02% haematoxylin.

183

#### 184 *qPCR Analysis*

185 Total RNA was isolated from the GCs using the RNeasy<sup>®</sup> mini kit (Qiagen<sup>®</sup>) and protocol.  
186 First strand cDNA synthesis was performed using Revertaid<sup>™</sup> H-minus reverse  
187 transcriptase (Thermo Scientific<sup>™</sup>) according to the prescribed protocol. Unless otherwise  
188 stated all qPCR, reagents were supplied by Applied Biosystems<sup>™</sup> (Warrington, UK). All  
189 quantitative PCR (qPCR) was carried out on Applied Biosystem's 7500 FAST<sup>®</sup> Real-time  
190 thermocycler. Target genes VEGF<sub>120</sub>, VEGF<sub>164</sub>, NPPA, NPPB, NPPC, NPRA and NPRC,  
191 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<sup>®</sup> real-time primer/probe sets and  
195 followed the prescribed Taqman<sup>®</sup> 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 94°C for 10 minutes and cycled 35  
199 times at 94°C for 30 seconds, 57°C for 30 seconds and 72°C 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 17 $\beta$  (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<sup>®</sup>,  
207 Cambridge, UK) or P4 antibody (R7044X, Scottish Antibody Production Unit<sup>®</sup>, 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 4°C. 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  $\mu$ l 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<sup>®</sup> Company, Portland, Oregon, USA). The  
214 assay plates were then incubated for 2 hours on an orbital shaker at 170 rpm. This was

215 followed by five washes (as above) and addition of 50  $\mu$ L of HRP substrate (3,3',5,5'-  
216 Tetramethylbenzidine, TMB). After about 15 minutes (or after the development of a blue  
217 colouration) the HRP enzymatic reaction was halted by addition of 50  $\mu$ L of 'Stop' solution  
218 (1 N sulphuric acid) and the plate read at 450 nm. The sensitivities of the E2 and P4 assays  
219 were both 39 pg/ml (~90% of zero binding value) and the inter- and intra-assay coefficients  
220 of variation were both <10%.

221

### 222 *Statistical analysis*

223 Production of E2 and P4 were calculated as the concentration per  $1 \times 10^3$  cells seeded in  
224 the cultures. The qPCR results were analyzed using a 'relative standard curve' method  
225 according to Applied Biosystems' analysis software package, version 2.0. Semi-quantitative  
226 comparisons between sample groups were made after the target gene expression had been  
227 normalised against an 18S endogenous control. The results shown are the means  $\pm$  SEMs  
228 of at least three independent experiments with each experiment having been carried out in  
229 at least triplicate. 'Repeated measures ANOVA' was performed (SPSS software  
230 version 16.0) to determine the level of significance between sample groups.

231

### 232 **Results**

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

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

243

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

254

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

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

265

266 In other ovine GC cultures, set up in 24-well plates (Methods) the production of the major  
267 steroid hormones, oestradiol 17 $\beta$  (E2) and progesterone (P4) were measured over time by  
268 ELISA (Methods) and the effects of 100 nM ANP or CNP were compared. In the untreated  
269 cultures E2 production over the 48 hours of culture preceding the 96 hours time-point was  
270 noted to more than double ( $P<0.05$ ) the production over the first 48 hours of culture (Fig.  
271 7A). However, in the 48 hours following the 96 hour time-point production was noted to  
272 fall back markedly ( $P<0.05$ ) to similar levels to that produced in the first 48 hours of culture.  
273 While CNP was shown to have no effect on this profile, production was markedly ( $P<0.05$ )  
274 reduced it in the ANP-treated cells, over the 48 hours of culture preceding the 96 hour time-  
275 point. Over the same 144 hours of culture P4 production showed a trend of increasing  
276 production (Fig. 7B), with a 4-fold increase ( $P<0.05$ ) noted after 144 hours of culture over  
277 the levels noted after the first 48 hours of culture. The addition of CNP to the culture media  
278 did not have a significant effects on the 'no treatment' profile.

279

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

284 clusters (High-density seeded, HD) previously reported to induce naturally increasing  
285 levels of pericellular hypoxia (Marsters et al., 2014). In the LD cultures no significant  
286 effects of ANP or CNP treatment were noted, so those outcomes have not been included in  
287 Fig. 8. However, in the HD seeded GCs, though neither ANP nor CNP had a significant  
288 effect on the expression levels of VEGF<sub>120</sub> (Fig. 8A) over the first 48 hours of culture, that  
289 variant's expression increased by almost 6-fold ( $P<0.05$ ) in the ANP-stimulated cells, over  
290 the next 48 hours to 96 hours of culture. In contrast CNP had no effect on the 120 variant's  
291 expression level over the whole 96 hours of culture. In the untreated HD-seeded GCs, the  
292 expression profile of VEGF<sub>164</sub> (Fig. 8B) was distinctly different than that of VEGF<sub>120</sub>,  
293 which appeared to be expressed at a low level throughout the time course. Whereas, after  
294 24 hours of culture the expression levels of VEGF<sub>164</sub> increased by over 5-fold ( $P<0.05$ )  
295 over the following 24 hours of culture, but fell away markedly ( $P<0.05$ ) thereafter to  
296 starting levels by 96 hours of culture. This surge of expression in the untreated GCs,  
297 measured at 48 hours of culture, was not apparent in either of the ANP-stimulated or the  
298 CNP-stimulated cells.

299

300

### 301 **Discussion**

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

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

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

334

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

352

353 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-

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

382

383 A number of studies (Campbell et al., 1996; Gutierrez et al., 1997; Campbell et al., 1998;  
384 Marsters et al., 2003) have confirmed that in GCs, taken from the small antral follicles of  
385 large ruminants and cultured under the optimal conditions described by Campbell et al.,  
386 (1996), E2 production remains non-existent or at low levels for up to 48 hours of culture.  
387 Around this point cell differentiation occurs marked by E2 levels up-regulating, usually  
388 peaking after at least a further 48 hours of culture and this concurs with the outcomes of  
389 this present study showing E2 increased sharply between 48 and 96 hours of culture. It has  
390 been assumed that the subsequent marked decrease after that point is due to a limiting  
391 steroidogenesis precursor such as cholesterol (not included in the specialised culture  
392 medium). From earlier reports (ibid) and this current study it is tempting to hypothesise  
393 that E2 production may be initiated as a result of cells coming together and forming gap-  
394 junction-coupled clusters in which the pericellular environment undergoes steadily  
395 increasing hypoxia. However this may be quite transient as work reported by Marsters et  
396 al., (2014), along with findings reported here indicate that when numbers and sizes of cell  
397 clusters are well advanced the heightened density of large multi-laminar cell masses are  
398 concomitant to, and may induce, E2 inhibition. Utilising ANP to stimulate a NPRA-  
399 mediated response, the E2 'spike' was noted to be markedly less than in the NT cultures.

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

405

406 This work has produced novel and compelling evidence that the regulation of NP-  
407 responsiveness in the GC and TC compartments of sheep is complex and multi-layered,  
408 and may involve ligand-mediated receptor desensitisation, receptor-mediated ligand  
409 clearance and cell-specific mechanisms for the production of receptors, and active ligand.

410 Taken together these data suggest that this super family of ligands and receptors are highly  
411 active in the developing follicles of a monovulatory species and may have a number of  
412 important roles besides those already described in the literature.

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558 **Tables**

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**Table 1. Antibodies - Proximity Ligation Assay (PLA).** <sup>561</sup>

<b>Antibody</b>	<b>Description</b>	<b>I.D.</b>	<b>Dilution</b>
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/D UO92004minus	1:5
PLA probe-anti-rabbit plus/PLA probe-anti-rabbit minus	Duolink® PLA Donkey anti-rabbit IgG Probe (Sigma™)	DUO92002plus/D UO92005minus	1:5

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**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' - TCGGCTTGTCACATTTTCTTG - 3'	AF250375
oVEGF164.R	Exon 5/7a	R: 5' - CAAGGCCACAGGGATTTTC - 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



581 Figures

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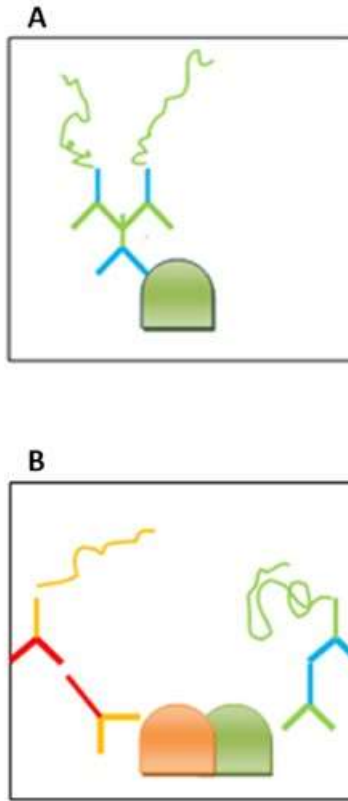


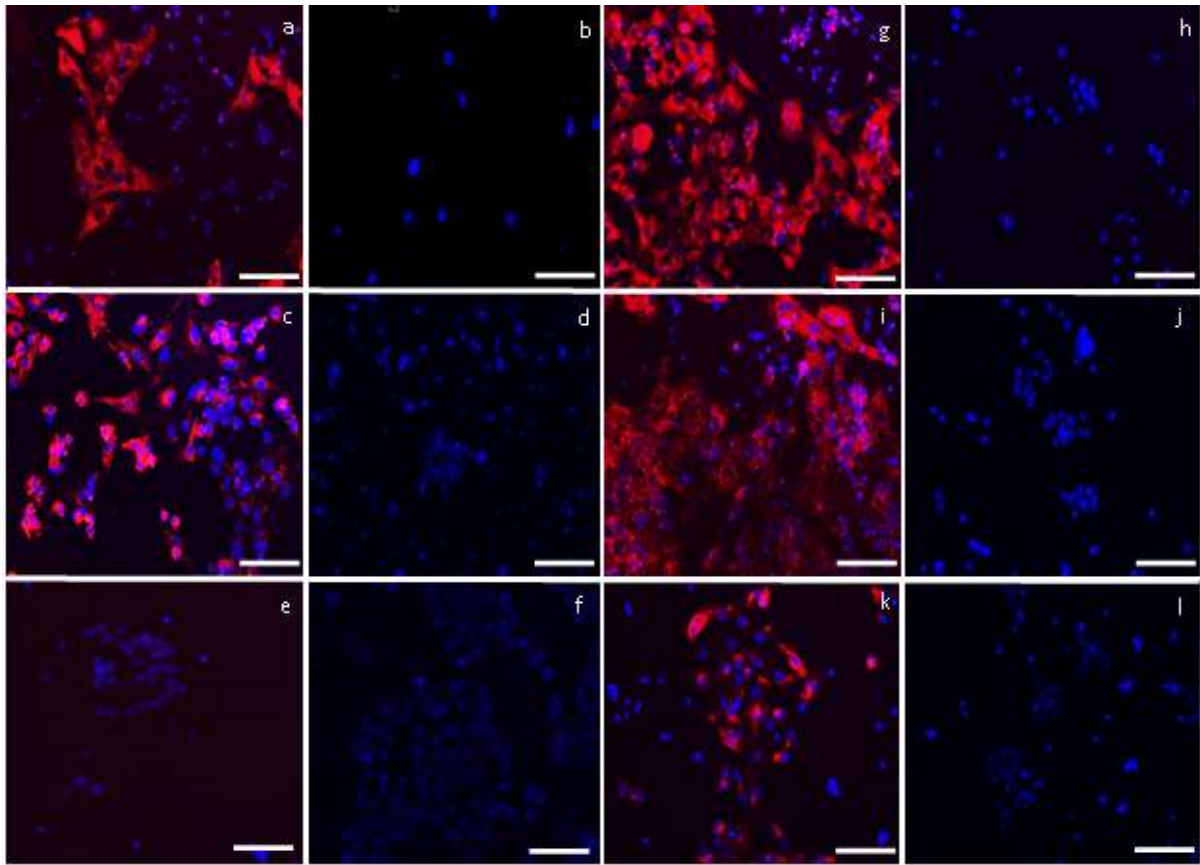
Figure 1. Proximity Ligation Assay (PLA). DNA strands attached to secondary Abs, which are primary Ab-specific, need to be in close contact in order for PCR amplification and detection-entity tagged nucleotide incorporation to occur (Methods). This was achieved using target-specific primary Abs and, 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 two target proteins and the plus and minus secondary Abs are each species-specific to target each of the primary Abs.

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 609 Figure 2. Cultured ovine TC and GC showing immunolocalisation (red fluorescence) of natriuretic  
 610 peptide receptors; NPRA (a, TC) and (g, GC), NPRB (c, TC) and (i, GC), and NPRC (e, TC) and (k,  
 611 GC). The respective, non-primary Ab negative controls (b, h, d, j, f and l), control against non-  
 612 specific binding by secondary Abs. Cell nuclei were counter-stained blue using DAPI and bar = 100  
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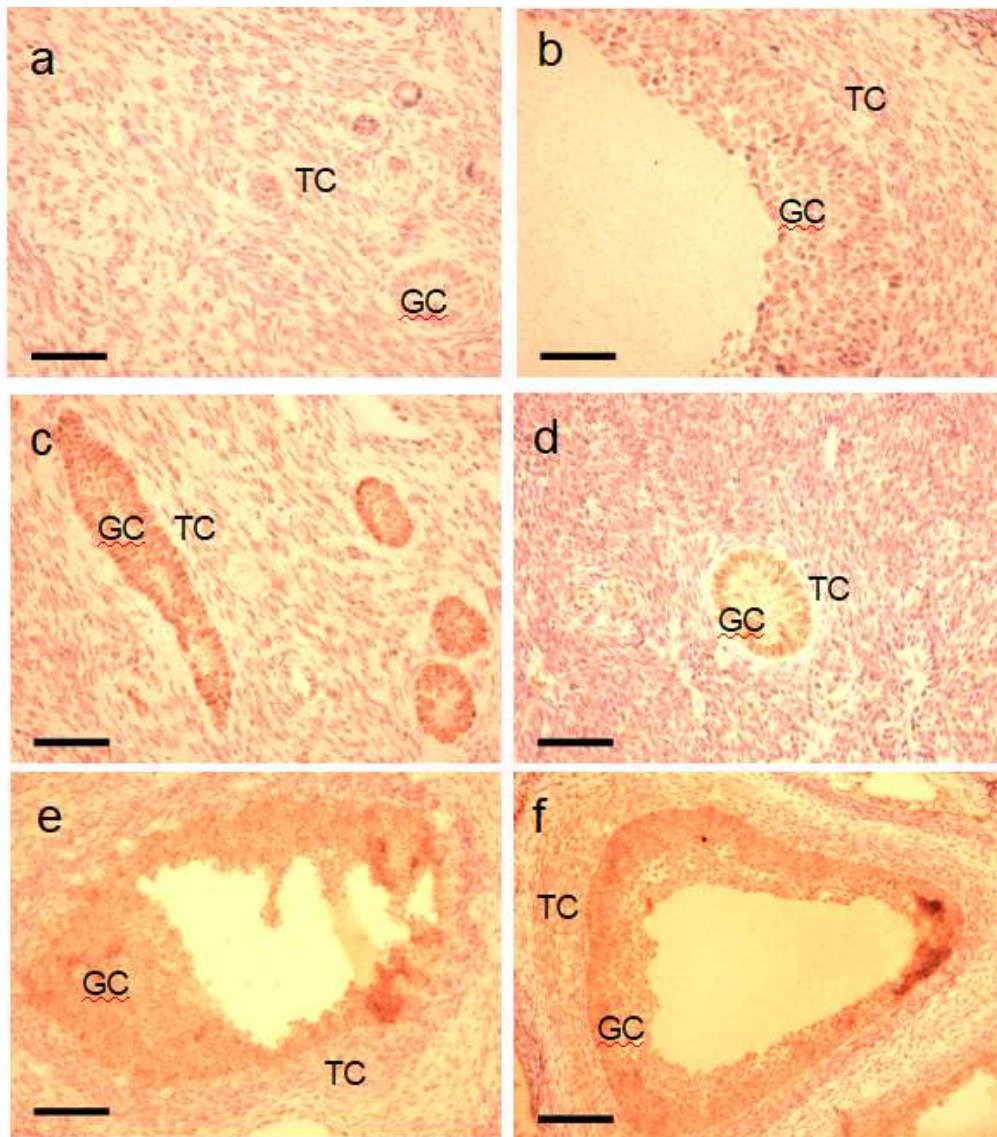
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652 Figure 3. Immuno-histochemistry (IHC) analysis in ovine ovary sections localising anti- NPRA (c &  
 653 e) and anti-NPRB (d & f) to various sized follicles Positive immunocalisations depicted in darker  
 654 red. Primary Ab absent, negative controls (a & b) are included for NPRA and NPRB respectively.  
 655 Theca and Granulosa cell compartments indicated by TC and GC respectively and bar =50µm

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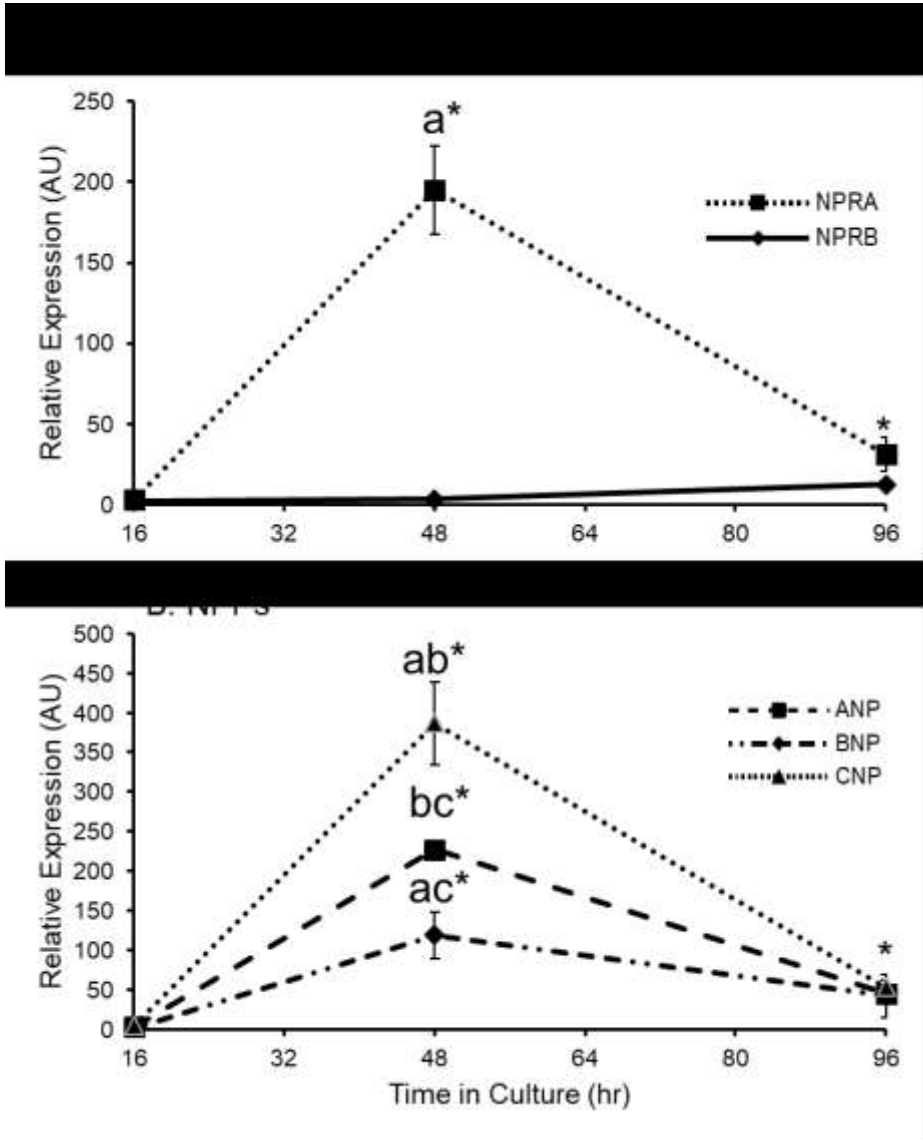
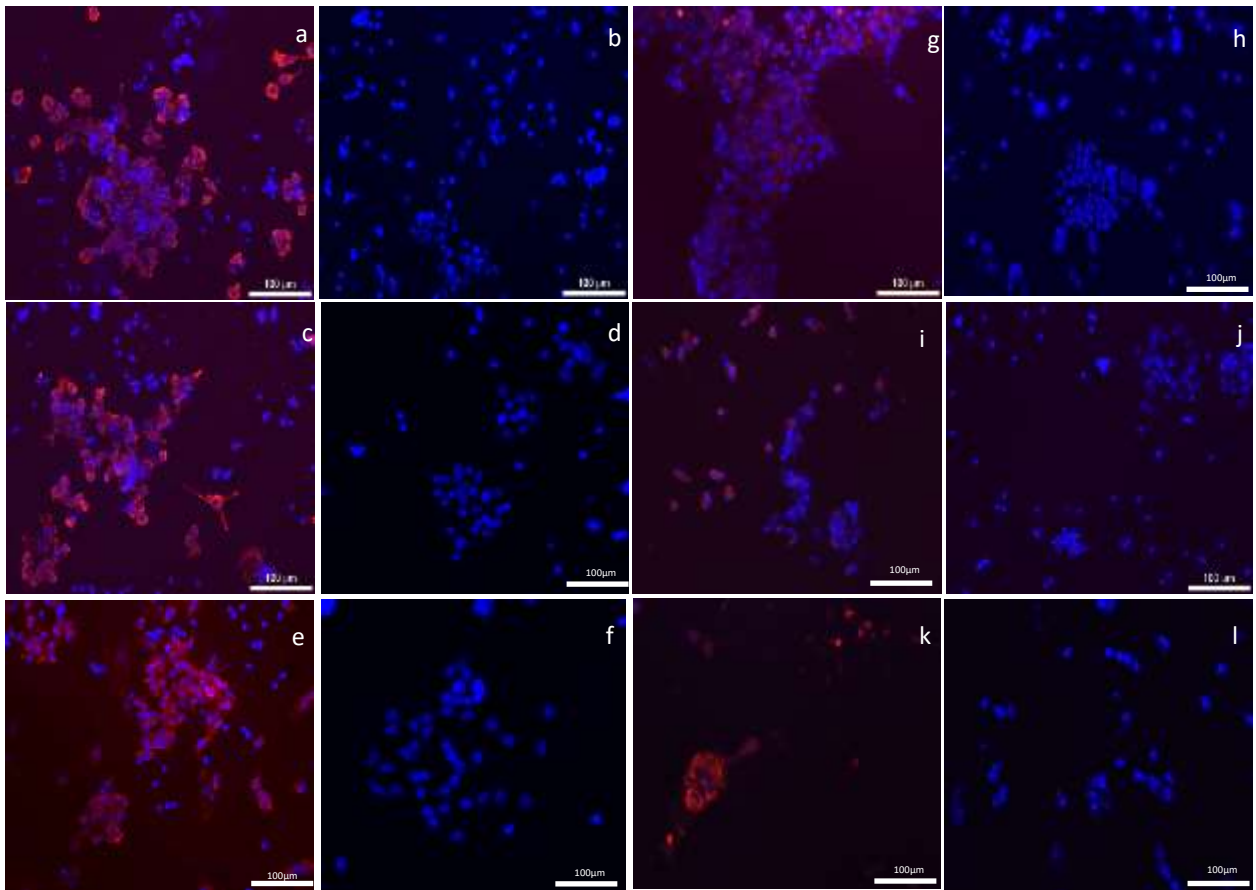


Figure 4. Expression in high or low density cultured cells (HD or LD respectively) of (A) NPRA (dotted line) and NPRB (filled line) and (B) NPPA (dashed line), NPPB (dot & dash line) and NPPC (dotted line) relative to an endogenous control (18S rRNA) in ovine GC cultured over time. The graphs represent 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).

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699 Figure 5. Cultured ovine TC and GC showing immunolocalisation (red fluorescence) of natriuretic  
700 peptide forms. ANP forms (a, TC) and (g, GC), BNP forms (c, TC) and (i, GC), and CNP forms (e,  
701 TC) and (k, GC). The respective, non-primary Ab negative controls (b,h,d,j,f and l), control against  
702 non-specific binding by secondary Abs. Cell nuclei were counter-stained blue using DAPI, and bar  
703 = 100 µm

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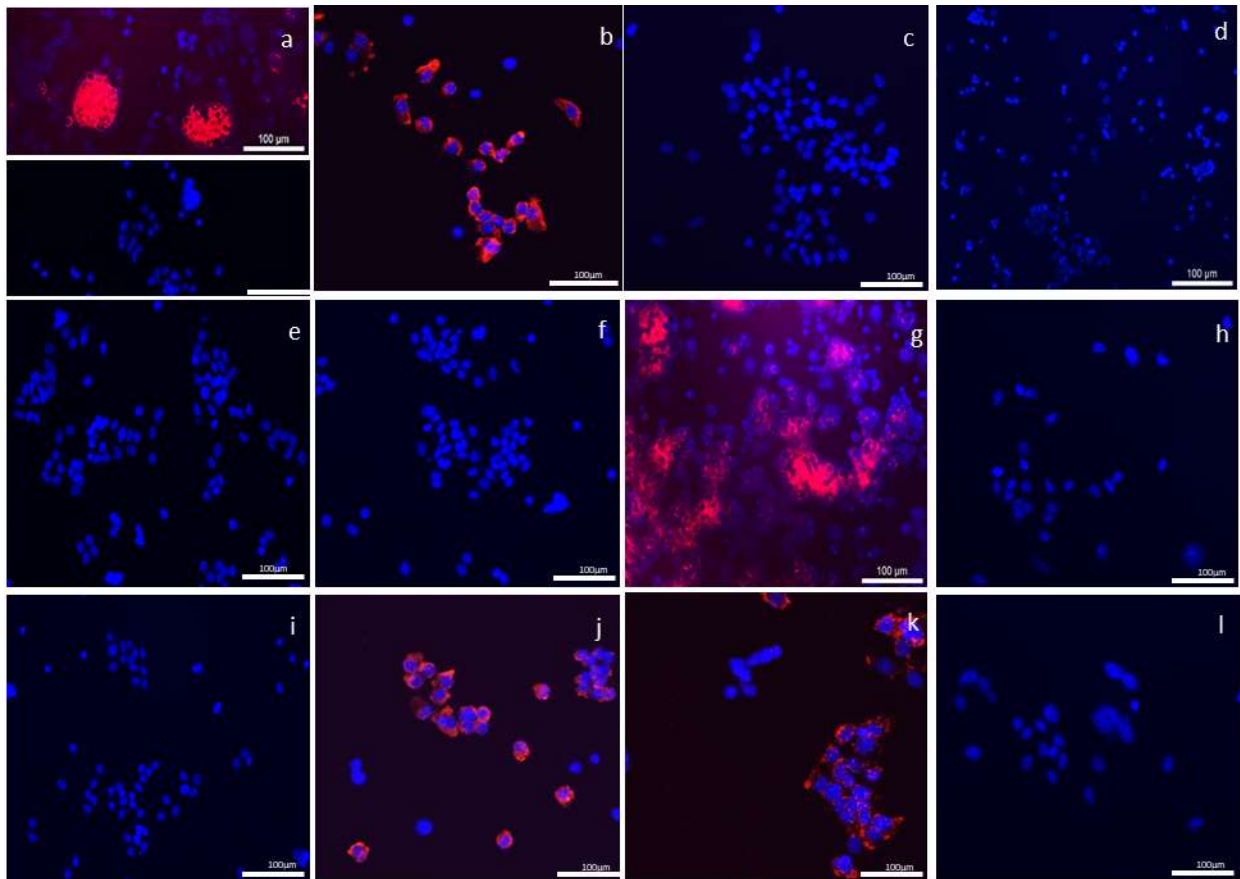
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 715 Figure 6. Cultured ovine TC and GC showing locally produced NP ligand associations with NPRs  
 716 (red fluorescence) utilizing PLA detection and signal enhancement. Sets of target-specific primary  
 717 antibodies were used which were specific for NPRA with ANP (a) with exogenous ANP in upper,  
 718 BNP (b), and CNP (c); NPRB with ANP (e), BNP (f) and CNP (g); and NPRC with ANP (i), BNP (j)  
 719 and CNP (k). The respective non-primary Ab negative controls (d, h and l), controls against non-  
 720 specific binding by secondary Abs. Cell nuclei were counter-stained blue using DAPI and bar = 100  
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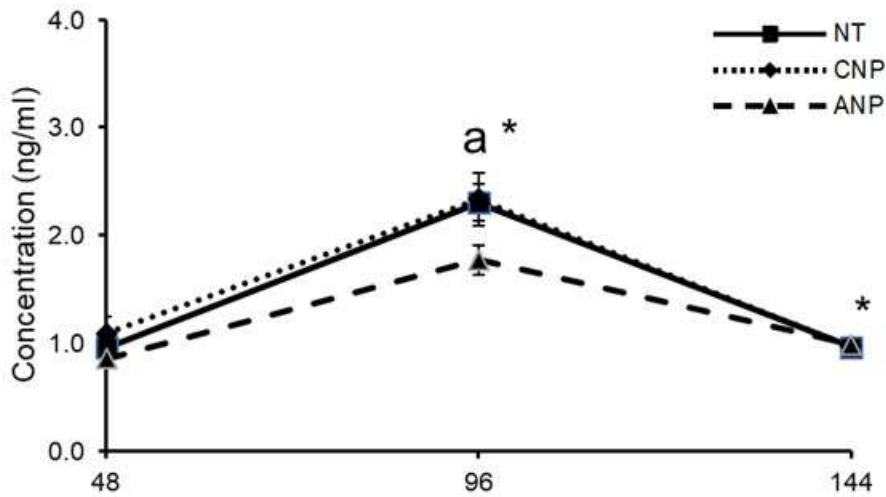
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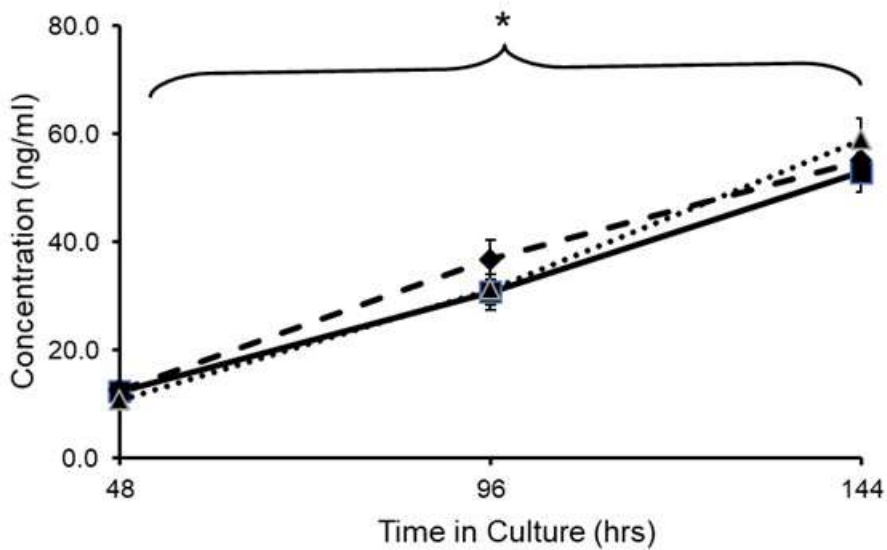
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A. Oestradiol (E2)



B. Progesterone (P4)



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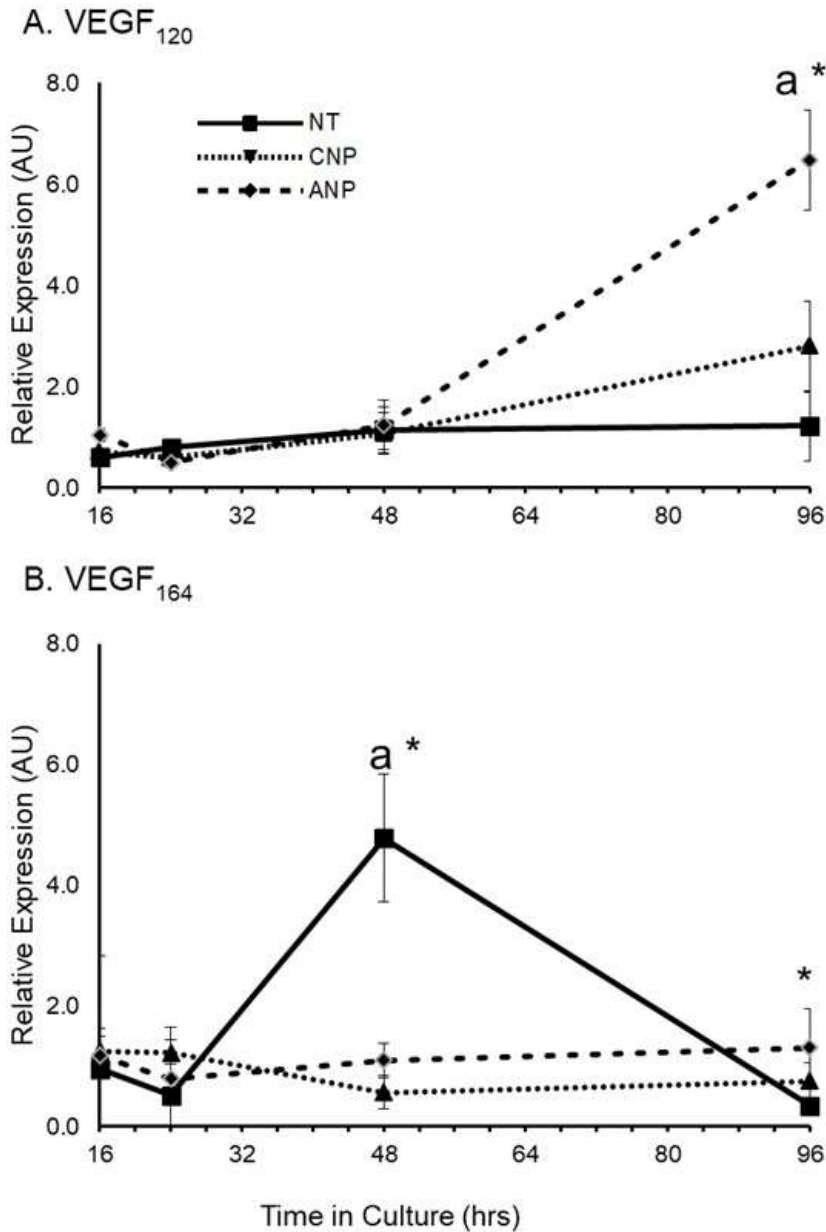


Figure 8. Expression of VEGF<sub>120</sub> (A) and VEGF<sub>164</sub> (B) relative to an endogenous control (18S rRNA) in ovine GC cultured over time in media treated with either ANP (dotted line), CNP (dashed line) and without treatment (NT, filled line). Graphs represent the means, +/- the standard 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'.