Novel Functions of Natriuretic Peptides in Mammalian Cumulus-Oocyte Complexes

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Abstract

Natriuretic peptides are physiological regulators of oocyte meiosis, existing within ovarian follicles and are being exploited to reveal the mechanisms regulating meiosis. To date, assessment of natriuretic peptides have been examined during culture of COCs from many mammals including mice, rats, pigs, cows, goats, horses and cats. Mouse models have dominated this research, elucidating the functions of CNP and NPR2 signalling within the COC and which maternal and oocyte-derived factors regulate their activity. Three *in vitro* models are utilised to explore novel functions of natriuretic peptides. These include: sheep IVM, pig IVM from large antral follicles, and pig IVM from small developmentally incompetent follicles.

This thesis shows that *NPR2* is the predominant cGMP-generating natriuretic peptide receptor in cumulus cells and is likely responsible for the majority of cGMP produced in the sheep ovarian follicle. OSFs and IGF-1 may inhibit *NPR2* expression in cumulus cells whilst promoting *NPR1* expression. Furthermore, CNP has the ability to inhibit the resumption of meiosis in ovine COCs *in vitro*, however is dose dependant.

Using pig COCs, I determine CNP and cGMP have the ability to activate ERK1/2 in COCs from large follicles cultured *in vitro*. This action was independent of cAMP and CREB activation. However, it required functional MMPs and EGFR without altering the expression of the EGF-like peptides; AREG, EREG or EGFR. CNP and cGMP also induced the breakdown of gap junctions in COCs from large follicles. In pig COCs from small follicles, CNP and cGMP had no effect of ERK1/2 activation or gap junction communication. In COCs collected from both small and large follicles, CNP promoted meiotic resumption of oocytes cultured *in vitro*. However, when cultured in the presence of a highly stimulated system (FSH+pFF), CNP could inhibit the meiotic resumption of oocytes *in vitro*. I further show CNP stimulates ERK1/2 activation to levels comparable to FSH and pFF, which therefore could explain why my results may vary from current published research.

Our knowledge of the molecular mechanisms which regulate oocyte maturation has grown significantly over the past few years. The findings in this thesis illustrate a major difference between cellular signalling between porcine COCs from small follicles, compared to COCs collected from larger follicle and therefore have importance to research into ovarian follicle development. Furthermore, this thesis supports the notion that there may be more to the CNP/cGMP pathway than simply the inhibition of meiosis; it may promote oocyte development by propagating cumulus cell signals. This dissertation advances our understanding of the process of oocyte maturation, and could be applied to provide better opportunities for the application of IVM in assisted reproductive technology for humans, and in agriculture.

Declaration

I certify that this thesis does not contain any material which has been previously been offered for any other degree or diploma at any other university, nor to the best of my knowledge does it contain previously published material except where due reference has been made.

In addition, I certify that no part of this work will, in the future, be used in any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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Ryan David Rose

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Abbreviations

8-Br-cGMP	8-Bromoguanosine 3',5'-cyclic monophosphate
8pCTPcGMP	8-(4-Chlorophenylthio)-guanosine 3′,5′-cyclic monophosphate
ACTB	B-actin
ADAM	A disintegrin and metalloprotease domain
ADAM17	ADAM metallopeptidase domain 17
ADCY	Adenylate cyclase
ALK	Activin receptor-like kinases
ANOVA	Analysis of variance
ANP	Atrial natriuretic peptide
AREG	Amphiregulin
ART	Assisted reproductive technology
ATI	Anaphase/telophase I
BMP15	Bone morphogenetic protein 15
BMPRII	Bone morphogenetic protein receptor type-II
BNP	Brain natriuretic peptide
BSA	Bovine serum albumin
BTC	Betacellulin
cAMP	Cyclic adenosine monophosphate
CBX	Carbenoxolone
CC	Cumulus cell
Cdc	Cell division cycle
cDNA	Complementary deoxyribonucleic acid
cGMP	Cyclic guanosine monophosphate
CNP	C-type natriuretic peptide
CO_2	Carbon dioxide
COC	Cumulus-oocyte complexes
COX-2	Cyclooxygenase-2
CREB	cAMP-response binding protein
c-Src	Proto-oncogene tyrosine-protein kinase
cx37	Connexin-37
cx43	Connexin-43
Cyp19a1	Cytochrome P450 family 19 subfamily A member 1
DAPI	6-diamidino-2-phenylindole
DNA	Deoxyribonucleic acid
DO	Denuded oocyte

E2	Oestradiol
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGFp	Epidermal growth factor-like peptides
EGFR	Epidermal growth factor receptor
EREG	Epiregulin
ERK	Extracellular response kinase
FGF	Fibroblast growth factor
FP	Foot processes
FSH	Follicle stimulating hormone
FSHR	Follicle stimulating hormone receptor
G	G-protein
GC	Granulosa cells
GC-B	Guanylate cyclase B
GDF9	Growth differentiation factor 9
GJ	Gap junction
GJA	Gap junction alpha protein
GJC	Gap junction communication
GnRH	Gonadotropin-releasing hormone
GPR	G-protein-coupled receptors
GTP	Guanosine-5'-triphosphate
GV	Germinal vesicle
GVBD	Germinal vesicle break down
h	Hour
HA	Hyaluronic acid
Has2	Hyaluronan synthase 2
hCG	Human chorionic gonadotropin
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IGF	Insulin-like growth factors
IgG	Immunoglobulin G
IMP	Inosine monophosphate
IMPDH	Inosine monophosphate dehydrogenase
IVF	*In vitro* fertilisation
IVM	*In vitro* maturation
kBq	Kilobecquerel

kDa	Kilodalton
L	Litre
LH	Luteinising hormone
LHR	Luteinising hormone receptor
LY	Lucifer yellow
MAPK	Mitogen activated protein kinases
MAS	Meiosis activating sterol
mg	Milligram
MGC	Mural granulosa cells
MI	Metaphase I
MII	Metaphase II
min	Minute
mL	Millilitre
mM	Millimolar
µg	Microgram
µL	Microliter
µM	Micromolar
mm	Millimetre
MMP	Matrix metalloproteinase
MPF	Maturation promoting factor
mRNA	Messenger RNA
MYT1	Myelin transcription factor 1
N_2	Nitrogen
NaCl	Sodium chloride
ng	Nanogram
nm	Nanometres
nM	Nanomolar
NO	Nitric oxide
NPPC	Natriuretic peptide precursor type C
NPR1	Natriuretic peptide receptor 1
NPR2	Natriuretic peptide receptor 2
NPR3	Natriuretic peptide receptor 3
^{o}C	Degrees Celsius
OHSS	Ovarian hyperstimulation syndrome
OMI	Oocyte maturation inhibitor
OOX	Oocytectomised cumulus-oocyte complex

OSF	Oocyte secreted factors
P	Probability
p38MAPK	P38 mitogen-activated protein kinase
PBS	Phosphate buffered saline
PCOS	Polycystic ovarian syndrome
PCR	Polymerase chain reaction
pCREB	Phosphorylated cAMP response binding protein
PDE	Phosphodiesterase
PDE3A	Phosphodiesterase 3A
pERK1/2	Phosphorylated extracellular response kinase 1/2
pFF	Porcine follicular fluid
PGE2	Prostaglandin E2
PKA	Protein kinase A
PKC	Protein kinase C
PKG	Protein kinase G
PMA	Phorbol-12-myristate-13-acetate
pmol	Picomol
Ppia	Peptidylprolyl isomerase A
Ptger2	Prostaglandin E2 receptor 2
Ptgs2	Prostaglandin-endoperoxide synthase 2
Ptx3	Pentraxin-related protein 3
RIPA	Radio-immuniprecipitation Assay
RNA	Ribonucleic acid
RT	Room temperature
RT-PCR	Reverse transcription polymerase chain reaction
RT-QPCR	Quantitative reverse transcription polymerase chain reaction
s	Second
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SFK	Src –family kinases
siRNA	Small interfering ribonucleic acid
SPSS	Statistical package for the social sciences
Src	Sarcoma
TACE	Tumour necrosis factor converting enzyme
TBST	Tris-buffered saline with Tween 20

TCM	Tissue culture medium
TGFβ	Transforming growth factor β
TNFa	Tumour necrosis factor alpha
Tnfaip6	Tumour necrosis factor, alpha-induced protein 6
TZP	Transzonal process
U	Units
V	Volume
v/v	Volume per volume
vol	Volume
w/v	Weight per volume
WB	Western blot
wt	Weight
ZP	Zona pellucida

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CHAPTER 1:

LITERATURE REVIEW

1.1 Ovarian Follicle Formation and Development

The mammalian ovary is the female gonad responsible for the production of germ cells (oocytes) and the production of the steroids, oestrogen and progestins, and peptide growth factors. These are all critical for ovarian function, regulation of the hypothalamic-pituitary-ovarian axis, and development of secondary sexual characteristics (Edson *et al.* 2009).

During the fetal stages of life primordial germ cells are formed, which undergo a series of cellular transformations to form oogonia and progress to form the female germ cell, or oocyte. Primordial germ cells become oogonia when gonadal differentiation initiates, during which the oogonia are mitotically dividing and migrating (Beaumont 1961; Chretien 1966; Jost and Prepin 1966). After rapid mitotic division and migration, oogonia enter meiosis (Mauleon 1967; Challoner 1974). The major consequence of this early termination of oogonial mitosis is that by the time of birth, or shortly after in some species, there remains a finite population of oocytes for the duration of a female's life. The entry into meiosis coincides with the formation of the primordial follicle, which constitutes the fundamental functional unit of the ovary. Primordial follicles advance with the recruitment of surrounding granulosa cells derived from invading sex cords. The oocytes, halted at prophase I of meiosis, are surrounded by a layer of squamous granulosa cells which possess cytoplasmic projections that connect with the oocyte membrane (Byskov 1986; Pepling 2006). Primordial oocytes remain in a state of meiotic arrest for up to 50 years, dependent on the species, until the appropriate signalling cascade of follicle growth, and finally, meiotic resumption is initiated (Edson *et al.* 2009). Oocytes not contained within a primordial follicle degenerate, which occurs in the majority of primary oocytes. An event, which remains elusive, triggers the activation of the primordial follicles, and represents the beginning of folliculogenesis.

Folliculogenesis is a highly regulated process whereby a primordial follicle initiates its growth phase, with initial proliferation of the surrounding somatic cells (described as a transition from a primary follicle to a pre-antral or secondary follicle) followed by establishment of a fluid-filled antrum and a blood vascular network (described as antral or Graafian follicle), and culminates in the process of ovulation, where a fully mature oocyte is expelled from the ovary in preparation for fertilisation by sperm (see Figure 1.1). This process is regulated by autocrine, paracrine and endocrine factors, which maintain a fine balance between stimulatory and inhibitory actions. Follicle classification is based on several criteria including the size of the follicle and oocyte, the number of granulosa cell and somatic cell layers, the presence or absence of an antrum, and the dependency and sensitivity to gonadotrophins.

The first definitive morphological change in primordial follicle activation is the rounding of the flattened granulosa cells, and slow proliferation. In secondary to pre-antral follicle development, proliferating granulosa cells (GCs) progressively form several layers around the oocyte (secondary to late preantral follicles). From this stage, a layer of theca cells surrounds the follicle, and start to produce oestrogens (Figure 1.1). Theca cells produce androgens, which are converted into oestrogens by granulosa cells (Georges *et al.* 2014). Growth of preantral follicles is dependent on autocrine and paracrine regulatory factors but appears to be gonadotropin-independent, and the complex bidirectional communication between the oocyte and the somatic compartments of the follicle becomes more apparent (Eppig *et al.* 2002; Edson *et al.* 2009).

The bulk of oocyte growth occurs prior to antral formation and corresponds with increased number of mitochondria and intense metabolic activity, with half the protein required for this growth being synthesised by the oocyte, with the remainder supplied by the follicular cells (Schultz *et al.* 1979). Synthesis of core proteins responsible for the resumption of meiosis, like mitogen activated protein kinases (MAPK) and cyclin B2, are also synthesised during this phase (Wassarman and Mrozak 1981; Taieb *et al.* 1997). Furthermore, zona pellucida (ZP) and cortical granule synthesis mainly take place during the oocyte growth phase (Philpott *et al.* 1987; Fair *et al.* 1997).

The transition from the pre-antral to antral follicle is gradual and strictly depends on follicle stimulating hormone (FSH) stimulation. In brief, GC continue to proliferate, the antrum is formed and separates *de facto* granulosa cells into two general populations: cumulus and mural GCs (Georges *et al.* 2014), a process controlled by a complex network of autocrine/paracrine growth factors, requiring particular members of the transforming growth factor β (TGFβ) superfamily (Li *et al.* 1995; Yoshida *et al.* 1997). Mural granulosa cells (close to the basement membrane) become pseudo-stratified, and under the influence of FSH, develop luteinizing hormone receptors (Gougeon 1996). Granulosa cells develop cytoplasmic processes that extend between cells and the corona radiata, and transzonal processes are established (TZP) that form indentations in the oolemma to facilitate the exchange of molecules (discussed in Cumulus Oocyte Complex – Structure and Communication) (Albertini *et al.* 2001).

Among the cohort of growing antral follicles, only one (or a limited number of follicles in poly-ovular species) continues to grow to the pre-ovulatory stage, while others undergo atresia. Approaching ovulation, the pre-ovulatory follicle endures changes to the follicular envelope

and surface epithelium. Corona radiata acquire their characteristic columnar shape during the final maturation phase. Prior to follicular rupture, the entire follicle expands, now adjacent to the ovary surface, bulges externally and a localised part of the follicle wall dissipates and releases the cumulus-oocyte complex (Russell and Robker 2007). After ovulation, which is triggered by a peak of FSH and luteinizing hormone, theca cells and mural granulosa cells luteinise to form the corpus luteum which produces progesterone (Edson *et al.* 2009).

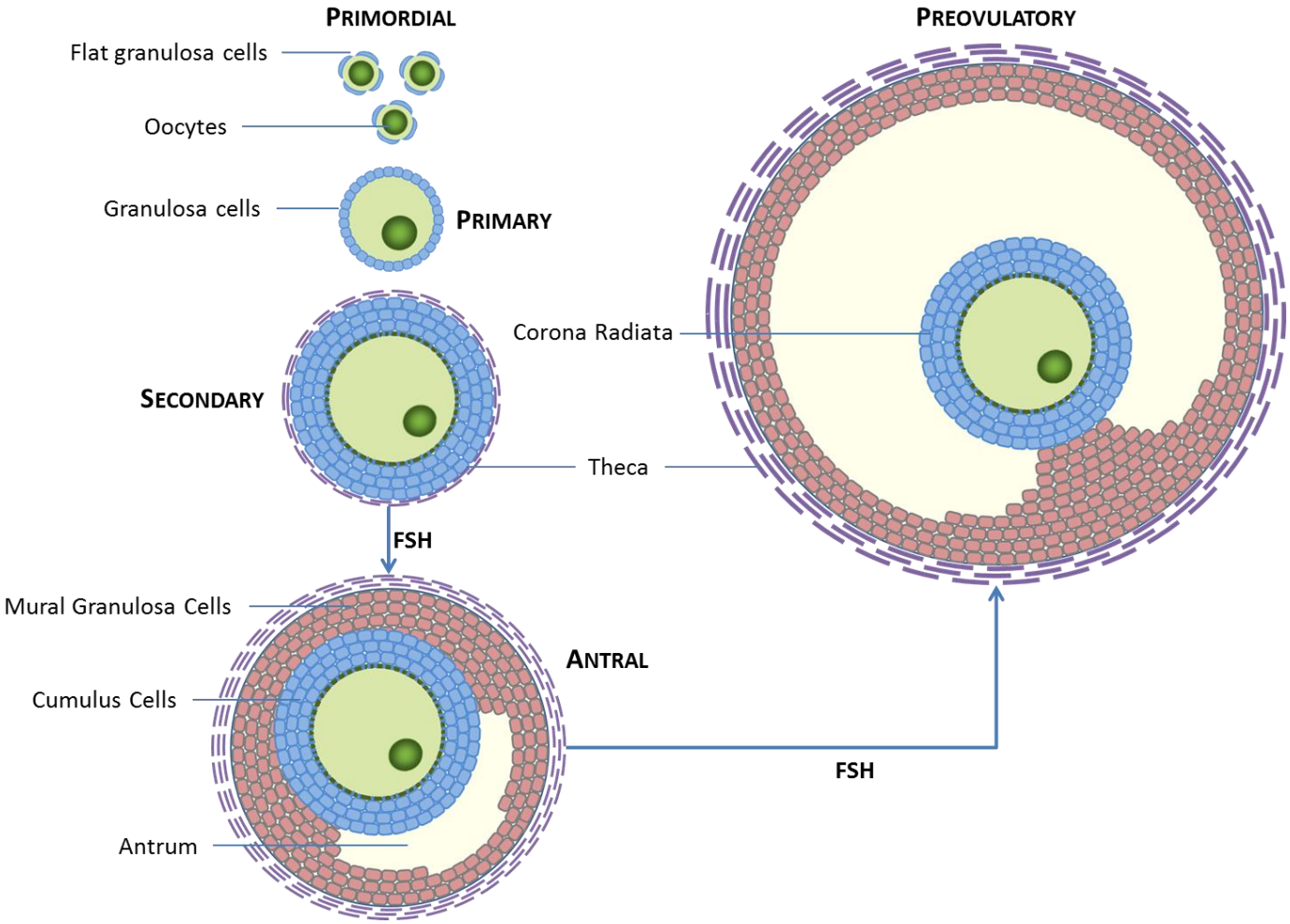

Figure 1.1 - Outline of the main steps of folliculogenesis. Folliculogenesis starts with primordial follicles that are subsequently recruited to become primary follicles. GCs proliferate and form layers around the oocyte (secondary follicles). From this stage, theca cells surround the follicle, and the follicles start to produce oestrogens. Follicular growth to the secondary follicle stage is independent of gonadotrophins; however progression beyond this stage strictly depends on FSH stimulation. As GC continue to proliferate, the antrum is formed and separates de facto granulosa cells into two general populations: cumulus (blue) and mural GCs (red) (antral follicles). At this stage, selection occurs between growing follicles, and in mono-ovular species only a single follicle continues growing to the pre-ovulatory stage whilst others undergo atresia.

1.2 Hormonal Control of Folliculogenesis

The main hormones that influence folliculogenesis are FSH, LH, androgens and oestrogen (Eppig 2001). The endocrine regulation of folliculogenesis is now well described, but some aspects of folliculogenesis are not fully understood, especially the daily selection and recruitment of primordial follicles. It is clear that preantral follicular development can occur independent of direct extra-ovarian follicular controls; however mRNA for FSH receptor (FSHR) has been detected on the granulosa cells of primary and secondary follicles (Oktay *et al.* 1997). Furthermore, the rate of preantral development is sensitive to gonadotrophins, with gonadotrophin-releasing hormone (GnRH) antagonists and FSH causing changes to the development of primordial and primary follicles (Gulyas *et al.* 1977; McGee *et al.* 1997b). Development of primordial and primary follicles can occur in the absence of gonadotrophins, and it is probable that follicles become progressively responsive to FSH towards antrum formation. The ordered growth and differentiation of antral follicles is dependent on a functional relationship between the hypothalamus, pituitary and ovary. The hypothalamic-pituitary unit secretes gonadotrophins in pulses with low pulsatile secretions maintaining sufficient hormone levels in blood to support follicular growth and high cyclic pulsatile patterns of secretions initiating the final differentiation of the follicle and ovulation. FSH is necessary for antrum formation, and the follicle is then dependent on gonadotrophins with follicular atresia occurring if FSH falls below a certain minimum (Baird 1983). The increased requirement for FSH is satisfied by the dominant follicle(s) developing a greater FSH receptivity through an improved vasculature and an increase in the number of granulosa cells, rather than an increase in the number of receptors per cell (Louvet and Vaitukaitis 1976).

Both FSH and LH have significant effects on oestrogen synthesis. In antral follicles, FSH stimulates granulosa cells to metabolise androgens and oestrogens whilst LH pulses become more frequent to meet the demands for an increased LH requirement by theca cells, resulting in increased production of androgens and oestrogens (Peters and McNatty 1980). The large antral follicle is the major source of oestrogens, with secretion of oestradiol-17β from follicle GC and theca cells into ovarian venous blood. Production of oestrogens is essential for further growth, increased LH receptor expression, antrum formation, development of gap junctions between cumulus cells and the oocyte, and for the prevention of atresia (Baird and Fraser 1974; McNatty *et al.* 1976a; McNatty and Baird 1978; Emmen *et al.* 2005). Progesterone is secreted by theca cells of pre-antral and larger follicles, however the major source of progesterone in the follicular fluid is from granulosa cells (Peters and McNatty 1980; Juengel

et al. 2006). The capacity of granulosa cells to secrete progesterone is influenced in some species (e.g. sheep) by prolactin in the follicular fluid, which is present in the follicular fluid throughout follicular development (McNatty *et al.* 1974; McNatty *et al.* 1976b).

Growth factors, such as insulin-like growth factors (IGF), epidermal growth factors (EGF), and fibroblast growth factors (FGF), are expressed throughout follicle and oocyte development and play important roles in stimulation, proliferation and differentiation of follicular cells and the inhibition of differentiation (Monget *et al.* 1993; Monget and Monniaux 1995).

1.3 Somatic Cell Differentiation

Granulosa cells are relatively homogenous before antral formation. Preantral granulosa cells are common precursors, and progression of follicle growth and maturation triggers the differentiation of two functionally unique populations of granulosa cells: the mural granulosa cells and the cumulus cells. Mural granulosa cells (MGCs) are principally involved in steroid production and differentiate into luteal cells following a trigger from LH, whilst cumulus cells are required to support the development of the oocyte and to facilitate ovulation and fertilisation (Gilchrist *et al.* 2008). Antrum formation physically separates mural granulosa cells, which line the follicle wall, and cumulus cells, which surround the oocyte. MGCs differentiate to acquire LH receptors under the influence of FSH, and increase their aromatase activity and inhibin production (Erickson *et al.* 1979). Differentiation of GC beyond the preantral phenotype requires FSH secreted by the pituitary and factors secreted by the oocytes (Dierich *et al.* 1998).

It is recognised that oocytes facilitate the differentiation of their adjacent GCs to CCs, through the paracrine actions of oocyte secreted factors (OSFs). Many changes of GC genes and functions,which are necessary for its differentiation to CCs, are controlled by OSFs (reviewed by Gilchrist *et al.* 2008). Cumulus cells lose their distinct phenotype when oocyte paracrine signalling is removed via either oocytectomy or loss of oocyte activated SMAD signalling, resulting in a phenotype more closely associated with MGCs (Eppig *et al.* 1997b; Li *et al.* 2000; Gilchrist *et al.* 2006; Diaz *et al.* 2007; Dragovic *et al.* 2007). Furthermore, oocytes actively inhibit luteinisation of cumulus cells via the suppression of FSH-induced *LHR* mRNA expression (el-Fouly *et al.* 1970; Eppig *et al.* 1997b). *In vivo,* the pre-ovulatory surge of LH initiates further transition of cumulus cells, leading to a cascade of events which results in cumulus expansion. *In vitro,* this can be mimicked by EGF or FSH treatment and all involve

MAPK3/1-dependent upregulation of cumulus mucification/expansion mRNAs *Has2, Ptgs2, Ptx3 and Tnfaip6* (Su *et al.* 2003; Diaz *et al.* 2006).

1.4 Cumulus Oocyte Complex – Structure and Communication

Female fertility relies on the intimate communication between the oocyte and the cells of the ovarian follicle. Cumulus-oocyte complexes (COC) are unique, functional units encompassing a dynamic three dimensional cellular architecture and complex signalling mechanisms. The prolonged, tightly regulated development of these structures are the result of sophisticated junctional and paracrine interactions between the oocyte and its surrounding somatic cells. Communication between the oocyte and its surrounding cumulus cells is bi-directional, and is crucial for transfer of signalling molecules and metabolites.

Transzonal projections (TZPs) are granulosa and cumulus cell cytoplasmic extensions that transverse the zona pellucida and terminate on the oocyte cell surface (Albertini *et al.* 2001). They have been well characterised in mammals and illustrated in humans at specific stages of follicular development in various forms and numbers (Anderson and Albertini 1976; Motta *et al.* 1994). Numerous adherens and gap junction contacts form TZPs with deep invaginations at the oocyte surface and plasma membrane during periods of peak oocyte growth (see figure 1.2). Further development and maturation is accompanied by TZP retraction and fewer terminal connections with the oocyte. During the peri-ovulatory cascade, the cumulus cells possess highly specialised trans-zonal cytoplasmic projections which pass through the zona pellucida and form gap junctions at their tips with the oocyte, forming the elaborate structure known as the cumulus–oocyte complex (COC) (Albertini *et al.* 2001; Gilchrist *et al.* 2008).

Follicular somatic cells and their oocyte have a metabolic partnership which ensures necessary substrate provisions to support growth and development of the oocyte. Cumulus cells transport small molecules, amino acids, cyclic nucleotides and other low molecular weight peptides to the oocyte and to neighbouring cumulus cells via gap junctions at the termini of TZPs (Kidder and Mhawi 2002; Van Soom *et al.* 2002). This transport is essential for the development and coordination of oocyte maturation and competence. Among the family of gap junction proteins, two proteins have been identified that are vital in mammalian COC development: GJA1, also known as connexin-43 (cx43), and GJA4, also known as connexin-37 (cx37). Mammalian models where either *Gja1* or *Gja4* are deleted or knocked out, experience the cessation of follicular development and meiotic incompetence (Simon *et al.* 1997; Ackert *et al.* 2001; Richard and Baltz 2014). Gap junction communication (GJC) is

crucial to the maintenance of meiotic arrest, which is essential for oocyte maturation and competence, and the breakdown of GJC results in the resumption of meiosis (Larsen *et al.* 1986; Thomas *et al.* 2004).

Paracrine signalling is active throughout all stages of oocyte growth and development and plays important roles during maturation. Oocytes have the capacity to regulate follicle growth, granulosa cell growth, differentiation, proliferation and expansion through the production of soluble paracrine growth factors called oocyte secreted factors (OSFs). Two of the most important OSFs are growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15) (Edson *et al.* 2009; Gilchrist 2011). Produced by the oocyte, GDF9 and BMP15 act on granulosa and cumulus cells via an interaction with bone morphogenetic protein receptor type-II (BMPRII) and activin receptor-like kinases (ALKs), to activate Sma- and Mad-related (SMAD) intracellular signal transducers (Kaivo-oja *et al.* 2006).

Follicle development requires, in a species-specific manner, the actions of GDF9 and BMP15. This is evident from, 1) observations where the addition of the OSFs *in vitro* can promote follicle growth in both bovine and murine models (Fenwick *et al.* 2013; Passos *et al.* 2013) and, 2) deficiencies in GDF9 and BMP15 lead to alterations in follicle development and fecundity in ovine and murine species (Dong *et al.* 1996; Galloway *et al.* 2000). GDF9 and BMP15 are responsible for modulation of energy metabolism and cholesterol biosynthesis (Sugiura *et al.* 2005; Sugiura *et al.* 2007; Su *et al.* 2008) which are operational in granulosa and cumulus cells, but some aspects of which are fundamentally absent in the oocyte. Furthermore, they have major roles in the proliferation and the expansion of granulosa and cumulus cells. This has been thoroughly studied with the use of [^{3}H]-thymidine uptake assays which indicate DNA synthesis in cells (Vanderhyden *et al.* 1992; Li *et al.* 2000). Mouse cumulus expansion requires oocyte involvement, as oocytectomy of cumulus-oocyte complexes (OOXs) via microsurgical removal of the oocyte, generates OOXs that are unable to undergo cumulus expansion and synthesise hyaluronic acid (HA) in response to FSH (Buccione *et al.* 1990a). This can be overcome by the addition of native OSFs (co-culture of denuded oocytes) or oocyte conditioned medium (Buccione *et al.* 1990b; Salustri *et al.* 1990; Gilchrist *et al.* 2008). Native OSFs and recombinant OSFs can induce expression of matrix genes in OOXs; *Has2, TNFalp6, Ptgs2* and *Ptx3* to increase production of extracellular matrix (Dragovic *et al.* 2005; Dragovic *et al.* 2007). Interestingly, these observations are not conserved across all mammalian species. It appears the non-rodent species studied to date

are not dependent on OSFs for cumulus expansion (Vanderhyden 1993; Gilchrist and Ritter 2011).

Oocyte secreted factors can prevent cumulus cell apoptosis, with Hussein et al (2005) demonstrating the OOXs co-cultured with increasing concentrations of native OSFs linearly decreased the level of apoptosis (Hussein *et al.* 2005). Steroidogenesis, luteinisation and granulosa differentiation are also regulated by OSFs, with production of progesterone and *lhr* mRNA suppressed by OSFs (Eppig *et al.* 1997b; Li *et al.* 2000). Moreover, oestradiol production is also regulated by OSFs (Vanderhyden *et al.* 1993; Glister *et al.* 2003).

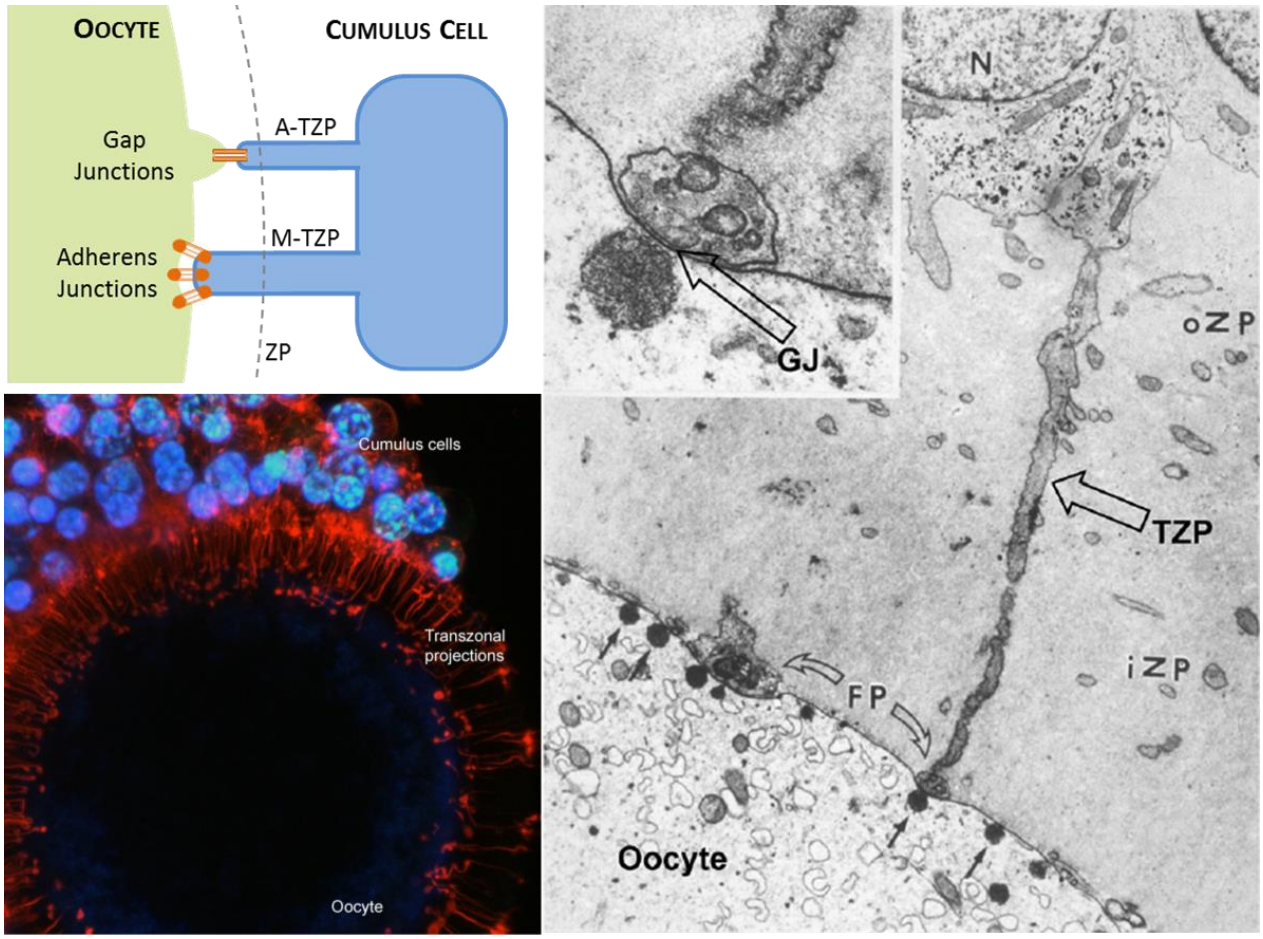

Figure 1.2 - Interaction of the oocyte with cumulus cells. Top Left) Schematic of the cumulus-oocyte interface. Transzonal projections (TZP) from cumulus cells penetrate the zona pellucida (ZP) and form adherens junctions at invaginations at foot processes (FP) at the oocyte's surface and/or gap junctions at the oocyte plasma membrane. TZPs can be rich in F-actin (A-TZP), or they are composed primarily of microtubules (M-TZP). Bottom Left) Confocal microscopy of TZP (stained with phalliodin, in red) on a cow oocyte with cumulus cell nuclei stained in blue (Hoechst 3342) (Gilbert et al. 2015). Right) Transmission electron microscopy showing a TZP, traversing the ZP with gap junctions (GJ) present at the point of contact of the two membranes. Source Gilchrist *et al.* (2008).

1.5 Natriuretic Peptides

Natriuretic peptides are a family of structurally related, but genetically distinct hormones/paracrine factors that regulate blood volume and pressure, ventricular hypertrophy, pulmonary hypertension, fat metabolism, and long bone growth. The most common of these are atrial (ANP), brain (BNP) and C-type (CNP) natriuretic peptides. These will be the main focus of this review; however others also include dendroaspis natriuretic peptide and urodilatin. ANP and BNP bind preferentially to natriuretic peptide receptor 1 (NPR1 also known as NPRA) and CNP binds preferentially to natriuretic peptide receptor 2 (NPR2 or NPRB). NPR1 and NPR2 represent two of the five transmembrane guanylate cyclases which convert GTP to cGMP (Potter 2005). A third receptor, NPR3 or NPRC, also known as the clearance receptor, does not possess any known intrinsic enzymatic activity (Potter *et al.* 2006). The physiological effects of natriuretic peptides are elicited through cGMP and cGMP-binding proteins including cGMP-dependant protein kinase (PKG or cGKI), cGMP-regulated phosphodiesterases, and cGMP-gated ion channels (Potter *et al.* 2006).

All three major natriuretic peptides, which were originally discovered in the atrium of the heart, the porcine brain, and the central nervous system, have been demonstrated to have important functions in female reproduction. In 2010, Zhang *et al.* illustrated that CNP is necessary for oocyte meiotic arrest and for fertility (Zhang *et al.* 2010). This was a pivotal publication in oocyte biology and for the recognition of the importance of natriuretic peptides in the regulation of the cumulus oocyte complex, which will be explored in the subsequent sections.

1.6 Oocyte Meiotic Arrest

In vivo, the oocyte is meiotically arrested at the prophase I stage of meiosis throughout all of folliculogenesis until the peri-ovulatory events. Growing oocytes become capable of undergoing meiotic maturation with the acquisition of meiotic competence occurring prior to the surge of gonadotrophic luteinising hormone (LH) (Bentley and Beavo 1992; Downs 2010). In response to the LH surge, meiosis resumes and oocytes progress through the first meiotic division and arrest at metaphase II awaiting fertilisation. However, it is the MGCs that exhibit LH receptors whilst cumulus cells and the oocyte do not (Eppig *et al.* 1997a). Therefore, the mechanism by which LH stimulates oocyte maturation is indirect, and involves paracrine signalling within the follicle.

It is apparent that the follicular unit provides the environment necessary for meiotic arrest of the oocytes at the germinal vesical (GV) stage (prophase I) and for meiotic resumption. This

is because *in vivo* oocytes undergo meiotic resumption in response to the pre-ovulatory gonadotrophic surge of gonadotrophins, and the phenomenon of the spontaneous resumption of meiosis *in vitro*, originally observed by Pincus and Enzmann where the removal of oocytes from their follicular environment induced the resumption of meiosis (Pincus and Enzmann 1935; Edwards 1965). It is well established that intra-oocyte cyclic adenosine monophosphate (cAMP) plays a significant role in regulating mammalian meiotic maturation.

Cyclic AMP plays a critical role in the transition from meiotic arrest to the completion of meiosis II. A physiological decrease in oocyte cAMP precedes geminal vesicle breakdown (GVBD) (Schultz *et al.* 1983; Vivarelli *et al.* 1983; Dekel *et al.* 1984), and many studies have shown that high intra-oocyte cAMP, mimicked by exogenous analogues, PDE inhibitors or adenylate cyclase activators, hold the oocyte in reversible meiotic arrest. High intra-oocyte cAMP concentration is produced endogenously via the constitutively active G-protein (Gs) by the G-protein-coupled receptors (GPR) 3 and 12 (Mehlmann *et al.* 2002; Mehlmann 2005), and also is generated by the cumulus cells and transported to the oocyte via gap junctions (Bornslaeger and Schultz 1985; Webb *et al.* 2002). High intra-oocyte cAMP activates protein kinase-A (PKA) (Viste *et al.* 2005), which promotes the phosphorylation of key kinases WEE1 and MYT1. These kinases, in turn, phosphorylate the maturation promoting factor (MPF). Maturation promoting factor consists of Cdc2 and cyclin B, where phosphorylated MPF is inactive and holds the oocyte meiotically inactive (reviewed (Conti *et al.* 2012)). Maintenance of cAMP within the COC is achieved through a balance of production, degradation and secretion from the COC. This balance is achieved with the synthesis of cAMP by adenylate cyclases and GPR. Degradation of COC cAMP is mainly the result of phosphodiesterases (PDEs), which hydrolyse cAMP to AMP (See Figure 1.3).

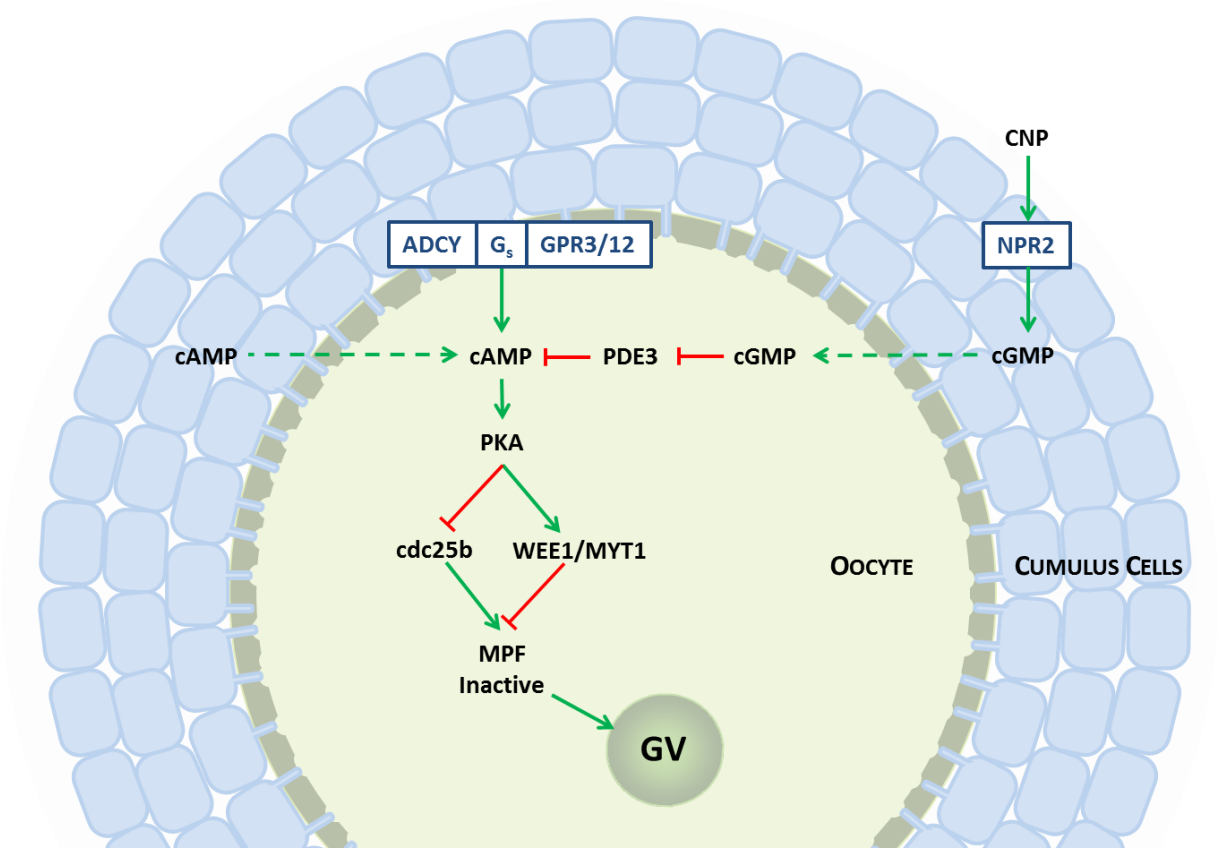

Figure 1.3 - Schematic of intra-oocyte signalling necessary for meiotic arrest. Increased intra-oocyte cAMP concentration occurs by means of local production, via activated GPR3/12 which activates Gs and stimulates ADCY production of cAMP or is transferred from adjacent CCs via gap junctions. Transport of cGMP maintains high intra-oocyte cAMP through the inhibition of PDE3A and reduced cAMP degradation. cAMP activates PKA which activates WEE1/MYT1 and inactivates cdc25b. In turn, kinase phosphorylation renders MPF inactive and prevents meiotic resumption.

Cyclic guanylate monophosphate (cGMP) is also a key regulator of oocyte maturation. cGMP is produced from GTP in granulosa cells by guanylyl cyclases, and GTP is produced from inosine monophosphate (IMP) by a series of enzymatic reactions beginning with the activity of inosine monophosphate dehydrogenase (IMPDH), and the rate limiting enzyme of guanylyl metabolism (Wigglesworth *et al.* 2013). It has been well documented that cGMP levels drop in the somatic compartment of the follicle in response to the LH surge *in vivo* (Hubbard and Greenwald 1982; Norris *et al.* 2009; Vaccari *et al.* 2009). Studies in the 1980s suggested that the granulosa cells secrete an "oocyte maturation inhibiting" (OMI) factor into the follicular fluid and that this OMI factor was a peptide of less than 2000 Da (Tsafriri and Pomerantz 1986). Recent studies have demonstrated that CNP, encoded by the natriuretic peptide precursor type C (NPPC) in the mural granulosa cells stimulate the production of cGMP via its receptor, natriuretic peptide receptor 2 (NPR2) or guanylate cyclase B (GC-B) (Zhang *et al.* 2010). Cyclic GMP originates in the somatic cell compartment of the follicle and diffuses through gap junctions to the oocyte where it inhibits the activity of the very potent PDE3A, the main cAMP degrading phosphodiesterase in the mouse oocyte, and assists in maintaining meiotic arrest through high intra-oocyte cAMP concentrations (Norris *et al.* 2009; Vaccari *et al.* 2009). The maintenance of meiotic arrest relies on cGMP production and transport to the oocyte to promote elevated cAMP levels. *In vivo,* the inhibition of the rate-limiting enzyme IMPDH, with mycophenolic acid or mizoribine, induces rapid gonadotrophin-independent precocious resumption of meiosis (Downs and Eppig 1987; Downs 1993). The predominant guanylyl cyclase in the ovarian follicle is NPR2 in granulosa cells, and without IMPDH, meiosis-inhibiting levels of cGMP are not produced (Wigglesworth *et al.* 2013). It is of note that OSFs promote the expression of *Impdh* and IMPDH protein (Wigglesworth *et al.* 2013), and promote the expression of *Nppc* and *Npr2* and increase receptor activity/binding (Zhang *et al.* 2010; Zhang and Xia 2012). These findings illustrate that granulosa cell cGMP production is, in part, regulated by the oocyte.

An important component of cGMP regulation in smooth muscle is the cGMP specific phosphodiesterase type 5 (PDE5) (Rybalkin *et al.* 2002). The expression of PDE5 has been detected in mouse cumulus cells (Wang *et al.* 2008) and follicular somatic cells (Vaccari *et al.* 2009), and as such may regulate meiosis by hydrolysis of cumulus cell cGMP and reduced intra-oocyte cGMP. However, cGMP specific PDE activity is unaffected by LH-stimulation (Patwardhan and Lanthier 1984; Vaccari *et al.* 2009) and in cultured granulosa cells cGMP hydrolysis also is unaffected (Robinson *et al.* 2012). Furthermore, PDE5 inhibitors produce no significant effects on GVBD and intra-oocyte cGMP concentrations (Zhang *et al.* 2005b; Wang

et al. 2008; Vaccari *et al.* 2009; Kawamura *et al.* 2011). Therefore, cGMP synthesis is likely to decrease in response to LH rather than increasing PDE activity (Patwardhan and Lanthier 1984) and CNP-induced production of cGMP in CCs, surpasses PDE5 hydrolysis of cGMP and maintains adequate levels of cGMP in oocytes to inhibit meiotic resumption prior to the LH surge (Kawamura *et al.* 2011).

The capacity of CNP to inhibit meiosis and induce CC cGMP production is transient without the steroid, oestradiol, *in vitro* (Zhang *et al.* 2011). Oestradiol cooperates with FSH to increase expression of *Nppc* in MGCs and is essential for *Npr2* mRNA expression in CCs. Notably, Zhang *et al.* demonstrated an oocyte-specific role in cGMP production by; 1) The co-culture of oocytectomised cumulus cells with DOs increased the expression of *Npr2* mRNA levels in cumulus cells, and 2) The addition of oocyte secreted factors BMP15, plus either GDF9 or FGF8, generated a synergistic increase in *Npr2* mRNA levels in cumulus cells to levels comparable to oocytectomised cumulus cells co-cultured with DOs (Zhang *et al.* 2010). Mouse MGC expressed similar levels of *Nppc* and *Npr2,* however in mouse cumulus cells, *Nppc* levels were 10-fold less compared to MGC, and *Npr2* expression levels were nearly twice that in cumulus cells compared to MGC (Zhang *et al.* 2010). This suggests an important and functional relationship of NPPC and NPR2 between the cell types and that the oocyte may play an active role in this regulatory network.

1.7 Ovulation and Resumption of Meiosis

LH promotes the resumption of meiosis, and the rapid remodelling of the follicle and cumulus complex in preparation for ovulation. As previously described, receptors for LH are predominantly found on the MGCs and theca cell layers, meaning that these cells are the first to respond following the surge, and transmit a secondary signal to the cumulus cells inducing numerous changes. This differs between species, but generally, the LH surge prompts the activation of adenylate cyclases to produce cAMP in the MGCs to initiate all these processes. Synchronously, resumption of meiosis is triggered in the oocyte, the COC begins the synthesis of extracellular matrix, and the follicle is rapidly remodelled, with the basement membrane breaking down to allow for vascular infiltration and luteal formation. The major factor that induces the resumption of meiosis is MPF. LH-induced activation of adenylate cyclases increases intracellular cAMP and results in activation of cAMP-dependant protein kinase A (PKA) (Marsh 1976; Richards 1994). Following PKA activation, the cAMP response binding (CREB) protein is phosphorylated at serine-133 and recruits the CBP/p300 transcriptional activator (Arias *et al.* 1994; Mukherjee *et al.* 1996; Russell *et al.* 2003). cAMP is thought to be

a major mediator of LH-induced resumption of meiosis as pulsing COCs with cAMP analogues or cAMP-elevating agents triggers the resumption of meiosis (Tsafriri *et al.* 1972; Hillensjo *et al.* 1978; Dekel *et al.* 1981). However, a decrease in oocyte cAMP has been shown to precede GVBD (Schultz *et al.* 1983; Vivarelli *et al.* 1983; Dekel *et al.* 1984; Racowsky 1984; Sirard and First 1988). This illustrates a dual role for cAMP in meiotic regulation, with inhibition of meiosis maintained by high intra-oocyte cAMP above an inhibitory threshold, and the resumption of meiosis induced by a transient pulse of cAMP within the granulosa cells (Dekel *et al.* 1988; Downs 2010).

Granulosa cells respond to the LH surge with the PKA-dependent, rapid stimulation of the extracellular regulated kinase (ERK 1/2) pathway (Das *et al.* 1996; Seger *et al.* 2001; Salvador *et al.* 2002; Russell *et al.* 2003; Sela-Abramovich *et al.* 2005). Importantly, Fan *et al.* outlined ERK1/2 as a crucial mediator of pre-ovulatory signalling in response to the LH surge, with ERK1/2 knockout mice exhibiting inhibited meiotic resumption, cumulus expansion, ovulation and luteinisation (Fan *et al.* 2009). The mechanism underlying the effects of ERK1/2 on the resumption of meiosis remains unclear (Conti *et al.* 2012). Reports indicate LH-stimulated MAPK activation cause decreased permeability of gap junctions (Norris *et al.* 2008; Conti *et al.* 2012), reduced transport of cGMP to the oocyte (Norris *et al.* 2010), likely via the phosphorylation of connexin 43 (Norris *et al.* 2008; Andric *et al.* 2010; Norris *et al.* 2010; Hsieh *et al.* 2011). Reduced gap junction communication is sufficient to induce oocyte maturation (Sela-Abramovich *et al.* 2006), which would lead to reduced transfer of cumulus cell cGMP to the oocyte and other CC inhibitory substances. Contrary to this, the resumption of meiosis can occur preceding gap junction breakdown (Motlik *et al.* 1986; Conti *et al.* 2012; Zhang and Xia 2012). Activation of MPF and the resumption of meiosis occurs in response to meiosis activating sterol (MAS) or MAPK, in the presence of functional gap junctions (Liang *et al.* 2007). Furthermore, considering the inhibition of gap junction closure with U0126, a potent MAPK inhibitor, only lessens LH-stimulated oocyte maturation *in vitro* (Su *et al.* 2003). The primary contributor for LH-induced resumption of meiosis may be a decrease in cGMP due to decreased granulosa cell cGMP synthesis via inactivation of the guanylyl cyclase, NPR2 (Robinson *et al.* 2012). However, LH-induced MAPK activation inhibits the expression *Cyp19α1*, which encodes aromatase, which is responsible for the conversion of androgens to oestradiol, resulting in decreased E2 concentrations (McRae *et al.* 2005; Andric and Ascoli 2006; Su *et al.* 2006), possibly triggering a decrease in expression and activity of NPPC and NPR2 in granulosa cells (Liu *et al.* 2013).

Although early observations indicated LH may regulate follicular cGMP concentrations (Davis and Sheppard 1986), recent data illustrates that similar to cAMP, the surge of gonadotrophins results in dramatic decrease of follicular and intra-oocyte cGMP (Norris *et al.* 2009; Vaccari *et al.* 2009). This is the result of reduced production of NPPC by granulosa cells, inhibition of NPR2 activity, and suppression of gap-junction mediate transport of cGMP (Kawamura *et al.* 2011; Robinson *et al.* 2012; Liu *et al.* 2014). Prior to the LH surge, cGMP concentration is uniformly high across the follicle, however the LH stimulus results in rapid cGMP decreases in granulosa cells, which causes oocyte cGMP to diffuse to the surrounding cells, ensuing decreased intra-oocyte cGMP (Shuhaibar *et al.* 2015). LH signalling is known to decrease CNP in ovaries of rodents (Jankowski *et al.* 1997; Kawamura *et al.* 2011; Robinson *et al.* 2012; Liu *et al.* 2014) and in porcine and human follicular fluid (Kawamura *et al.* 2011; Zhang *et al.* 2014a). LH-induced decreases in CNP are associated with decreases in *Nppc* mRNA expression (Kawamura *et al.* 2011; Tsuji *et al.* 2012; Liu *et al.* 2014). However, decreased ovarian cGMP precedes decreases in ovarian CNP (Egbert *et al.* 2014; Liu *et al.* 2014). Guanylyl cyclase activity is rapidly reduced in response to the LH stimulus, and by the time CNP declines, NPR2 activity is less than half (Robinson *et al.* 2012; Egbert *et al.* 2014; Liu *et al.* 2014). Corresponding to these studies is the observation demonstrating cGMP production significantly decreases in granulosa cells, even though CNP concentrations are constant (Liu *et al.* 2014). LH induces the rapid dephosphorylation of NPR2 and the phosphorylation/activation of the cGMP degrading PDE5 (Egbert *et al.* 2014). Conclusively, the pre-ovulatory surge of gonadotrophins removes cGMP inhibition of oocyte PDE3A, resulting in decreased cAMP and promotion of GVBD via MPF. LH induces dephosphorylation of NPR2 and the activation of PDE5, resulting in cGMP decrease in the granulosa and cumulus cells. Through gap junctions, cGMP in the oocyte diffuses down its concentration gradient to the cumulus cells relieving PDE3A inhibition in the oocyte (Shuhaibar *et al.* 2015). Thus, cGMP inhibition is removed in a two-step process, an initial rapid decrease followed by a slower extended decrease of cGMP (Egbert *et al.* 2014; Liu *et al.* 2014).

The EGF-family of proteins are low-molecular weight proteins with similar structures and function. They act by binding with high affinity to the EGFR to induce signalling cascades. Historically, EGF was found in follicular fluid (Westergaard and Andersen 1989) and although it's presence and function was debated, it was shown to induce cumulus expansion and oocyte maturation (reviewed (Hsieh *et al.* 2009)). More recently, research demonstrates the pre-ovulatory signals are mediated by members of the EGF growth factor family, in particular the EGF-like peptides (EGFp); amphiregulin (AREG), epiregulin (EREG), and to a lesser extent,

beta-cellulin (BTC) (Park *et al.* 2004). Expressions of EGFps in mural granulosa and cumulus cells are induced in response to the pre-ovulatory LH surge (Espey and Richards 2002; Park *et al.* 2004). Binding of EGFps to EGFR in the granulosa cells (Shimada *et al.* 2006a) have meiosis-inducing actions (Hsieh *et al.* 2009). During the pre-ovulatory period, EGFps and prostaglandin E_2 (PGE_2) expression is auto-amplified via autocrine mechanisms and between MGC and CC via paracrine communication, to induce p38MAPK-dependant, ERK1/2-dependent gene transcription. Studies knocking out EGFps in mice support their critical role in female reproduction, resulting in compromised ovulation and meiotic resumption (Conti *et al.* 2006; Shimada *et al.* 2006b), as well as a myriad of other phenotypes.

1.8 EGFp Shedding and Metalloproteases

The EGF-like peptides; AREG, EREG, and BTC are synthesised as integral membrane-bound proteins and cleavage of the precursor form at one or more sites in the extracellular domain is required to release the soluble EGF-domain (Lee *et al.* 2003; Sahin *et al.* 2004). Interactions with membrane bound receptors, like EGFR, require matrix metalloprotease (MMP) activity. Inhibition of MMPs with protease inhibitors can block LH signal transmission, and can be overcome with exogenous EREG and other EGFps (Ashkenazi *et al.* 2005; Yamashita *et al.* 2007). Tumour necrosis factor alpha (TNFα)-converting enzyme (TACE), also known as ADAM17, is a member of a disintegrin and metalloprotease (ADAM) family of proteases and is the main sheddase of AREG and EREG in mouse embryonic cells (Sahin *et al.* 2004). Mouse cumulus cells express *Tace/Adam17* mRNA during ovulation (Hernandez-Gonzalez *et al.* 2006). Research indicates TACE/ADAM17 is responsible for the cleavage of pro-EGFp to active ligands which enable them to bind to membrane bound EGFR in cumulus cells, resulting in downstream activation of ERK1/2, cumulus expansion and the resumption of meiosis (Yamashita *et al.* 2007). Gonadotrophin-induced, cAMP-dependant signalling, PKA, and p38MAPK, positively regulate *Areg* expression in cumulus cells (Yamashita *et al.* 2009). FSH can also induce expression of TACE/ADAM17 and EGFps in porcine granulosa and cumulus cell cultures (Yamashita *et al.* 2011). Furthermore, gonadotrophins can induce ligand activation of EGFR leading to the phosphorylation and activation of ERK1/2 resulting in the resumption of meiosis (Yamashita *et al.* 2007). Porcine granulosa cells and COCs cultured in the presence of TAPI-2, a semi-selective inhibitor of TACE/ADAM17, significantly inhibit GnRH-induced ERK1/2 phosphorylation and delay oocyte nuclear maturation (Yamashita *et al.* 2007). The inhibitory response was overridden with the addition of EGFps. In rodents, LH-induced ERK1/2 phosphorylation in granulosa cells was downregulated with transfection of

Tace/Adam17 siRNA (Yamashita *et al.* 2007). Hence, expression of TACE/ADAM17 and other MMPs in granulosa/cumulus cells facilitate the cleavage of pro-EGF peptides to active ligand, allowing binding to EGFR, inducing the phosphorylation of ERK1/2, and initiating activation of its signalling network. In porcine COC, increased expression of *Tace/Adam17*, *Areg* and *Ereg*, in response to FSH is associated with increased expression of *Ptgs2* and *Ptger2* (Yamashita *et al.* 2011), which encodes cyclooxygenase-2 (COX-2) and prostaglandin E_2 (PGE_2) receptor-2 (PGER2).

Peri-ovulatory follicles synthesise and accumulate PGE_2 in follicular fluid, and mice deficient in *Ptgs2* and *Ptger2* have a significant reduction in number of oocytes ovulated and abolished fertilisation potential (Matsumoto *et al.* 2001). Inhibition of PGE_2 with NS398, significantly reduced the expression of *Tace/Adam17* and *Areg,* and significantly inhibited ERK1/2 phosphorylation, although only after extended culture (Yamashita *et al.* 2011). Therefore, it was concluded the initial expression of TACE/ADAM17 and EGFps are induced by gonadotrophins however, are sustained by a positive feedback loop involving the PGE_2 - PGER2 pathway (Yamashita *et al.* 2011; Yamashita and Shimada 2012).

Gonadotrophins similarly induce signalling cascades to activate protein kinase C (PKC) members of the rous sarcoma oncogene (SRC) family kinases (SFK) (Su *et al.* 1999; Downs *et al.* 2001; Fan *et al.* 2004; Talmor-Cohen *et al.* 2004; Rozengurt 2007; Downs 2010; Levi *et al.* 2010; Sasseville *et al.* 2010). Phosphorylated c-Src promotes TACE/ADAM17 enzyme activity, via a PKC-dependant mechanism (Yamashita *et al.* 2014). Interestingly, inhibitors of c-Src and PKC, PP2 and CalC respectively, inhibited FSH-induced phosphorylation of ERK1/2 and suppressed nuclear maturation, steroidogenesis and TACE/ADAM17 activity, yet had no effect on FSH-induced *Areg* or *Tace/Adam17* mRNA expression (Wayne *et al.* 2007; Yamashita *et al.* 2014). Furthermore, the PKC activator phorbol-12-myristate-13-acetate (PMA), can induce phosphorylation of TACE/ADAM17 (Yamashita *et al.* 2014), induce the resumption of meiosis in porcine COCs (Chen *et al.* 2008), and promotes oocyte developmental competence (Ali and Sirard 2005). Therefore, gonadotrophin-induced TACE/ADAM17 activity is somewhat regulated by a PKC, c-Src-dependant mechanism, whilst increased mRNA expression is regulated by PKA-dependant and p38MAPK-dependant signalling. In conclusion, this growing body of evidence demonstrates the importance of EGFp and MMP signalling mechanisms are vital for oocyte maturation, ovulation, and in the propagation pre-ovulatory LH-stimulus.

1.9 Conclusion and Objectives

The oocyte is a unique cell which carries the responsibility for generational renewal. At birth, the ovary contains a finite population of oocytes, compartmentalised with supporting somatic cells called granulosa cells, which together comprise the primary functional unit of the ovary, the follicle. It is well established that the oocyte is dependent on the neighbouring granulosa cells along with other local and distant factors to grow and develop. What constitutes oocyte developmental competence still eludes reproductive and developmental biologists therefore, a protocol to achieve absolute developmental competence is yet to be written and we are likely to have a long way to go in terms of fully understanding the secrets enclosed within the oocyte.

Our knowledge of molecular mechanisms which regulate oocyte maturation has grown significantly over the past few years and advancing our knowledge promotes future research in establishing mechanisms of improving oocyte developmental competence. Understanding the signalling mechanisms of the follicular environment and the cumulus oocyte complex provides the knowledge to potentially replicate and manipulate the process external from its *in vivo* environment. This is important as an ever increasing proportion of human pregnancies are generated from oocytes that are manipulated *ex vivo.*

One of the most exciting developments in mammalian oocyte biology over the past decades has been the discovery that natriuretic peptides are central regulators of oocyte maturation (Zhang *et al.* 2010; Zhang and Xia 2012). Intriguing comparisons arise when one compares the effect of the natriuretic peptides between species. To date, natriuretic peptides have been used to culture COCs from many mammals including mice (Zhang *et al.* 2010; Zhang *et al.* 2011; Wigglesworth *et al.* 2013; Richard and Baltz 2014; Zhang *et al.* 2014b; Zhang *et al.* 2015b; Romero *et al.* 2016), rats (Tornell *et al.* 1990b), pigs (Zhang *et al.* 2005a; Zhang *et al.* 2005b; Hiradate *et al.* 2014; Santiquet *et al.* 2014), cows (Bilodeau-Goeseels 2007; Franciosi *et al.* 2014; Cesaro *et al.* 2015), goats (Zhang *et al.* 2015a), horses (Mugnier *et al.* 2009), and cats (Zhong *et al.* 2015). Most recently, COCs from unprimed prepubertal mice cultured *in vitro* with CNP as a vital component have resulted in meiotic arrest and improved developmental competence (Romero *et al.* 2016). Mouse models have dominated the research, elucidating the functions of CNP and NPR2 signalling in the COC and the factors, both maternal and oocyte derived, that regulate their activity. The roles and regulation of natriuretic peptides in higher mammals is yet to be fully established. Considering the mouse uses significantly different mechanisms in oocyte meiosis compared to human, and other non-rodent models, utilising non-rodent mammalian species for oocyte research, allows closer

analogies with the human. Consequently, we chose to examine two important and established domestic animal models; the sheep and pig. The first objective of this thesis is to investigate factors regulating the expression of the natriuretic peptide receptors in sheep, and the effect of natriuretic peptides on the resumption of meiosis.

Indisputably, the production of cumulus cell cGMP, by binding of CNP to NPR2, is transported through TZPs to the oocyte where it suppresses the cAMP degrading PDE3A and inhibits the breakdown of cAMP, consequently delaying meiotic resumption by suppression of MPF. However, whether CNP-regulated cGMP can regulate other mechanisms involved in oocyte maturation like ERK1/2 activation (porcine - Zhang *et al.* 2005b; Blaha *et al.* 2015) and gap junction signalling (bovine - Franciosi *et al.* 2014; porcine - Santiquet *et al.* 2014) are debated and unclear. Therefore, this thesis will investigate the effects of CNP and cGMP on porcine COCs and their effect on the key regulatory networks involving ERK1/2. Subsequent investigations will take place in porcine COCs retrieved from less competent mid-sized antral follicles. Lastly, this thesis will investigate the effect of CNP on the resumption of meiosis from porcine oocytes retrieved from different sized follicles.

1.10 Hypotheses and Aims

Hypothesis 1 – Functional CNP and NPR2 systems are present in sheep COCs and inhibit the resumption of meiosis *in vitro*.

Aim: To determine the expression and regulation of the natriuretic peptide receptors in sheep COCs.

Aim: To determine the effect of natriuretic peptides on the resumption of meiosis of sheep oocytes *in vitro*.

Hypothesis 2 - CNP, via cGMP dependent signalling, regulates ERK1/2 activation via mechanisms common to FSH signalling in porcine COCs, and that the capacity of CNP to activate COC ERK1/2 is dependent on the developmental stage of the follicle.

Aim: To determine if CNP/cGMP can induce the phosphorylation of ERK1/2 of porcine COCs *in vitro.*

Aim: To determine what mechanisms are involved in CNP-induced activation of ERK1/2 in COCs.

Aim: To determine if CNP-induced ERK1/2 activation of ERK1/2 is altered in COCs retrieved from different sized follicles.

Hypothesis 3: Functional CNP and NPR2 systems in porcine COCs inhibit the resumption of meiosis *in vitro*.

Aim: To determine the effect of CNP on the resumption of meiosis from porcine oocytes retrieved from different sized follicles.

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CHAPTER 2:

MATERIALS AND METHODS

2.1 Chemicals

Unless otherwise specified all chemicals used in the following protocols were purchased from Sigma (St. Louis, MO, USA).

2.2 Media Composition

All media were prepared in house. Handling medium; 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-tissue culture medium 199 (H-TCM-199; GIBCO® Life Technologies, Mulgrave, Australia), was supplemented with 50 µg/mL kanamycin and 4 mg/mL (ovine) or 3 mg/mL (porcine) bovine serum albumin (BSA; H-TCM-199/BSA). Culture medium; bicarbonate buffered TCM-199 (B-TCM-199) was supplemented with 50 µg/mL kanamycin, 100 U/mL penicillin, 0.1 mg/mL streptomycin, and 100 µM cysteamine.

2.3 Collection and Preparation of Oocytes

Ovaries were obtained from an abattoir and transported in warm (29 – 32°C) saline (0.9% wt/vol NaCl; Baxter Healthcare) supplemented with antibiotics (100 U/mL penicillin G and 0.1 mg/mL streptomycin sulfate) and delivered to the laboratory within 3-4 h of slaughter. Ovine ovaries were collected from cycling ewes at Thomas Foods Abattoir (South Australia; latitude -35.109 and longitude139.262), whilst porcine ovaries were collected from gilts at Big River Pork (South Australia; latitude -35.201 and longitude 139.298). Follicle size was measured with a Vernier calliper. Ovine antral follicles of 2-8 mm in size or porcine antral follicles of either 2-4 mm or >4 mm (see chapter experimental design) were aspirated using a 20-gauge needle and constant suction (1 L/min) into vacutainer tubes. Cellular sediment was transferred to 100 mm Petri dishes and searched under a dissecting microscope for intact cumulus-oocyte complexes (COCs) with ooplasms of uniform appearance. Oocytes which were surrounded by >3 cumulus cell layers were collected and washed once in handling medium and twice in culture medium.

2.4 In Vitro Maturation (IVM)

Cumulus-oocyte complexes were pooled and randomly selected into groups of up to ten COCs, then were transferred into pre-equilibrated 100 µL drops of maturation medium overlaid with mineral oil and incubated at 38.5°C with 6% CO_2 humidified air for IVM. For each experimental replicate, equal number of COCs were used per treatment group. All non-standard media additives will be outlined for each specific experiment in subsequent chapters.

IVM was performed for varying lengths of time, dependent on experimental plan. Whilst we recognise variations in atmospheric O_2 levels may alter some of the IVM outcomes illustrated, due to the short culture times and absence of zygote generation, these would be minor.

2.5 Primary Cell Culture

Mural granulosa cells (MGCs) were collected as above and washed twice in B-TCM-199 then transferred to a 96 well plate (Falcon; Becton Dickinson, Franklin Lakes, NJ, USA) with 25,000 cells in a final volume of 125 µL. MGCs were cultured at 38.5°C with 6% CO_2 humidified air. For specific experiments, cumulus cells were denuded from COCs via continuous pipetting; no chemical or enzymatic methods were used to aid isolation. Cumulus cells were collected and washed twice in B-TCM-199 then transferred to a 96 well plate with 25,000 cells in a final volume of 125 µL. Cumulus cells were cultured at 38.5°C with 6% CO_2 humidified air.

2.6 Cumulus Expansion Assessment

Cumulus expansion was examined at 24 h of IVM and assessed according to the scoring system of Vanderhyden et al. (Vanderhyden et al. 1990). Briefly, a score of 0 indicates no detectable response; +1 indicates minimum response, with cells in the peripheral two layers beginning to expand; +2 indicates expansion extending inwards to several layers; +3 indicates expansion of all layers of cumulus except the corona radiata cells; and +4 indicates expansion of the entire cumulus including corona radiata cells.

2.7 Oocyte Meiotic Assessment

At the end of defined periods of ovine and porcine oocyte *in vitro* maturation, COCs were mechanically denuded of cumulus cells by repeated pipetting, then fixed in 4% paraformaldehyde overnight at 4°C. Timing of meiotic assessment experiments correspond to time of germinal vesicle break down (GVBD) in the different species examined. Fixed oocytes were then incubated in permeabalisation solution (0.5% Triton X-100) for 15 – 30 min, followed by incubation in 3 µM 4',6-diamidino-2-phenylindole (DAPI) solution in darkness for 15 min. Oocytes were washed in 0.01% (w/v) BSA in phosphate buffered saline (PBS), and mounted on a slide with glycerol and antifade (Prolong, Invitrogen, Carlsbad, California) (3:1), and chromosome configurations were assessed using a Nikon Eclipse TE2000-E Microscope (330-380 nm excitation wavelength, Nikon, Melville, NY, USA). Results were represented as total GVBD and encompassed all oocytes not at the geminal vesicle stage of meiosis (GVBD = GVBD + metaphase I + anaphase/telophase I + metaphase II, see Figure 2.1).

2.8 [^{3}H] Thymidine Incorporation Assay

Granulosa cell bioassays were performed as previously described (Gilchrist *et al.* 2001; Gilchrist *et al.* 2004; Gilchrist *et al.* 2006). Mural granulosa and cumulus cells were recovered and processed for culture as described above. Cells were cultured for 18 h, followed by a further 6 h pulse of 15.4 kBq [^{3}H] thymidine (ICN, Costa Mesa, CA, USA) in an atmosphere of 37°C with 5% CO_2 humidified air. MGCs and CCs were harvested post culture and the incorporated [^{3}H] thymidine was quantified using a scintillation counter as an indicator of the proportion of cells in S phase and thus an indication of the level of DNA synthesis and MGC proliferation (Lee *et al.* 2001). A reduction in temperature from primary cell culture to GC bioassay (38.5 vs 37ºC, respectively) may result in variability of results, yet follow published protocols and were comparable. Treatments were performed in duplicate, and at least three experimental replicates performed for all treatments.

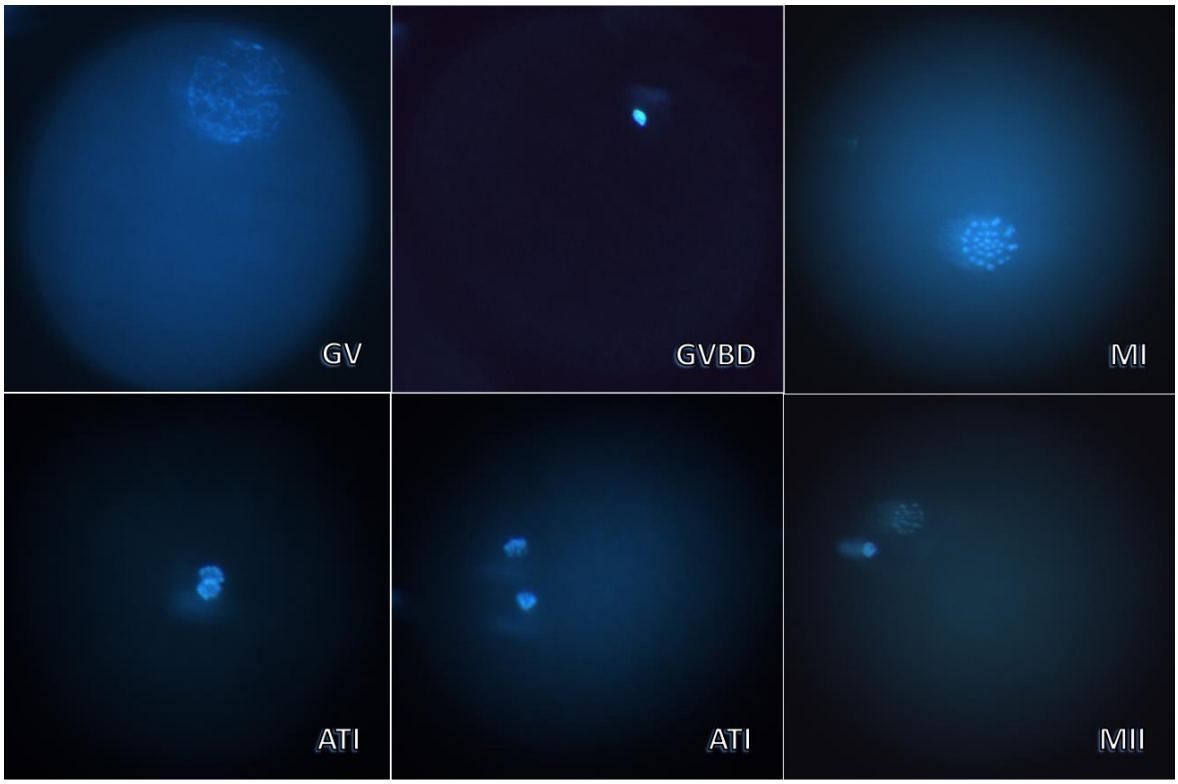

Figure 2.1 - Assessment of chromosome configurations using DAPI staining protocol in ovine oocytes. Meiotic stages are GV = geminal vesicle, GVBD = geminal vesicle breakdown, MI = metaphase I, ATI = anaphase/telophase I and MII = metaphase II.

2.9 Western Blot Immunodetection

COCs were collected as described above and suspended in Radio-Immunoprecipitation Assay (RIPA) buffer (10 mM Tris, 150 mM NaCl, 1mM EDTA, 1% Triton X-100) containing phosphatase (Roche, Penzberg, Germany) and protease inhibitor cocktails (Roche), then snap frozen in liquid nitrogen and stored at −80°C. Samples were mixed with loading buffer containing 100 mM dithiothreitol, heated at 100°C for 5 min, and proteins separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) at 7.5% (separating) and 4% (stacking). Gels were run at 150 V for ~65 min for band resolution. Molecular weight markers ranging from 10 – 250 kDa (Precision Plus Protein Dual Colour Standards, Bio-Rad, CA, USA) were run on every gel. Following electrophoresis, proteins were transferred to Hybond-ECL membranes (GE Healthcare, Waukesha, USA) at 100 V for 70 min. Membranes were washed, blocked with 2% blocking reagent (supplied in an ECL Advance kit; GE Healthcare), diluted in Tris-buffered saline containing 0.1% (v/v) Tween 20 (pERK/ERK) or in Odyssey™ Blocking Buffer (LI-COR, Licor Bioscience, Lincoln, NE) diluted 1:2 with PBS (pCREB/CREB, TACE/ADAM17/βactin) for 1 h at room temperature (RT). The membrane was incubated with two antibodies simultaneously (Table 2.1), at 4°C for 22-26 h, followed by incubation with goat anti-mouse IgG IRDye™ 680 (LI-COR) and goat anti-rabbit IgG IRDye™ 800 (LI-COR) for 1 h at room temperature (Dilutions, Table 2.1). Membranes were washed with TBST then scanned and quantified using Odyssey™ infrared imaging system (LI-COR). Band intensities for pERK1/2 were normalized to ERK1/2, pCREB were normalised to CREB, and TACE/ADAM17 were normalised to β-actin (ACTB). All treatments were standardised relative to untreated controls. For experimental controls, standard error of the mean (SEM) was calculated by the standardised fluoresce of the control divided by the average standardised fluoresce of all replicate controls. Then SEM was calculated from standard deviation of above divided by the square route of the number of replicates.

Table 2.1 - List of Antibodies Used for Western Blot Analyses

Primary Antibody	**Company**	**Dilution Used**
Monoclonal anti-MAPK, activated (diphosphorlyated ERK 1&2) – Mouse	SIGMA (M8159)	1 : 1200[1]
Anti-MAPK (ERK1&2) – Rabbit	SIGMA (M5670)	1 : 10000[2]
Phospho-CREB (Ser133) (1B6) Mouse	Cell Signalling (9196)	1 : 500[3]
CREB (48H2) – Rabbit	Cell Signalling (9197)	1 : 1000[4]
Anti-ADAM17 antibody – activation site	Abcam (ab39163)	1 : 5000[5]
Monoclonal anti-β-actin−peroxidase antibody – Mouse	SIGMA (A3854)	1 : 40000[1]
Secondary Antibody	**Company**	**Dilution Used**
IRDye® 680LT goat anti-mouse IgG	LI-COR (926-68020)	1 : 50000[1] 1 : 10000[3]
IRDye® 800CW goat anti-rabbit IgG	LI-COR (926-32211)	1 : 200000[2] 1 : 50000[4] 1 : 20000[5]

Primary antibody dilution used with the secondary antibody dilution with matching superscript (1,2,3,4,5)

2.10 RNA Extraction and Real Time RT-PCR Analysis

Cells were collected as above and transferred to 1.7 mL Eppendorf tubes where they were centrifuged at maximum speed for 3 min and the supernatant (media) removed. Cell pellets were snap frozen in liquid N_2 and stored at -80°C. Cells were lysed using TRI Reagent®, then chloroform was added (5:1) and the homogenate was allowed to separate. The clear upper aqueous layer (containing RNA) was transferred to new tubes containing 70% ethanol (1:1), then the total RNA was extracted using the RNeasyMicro Kit (QIAGEN, Valencia, CA) according to the manufacturer's instructions. Residual genomic DNA was removed by digesting with recombinant RNase-free DNase I (QIAGEN). RNA was quantified using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Scoresby, VIC, Australia). 300 ng of RNA was reverse transcribed with random primers (Invitrogen; Life Technologies) using Superscript III Reverse Transcriptase (Invitrogen). Quantitative real-time PCR analysis was performed on Corbett Rotor-Gene 6000 (QIAGEN) in 10 µl reaction volume containing; primers at 5 pmol/reaction (see tables 2.2 & 2.3), 1.5 µL cDNA and SYBR Green (Applied Biosystems, Mulgrave, Australia). All reactions were carried out in duplicate. Universal thermal cycling parameters (initial step of 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 60 s at 60°C) were used to quantify the expression of all genes. A five point serial dilution standard curve was produced for each transcript with cDNA derived from COCs and granulosa cells. Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method and results were normalised to the geometric mean of the housekeeping genes Peptidylprolyl isomerase A (*PPIA)* and Beta-actin (*ACTB*) in ovine or only to *PPIA* in porcine. To validate primers, PCR products generated from reactions with ovine granulosa cell or porcine COC/granulosa cell cDNA was run on a 3% agarose gel and considered valid when a single product of the correct size was observed and primer efficiency was ~95%. For experimental controls, standard error of the mean (SEM) was calculated by the standardised cycle threshold (CT) of the control divided by the average CT of all replicate controls. Then SEM was calculated from standard deviation of above divided by the square route of the number of replicates.

Table 2.2 - List of Primers Used for Quantitative RT-PCR Analysis in Ovine

mRNA	*Primer Sequences (5' – 3')*	*Accession #*	*Product*
Npr1	F : TACTTCAGTGACATTGTGGGTTTCA R : TCAAAGTTGTCTATGACAGCATCAAA	NM_000906.3	116
Npr2	F : GCGTATTCGCTGGGAAGAACT R : GTCATGAGCGAGCCGTAACTG	NM_003995.3	111
Ppia	F : CAAATGCTGGACCCAACACA R : TATTCATGCCTTCTTTCACTTTGC	NM_021130.4	111
Actb	F : TCATCACCATCGGCAATGAG R : ATGATGGAATTGAAGGTAGTTTCG	NM_001009784.1	109

Table 2.3 - List of Primers Used for Quantitative RT-PCR Analysis in Porcine

mRNA	*Primer Sequences (5' – 3')*	*Accession #*	*Product*
NPR2	F : GCGTATTCGCTGGGAAGAACT R : GTCATGAGCGAGCCGTAACTG	NM_003995.3	111
AREG	F : AGGTGCAGCTGAAGAACGAA R : CAGTGACCCCGATCTGCTAC	NM_214376.1	103
EGFR	F : GCCGGCTACGTGCTCATC R : AAGGCATGGGTGTTTTCATACAG	NM_214007.1	101
HAS2	F : AGTTTATGGGCAGCCAATGTAGTT R : GCACTTGGACCGAGCTGTGT	NM_214053.1	101
PTGS2	F : AGCAATTCCAATACCAAAACCGTAT R : TGTACTCGTGGCCATCAATCTG	NM_214321.1	102
PPIA	F : CATTTGCACTGCCAAGACTGA R : GGACCCAAAGCGCTCCAT	NM_214353.1	103
EREG	F : AAGACAATCCACGTGTGGCTCAAG R : CGATTTTTGTACCATCTGCAGAAA	*(Yamashita et al. 2009)*	

2.11 Gap Junctional Communication Assay

The degree of gap junction communication in COCs was assessed using Lucifer yellow (LY; Sigma) dye microinjected into the ooplasm, as previously described (Luciano *et al.* 2004). Briefly, after 0, 2, 6, 12 and 24 h of IVM, 3% LY in 5 mM lithium chloride (Sigma) was microinjected into the oocyte of intact COCs in 5 µl of wash medium (IVF Vet Solutions, Adelaide, Australia) overlaid with mineral oil. The spread of dye into the surrounding cumulus cells was then assessed with a confocal microscope (Nikon) within 15 min of injection. As a negative control, COCs were cultured in IVM medium for 24 h supplemented with the gap-junction inhibitor, Carbenoxolone (CBX, 100µM; Sigma), and assessed at 24 h. COCs were scored, as previously described (Luciano *et al.* 2004) as +2 when the dye was completely transferred to the entire cumulus mass, +1 when the dye was transferred to limited number of cumulus cell layers just beyond the corona radiata, and 0 when the dye was transferred to only the corona radiata cells or was not transferred to any cumulus cells at all (Figure 2.2).

2.12 TACE/ADAM17 Activity

COCs were collected as described above and lysed in 200 µL of lysis buffer (25 mM Tris (pH 7.4), 1% Triton X-100) containing 3 µg/mL aprotinin, 1 mM phenylnethylsufonyl fluoride (PMSF) and 0.1 mM leupeptin. Lysed samples were snap frozen in liquid nitrogen and stored at −80°C. Samples were centrifuged and supernatants removed to determine protease activity with 10 µM Fluorogenic Peptide III (R&D Systems) as a substrate. The TACE/ADAM17 cleavage site is the bond between Ala and Val peptides, which separates the fluorochrome from the quencher allowing fluorescent detection (Yamashita *et al.* 2014). The plate was incubated in the Synergy H1 Hybrid Micro-plate Reader (BioTek, Winooski, VT) for 3 h at 37°C and with fluorescent detection every 15 min using excitation wavelength of 320 nm and emission wavelength of 405 nm.

2.13 Statistics

Data were tested for normality and significance using SPSS version 18.0.2 (Predictive Analytics Software (PASW), IBM, NSW, Australia). Details of the statistics used will be explained for all experiments in each chapter.

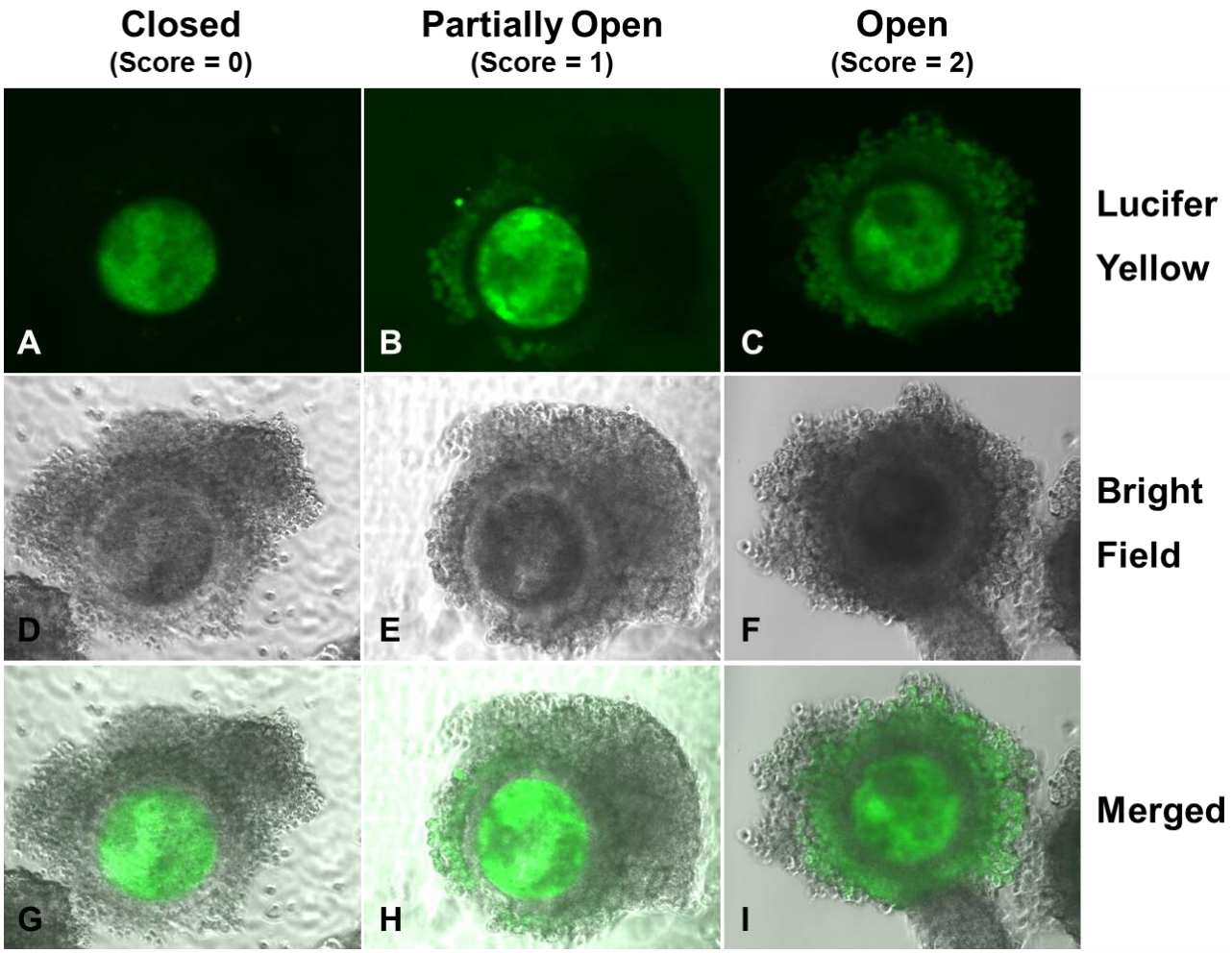

Figure 2.2 - Assessment of COC gap junction communication by microinjection of LY dye into oocytes. Fluorescent (A,B,C), bright field (D,E,F) and merged (G,H,I) images of COCs with closed gap junctions (A,D,G) having no fluorescent cumulus cells scored as 0, partially open gap junctions (B,E,H) having some of the corona radiata and its cumulus fluorescent scored as 1, and open gap junctions (C,F,I) where all of the corona radiata and most of its cumulus fluorescent scored as 2. Figure adapted from (Sugimura et al. 2014).

2.14 References

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CHAPTER 3:

OOCYTE SECRETED FACTORS AND NATRIURETIC PEPTIDES IN OVINE FOLLICULAR CELLS

3.1 Abstract

Atrial (ANP), Brain (BNP), and C-Type (CNP) natriuretic peptides stimulate cGMP production by binding to their cognate receptors natriuretic peptide receptor 1 (NPR1) and NPR2, which play an essential role in mouse and porcine oocyte meiotic arrest, however their role and regulation in sheep cumulus oocyte complexes are unexplored. In the present study, we explored the presence of and regulation of *NPR1* and *NPR2* in sheep ovarian follicle cells. We found that *NPR1* mRNA was expressed at low levels in cumulus cells (CCs) and granulosa cells (GCs), and was more abundant in GCs. Similarly, *NPR2* was more abundant in both cells types and higher in CCs compared to GCs ($P<0.05$). Native oocyte secreted factors (OSFs) increased *NPR1* expression in cumulus cells in the presence of IGF1 ($P<0.05$). Neither native nor exogenous OSFs effected *NPR2* mRNA expression in sheep CCs or GCs, however IGF1 negatively regulated *NPR2* expression in CCs (main effect $P<0.05$). During IVM, the spontaneous resumption of meiosis was unaffected by ANP, BNP or the cGMP analogue 8pCPTcGMP, but was significantly inhibited by 12.5nM of CNP ($P<0.05$). This did not occur at higher concentrations. In summary, we observed the differing expression of NPR mRNA in sheep granulosa and cumulus cells, and that CNP could maintain oocyte meiotic arrest in sheep oocytes. This suggests the CNP/NPR2 system is active in sheep ovarian follicles and likely contributes to the maintenance of oocyte meiotic arrest. However, this work also illustrates key species differences relating to regulation of the natriuretic peptide receptors and dose-dependent inhibition of CNP on meiotic resumption.

3.2 Introduction

Mammalian oocytes begin meiosis during fetal life and are then arrested at the diplotene stage of the first meiotic prophase for an extended period of time; up to 50 years in humans. This germinal vesicle stage is characterised by partial condensation of the chromosomes (Erickson and Sorensen 1974; Sorensen and Wassarman 1976). The pre-ovulatory surge of luteinising hormone (LH) from the pituitary gland re-initiates meiosis and oocytes undergo germinal vesicle breakdown (GVBD) (Ducibella 1996; Mehlmann *et al.* 1996; Ducibella 1998), mediated by actions of the intracellular second messenger, cyclic adenosine monophosphate (cAMP).

In the oocyte, G-protein-coupled receptors (GPR) 3 and 12 are the main cAMP generating receptors and are considered to be constitutively active (Mehlmann *et al.* 2004; Freudzon *et al.* 2005; Hinckley *et al.* 2005). Oocyte cAMP concentrations are regulated by phosphodiesterase 3A (PDE3A) which in turn is inhibited by cyclic guanosine monophosphate (cGMP) (Norris *et al.* 2009). Over the past decade, evidence has revealed cGMP as a major player in mammalian oocyte maturation. cGMP originates in the somatic cells surrounding the oocyte and diffuses through gap junctions to the oocytes where it inhibits the activity of PDE3A, which in the mouse, is the main cAMP degrading phosphodiesterase in the oocyte. Meiotic arrest is achieved by high intra-oocyte cAMP concentrations and maintained by high follicular and oocyte cGMP (Norris *et al.* 2009; Vaccari *et al.* 2009). It has been well documented that cGMP levels drop in the somatic compartment of the follicle in response to the gonadotrophic LH surge in vivo (Hubbard and Greenwald 1982; Norris *et al.* 2009; Vaccari *et al.* 2009).

In mice, C-type natriuretic peptide (CNP), encoded by the natriuretic peptide precursor type C (NPPC) in the mural granulosa cells, stimulates the production of cGMP via its receptor, natriuretic peptide receptor 2 (NPR2) or guanylate cyclase B (GC-B) (Zhang *et al.* 2010). Atrial Natriuretic Peptide (ANP) has been shown to stimulate cGMP production in the cumulus-oocyte complex (COC) and prevents oocyte maturation in the rat, despite the fact that it elicits its effects through a different receptor (Tornell *et al.* 1990a). It has also been suggested that the oocyte assists with cGMP-mediated meiotic arrest by promoting the expression of CNP receptor NPR2 in cumulus cells (Zhang *et al.* 2010). Zhang *et al.* was able to demonstrate an oocyte-specific role in cGMP production by cumulus cells; 1) by showing that co-culture of oocytectomised cumulus cells with denuded oocytes (DOs) increased the expression of *NPR2* mRNA levels in cumulus cells, and 2) the addition of oocyte secreted factors BMP15 with either GDF9 or fibroblast growth factor 8 (FGF8) generated an increase in *NPR2* mRNA levels

in cumulus cells to levels comparable to oocytectomised cumulus cells co-cultured with DOs(Zhang *et al.* 2010). Mouse GCs express similar levels of *NPPC* and its cognate receptor *NPR2*, however expression of *NPR2* receptor is nearly two fold higher in cumulus cells compared to GCs (Zhang *et al.* 2010). Goat COCs demonstrate a similar localisation, with approximately 3-fold higher expression of *NPR2* in COCs compared to MGCs (Zhang *et al.* 2015a).

To our knowledge, few studies have explored the natriuretic peptide system and its effect in primates or ruminant oocyte nuclear maturation. This is important as there are differences between species in cAMP control of oocyte maturation. For example, PDE8 is a major PDE within cattle COCs (Sasseville *et al.* 2009a) compared to PDE4 in rodents (Tsafriri *et al.* 1996) and very limited data is available regarding the natriuretic peptide signalling in human COCs, with only a single paper describing CNP levels in ovarian follicular fluid in response to hCG treatment (Kawamura *et al.* 2011). Therefore, examination of this signalling system in a broad range of species is required. The goals of the present investigations were to examine the expression of the natriuretic peptide receptors in sheep granulosa and cumulus cells, to investigate whether NPR expression is effected by oocyte secreted factors and IGF1, and to determine the effect of natriuretic peptides during IVM on meiotic resumption of sheep cumulus oocyte complexes.

3.3 Materials and Methods

Recombinant mouse mature region GDF9 and human mature region BMP15 were purchased from R&D Systems (Minneapolis, MN, USA). Recombinant human IGF-1 was purchased from Gropep (Adelaide, Australia) and unless otherwise specified all chemicals used in the following protocols were purchased from Sigma (St. Louis, MO, USA).

3.3.1 - Collection, Preparation and Culture of Oocytes

Ovine ovaries were collected from cycling ewes (approximately 1-2 years of age) at Thomas Foods Abattoir (South Australia; -35.109, 139.262) and transported in warm (29 – 32°C) saline (0.9% wt/vol NaCl; Baxter Healthcare) supplemented with antibiotics (100 U/mL penicillin G and 0.1 mg/mL streptomycin sulphate) within 3-4 h of slaughter. Seasonal variations may impact results obtained from these animals, however would be expected to be minimal due to the age of the animals. Antral follicles were aspirated using a 20-gauge needle and constant suction (1 L/min) into vacutainer tubes. Cellular sediment was transferred to 100 mm Petri dishes and searched for COCs with ooplasms of uniform appearance and surrounded by >3

cumulus cell layers, which were collected and washed once in handling medium (HEPES buffered culture medium H-TCM-199; GIBCO® Life Technologies, Mulgrave, Australia) containing 50 µg/mL kanamycin and 4 mg/ml low fatty acid BSA, and twice in culture medium (bicarbonate-buffered tissue culture medium (B-TCM-199) supplemented with 50 µg/mL kanamycin, 100 U/mL penicillin, 0.1 mg/mL streptomycin, 100 µM cysteamine and 4 mg/ml low fatty acid BSA). Groups of up to 10 COCs were transferred into pre-equilibrated 100 µl drops of maturation medium overlaid with mineral oil and incubated at 38.5°C with 6% CO_2 humidified air.

3.3.2 - Somatic Cell Culture

Mural granulosa cells were collected as above and washed twice in B-TCM-199, then transferred to a 96 well plate (Falcon; Becton Dickinson, Franklin Lakes, NJ, USA) with 25,000 cells in a final volume of 125 µL. MGCs were cultured at 38.5°C with 6% CO_2 humidified air for 18h with respective treatments. COCs were denuded of cumulus cells via continuous pipetting; no chemical or enzymatic methods were used to aid isolation. Cumulus cells were collected and washed twice in B-TCM-199, then transferred to a 96 well plate with 25,000 cells in a final volume of 125 µL. Cumulus cells were cultured at 38.5°C with 6% CO_2 humidified air for 18h with respective treatments.

3.3.3 - [^{3}H] Thymidine Incorporation Assay

Granulosa cell bioassays were performed as previously described (Gilchrist *et al.* 2001; Gilchrist *et al.* 2004; Gilchrist *et al.* 2006). Mural granulosa and cumulus cells were recovered and processed for culture as described above. Cells were cultured for 18 h, followed by a further 6 h pulse of 15.4 kBq [^{3}H] thymidine (ICN, Costa Mesa, CA, USA) in an atmosphere of 37°C with 5% CO_2 humidified air. MGCs and CCs were harvested post culture and the incorporated [^{3}H] thymidine was quantified using a scintillation counter as an indicator of the proportion of cells in S phase and thus an indication of the level of DNA synthesis and MGC proliferation (Lee *et al.* 2001). Treatments were performed in duplicate, and at least three experimental replicates performed for all treatments.

3.3.4 - RNA Extraction and Real Time RT-PCR Analysis

COCs were denuded of cumulus cells as above. Ovine oocytes, cumulus and granulosa cells were collected and frozen as cell pellets and then snapped frozen in liquid nitrogen and stored at −80°C. Groups of 30 COCs were lysed using TRI Reagent® then chloroform was added

(5:1) and the homogenate was allowed to separate. The clear upper aqueous layer (containing RNA) was transferred to a new tube containing 70% ethanol (1:1) then the total RNA was extracted using the RNeasy Micro Kit (QIAGEN, Valencia, CA) according to manufacturer's instructions. Residual genomic DNA was removed by digesting with recombinant RNase-free DNase I (QIAGEN). RNA was quantified using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Scoresby, VIC, Australia). RNA was reverse transcribed with random primers (Invitrogen; Life Technologies) using Superscript III reverse transcriptase (Invitrogen). Quantitative real-time PCR analysis was performed on a Corbett Rotor-Gene 6000 (QIAGEN) in 10 µl reaction volume containing; primers at 5 pmol/reaction (see Table 2.2), 1.5 µl cDNA and SYBR Green (Applied Biosystems, Mulgrave, Australia). All PCR reactions were carried out in duplicate. Universal thermal cycling parameters (initial step of 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 60 s at 60°C) were used to quantify the expression of all genes. A five point serial dilution standard curve was produced for each transcript with cDNA derived from COCs and granulosa cells. Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method and results were normalised to the geometric mean of the housekeeping genes peptidylprolyl isomerase A (*PPIA)* and Beta-actin (*ACTB).* To validate primers, PCR products generated from reactions with ovine ovarian tissue, COCs and granulosa cell cDNA were run on a 3% agarose gel and considered valid when a single product of the correct size was observed and primer efficiency was ~95%.

3.3.5 - Statistical Analyses

In all experiments, differences between treatments were analysed by one-way ANOVA, followed by Tukey's post hoc comparison. Factor interactions were determined by a general linear model with two or more factors analysed and main effects adjusted for Bonferroni comparison. $P \leq 0.05$ was considered statistically significant.

3.4 Results

3.4.1 – The expression of natriuretic peptide receptor mRNAs in granulosa and cumulus cells

To determine the presence of the natriuretic peptide receptors 1 and 2 (*NPR1* and *NPR2*) in the sheep COC, RNA extraction, and RT-QPCR was performed. *NPR1* levels were five-fold higher in granulosa cells compared to cumulus cells (1.00±0.01 vs 0.19±0.07, respectively $P<0.05$). Expression of *NPR2* was significantly higher than *NPR1* in cumulus cells; however *NPR2* expression did not differ between granulosa and cumulus cells. Neither *NPR1* nor *NPR2* were expressed in the oocyte (Figure 3.1).

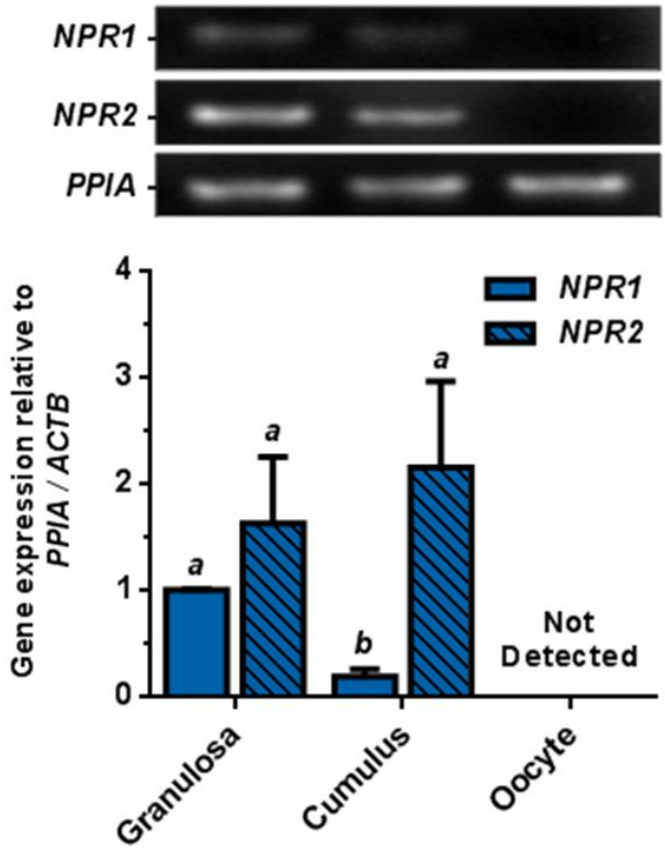

Figure 3.1 - The expression of natriuretic peptide receptor mRNAs in granulosa and cumulus cells. Relative mRNA expression of NPR1 and NPR2 in ovine granulosa cells, cumulus cells and oocytes by real-time PCR analysis. Expression was normalised to that of PPIA and ACTB and represented as fold change from GC-*NPR1*. PCR products confirmed using gel electrophoresis (Top). Graph represents 7 experimental replicates. Columns represent mean ± SEM, means without a common superscript differed (^{ab,} $P < 0.05$).

3.4.2 – Activity of exogenous recombinant GDF9 and BMP15 on ovine granulosa cells

To examine the activity of the exogenous GDF9 and BMP15 preparations on sheep ovarian cells, we assessed [^{3}H]-thymidine incorporation in sheep granulosa cell cultures. The addition of GDF9 and BMP15 had no effect on thymidine incorporation unless cultured in the presence of IGF1. GDF9 at 50 ng/mL + 10 ng/mL IGF1 significantly increased thymidine incorporation compared to untreated control (P=0.05, Figure 3.2B). BMP15 at either 12.5 ng/mL or 50 ng/mL + 10 ng/mL IGF1 significantly increased thymidine incorporation compared to controls. Treating granulosa cells with GDF9 and BMP15 combined at 12.5 ng/mL or 50 ng/mL with IGF1 significantly increased thymidine incorporation compared to any other treatment (Figure 3.2). IGF-1 alone did not affect the level of thymidine incorporation.

3.4.3 – The effect of exogenous and native oocyte secreted factors on the expression of natriuretic peptide receptors in ovine mural granulosa cells

Culture of MGCs with the exogenous OSFs, GDF9 and BMP15, had no effect on *NPR1* or *NPR2* mRNA expression (Figure 3.3). Additionally, native OSFs also had no effect on *NPR1* or *NPR2* mRNA expression in granulosa cells. Supplementation with IGF1 did not affect these results (Figure 3.3).

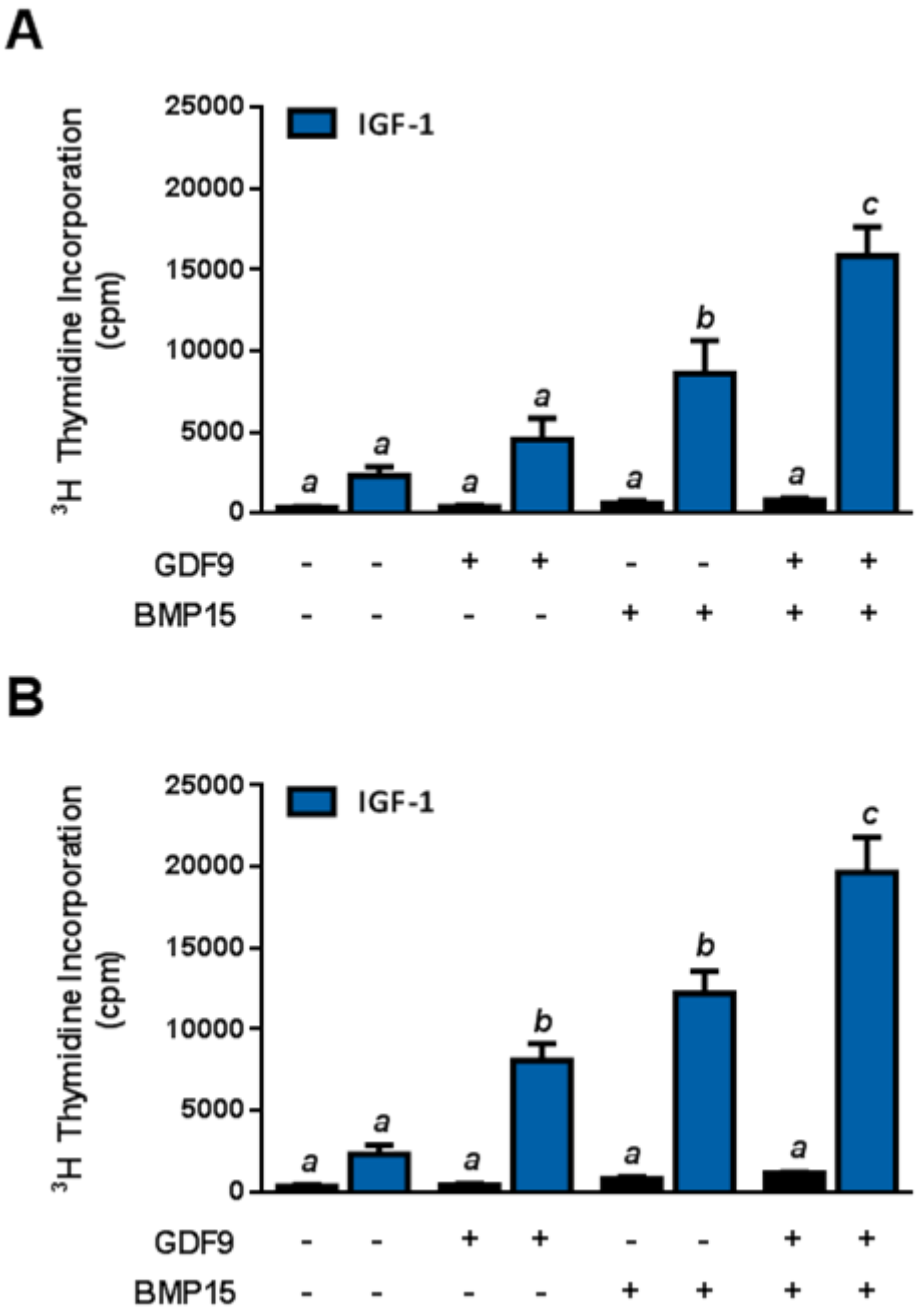

Figure 3.2 - Activity of exogenous recombinant GDF9 and BMP15 on ovine granulosa cells. Ovine mural granulosa cell bioassay (A) GDF9 and BMP15 at 12.5 ng/mL, and (B) GDF9 and BMP15 at 50 ng/mL, with or without 10ng/ml IGF-1 (blue). Each graph reflects 3 experimental replicates. Columns represent mean ± SEM. Means without a common superscript differed (^{abc,} $P \leq 0.05$).

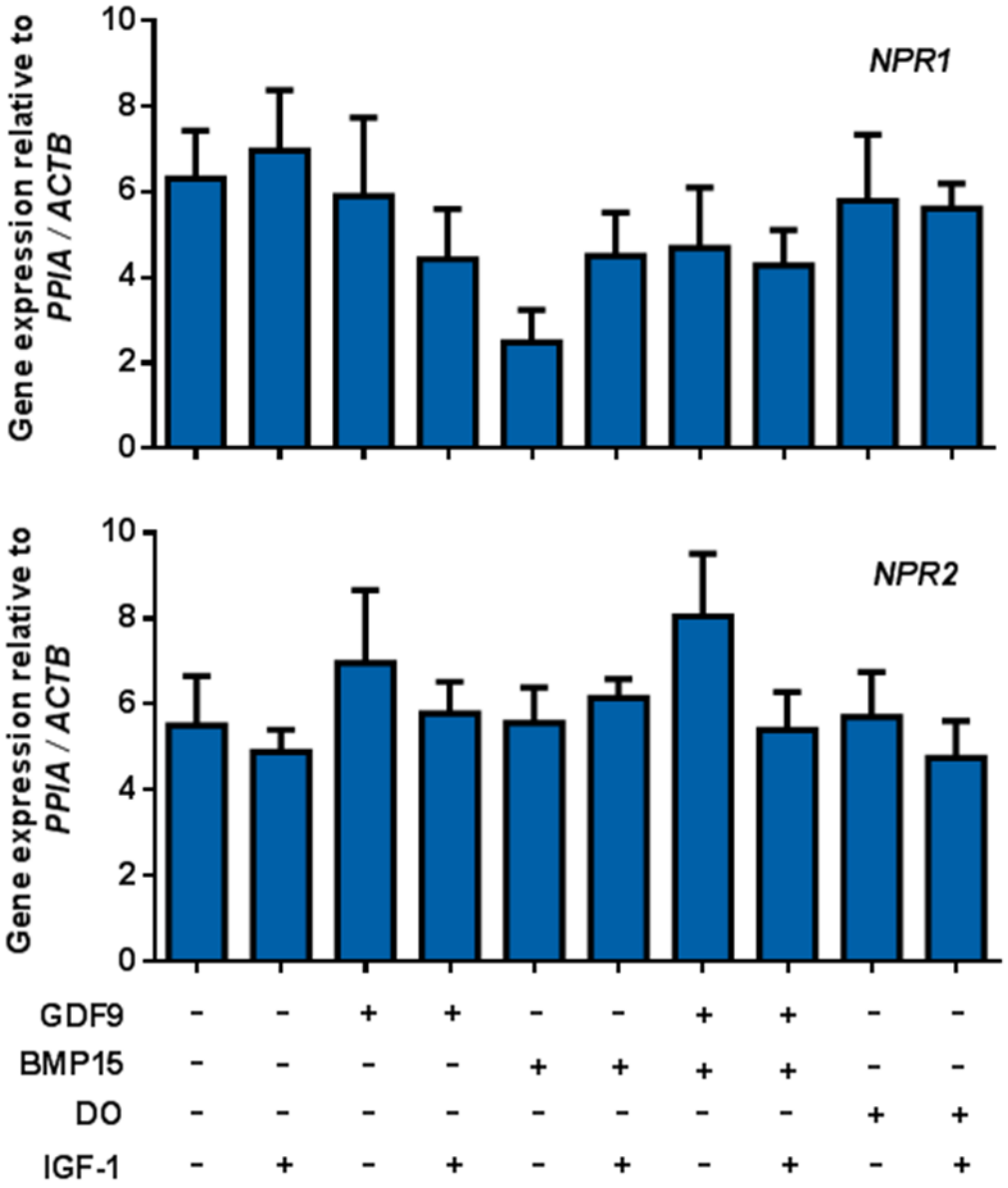

Figure 3.3 - The effect of exogenous and native oocyte secreted factors on the expression of natriuretic peptide receptor (NPR) in ovine mural granulosa cells. Messenger RNA expression of *NPR1* (Top) and *NPR2* (Bottom) in granulosa cells by real-time PCR analysis. MGCs were plated in 5 wells per treatment and pooled for RNA extraction; each graph contains 5 experimental replicates. Expression was normalised to that of *PPIA* and *ACTB* and represented as fold change from internal control. Columns represent mean ± SEM.

3.4.4 – The effect of exogenous oocyte secreted factors on the expression of natriuretic peptide receptors in ovine cumulus cells

Treatment of cumulus cells with the exogenous OSFs, GDF9 and BMP15, had no effect on *NPR1* or *NPR2* mRNA expression. IGF1 had no main effect on *NPR1,* but IGF1 significantly decreased *NPR2* expression irrespective of GDF9 and BMP15 treatments (2-way ANOVA; main effect P<0.05, Figure 3.4).

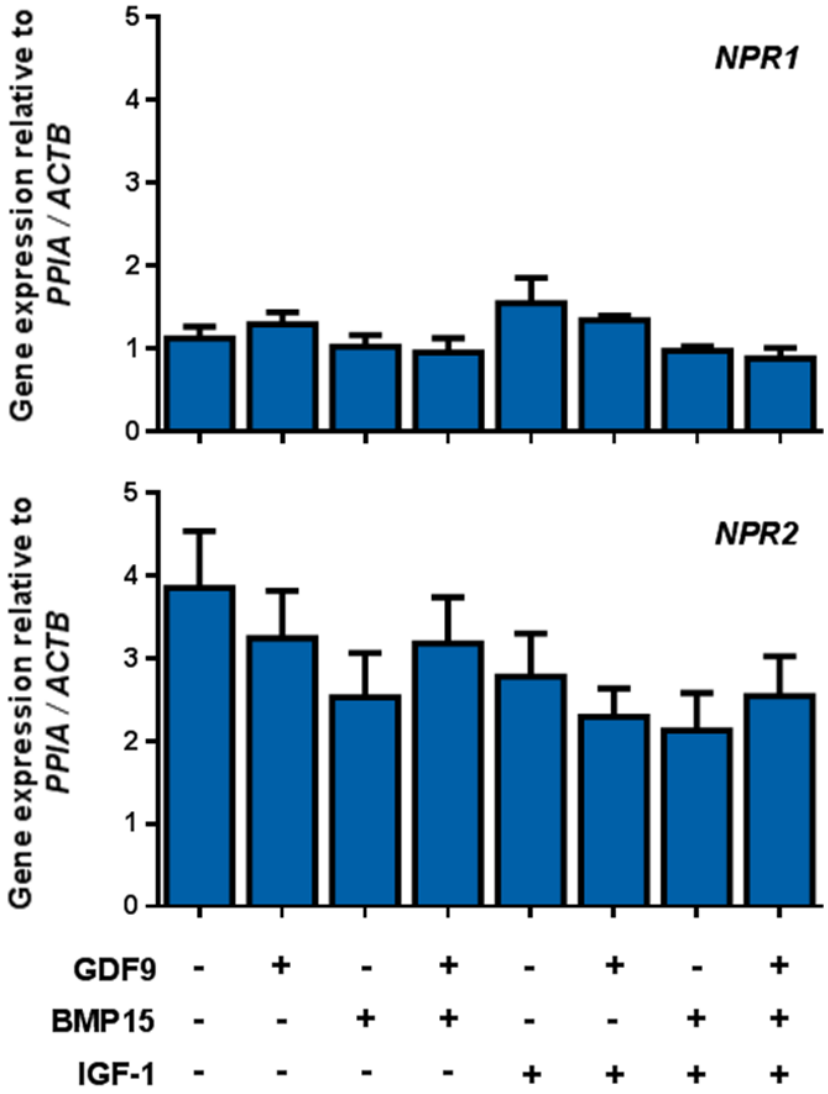

Figure 3.4 - The effect of exogenous oocyte secreted factors on the expression of natriuretic peptide receptor (NPR) in ovine cumulus cells. Relative mRNA expression of *NPR1* (Top) and *NPR2* (Bottom) in cumulus cells by real-time PCR analysis. Expression was normalised to that of *PPIA* and *ACTB* and represented as fold change from internal control. Cumulus Cells were plated in 5 wells per treatment and pooled for RNA extraction; each graph contains 5 experimental replicates. Columns represent mean ± SEM.

3.4.5 – The effect of native oocyte secreted factors on the expression of natriuretic peptide receptors in ovine cumulus cells

The effect of treating cumulus cells with native oocyte-secreted factors with and without IGF1 on expression of natriuretic peptide receptors was assessed. Combining native OSFs and IGF1 tended to produce the highest mRNA expression of *NPR1* but the lowest *NPR2* mRNA expression compared to all treatment groups (Figure 3.5). Treatment with DOs and IGF1 significantly increased *NPR1* expression compared to IGF1 alone, and significantly decreased *NPR2* expression from treatments without IGF1 (± DO – IGF-1, $P < 0.05$, Figure 3.5).Irrespective of IGF1 treatment, DOs co-cultured with CCs significantly increased *NPR1* expression (2-way ANOVA, main effect $P < 0.05$), but had no effect on *NPR2* expression. IGF1 had no main effect on *NPR1* but significantly decreased *NPR2* expression (2-way ANOVA, main effect $P < 0.05$, Figure 3.5).

3.4.6 - The effect of natriuretic peptides on the spontaneous resumption of meiosis in ovine cumulus-oocyte complexes

Natriuretic peptides are known to increase cGMP and inhibit meiosis in many species (Zhang *et al.* 2005b; Zhang *et al.* 2010). We assessed the effects of natriuretic peptides ANP, BNP, CNP, and the cGMP analogue 8pCPTcGMP on the resumption of meiosis in ovine oocytes (Figure 3.6 A, B, D). Oocyte meiotic resumption was unaffected by treatment with ANP, BNP, or 8pCPTcGMP *in vitro*. In contrast, CNP inhibited the resumption of meiosis in a dose dependent manner. Significantly fewer oocytes entered the GVBD stage with the lowest (12.5 nM) dose of CNP compared to 50 nM CNP (22.9±3.9 vs 65.9±7.9%, $P<0.05$). Although 12.5 nM CNP did not significantly reduce the percentage of oocytes at GVBD compared to the control, this was a notable trend ($P<0.07$) with a 2.8-fold reduction in meiotic resumption. Doses of CNP greater than 12.5 nM led to equivalent proportions of oocytes resuming meiosis as in the control (Figure 3.6C).

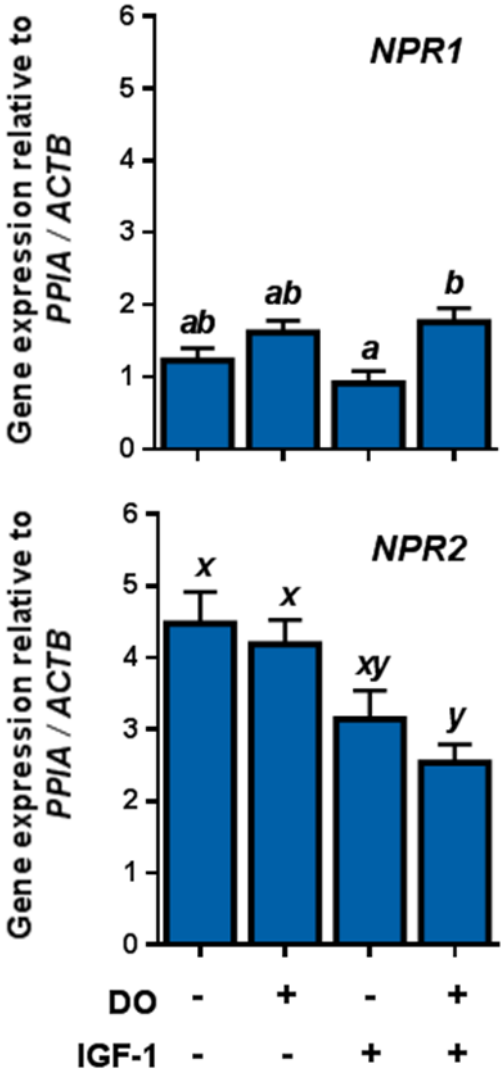

Figure 3.5 - The effect of native oocyte secreted factors on the expression of natriuretic peptide receptors (NPR) in ovine cumulus cells. mRNA expression of *NPR1* (Top) and *NPR2* (Bottom) in cumulus cells by real-time PCR analysis. Expression was normalised to that of *PPIA* and *ACTB* and represented as fold change from internal control. Cumulus Cells were plated in 5 wells per treatment and pooled for RNA extraction; each graph contains 5 experimental replicates. Columns represent mean ± SEM, within receptors means without a common superscript differed ($^{ab, xy,}$ $P < 0.05$)

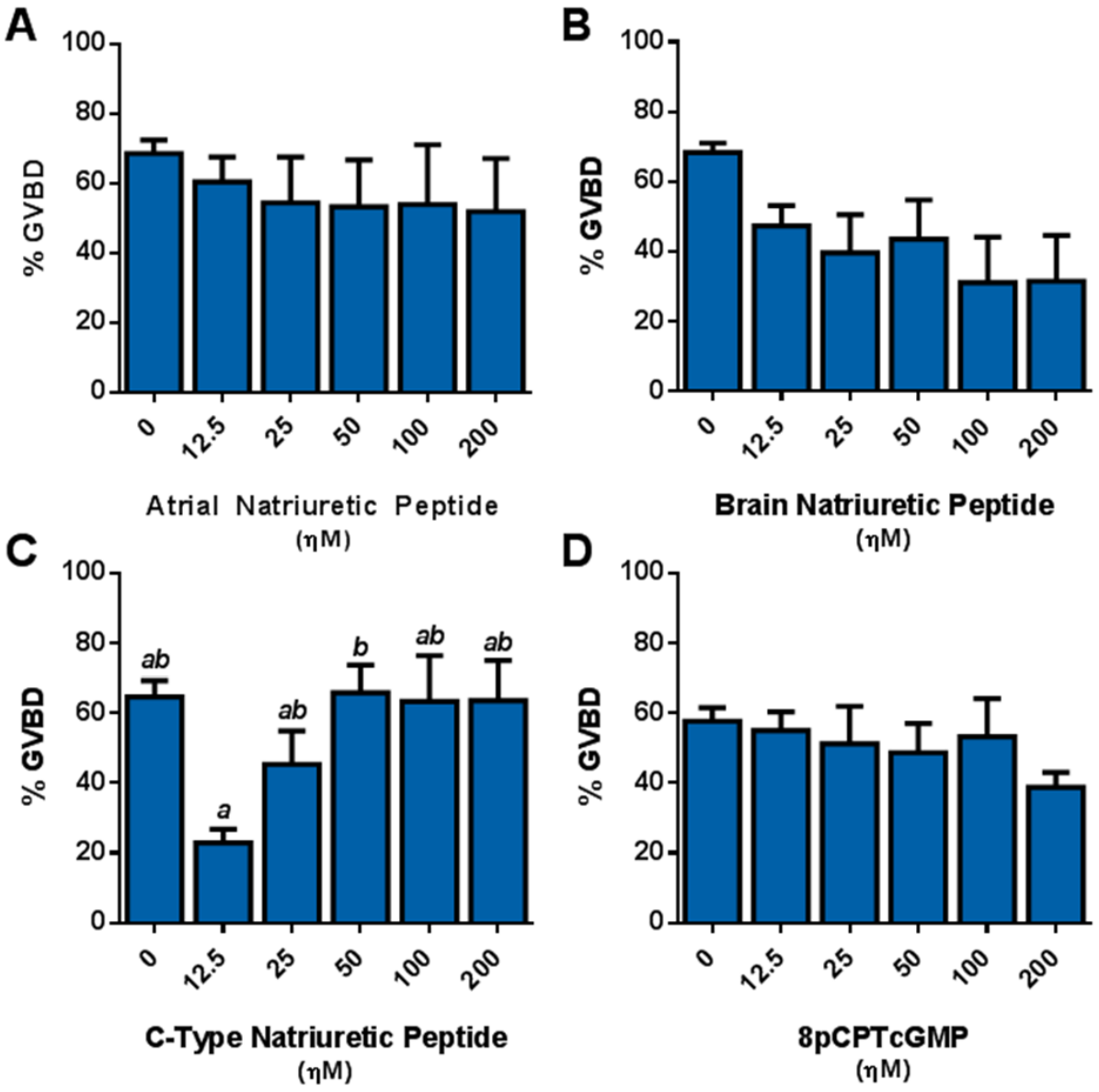

Figure 3.6 - The effect of natriuretic peptides on the spontaneous resumption of meiosis in ovine cumulus-oocyte complexes. Effect of increasing concentrations of natriuretic peptides atrial (A), brain (B) and c-type (C) and the cGMP analogue 8pCPTcGMP (D), on the nuclear maturation of ovine oocytes after 9 hours of culture. Each graph consists of 5 replicates, average of 30 oocytes per treatment. Columns represent mean ± SEM within a figure, means without a common letter differed ($P<0.05$).

3.5 Discussion

In the present study, mRNA expression encoding for the natriuretic peptide receptors 1 and 2 was characterised in ovine granulosa, cumulus cells, and oocytes, and the effect of OSFs and IGF1 on the modulation of their levels of expression *in vitro* was assessed. Additionally, the capacity of ANP, BNP, CNP, and 8pCPTcGMP to inhibit the resumption of ovine oocyte meiosis was described. Our main findings were as follows: 1) *NPR1 and NPR2* mRNA was differentially expressed in sheep granulosa and cumulus cells but was absent from sheep oocytes. 2) *In vitro*, native oocyte secreted factors regulated *NPR1* expression in cumulus cells in the presence of IGF1. 3) IGF1 negatively regulated *NPR2* mRNA expression in cumulus cells. 4) The resumption of meiosis was inhibited by low concentrations of CNP *in vitro*. These results show that CNP can inhibit the resumption of meiosis of sheep COCs *in vitro*, likely via increasing cGMP production by binding to and activating the guanylate cyclase NPR2, a process well described in many species (reviewed (Zhang and Xia 2012; Liu *et al.* 2013)).

Research evidence suggests CNP acts on cumulus cells but not the oocyte. QT-PCR analysis demonstrated that expression of *Npr2* in mice is much higher in cumulus compared to granulosa cells, confirmed by *in situ* hybridisation where *Npr2* was localised to cumulus cells with a decreasing gradient of expression relative to the position of the oocyte (Zhang *et al.* 2010). A similar observation was made in porcine ovarian follicles with *NPR2* mRNA expressed in porcine cumulus cells but not in oocytes (Hiradate *et al.* 2014). We examined the expression of *NPR1* and *NPR2* mRNA in ovine granulosa, cumulus cells, and oocytes. As with mice (Zhang *et al.* 2010; Tsuji *et al.* 2012) and pig (Zhang *et al.* 2015b), but in contrast to cow (Cesaro *et al.* 2015), neither natriuretic peptide receptor was observed in oocytes of sheep, indicating natriuretic peptide actions are constrained to the granulosa and cumulus cells. In contrast to mice, we found *NPR1* mRNA expression in sheep granulosa cells was similar to *NPR2,* whereas in mice, it is generally found that *NPR1* expression in granulosa cells is lower than *NPR2* (Robinson *et al.* 2012). Considering the biological activity of natriuretic peptides is attributed to a cGMP-dependent cascade (Potter *et al.* 2006), and ANP and/or CNP can inhibit the resumption of meiosis in multiple species (Tornell *et al.* 1990b; Zhang *et al.* 2005a; Zhang *et al.* 2010; Hiradate *et al.* 2014), the presence of these receptors in sheep ovarian cells indicates natriuretic peptide-generated cGMP should inhibit the meiotic resumption of sheep oocytes. The participation of natriuretic peptides in the resumption of meiosis differs amongst species. In sheep oocytes, neither ANP nor BNP inhibited the

spontaneous resumption of meiosis *in vitro* (present study); consistent with a previous observation with ANP alone (Ledda *et al.* 1996). Likewise, meiosis is unaffected in mouse COCs cultured with ANP or BNP (Zhang *et al.* 2010; Zhang *et al.* 2011), or bovine COCs treated with ANP (Bilodeau-Goeseels 2007), or BNP (Cesaro *et al.* 2015). This lack of effect is most likely because of a lack of or negligible expression of NPR1, the specific receptor for ANP and BNP in cumulus cells in sheep (present study), mice (Zhang *et al.* 2014b), and cows. By contrast, inhibitory effects of CNP on oocyte maturation have been described in mice (Zhang *et al.* 2010), pigs (Hiradate *et al.* 2014; Zhang *et al.* 2015b), cows (Franciosi *et al.* 2014), goats (Zhang *et al.* 2015a), and cats (Zhong *et al.* 2015). The present study demonstrates that CNP inhibits the spontaneous resumption of meiosis in sheep COCs but only at low concentrations *in vitro*, an observation exclusive to sheep. In mouse models, the transient response of CNP in inhibiting meiosis could be explained by decreasing *NPR2* levels due to *in vitro* culture, which is overcome by exogenous oestradiol (Zhang *et al.* 2011; Zhang *et al.* 2014b). In this study, CNP significantly inhibited meiotic progression at low doses. Furthermore, IGF1, a known promoter of oestradiol synthesis (Campbell *et al.* 1995), decreased *Npr2* in our system, and hence would unlikely promote further meiotic inhibition. A plausible assumption could be that at low levels of CNP, the increased cGMP produced is transported into the oocyte, thereby maintaining meiosis-inhibitory cAMP levels. Meanwhile, higher concentrations trigger higher cGMP levels, which may have alternative actions, such as the activation of ERK1/2 signalling (see Chapter 4), potentially overriding the cGMP-inhibition of meiosis. This is supported by the fact that the cGMP analogue 8pCPTcGMP does not inhibit the spontaneous resumption of meiosis of sheep COCs *in vitro*, as it promotes ERK1/2 activation in porcine COCs (Chapter 4) and cGMP-dependant protein kinase activation with minimal action or cross-activation of phosphodiesterases (Sirotkin *et al.* 2000; Stahl *et al.* 2015).

In vitro studies investigating the effects of recombinant GDF9 and BMP15 on thymidine incorporation, which is reflective of cell DNA synthesis in granulosa cells, have shown that these factors have a synergistic effect when present together in culture (McNatty *et al.* 2005; McIntosh *et al.* 2008; Mottershead *et al.* 2012). In the present study, both GDF9 alone at 50 ng/mL and BMP15 alone at 12.5 or 50 ng/mL, stimulated thymidine incorporation in ovine granulosa cells, but only in the presence of IGF1. This suggests that the actions of OSFs to increase DNA synthesis compared to control requires IGF1 in our system, consistent with previous studies using sheep granulosa cells (McNatty *et al.* 2005).

The expression of *NPR2* is highly regulated in cumulus cells of mice by oestradiol, testosterone and progesterone (Zhang *et al.* 2011). In addition, in mice, expression of cumulus cell *Npr2* mRNA can be promoted by the oocyte-secreted factors GDF9, BMP15, and FGF8B *in vitro*, and the combination of the three proteins promotes levels of *Npr2* mRNA expression equivalent to those promoted by native OSFs (Zhang *et al.* 2010). Porcine COCs show a similar response to native OSFs. Porcine oocytectomised COCs (OOX) lose *NPR2* mRNA expression within cumulus cells, however co-culturing these OOXs with denuded oocytes promotes *NPR2* mRNA expression equivalent to COCs (Hiradate *et al.* 2014). These studies suggest the oocyte plays an important role in the regulation of *NPR2.* The regulation of *NPR2* in ruminants is still unknown. Here, we found that although exogenous OSFs, BMP15, and GDF9 were biologically active in sheep granulosa cells, as determined by thymidine incorporation, they did not alter the mRNA expression of *NPR1* or *NPR2* in either granulosa or cumulus cells collected from antral sheep follicles. Furthermore, native OSFs had a negligible effect on the expression of *NPR2* mRNA in granulosa and cumulus cells from these follicles. Only native OSFs in the presence of IGF1 could significantly increase cumulus *NPR1* mRNA expression, even though *NPR1* mRNA expression is much lower than *NPR2* mRNA in cumulus cells and native OSFs had no effect on *NPR2* mRNA expression. IGF1 significantly decreased *NPR2* mRNA expression in sheep cumulus cells, which suggests another functional role of IGF1 in the resumption of meiosis. Since IGF1 concentrations increase in large antral follicles during the peri-ovulatory period (Monget *et al.* 1993), IGF1 in this study is likely to either: 1) Help promote meiotic resumption by decreasing *NPR2* expression and increasing FSH receptor expression (Minegishi *et al.* 2000) and EGF transactivation, or 2) Help to maintain *NPR2* mRNA expression by increasing oestradiol synthesis (Campbell *et al.* 1995), a known promoter of *Npr2* mRNA expression in mouse cumulus cells (Zhang *et al.* 2011). Nonetheless, under the conditions in this study, oestradiol levels may be at insufficient concentrations to maintain such levels.

In conclusion, sheep granulosa cells and COCs have a functional natriuretic peptide signalling system. Consistent with other species, *NPR1* expression is low in cumulus cells, making NPR2 and CNP the predominant receptor and ligand, respectively, in sheep cumulus oocyte complexes. Expression of these receptors is altered in response to OSFs and IGF1, and spontaneous resumption of meiosis is prevented in vitro by low concentrations of CNP. Further research is warranted to elucidate functional actions of natriuretic peptides in sheep ovarian follicles.

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CHAPTER 4:

CNP/cGMP ACTIVATION OF ERK1/2 SIGNALLING IN THE PORCINE CUMULUS OOCYTE COMPLEX

4.1 Abstract

Research conducted in recent years has led to great advances in our understanding of the participation of cyclic guanosine monophosphate (cGMP) in meiosis. It is clear that intra-oocyte cGMP inhibits meiosis, however the cGMP effects on ERK1/2 signalling in the cumulus oocyte complex (COC) is less understood. Abattoir-derived gilt porcine ovaries were collected, antral follicles aspirated, and oocytes collected and cultured in medium. RT-PCR analysis demonstrated significantly more natriuretic peptide receptor 2 (NPR2) expression in cumulus cells compared to granulosa cells. Amphiregulin (AREG), FSH, c-type natriuretic peptide (CNP) and a cGMP analogue, 8-(4-Chlorophenylthio)-guanosine 3′,5′-cyclic monophosphate (8pCPTcGMP) induced significant increases in the phosphorylation of ERK1/2 in the COC by 2 h of in vitro culture compared to controls. cGMP-dependent protein kinase (PKG) and cAMP-dependent protein kinase (PKA) inhibitors, Rp-8pCPTcGMP and H89 respectively, had no effect on CNP-/cGMP-induced ERK1/2 phosphorylation. Phosphorylation of cAMP-response binding protein (CREB) was not effected by CNP/cGMP, and the expressions of the AREG, epiregulin (EREG), cumulus expansion genes (HAS-2 and PTGS2), or epidermal growth factor receptor (EGFR) were also unaffected. CNP/cGMP induced ERK1/2 phosphorylation was abolished by the inhibition of metalloproteinases (MMPs) with TAPI-2, and by the EGFR antagonist AG1478, but not by inhibition of src-family kinases (SFKs) with PP2. Neither TACE/ADAM17 expression nor activity was significantly altered in the COC in response to CNP/cGMP. *In vitro* culture of COCs with AREG, FSH, CNP, or 8pCPTcGMP significantly decreased gap junction communication by 12 and 24 h compared to controls. Therefore, cGMP induces the phosphorylation of ERK1/2 in porcine COCs by EGFR-dependent signalling requiring metalloproteinases and could result in the breakdown of oocyte-cumulus gap junction communication. This data suggests a mechanism of CNP/cGMP regulation of ERK1/2 implicating this pathway in the development and maturation of the ovarian follicle and its oocyte.

4.2 Introduction

To achieve reproductive success in mammals, females require gonadotrophins for the development of their ovarian follicles, differentiation of granulosa cells (GC), ovarian release of the cumulus oocyte complex (COC), and the resumption of oocyte meiosis (Richards 1994; Matzuk *et al.* 2002; Hunzicker-Dunn and Maizels 2006). Understanding how gonadotrophins regulate these mechanisms is important as it impacts on fertility and healthy development of subsequent offspring.

Gonadotrophin hormones LH and FSH activate ERK1/2 signalling cascades in ovarian granulosa cells. In essence, granulosa cells respond to gonadotrophins with the elevation of intracellular cAMP and the PKA-dependent rapid stimulation of ERK 1/2 pathway (Das *et al.* 1996; Seger *et al.* 2001; Salvador *et al.* 2002; Russell *et al.* 2003; Sela-Abramovich *et al.* 2005). Activation of ERK1/2 in cumulus cells is essential for inducing the expression of the cumulus expansion genes hyaluronan synthase 2 (*HAS2*), prostaglandin-endoperoxide synthase 2 (*PTGS2*), and tumour necrosis factor alpha induced protein 6 (*TNFA1P6*) (Shimada *et al.* 2006b; Fan *et al.* 2009; Yamashita *et al.* 2009; Prochazka *et al.* 2012). As such, ERK1/2 knockout mice exhibit inhibited cumulus expansion, meiotic resumption, ovulation, and luteinisation (Panigone *et al.* 2008; Fan *et al.* 2009).

The pre-ovulatory surge of LH, induces rapid and transient expression of the epidermal growth factor (EGF)-like peptides (EGFp); amphiregulin (AREG), epiregulin (EREG), and betacellulin (BTC) in the somatic compartment of the follicle, a process which propagates ERK1/2 activity in COCs and is essential for fertility (Park *et al.* 2004; Ashkenazi *et al.* 2005; Chen *et al.* 2008). EGFps are synthesised as membrane bound proteins and cleavage of the precursor form at one or more sites in the extracellular domain is required for release of a soluble EGF-domain (Lee *et al.* 2003; Sahin *et al.* 2004). Interactions with membrane bound receptors like epidermal growth factor receptor (EGFR) require matrix metalloproteinase (MMP) activity. Inhibition of cleavage enzymes with protease inhibitors can block FSH and LH signal transmission, including increases in ERK1/2 activity, however can be overcome with exogenous EGFps (Ashkenazi *et al.* 2005; Yamashita *et al.* 2007; Panigone *et al.* 2008).

Over the past decade, research has shown in the ovarian follicle that c-type natriuretic peptide (CNP) significantly inhibits meiotic progression of the oocyte by activating the guanylate cyclase natriuretic peptide receptor 2 (NPR2) and increasing intra-oocyte cGMP levels (Zhang *et al.* 2010). CNP is a potent meiotic inhibitor and is most likely the key somatic cell signal

responsible for maintaining oocyte meiotic arrest during follicular development. However, the effect of CNP on ERK1/2 signalling in granulosa cells and the COC is less clear. In porcine COCs, FSH-induced cumulus expansion and resumption of meiosis is significantly inhibited by the addition of ANP and 8-Br-cGMP in culture, attributed to the suppression of ERK1/2 activity (Zhang *et al.* 2005b). The cGMP-dependant protein kinase (PKG) inhibitor KT5823 completely reversed this effect (Zhang *et al.* 2005b), suggesting ANP function is dependent on PKG. Conversely, Blaha *et al.* demonstrated no effect of cGMP on FSH induced ERK1/2 activation with CNP and 8-CPT-cGMP producing comparable ERK1/2 phosphorylation to FSH culture (Blaha *et al.* 2015). On the other hand, in non-ovarian tissues, increasing cGMP levels by NO donors or cGMP analogues result in increased ERK1/2 activation in vascular smooth muscle cells (reviewed (Pilz and Casteel 2003)), osteoblasts (Rangaswami *et al.* 2009; Marathe *et al.* 2012), and cerebellar Purkinje cells (Endo and Launey 2003). Despite several reports of the phenomenon in different cell types, cGMP activation of ERK1/2 has yet to be observed in the ovarian follicular cells.

In this study we hypothesised that CNP, via cGMP dependent signalling, regulates ERK1/2 activation via mechanisms common to FSH signalling. To achieve this experimentally, we assessed the phosphorylation of ERK1/2 in porcine COCs in response to various established inhibitors as a measure of activity. Furthermore, because oocyte-cumulus cell gap junction communication (GJC) is terminated as a consequence of ERK1/2 activation, we used an established gap junction communication assay to determine if CNP/cGMP induced a breakdown in GJC.

4.3 Materials and Methods

Unless otherwise specified, all chemicals used in the following protocols were purchased from Sigma (St. Louis, MO, USA).

4.3.1 Collection, Preparation and Culture of Oocytes

Porcine gilt ovaries were obtained from an abattoir and transported in warm (29 – 32°C) saline (0.9% wt/vol NaCl; Baxter Healthcare) supplemented with antibiotics (100 U/mL penicillin G and 0.1 mg/mL streptomycin sulphate) within 3-4 h of slaughter. Large antral follicles, greater than 4mm in size, were aspirated using a 20-gauge needle and constant suction (1 L/min) into vacutainer tubes. Cellular sediment was transferred to 100 mm Petri dish and cumulus-oocyte complexes (COCs) with ooplasms of uniform appearance and surrounded by >3 cumulus cell layers were collected and washed once in handling medium (Hepes buffered tissue culture medium (H-TCM-199; GIBCO® Life Technologies, Mulgrave, Australia) + 3 mg/ml low fatty acid BSA), and once in culture medium (bicarbonate-buffered TCM-199 + 3 mg/ml low fatty acid BSA). Groups of up to 10 COCs were transferred into pre-equilibrated 100 µl drops of maturation medium overlaid with mineral oil and incubated at 38.5°C with 6% CO_2 humidified air.

4.3.2 Western Blot Immunodetection

COCs were collected as described above and suspended in RIPA buffer (10 mM Tris, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100) containing phosphatase (Roche, Penzberg, Germany) and protease inhibitor cocktails, snap frozen in liquid nitrogen and stored at −80°C. Samples were mixed with loading buffer containing 100 mM dithiothreitol, heated at 100°C for 5 min, and loaded onto a 7.5% (separating) and 4% (stacking) SDS–polyacrylamide gel for electrophoresis. Proteins were transferred to Hybond-ECL membranes (GE Healthcare, Waukesha, USA), and the membrane was cut horizontally below the 75kDa marker and the upper section was discarded. The remaining membrane was blocked with 2% blocking reagent (supplied in an ECL Advance kit; GE Healthcare) diluted in Tris-buffered saline containing 0.1% (v/v) Tween 20. The membrane was incubated with pairs of antibodies (See Table 2.1 for company/dilutions) simultaneously; pERK and ERK, or pCREB and CREB, or ADAM17 and β-ACTIN, at 4°C for 22-26 h, followed by incubation with goat anti-mouse IgG IRDye™ 680 (Licor Bioscience, Lincoln, NE) diluted 1:50000 and goat anti-rabbit IgG IRDye™ 800 (Licor Bioscience) for 1 h at room temperature (antibody dilutions see Table 2.1). Membranes were washed with Tris-buffered saline with Tween 20 (TBST), then scanned and quantified

using an Odyssey infrared imaging system (Licor Bioscience). Band intensities for pERK1/2 were expressed relative to ERK1/2, pCREB to CREB, and ADAM15 to β-ACTIN, and each were standardised relative to no treatment controls.

4.3.3 Gap Junctional Communication Assay

The degree of oocyte-cumulus cell gap junction communication in COCs was assessed using LY (Sigma) dye microinjected into the ooplasm, as previously described (Luciano *et al.* 2004). After 0, 2, 6, 12 and 24 h of culture, 3% LY in 5 mM lithium chloride (Sigma) was microinjected into the oocyte in 5 µl of wash medium (IVF Vet Solutions, Adelaide, Australia) overlaid with mineral oil. The spread of dye into the surrounding cumulus cells was assessed with a Olympus Fluoview FV10i laser scanning confocal microscope (Olympus, Tokyo, Japan) within 15 min of injection. As a negative control, COCs were cultured in oocyte maturation medium for 24 h supplemented with the gap-junction inhibitor, carbenoxolone (CBX, 100 µM; Sigma) and assessed at 24 h. Oocyte-cumulus cell GJC was scored as previously described (Luciano *et al.* 2004); +2 when the dye was completely transferred to the entire cumulus mass, +1 when the dye was transferred to limited number of cumulus cell layers just beyond the corona radiata, and 0 when the dye was transferred to only the corona radiata cells or was not transferred to any cumulus cells at all.

4.3.4 RNA Extraction and Real-Time PCR Analysis

COCs cultured for 0 or 2 h (see figure legends) were collected and frozen as cell pellets and snap frozen in liquid nitrogen and stored at −80°C. Groups of 30 COCs were lysed using TRI Reagent®, then chloroform was added (5:1) and the homogenate was allowed to separate. The clear upper aqueous layer (containing RNA) was transferred to a new tube containing 70% ethanol (1:1), then total RNA was extracted using the RNeasyMicro Kit (QIAGEN, Valencia, CA) according to the manufacturer's instructions. Residual genomic DNA was removed by digesting with recombinant RNase-free DNase I (QIAGEN). RNA was quantified using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Scoresby, VIC, Australia). RNA was reverse transcribed with random primers (Invitrogen; Life Technologies) using Superscript III reverse transcriptase (Invitrogen). Quantitative real-time PCR analysis was performed on a Corbett Rotor-Gene 6000 (QIAGEN) in a 10 µl reaction volume containing; primers at 5 pmol/reaction (see Table 2.3), 1.5 µl cDNA, and SYBR Green (Applied Biosystems, Mulgrave, Australia). All PCR reactions were carried out in duplicate. Universal thermal cycling parameters (initial step of 2 min at 50°C and 10 min at 95°C, followed by 40

cycles of 15 s at 95°C, and 60 s at 60°C) were used to quantify the expression of all genes. A five point serial dilution standard curve was produced for each transcript with cDNA derived from COCs and granulosa cells. Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method and results are presented relative to untreated control and normalised to the housekeeping gene peptidylprolyl isomerase A (*PPIA).* To validate primers, PCR products generated from reactions with porcine COCs and granulosa cell cDNA were run on a 3% agarose gel and considered valid when a single product of the correct size was observed and primer efficiency was ~95%.

4.3.5 TACE/ADAM17 Activity

COCs were collected as described above and lysed in 200 µL of lysis buffer (25 mM Tris (pH 7.4), 1% Triton X-100) containing 3 µg/mL aprotinin, 1 mM phenylnethylsufonyl fluoride (PMSF), and 0.1 mM leupeptin. Lysed samples were snap frozen in liquid nitrogen and stored at −80°C. Samples were centrifuged, and supernatants removed to determine protease activity with 10 µM fluorogenic peptide III (R&D Systems) as a substrate. The cleavage site targeted by TACE/ADAM17 is the peptide bond between Ala and Val, thus separating the fluorochrome from the quencher allowing fluorescent detection. The plate was incubated in a Synergy H1 Hybrid Micro-plate Reader (BioTek, Winooski, VT) for 3 h at 37°C, with fluorescent detection every 15 min using excitation wavelength of 320 nm and emission wavelength of 405 nm.

4.3.6 Statistical Analyses

In all experiments, differences between treatments were analysed by one-way ANOVA followed by Tukey post hoc comparison, or one-way ANOVA followed by LSD post hoc comparison (Figure 5).

4.4 Results

4.4.1 Expression of NPR2 in porcine follicular cells

To examine the presence of the CNP receptor, NPR2, in the pig COC, RNA extraction and QT-PCR were performed. *NPR2* was at significantly (P<0.05) higher abundance in cumulus cells compared to granulosa cells (6.7 ± 0.8 vs 3.3 ± 1.1-fold increase from internal control respectively; Fig. 4.1).

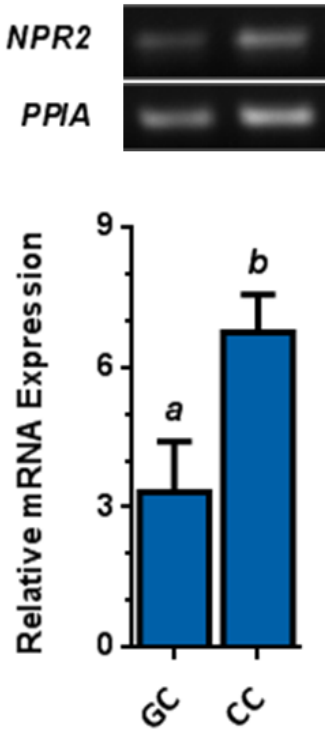

Figure 4.1 – Expression of NPR2 in porcine follicular cells. mRNA expression of natriuretic peptide receptor 2 (NPR2) in porcine granulosa (GC) and cumulus cells (CC). Untreated (0h) cumulus and granulosa cells were collected from follicles and mRNA expression of NPR2 was measured using quantitative real-time PCR and normalised to the stable housekeeper PPIA and represented as fold change from internal control. PCR products confirmed using gel electrophoresis (top). Columns represent means ± SEM of 4 replicate experiments, means without a common superscript differed (a,b P < 0.05).

4.4.2 Time-dependent phosphorylation of ERK1/2 in COCs by CNP and cGMP

The experiment examined the effect of CNP and cGMP on ERK phosphorylation in COCs. ERK phosphorylation was substantially increased within 15 mins in response the AREG (Fig. 4.2A), and was maintained at 2 h (Fig. 4.2B,C). Neither CNP nor the cGMP analogue, 8pCPTcGMP, showed any effect of ERK1/2 at 15 min (Fig. 4.2A), however by 2 h both produced a significant increase in the phosphorylation of ERK1/2 (Fig. 4.2B,C), suggesting a time dependent-effect and indirect activation. FSH, CNP, and FSH+CNP significantly increased the phosphorylation of ERK1/2 compared to control (Fig. 4.2C) suggesting CNP has a comparable effect to FSH on phosphorylation of ERK at the doses examined.

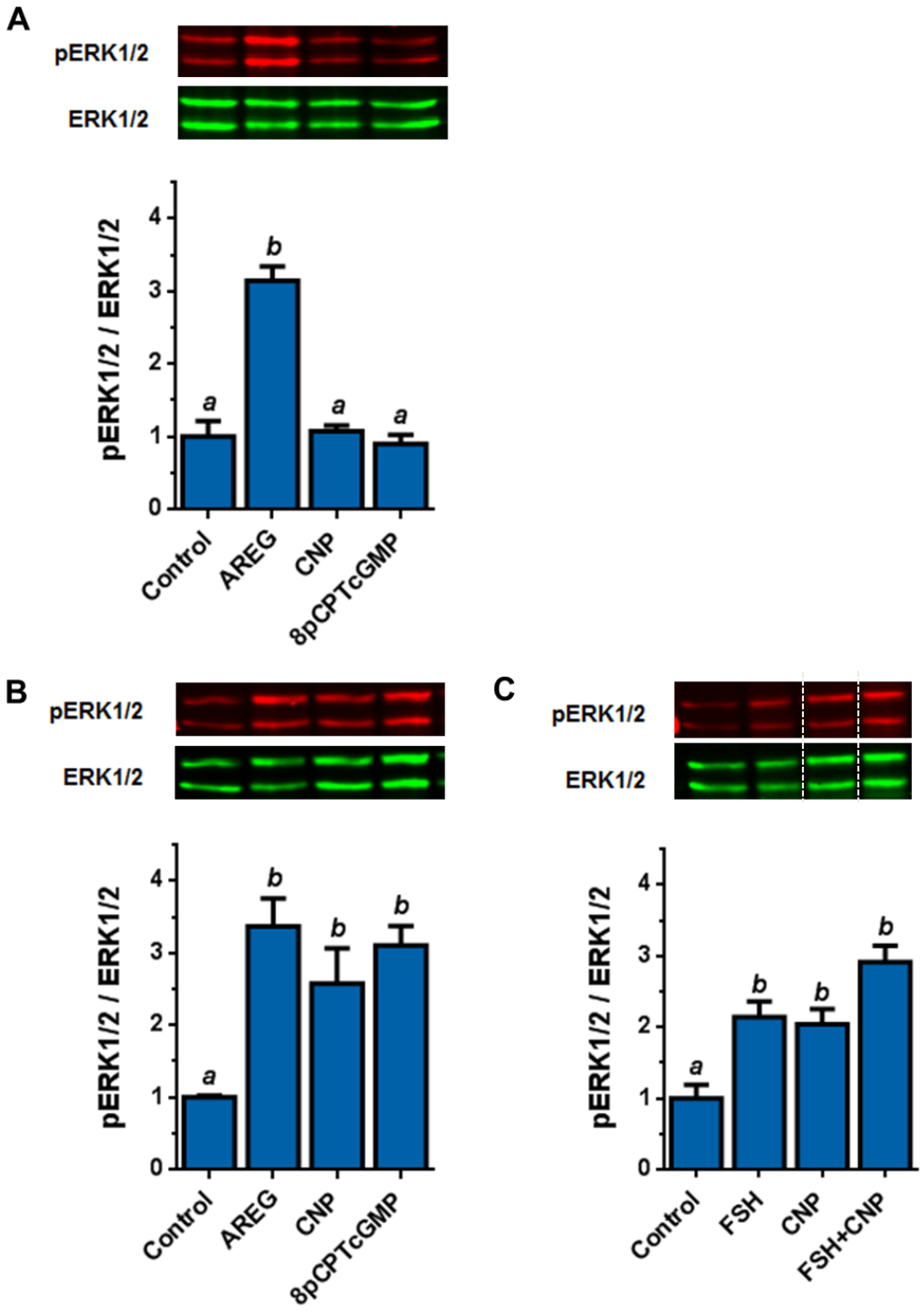

Figure 4.2 – Effect of CNP and cGMP on ERK1/2 activity in COCs. Western blot analysis of the phosphorylation of ERK1/2 relative to total ERK1/2 protein in porcine COCs after 15 mins (A), or 2 h (B,C) of culture in control medium or treated with AREG (100ng/mL), FSH (50mIU/mL), CNP (100nM), or 8pCPTcGMP (1µM). Columns are presented as fold-change from untreated control. Grey dashed lines on WB image (C) indicate non-continuous sections of the same gel. Columns represent means ± SEM of 4 replicates of 50 COCs/treatment/replicate, means within a graph without a common superscript differed (a,b $P<0.05$).

4.4.3 CNP and cGMP activate ERK1/2 independently of cyclic nucleotide-dependant protein kinases and CREB

Cyclic nucleotides acting via cAMP-dependant protein kinase (PKA) or the cGMP-dependant protein kinase (PKG) are known to phosphorylate ERK1/2 by a CREB-dependent mechanism (Pilz and Casteel 2003; Russell *et al.* 2003). Therefore, we looked at the activation of ERK1/2 in the presence of PKA/PKG inhibitors and assessed the phosphorylation ser-133 in CREB. COCs cultured with AREG, CNP, or 8pCPTcGMP, significantly ($P<0.05$) increased the phosphorylation of ERK1/2 compared to control (Fig. 4.3A,B). Inhibitors of PKG (Rp-8pCPTcGMP) and PKA (H89; Fig. 4.3B), did not prevent the increase in pERK induced by AREG, CNP, or 8pCPTcGMP, however in the absence of any treatment, Rp-8pCPTcGMP tended to lead to a small, non-significant, increase in pERK1/2 (Fig. 4.3A). Treatment of COCs with FSH or AREG, also significantly ($P<0.05$) increased the phosphorylation of the cAMP response element binding protein (CREB) compared to control. Both CNP and 8pCPTcGMP had no effect on the phosphorylation of CREB in COCs (Fig. 4.3D).

4.4.4 CNP and cGMP do not induce early EGF and cumulus expansion gene expression

The relative mRNA expression of *AREG, EREG, EGFR, HAS-2* and *PTGS2* was measured after culturing COCs in FSH, AREG, CNP, and 8pCPTcGMP for 2 h. COCs treated with FSH resulted in significantly higher *AREG, EREG, HAS-2* and *PTGS2* mRNA expression compared to all other treatments ($P<0.05$, Fig. 4.4). Addition of AREG resulted in significantly higher *AREG* mRNA expression compared to untreated controls and treatment with CNP or 8pCPT ($P<0.05$, Fig. 4.4). Neither CNP nor 8pCPTcGMP altered expression of any mRNA transcript examined more than 2-fold from untreated control. Expression of EGF receptor (*EGFR)* mRNA did not differ significantly between any treatments (Fig. 4.4).

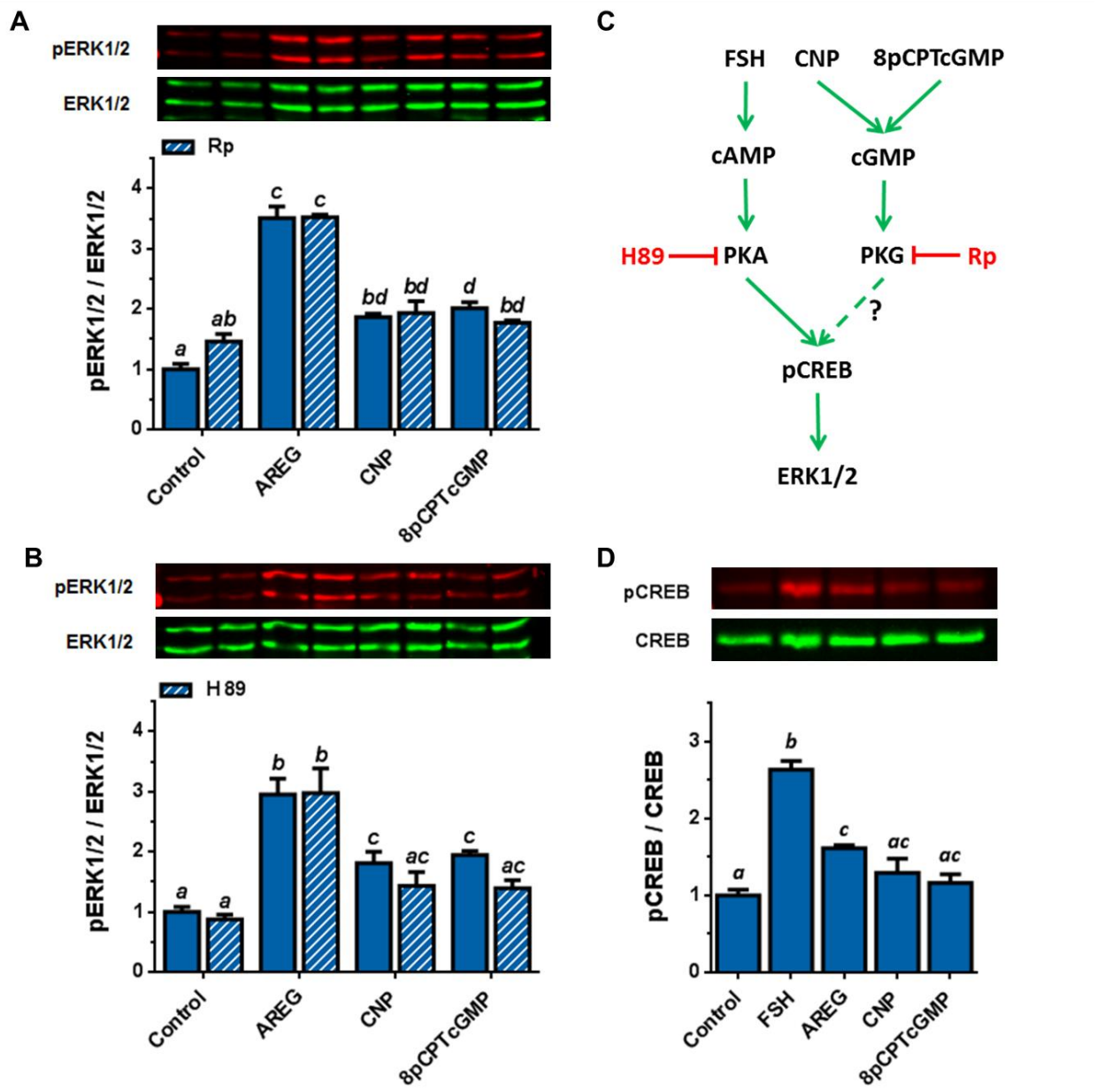

Figure 4.3 – Western blot analysis of the phosphorylation of ERK1/2 relative to total ERK1/2 protein in porcine COCs after 2 h culture with an inhibitor of PKG (A), Rp-8pCPTcGMP (20µM), and an inhibitor of PKA (B), H89 (20µM), and Western blot analysis of the phosphorylation of CREB relative to total CREB protein after 2 h (D). Treatments were control medium or AREG (100ng/mL), FSH (50mIU/mL), CNP (100nM), or 8pCPTcGMP (1µM). Treatments are presented as fold-change from untreated control. Columns represent mean ± SEM of 4 replicates with 50 COCs/treatment/replicate, means within a graph without a common superscript differed (a,b,c $P<0.05$).

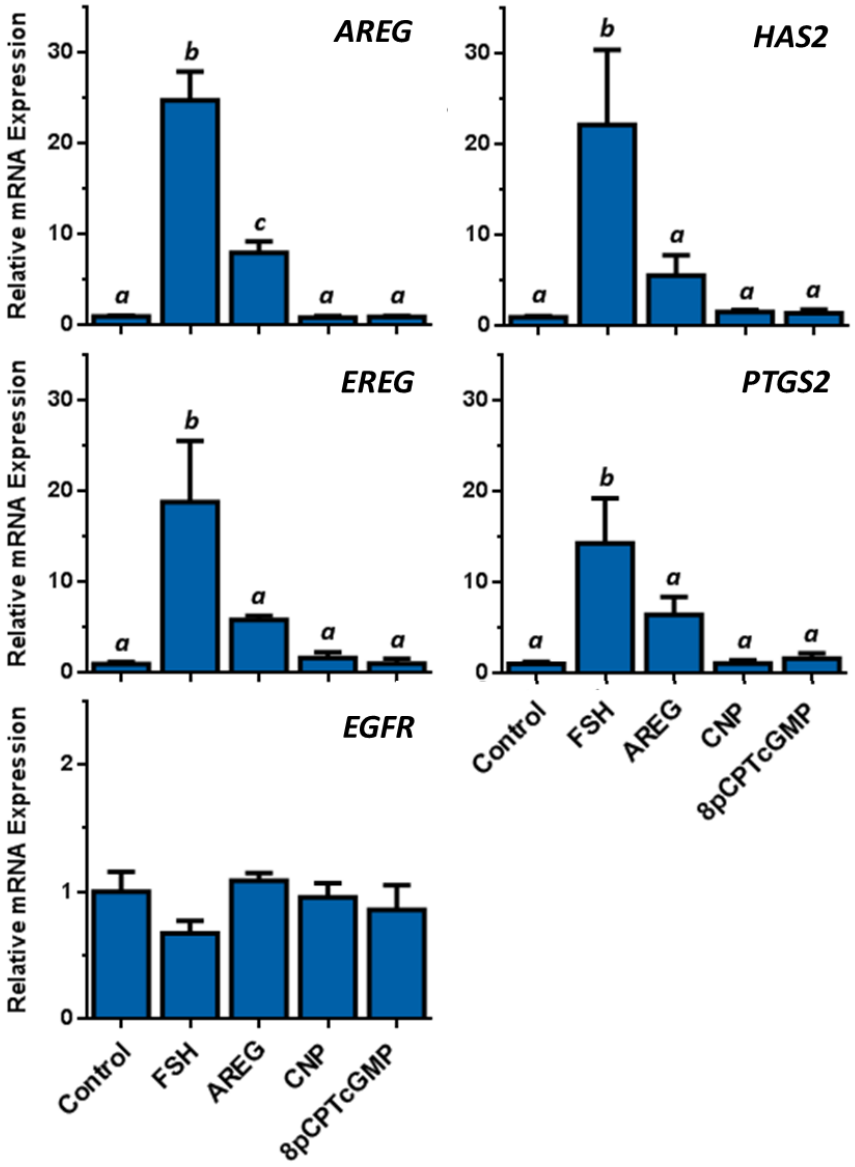

Figure 4.4 – The effect of CNP/cGMP on expression of EGF peptide and cumulus expansion genes. mRNA expression of *AREG*, *EREG*, *EGFR*, *HAS-2* and *PTGS2* in COCs after 2 h culture in control medium or AREG (100ng/mL), FSH (50mIU/mL), CNP (100nM), or 8pCPTcGMP (1µM). Expression was normalised to the stable housekeeper PPIA, and represented as fold change from untreated control. Columns represent mean ± SEM of 3 replicates, means within a graph without a common superscript differed (a,b,c $P<0.05$)

4.4.5 CNP and cGMP activate ERK1/2 via EGF signalling pathways

In porcine COCs, activation of ERK1/2 can occur by upstream mediators of EGFR via activation of Src-family kinases (Yamashita *et al.* 2007) and metalloproteinases (Yamashita and Shimada 2012), both of which can be activated by cGMP in other cell systems (Marathe *et al.* 2012). COCs cultured with FSH, CNP, or 8pCPTcGMP, significantly ($P<0.05$) increased the phosphorylation of ERK1/2 compared to control, each of which was completely antagonised by the addition of the metalloproteinase inhibitor TAPI-2 (Fig. 4.5A). AREG-induced phosphorylation of ERK1/2 was completely ablated by the EGFR inhibitor, AG1478 (Fig. 4.5B). Activation of ERK1/2 was not affected by the Src inhibitor, PP2 (Fig. 4.5C). Hence, CNP and cGMP phosphorylation of ERK1/2 appear to require metalloproteinase activity and EGFR functionality independent of Src-kinase activity.

4.4.6 CNP and cGMP do not increase TACE/ADAM17 expression or activity

Porcine COCs cultured *in vitro* with FSH is known to increase TACE/ADAM17 protein expression and activity (Yamashita *et al.* 2007), however in non-reproductive systems, a cGMP stimulus induces similar TACE/ADAM17 increases (Chanthaphavong *et al.* 2012). Treating COCs with FSH, AREG, CNP, or 8pCPTcGMP for 2 h had no effect on TACE/ADAM17 protein expression (Fig. 4.6A) or activity of the TACE/ADAM17 enzyme (Fig. 4.6B).

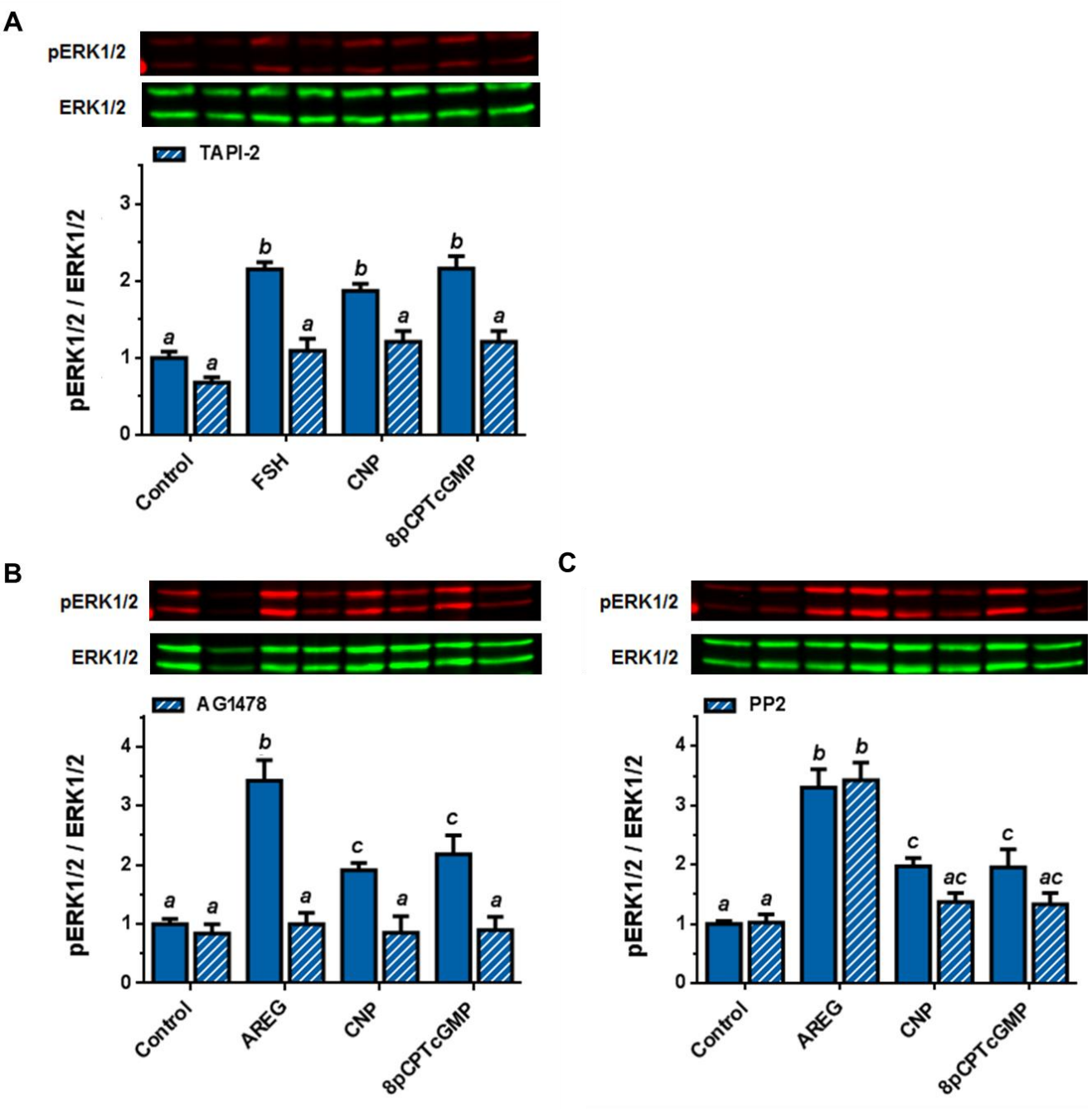

Figure 4.5 – Requirement of the EGF network for CNP/cGMP stimulation of ERK1/2. Western blot analysis of the phosphorylation of ERK1/2 relative to total ERK1/2 protein in porcine COCs after 2 h culture in control medium or FSH (50mIU/mL), AREG (100ng/mL), CNP (100nM), or 8pCPTcGMP (1µM), treated with inhibitors of metalloproteinases, TAPI-2 (20µM; A); EGFR, AG1478 (4µM; B); and of src-kinases, PP2 (10µM; C). Treatments are presented as fold-change from the untreated control. Columns represent mean ± SEM of 4 replicates with 50 COCs/treatment/replicate, means within a graph without a common superscript differed (a,b,c $P<0.05$).

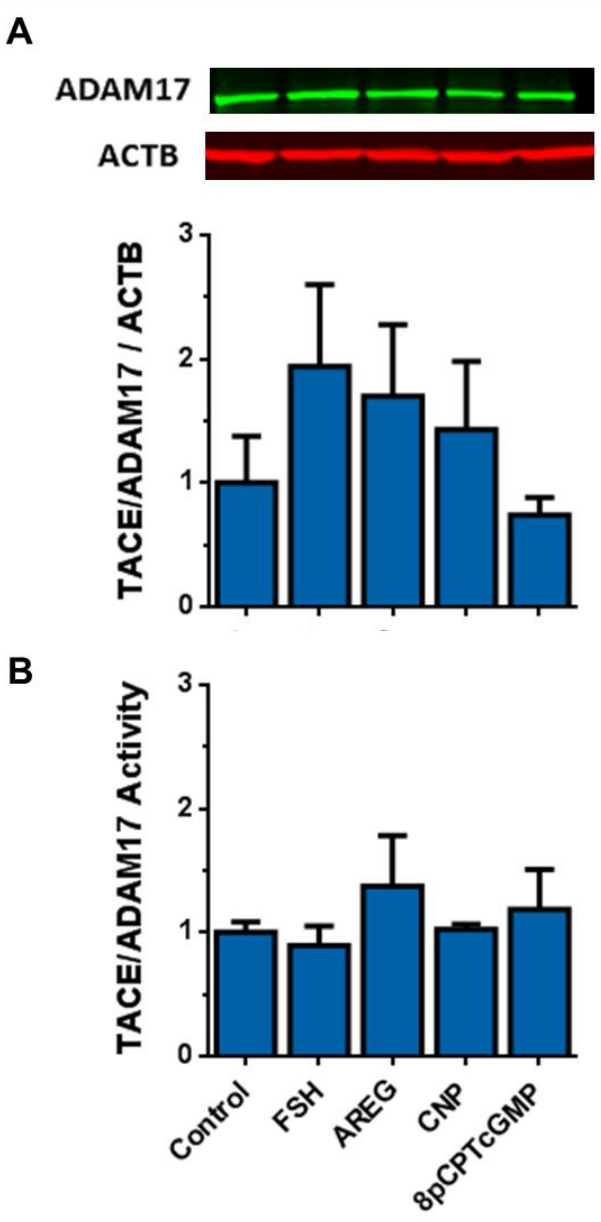

Figure 4.6 – Effect of CNP/cGMP on TACE/ADAM17 in porcine COCs. Western blot analysis of the TACE/ADAM17 protein relative to β-ACTIN protein (A), and TACE/ADAM17 activity (B), in COCs after 2 h culture in control medium or FSH (50mIU/mL), AREG (100ng/mL), CNP (100nM), or 8pCPTcGMP (1µM). Treatments are presented as fold-change from no-treatment control. Columns represent mean ± SEM of 4 replicates with 50 COCs/treatment/replicate.

4.4.7 CNP and cGMP promote gap junctional communication breakdown in COCs in vitro

It is well documented that the phosphorylation of ERK1/2 precedes gap junction closure and the breakdown of gap junctions is, at least in part, dependent on ERK1/2 (Dekel *et al.* 1981; Sela-Abramovich *et al.* 2005; Norris *et al.* 2008). As previously described (Sasseville *et al.* 2009b), porcine oocyte-cumulus cell GJC initially increased with culture time in all treatment groups (Fig. 4.7). AREG significantly decreased GJC compared to all other treatments after 6 h of culture ($P<0.05$). After 12 and 24 h of culture, FSH, AREG, CNP, and 8pCPTcGMP all significantly reduced the degree of GJC compared to control ($P<0.05$).

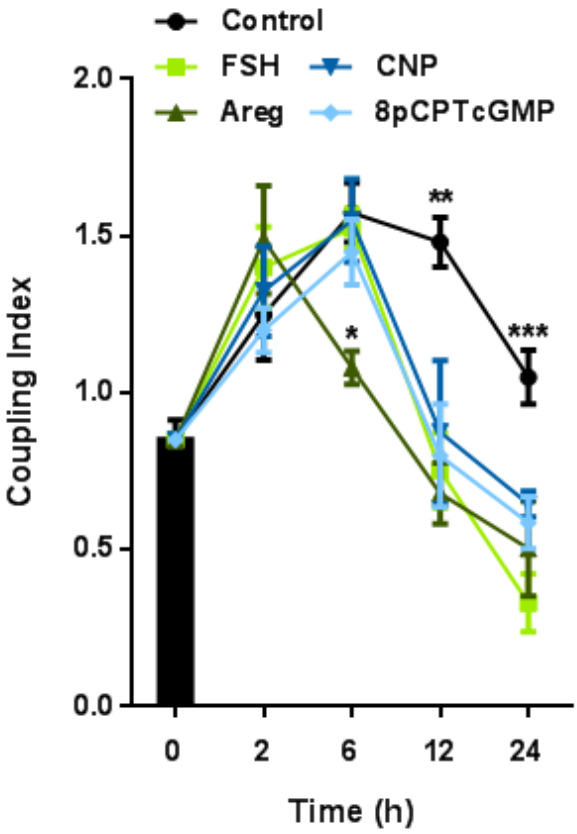

Figure 4.7 - Effect of FSH, AREG, CNP and 8pCPTcGMP on oocyte-cumulus cell gap junctional communication. After 0, 2, 6, 12, and 24 h of culture in control medium or FSH (50mIU/mL), AREG (100ng/mL), CNP (100nM), 8pCPTcGMP (1µM), diffusion of microinjected LY from the oocyte to cumulus cells was assessed. Treatments were scored as described in materials and methods. Columns represent mean ± SEM of 4 replicates with 10 COCs/treatment/time point/replicate. Asterisks indicate significant difference in the GJC index per time point (*,**,*** $P<0.05$).

4.5 Discussion

Pre-ovulatory follicles undergo dramatic changes in response to the surge of gonadotrophins, changes which are dependent on the activation of ERK1/2 in granulosa cells (Fan *et al.* 2009; Conti *et al.* 2012). Disruption of ERK1/2-dependent signalling in granulosa cells prevents COC expansion, oocyte meiotic resumption, and GC differentiation (Fan *et al.* 2009). The results from this study demonstrate upregulation of ERK1/2 activity via the CNP/cGMP pathway in porcine cumulus oocyte complexes and map a novel mechanism for CNP (Figure 4.8).

The guanylate cyclase NPR2 is responsible for the majority of cGMP synthesis in porcine follicles, and is mainly expressed in the cumulus cells (Figure 4.1), as is the case in other mammalian models (Zhang *et al.* 2010; Kawamura *et al.* 2011; Zhang *et al.* 2011). In rodents, natriuretic peptides facilitate ovarian growth and follicle development by increasing follicular cGMP concentrations (McGee *et al.* 1997a; Sato *et al.* 2012). In fact, during follicle growth expression of both *NPPC* and *NPR2* increase, with pre-ovulatory follicles expressing the highest levels of these genes (Sato *et al.* 2012), corresponding with a uniformly high level of cGMP in the pre-LH follicle (Shuhaibar *et al.* 2015). High oocyte concentrations of cGMP are responsible for the maintenance of meiotic arrest via inhibition of PDE3A thereby preventing degradation of cAMP (Norris *et al.* 2009; Zhang and Xia 2012). In the pre-ovulatory follicle, surging LH binds to its receptor in the granulosa cells; initiating a rapid decrease of cGMP in granulosa cells, followed by decreased intra-oocyte cGMP concentrations(Egbert *et al.* 2014). Decreased cGMP concentrations result from decreased production as a result of dephosphorylation of the guanylate cyclase NPR2 and decreased *NPPC* expression leading to reduced NPR2 activation by CNP (Sasseville *et al.* 2008; Vaccari *et al.* 2009; Egbert *et al.* 2014).

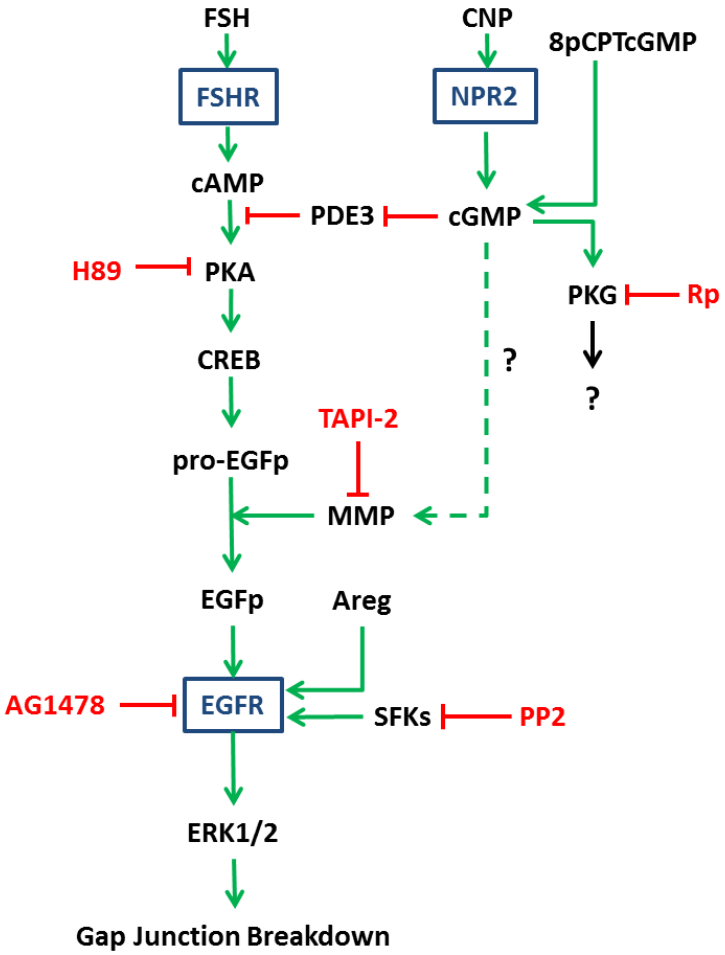

Figure 4.8 – Schematic hypothesis of CNP-, cGMP- and FSH-induced activation of ERK1/2 in porcine cumulus oocyte complexes in vitro leading to oocyte-cumulus gap junction closure. Green lines depict stimulatory processes, red lines represent inhibitory actions, and dashed line represents possible intermediates. In cumulus cells, FSH activation of the cAMP-PKA axis leads to phosphorylation of CREB, activation of p38MAPK (not shown), and expression of pro-EGF-like peptides (Reviewed (Gilchrist 2011)). Cleavage of the pro-EGF-peptide ectodomain by metalloproteinases initiates release of mature EGF-peptide, enabling EGFR binding leading to phosphorylation of ERK1/2. Addition of exogenous AREG or EREG also activated EGFR to induce this response. ERK1/2 phosphorylation induces mechanisms to breakdown gap junctions and induce oocyte geminal vesicle breakdown. This study suggests CNP/NPR2 production of cGMP leads to activation of ERK1/2, via an indirect mechanism not involving PKG, but requiring metalloproteinases and signalling via the EGF receptor. CNP/cGMP may induce extra-cellular shedding of mature EGF-like peptides by cumulus cells leading to activation of EGFR, induction of ERK1/2 phosphorylation, leading to gap junction breakdown. H89, inhibitor of PKA; Rp-8pCPTcGMP, inhibitor of PKG; TAPI-2, inhibitor of MMPs; PP2, inhibitor of SFKs; AG1478, inhibitor of EGFR.

Elevated pre-ovulatory concentrations of cGMP halts oocyte meiotic resumption and cumulus expansion, and this may occur via ERK1/2-dependent mechanisms, yet the actions of cGMP on ERK1/2 are unclear (Blaha *et al.* 2015). This report indicates CNP and the cGMP analogue 8-pCPTcGMP can significantly increase the phosphorylation of ERK1/2 after 2 h of COC culture, matching AREG- or FSH-induced ERK1/2 activation. However, LH-induced ERK1/2 activation is inhibited in rat follicles cultured in the presence of soluble guanylyl cyclase activators and nitric oxide donors (Sela-Abramovich *et al.* 2008). Conversely, inhibition of guanylyl cyclases by 1H-[1,2,4]oxadiazolo[4,3-a]quinoxaline-1-one induces activation of ERK1/2, similar to LH (Sela-Abramovich *et al.* 2008). In porcine COCs, FSH-induced ERK1/2 activation was partially reduced by ANP and 8-bromo-cGMP (Zhang *et al.* 2005b). However, other research suggests CNP-stimulated cGMP increases or cGMP analogues have no effect of FSH-induced ERK1/2 activation (Blaha *et al.* 2015). Considering the variations in this study and current literature, further research will be required to determine the role of cGMP in ERK1/2 activation. We hypothesis the opposing findings could be related to *in vitro* culture conditions, in particular media additives like follicular fluid or fetal calf serum used in other studies (Blaha *et al.* 2015), which up-regulate cAMP production *in vitro*, influencing a cGMP only response (see Chapter 6).

Activation of ERK1/2 by cAMP historically occurs via intermediates (Russell and Robker 2007; Yamashita *et al.* 2009; Conti *et al.* 2012; Prochazka and Blaha 2015) and research into others systems would suggest similarities to cGMP (Gudi *et al.* 2000; Pilz and Casteel 2003). Considering AREG can rapidly induce ERK1/2 activation by 15 min, and CNP/cGMP induced ERK1/2 activation only occurs after 2 h (Figure 4.2), this would suggest an indirect route of activation. LH promotes rapid activation of ERK1/2 in granulosa cells via cAMP/PKA-dependent signalling, requiring CREB and the EGF-like peptides (Salvador *et al.* 2002; Russell *et al.* 2003; Sela-Abramovich *et al.* 2005). In the cardiovascular system, increased cGMP activates cGMP-dependent protein kinase PKG, which in turn can activate PKA directly (Pilz and Casteel 2003), or induce the phosphorylation of CREB at ser-133 (Gudi *et al.* 2000). In the current study, inhibition of PKG by RP-8pCPTcGMP or PKA by H89 had no effect on AREG-, CNP-, or 8pCPTcGMP-induced ERK1/2 phosphorylation. Considering increasing cGMP via CNP or cGMP analogues was insufficient to induce CREB phosphorylation or the expression of *AREG* and *EREG* in COCs, and cAMP regulated cumulus expansion genes *HAS2* and *PTGS2*, it suggests cGMP-induced ERK1/2 activation is not dependent of cAMP.

LH-induced ERK1/2 activity is facilitated by proteolytic cleavage of EGFp ectodomains by matrix metalloproteases to enable mature ligand binding to EGFR. Inhibition of this cleavage event by MMP inhibitors, GM6001 and TAPI-1, blocked LH-induced resumption of meiosis and cumulus expansion (Ashkenazi *et al.* 2005; Panigone *et al.* 2008). Additionally, pharmacological inhibition of EGFR activity prevents LH-induced events (Park *et al.* 2004; Ashkenazi *et al.* 2005). Induced ERK1/2 phosphorylation by FSH+LH in porcine COCs is completely downregulated by the MMP inhibitor TAPI-2 (Yamashita *et al.* 2007). Likewise, we demonstrate CNP- or cGMP-induced ERK1/2 phosphorylation is prevented by the MMP inhibitor TAPI-2 and the EGFR inhibitor AG1478. Reports indicate the main sheddase for EGFp activation in porcine COCs is the MMP, TACE/ADAM17, and that it is responsible for EGFR/ERK1/2 activation (Yamashita and Shimada 2012). In hepatocyte cell cultures, increasing cGMP concentrations with 8-bromo-cGMP or NO-donors significantly increases the expression of activated TACE/ADAM17 (Chanthaphavong *et al.* 2012). Yet this study found in porcine COCs, CNP and cGMP did not induce TACE/ADAM17 protein expression or activity. Together these results suggest CNP/cGMP promote MMP activation, which in turn facilitates the proteolytic cleavage of EGFp ectodomains, allowing them to bind and activate EGFR to induce signals resulting in ERK1/2.

Phosphorylation of ERK1/2 precedes gap junction breakdown and modulation of GJC plays a pivotal role in oocyte maturation (Sela-Abramovich *et al.* 2005; Norris *et al.* 2008). Early after LH exposure, gap junction permeability is decreased (Norris *et al.* 2008; Conti *et al.* 2012) as a result of LH stimulated ERK1/2 phosphorylation of connexin-43 (Norris *et al.* 2008; Andric *et al.* 2010; Hsieh *et al.* 2011) coinciding with a decrease in granulosa cGMP. Consistent with previous studies, culturing porcine COCs *in vitro* promoted an initial increase in oocyte-cumulus gap junction permeability, known to be a result of mechanical rupture of the COC from its granulosa cell layer, which triggers up-regulation of Cx43 (Sasseville *et al.* 2009b). Decreased permeability of gap junctions was achieved at 6 h with AREG and but not until 12 h with FSH, CNP and cGMP. This corresponds to the rapid activation of ERK1/2 by AREG, but delayed activation by FSH, CNP, and cGMP. Similar reports indicate inhibitory roles of 8-pCPT-cGMP on GJC, however are not shared by CNP (Santiquet *et al.* 2014). Decreased granulosa cGMP concentration results in a cGMP gradient relative to the oocyte, which facilitates diffusion of oocyte cGMP outward to peripheral granulosa cells through a series of gap junctions down the concentration gradient (Shuhaibar *et al.* 2015). It is possible that in response to the LH surge, an initial increase in oocyte-cumulus GJC occurs, and decreased

granulosa cGMP synthesis promotes oocyte cGMP diffusion to cumulus cells to help sustain MMP activation, facilitating EGFR activation, whilst meiosis resumes.

These results also have potential importance to understanding ovarian follicle development. Concentrations of cGMP remain high in ovarian follicles until the LH surge. In fact, follicle development can be promoted by CNP and cGMP, and potentially substitute FSH in stimulating follicle growth (McGee *et al.* 1997a; Sato *et al.* 2012). Research shows ERK activity during follicle growth is critical to the survival of granulosa cells and oocyte development (Shiota *et al.* 2003). There is broad expression of MMPs throughout ovarian follicular development, and MMPs promote follicle growth and remodelling (Smith *et al.* 1999; Goldman and Shalev 2004; Kim *et al.* 2014). Therefore, during follicle development, CNP/cGMP pathways could promote follicle growth and remodelling by activating MMPs and promote granulosa cell proliferation and survival by activation of ERK1/2. The findings from the current study add further insights into natriuretic peptide regulation of the ovarian follicle and COC. We show that CNP/cGMP signalling has an important role beyond the inhibition of oocyte meiosis, by activating ERK1/2 which is fundamental to development and function of both the germ and somatic cell compartments of the ovarian follicle.

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CHAPTER 5:

CNP/cGMP HAS NO EFFECT ON ERK1/2 ACTIVATION IN PORCINE CUMULUS OOCYTE COMPLEXES FROM SMALL FOLLICLES

5.1 Abstract

Porcine oocytes collected from small antral follicles are known to be less competent than oocytes derived from larger follicles and produce lower fertilisation and blastocyst development rates. Whether the effects of CNP/cGMP on ERK1/2 activation are diminished in porcine oocytes from small follicles is unknown. Abattoir-derived gilt porcine ovaries were collected, small antral follicles (2-4mm in diameter) were aspirated, and oocytes collected and cultured in medium. RT-PCR analysis demonstrated significantly more natriuretic peptide receptor 2 (NPR2) expression in cumulus cells compared to granulosa cells. Amphiregulin (AREG) induced significant increases in the phosphorylation of ERK1/2 in the COC by 2 h of in vitro culture compared to controls, however C-type natriuretic peptide (CNP) and 8-(4-Chlorophenylthio)-guanosine 3′,5′-cyclic monophosphate (8pCPTcGMP) did not alter ERK1/2 phosphorylation compared to control. Expression of AREG, epiregulin (EREG), cumulus expansion genes (HAS-2 and PTGS2), and epidermal growth factor receptor (EGFR) mRNA in COCs were not affected by CNP/cGMP treatment. *In vitro* culture of COCs derived from small follicles, with AREG, CNP, and 8pCPTcGMP did not affect gap junction communication (GJC). Therefore, CNP and cGMP have no effect on the activity of ERK1/2 in porcine COCs derived from small follicles. These findings illustrate a major difference in intra-cellular signalling between cells in porcine COCs from small antral follicles, compared to COCs collected from larger follicles.

5.2 Introduction

The ovarian follicle has two important functions; to sustain oocyte development in preparation for fertilisation and the production of hormones. Fundamentally, oocytes need to be developmentally competent for successful fertilisation and embryo development. This is achieved in a plastic follicular environment with oocyte developmental competence improving with increasing follicle size (Marchal *et al.* 2002; Lequarre *et al.* 2005; Bagg *et al.* 2007). Follicular development is tightly orchestrated with the granulosa, cumulus, and oocyte driving maturation. Key ovarian signals between the granulosa and cumulus cells, and the oocyte, regulate the reinitiating and completion of meiosis and thus the final stage of oocyte development. These cells respond to the gonadotrophins, follicle stimulating hormone (FSH), and luteinising hormone (LH), to propagate a range of local growth factors and steroids, including natriuretic peptides, cyclic nucleotides, and epidermal growth factor (EGF)-like peptides (discussed in Chapter 1 and Reviewed (Conti *et al.* 2012)).

In pre-ovulatory follicles, expression of the EGF-like peptides amphiregulin (AREG), epiregulin (EREG), and beta-cellulin (BTC) are induced, in-turn activating downstream effectors of EGFR signalling, particularly extracellular signal-regulated kinases 1 and 2 (ERK1/2) and phosphatidylinositol-4,5-bisphosphate 3-kinase/protein kinase B (PI3K/AKT) (Park *et al.* 2004; Chen *et al.* 2013) which then stimulates the expression cumulus expansion genes (Shimada *et al.* 2006a; Yamashita *et al.* 2007; Prochazka *et al.* 2012). Subsequent cumulus expansion is associated with the breakdown in gap junction communication between oocytes and somatic cells (Norris *et al.* 2010).

Follicle size is positively correlated to oocyte meiotic competence and early embryo development in a number of species including pigs (Marchal *et al.* 2002; Lucas *et al.* 2003), sheep (Moor and Trounson 1977; Ledda *et al.* 1999), and cows (Torner *et al.* 2001; Lequarre *et al.* 2005). In most species, oocytes collected from small follicles (<3mm in size) result in low MII rates following *in vitro* culture, reflecting their immature nature, and meiotic incompetency (Abeydeera 2002). In pigs, oocytes collected from small follicles not only have lower MII rates, but fertilisation and blastocyst development rates are also significantly impaired compared to oocytes collected from larger (>3mm in size) follicles (Bolamba and Sirard 2000; Sun *et al.* 2001; Marchal *et al.* 2002; Lucas *et al.* 2003). These studies combined with other research suggest that crucial changes occur as the follicle grows in cumulus cell and oocyte derived factors, therefore, the mechanisms controlling oocyte development and maturity would be altered between oocytes from small versus large ovarian follicles.

The goal of the present investigation was to determine if CNP and cGMP addition to *in vitro* culture of porcine oocytes from small antral follicles effects CC signalling. Specifically can; 1) CNP/cGMP activate EGFR-ERK1/2 signalling cascades in small follicles? 2) CNP/cGMP induce EGF signalling and cumulus expansion gene expression? 3) CNP/cGMP alter gap junction communication between cumulus cells and the oocyte?

5.3 Materials and Methods

Unless otherwise specified, all chemicals used in the following protocols were purchased from Sigma (St. Louis, MO, USA). All experiments conducted in Chapter 4 were conducted in parallel with those in Chapter 5 for later comparative analysis.

5.3.1 Collection, Preparation and Culture of Oocytes

Porcine gilt ovaries were obtained from an abattoir and transported in warm (29 – 32°C) saline (0.9% wt/vol NaCl; Baxter Healthcare) supplemented with antibiotics (100 U/mL penicillin G and 0.1 mg/mL streptomycin sulphate) within 3-4 h of slaughter. Small antral follicles, 2-4mm in size, were aspirated using a 20-gauge needle and constant suction (1 L/min) into vacutainer tubes. Cellular sediment was transferred to 100 mm Petri dishes and cumulus-oocyte complexes (COCs) with ooplasms of uniform appearance and surrounded by >3 cumulus cell layers were collected and washed once in handling medium (Hepes buffered tissue culture medium (H-TCM-199; GIBCO® Life Technologies, Mulgrave, Australia) + 3 mg/ml low fatty acid BSA), and once in culture medium (bicarbonate-buffered TCM-199 + 3 mg/ml low fatty acid BSA). Groups of up to 10 COCs were transferred into pre-equilibrated 100 µl drops of maturation medium overlaid with mineral oil and incubated at 38.5°C with 6% CO_2 humidified air.

5.3.2 Western Blot Immunodetection

COCs were collected as described above and suspended in RIPA buffer (10 mM Tris, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100) containing phosphatase (Roche, Penzberg, Germany) and protease inhibitor cocktails, snap frozen in liquid nitrogen and stored at −80°C. Samples were mixed with loading buffer containing 100 mM dithiothreitol, heated at 100°C for 5 min, and loaded onto a 7.5% (separating) and 4% (stacking) SDS–polyacrylamide gel for electrophoresis. Proteins were transferred to Hybond-ECL membranes (GE Healthcare, Waukesha, USA), and the membrane was cut horizontally below the 75kDa marker and the upper section was discarded. The remaining membrane was blocked with 2% blocking reagent

(supplied in an ECL Advance kit; GE Healthcare) diluted in Tris-buffered saline containing 0.1% (v/v) Tween 20. The membrane was incubated with pairs of antibodies simultaneously (pERK and ERK) at 4°C for 22-26 h, followed by incubation with goat anti-mouse IgG IRDye™ 680 (Licor Bioscience, Lincoln, NE) diluted 1:50000 and goat anti-rabbit IgG IRDye™ 800 (Licor Bioscience) for 1h at room temperature. Membranes were washed with Tris-buffered saline with Tween 20 (TBST), then scanned and quantified using an Odyssey infrared imaging system (Licor Bioscience). Band intensities for pERK1/2 were expressed relative to ERK1/2 and standardised relative to no treatment controls.

5.3.3 Gap Junctional Communication Assay

The degree of oocyte-cumulus cell gap junction communication in COCs was assessed using LY (Sigma) dye microinjected into the ooplasm, as previously described (Luciano *et al.* 2004). After 0, 2, 6, 12 and 24 h of culture, 3% LY in 5 mM lithium chloride (Sigma) was microinjected into the oocyte in 5 µl of wash medium (IVF Vet Solutions, Adelaide, Australia) overlaid with mineral oil. The spread of dye into the surrounding cumulus cells was assessed with a Olympus Fluoview FV10i laser scanning confocal microscope (Olympus, Tokyo, Japan) within 15 min of injection. As a negative control, COCs were cultured in oocyte maturation medium for 24 h supplemented with the gap-junction inhibitor, carbenoxolone (CBX, 100 µM; Sigma) and assessed at 24 h. Oocyte-cumulus cell GJC was scored as previously described (Luciano *et al.* 2004); +2 when the dye was completely transferred to the entire cumulus mass, +1 when the dye was transferred to limited number of cumulus cell layers just beyond the corona radiata, and 0 when the dye was transferred to only the corona radiata cells or was not transferred to any cumulus cells at all.

5.3.4 RNA Extraction and Real Time RT-PCR Analysis

COCs cultured for 0, 2 or 6 h (see figure legends) were collected and frozen as cell pellets and snap frozen in liquid nitrogen and stored at −80°C. Groups of 30 COCs were lysed using TRI Reagent® then chloroform was added (5:1) and the homogenate was allowed to separate. The clear upper aqueous layer (containing RNA) was transferred to a new tube containing 70% ethanol (1:1), then total RNA was extracted using the RNeasyMicro Kit (QIAGEN, Valencia, CA) according to the manufacturer's instructions. Residual genomic DNA was removed by digesting with recombinant RNase-free DNase I (QIAGEN). RNA was quantified using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Scoresby, VIC, Australia). RNA was reverse transcribed with random primers (Invitrogen; Life Technologies)

using Superscript III reverse transcriptase (Invitrogen). Quantitative real-time PCR analysis was performed on a Corbett Rotor-Gene 6000 (QIAGEN) in a 10 µl reaction volume containing; primers at 5 pmol/reaction, 1.5 µl cDNA and SYBR Green (Applied Biosystems, Mulgrave, Australia). All PCR reactions were carried out in duplicate. Universal thermal cycling parameters (initial step of 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 60 s at 60°C) were used to quantify the expression of all genes. A five point serial dilution standard curve was produced for each transcript with cDNA derived from COCs and granulosa cells. Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method and results are presented relative to untreated control and normalised to the housekeeping gene peptidylprolyl isomerase A (*PPIA).* To validate primers, PCR products generated from reactions with porcine COCs and granulosa cell cDNA we run on a 3% agarose gel and considered valid when a single product of the correct size was observed and primer efficiency was ~95%.

5.3.5 Statistical Analyses

In all experiments, differences between treatments were analysed by one-way ANOVA followed by Tukey post hoc comparison, or one-way ANOVA followed by LSD post hoc comparison.

5.4 Results

5.4.1 Expression of *NPR2* in porcine follicular cells from small follicles

To illustrate the expression of natriuretic peptide receptor 2 in the pig COC from small antral follicles, RNA extraction and real time RT-PCR was performed. *NPR2* was expressed at significantly higher levels in cumulus cells compared to granulosa cells (7.9 ± 0.9 vs. 3.4 ± 0.4 –fold increase respectively) (Figure 5.1).

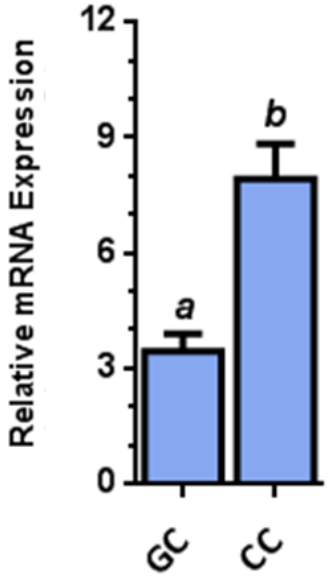

Figure 5.1 - Expression of NPR2 in porcine follicular cells from small follicles. Untreated (0h) cumulus and granulosa cells were collected from follicles and mRNA expression of NPR2 was measured using quantitative real-time PCR and normalised to the stable housekeeper PPIA and represented as fold change from internal control. Columns represent mean ± SEM, means without a common superscript differed (^{ab,} $P<0.05$).

5.4.2 Effect of AREG, CNP, and cGMP on the phosphorylation of ERK1/2

To establish whether CNP and cGMP could induce the activation of ERK1/2 in COC from small follicles, we assessed the phosphorylation of ERK1/2 in response to AREG, CNP, and cGMP. The phosphorylation of ERK1/2 was substantially increased within 15 min in response to AREG (Figure 5.2A), and was maintained at 2h (Figure 5.2B). Neither CNP nor 8pCPTcGMP showed any effect of ERK1/2 at 15 min or any substantial effect at 2 h compared to untreated control (Figure 5.2).

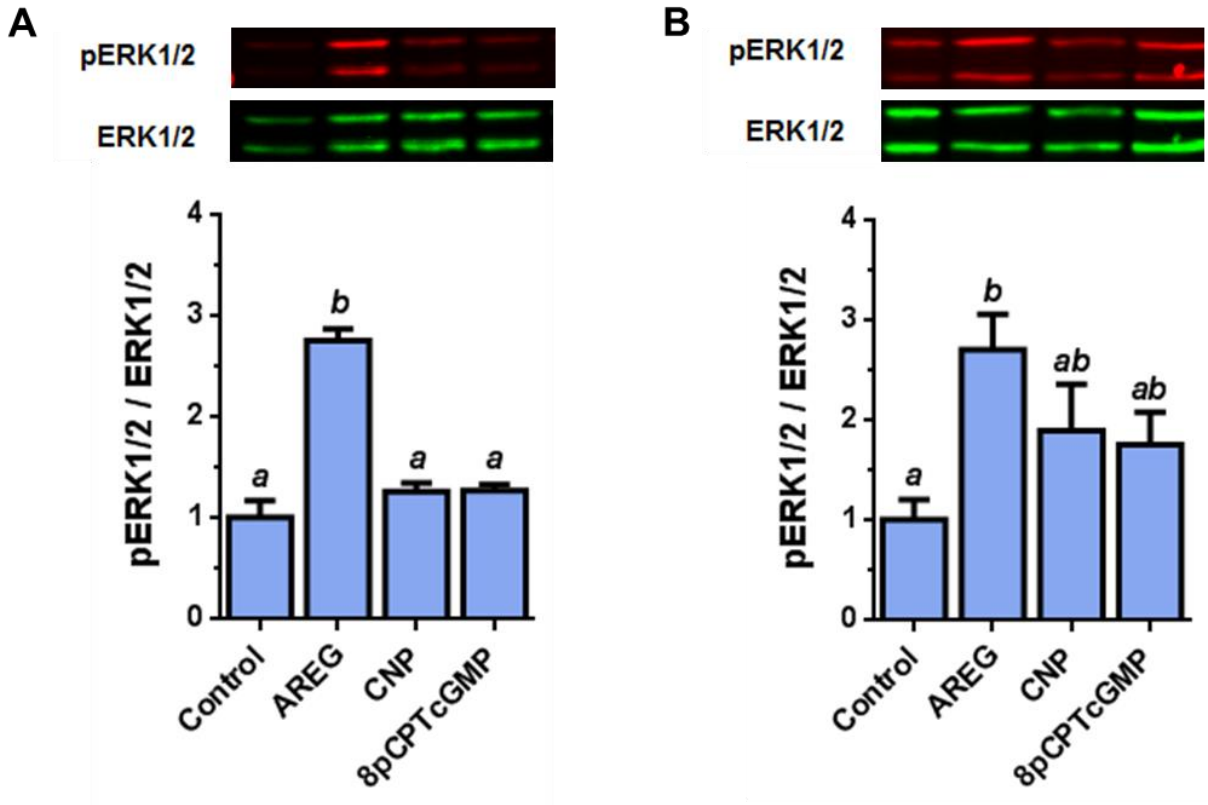

Figure 5.2 - Effect of AREG, CNP and cGMP on the phosphorylation of ERK1/2 in COCs. Western blot analysis of the phosphorylation of ERK1/2 relative to total ERK1/2 protein in porcine COCs after 15 mins (A) and 2 h (B) culture in control medium or treated with AREG (100 ng/mL), CNP (100 nM), or 8pCPTcGMP (1 µM). Columns are presented as fold-change from untreated control. Columns represent mean ± SEM of 4 replicates of 50 COCs/treatment/replicate, means within a graph without a common superscript differed (^{ab,} $P<0.05$).

5.4.3 Effect of AREG, CNP, and cGMP on the phosphorylation of ERK1/2 in the presence of AG1478

To explore the functionality of EGFR in COCs from small follicles, we determined the activity of ERK1/2 with the addition of a specific EGFR inhibitor AG1478. The phosphorylation of ERK1/2 was significantly increased in response to AREG compared to all other treatments ($P<0.05$), which was completely antagonised by AG1478 (Figure 5.3). Neither CNP nor 8pCPTcGMP showed any effect of ERK1/2 compared to untreated control. However, CNP significantly increased ERK1/2 phosphorylation compared to 8pCPTcGMP with AG1478 ($P<0.05$, Figure 5.3).

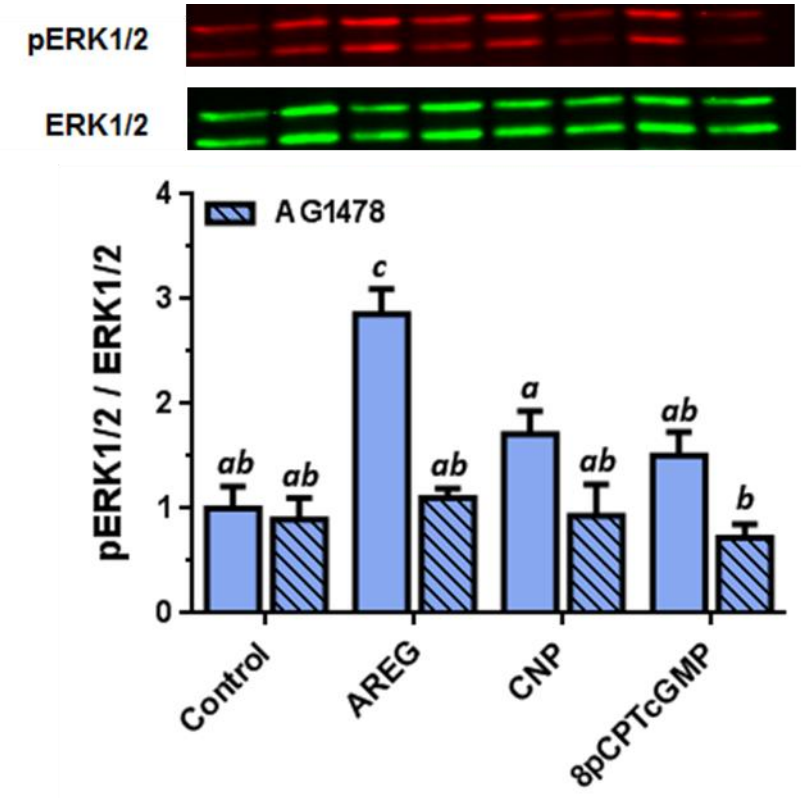

Figure 5.3 - Western blot analysis of the phosphorylation of ERK1/2 relative to total ERK1/2 protein in porcine COCs after 2 h culture in control medium or AREG (100 ng/mL), CNP (100 nM), or 8pCPTcGMP (1 µM) with an inhibitor of EGFR, AG1478 (4 µM). Treatments are presented as fold-change from untreated control. Columns represent mean ± SEM of 4 replicates with 50 COCs/treatment/replicate, means within a graph without a common superscript differed (^{abc,} $P<0.05$).

5.4.4 Effect of FSH, AREG, CNP, and cGMP to induce EGF-peptide and cumulus expansion gene expression

The relative mRNA expression of *AREG*, *EREG*, *EGFR*, *HAS2,* and *PTGS2* was measured after culturing COCs in FSH, AREG, CNP, and 8pCPTcGMP for 2 and 6 h. Porcine COCs cultured with FSH for 2 h (Figure 5.4), and 6 h (Figure 5.5) resulted in significantly higher *AREG*, *EREG*, *HAS2,* and *PTGS2* mRNA expression compared to all other treatments. At 2 h, AREG, CNP, and 8pCPTcGMP did not effect mRNA expression of any gene investigated (Figure 4). However at 6 h, AREG significantly increased *EREG* and *PTGS2* expression compared to control ($P<0.05$, Figure 5.5). Expression of *EGFR* mRNA did not differ significantly between any treatments at 2 h (Figure 5.4), yet at 6 h AREG significantly increased *EGFR* expression compared to FSH ($P<0.05$, Figure 5.5).

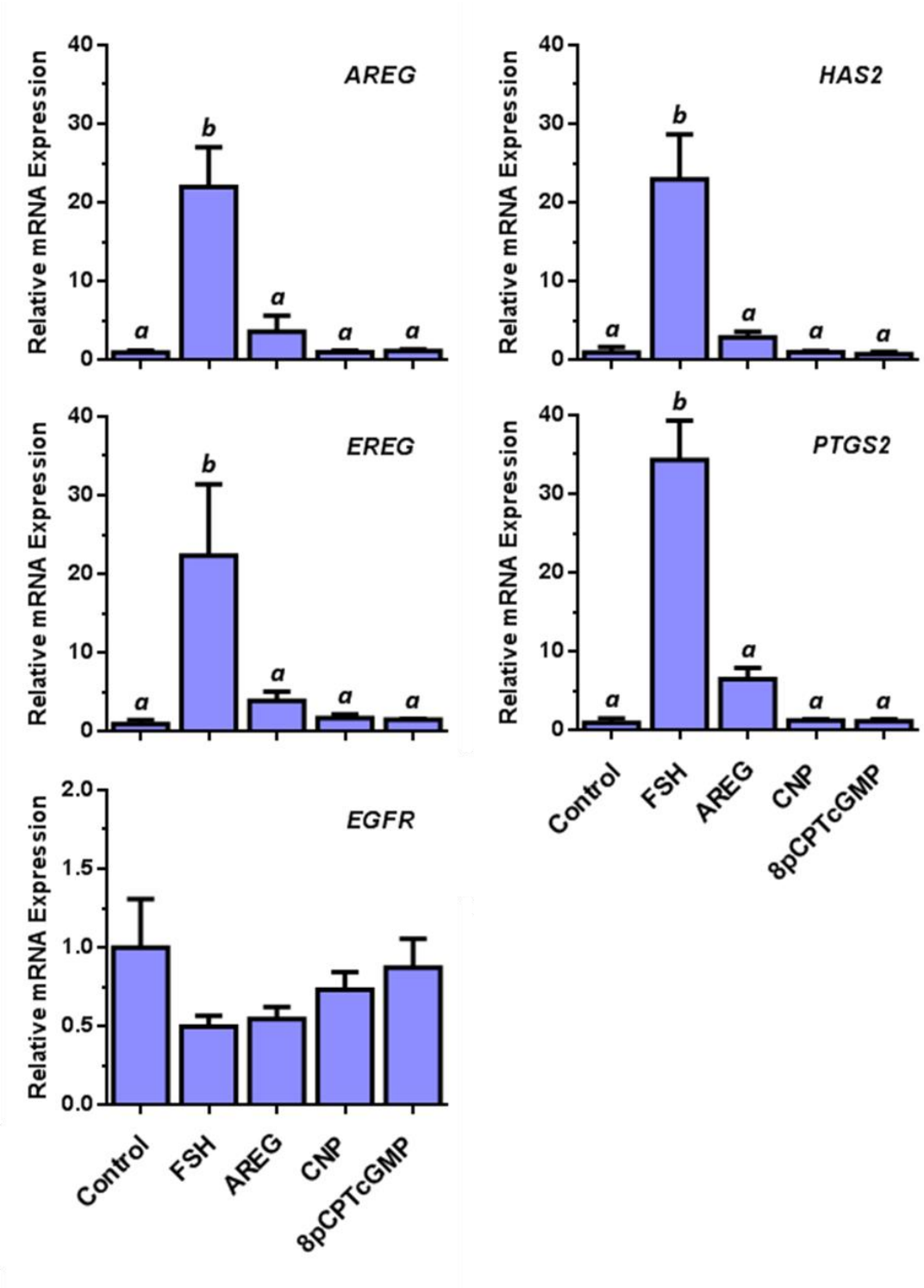

Figure 5.4 - The effect of FSH, AREG, CNP and cGMP on EGFp and cumulus expansion genes. mRNA expression of *AREG*, *EREG*, *EGFR*, *HAS-2,* and *PTGS2* in porcine COCs after 2 h culture in control medium or AREG (100 ng/mL), FSH (50 mIU/mL), CNP (100 nM), or 8pCPTcGMP (1 µM). Expression was normalised to stable housekeeper *PPIA*, and represented as fold change from untreated control. Columns represent mean ± SEM of 3 replicates, means within a graph without a common superscript differed (^{ab,} $P<0.05$).

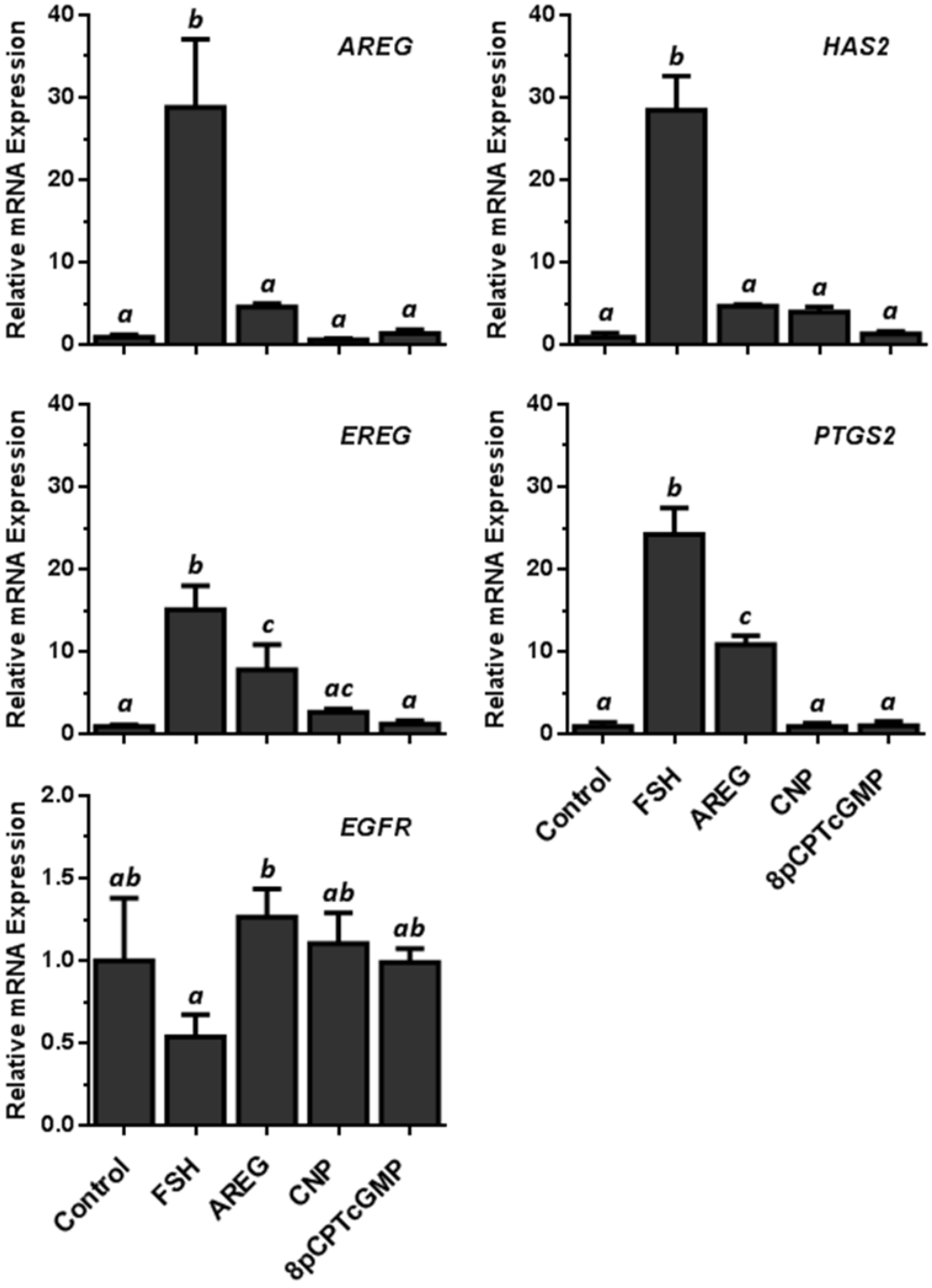

Figure 5.5 - The effect of FSH, AREG, CNP, and cGMP on EGFp and cumulus expansion genes. mRNA expression of *AREG*, *EREG*, *EGFR*, *HAS-2,* and *PTGS2* in porcine COCs after 6 h culture in control medium or AREG (100 ng/mL), FSH (50 mIU/mL), CNP (100 nM), or 8pCPTcGMP (1 µM). Expression was normalised to stable housekeeper *PPIA*, and represented as fold change from untreated control. Columns represent mean ± SEM of 3 replicates, means within a graph without a common superscript differed (^{abc,} P<0.05).

5.4.5 Effect of FSH, AREG, CNP, and cGMP on gap junctional communication in porcine COC *in vitro*

Culturing porcine COCs *in vitro* increased the coupling index between CCs and the oocyte at 0 h to 6 h ($P<0.05$), after which it declined. No differences between treatment groups were identified at 2, 6 and 12 h. At 24 h, FSH promoted the loss of gap junction communication compared to all other treatments ($P<0.05$, Figure 5.6).

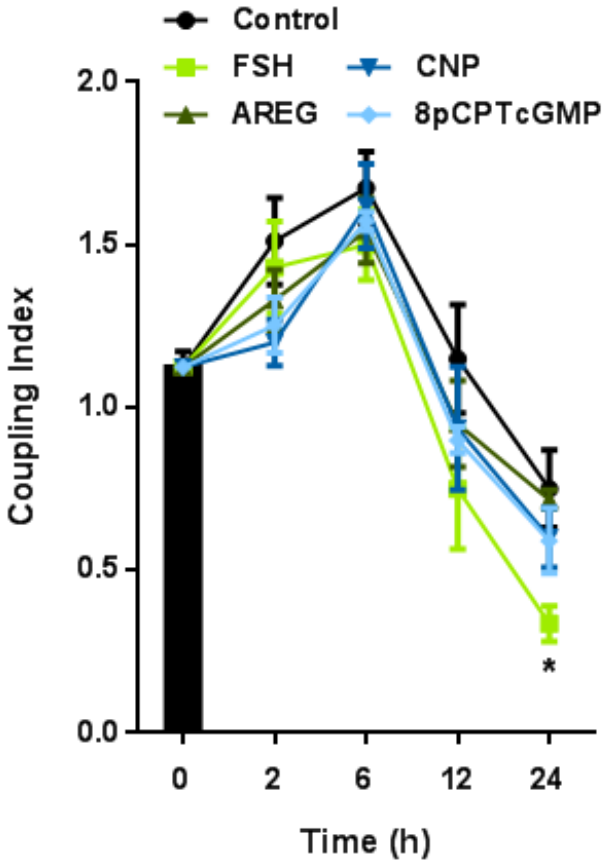

Figure 5.6 - Effect of FSH, AREG, CNP, and 8pCPTcGMP on gap junction communication. After 2, 6, 12, and 24 h of culture in control medium or FSH (50 mIU/mL), AREG (10 0ng/mL), CNP (100 nM), or 8pCPTcGMP (1 µM), LY diffusion of COCs was assessed with 0 h measured and presented as black bar. Treatments are scored as described in materials and methods. Columns represent mean ± SEM of 4 replicates with 10 COCs/treatment/time point/replicate. Asterisks indicate significant difference in the GJC index per time point (*, $P<0.05$).

5.5 Discussion

In the present study, we collected porcine COCs and granulosa cells from small antral follicles and determined the expression *NPR2* in these ovarian cells. We investigated the effects of CNP and cGMP on the phosphorylation of ERK1/2 via the EGF receptor and their effect on the expression of EGF signalling and cumulus expansion genes. Finally, we established whether CNP or cGMP could alter the gap junction communication between the cumulus cells and the oocyte. We found that *NPR2* was expressed in porcine cumulus and granulosa cells from small follicles, and that mRNA expression of *NPR2* was significantly greater in cumulus cells compared to granulosa cells. When COCs were treated with either CNP or 8pCPTcGMP during *in vitro* maturation of COCs from small follicles, there was no effect on the activation of ERK1/2. After IVM, CNP and cGMP had no effect on EGF signalling genes *AREG, EREG,* or *EGFR,* nor cumulus expansion genes *HAS-2* and *PTGS2.* CNP, cGMP, and AREG do not alter gap junction communication in porcine COCs from small follicles. Hence, despite our demonstration of the presence of the cognate receptor *NPR2* in granulosa and cumulus cells from small follicles, curiously these COCs failed to respond to CNP treatment as assessed by the major experimental read-outs we utilised in this study. This illustrates a major difference in regulatory mechanisms in COCs from different developmental stages, i.e. small versus large follicles (Chapter 4).

ERK1/2 activity in cumulus cells is required for gonadotropin-induced oocyte meiotic resumption and cumulus expansion (Su *et al.* 2002; Su *et al.* 2003). We show that AREG induced the phosphorylation of ERK1/2 in COCs from small follicles and was completely reversed by EGFR inhibition, consistent with observations of COCs from larger follicles (Prochazka *et al.* 2011). We also show CNP and cGMP lack the ability to regulate ERK1/2 activity in small follicles, however, is consistent with the hypothesis that small follicles lack some critical molecular machinery, because inappropriate activation of ERK1/2 in GCs of small growing follicles might disrupt normal follicular development and ovulation (Fan *et al.* 2009), and force major disruptions to female fertility and ovarian reserve (Fan *et al.* 2008).

The capability for COCs to undergo cumulus expansion is also achieved during advancing follicle growth. Naturally, cumulus expansion of porcine follicles requires the expression of cumulus expansion genes *HAS2, TNFA1P6,* and *PTGS2* (Fulop *et al.* 1997; Davis *et al.* 1999; Fulop *et al.* 2003), and each of these are naturally induced by EGFp. Nonetheless, we show that FSH is highly effective at mimicking this response even in COCs retrieved from smaller follicles, which is consistent with previous reports (pig (Ritter *et al.* 2015), bovine (Armstrong

et al. 1996)). Expression of these genes can be suppressed by specific inhibition of the EGF receptor and ERK1/2 (Yamashita *et al.* 2009; Gilchrist and Ritter 2011; Prochazka *et al.* 2012), indicating that *HAS2*, *TNFA1P6,* and *PTGS2* are regulated by a EGFR-ERK1/2 signalling mechanism in small follicles (Sugimura *et al.* 2015). Our results show that AREG can induce the phosphorylation of ERK1/2, however, it lacks the ability to adequately stimulate mRNA expression of *AREG, EREG, HAS2, and PTGS2* by 2 h, although there is moderate expression by 6 h of both *EREG* and *PTGS2* (Figure 4). It could be hypothesised that because EGFp expression is at lower levels in unstimulated COCs (Park *et al.* 2004; Ashkenazi *et al.* 2005), the increased mRNA transcripts would likely be delayed until EGF genes are up-regulated. Neither CNP nor 8pCPTcGMP affected the expression *AREG, EREG,* or *EGFR*, suggesting an intra-COC environment deficient in either functional NPR2-cGMP-signalling and/or EGF-signalling.

Gap junction communication between oocytes and cumulus cell do not drastically change in COCs from small follicles over the first 11 h of IVM (Bagg *et al.* 2009; Sasseville *et al.* 2009b). The initial increase in gap junction communication presented in this study is a common phenomenon in porcine COCs culture *in vitro,* and is a gonadotrophin independent response (Isobe *et al.* 1998; Sasseville *et al.* 2009b; Santiquet *et al.* 2012), with a decline in communication usually attributed to a gonadotrophin-dependant response (Sasseville *et al.* 2009b; Santiquet *et al.* 2012). Because gonadotropins and EGF are highly effective at promoting the breakdown of gap junction communication in COCs (Santiquet *et al.* 2012), it has been speculated that the EGFR-ERK1/2 pathway is involved in this process in pigs (Prochazka and Blaha 2015). However, in this study we induced a marginal, but statistically significant activation of ERK1/2 via the EGFR receptor in porcine COCs with exogenous AREG *in vitro,* and yet its presence has no effect on gap junction communication compared to controls. This is in contrast to Chapter 4 where AREG induced a significant breakdown in GJC in porcine COCs from large follicles, further supporting the hypothesis that COCs from small follicles respond poorly to AREG (Ritter *et al.* 2015; Sugimura *et al.* 2015).

These findings illustrate a major difference in intra-cellular signalling between cells in porcine COCs from small follicles, compared to COCs collected from larger follicles (Chapter 4). In the current study, CNP and cGMP had no effect on the activity of ERK1/2, mRNA expression or GJC in porcine COCs derived from small follicles. This could suggest porcine COCs from small follicles may lack a functional CNP-NPR2-cGMP axis and therefore, GVBD would need to be inhibited by some other means. On the contrary, current literature implicates CNP-cGMP

as the primary meiotic inhibiting mechanism in porcine oocytes (Hiradate *et al.* 2014; Zhang *et al.* 2014a; Blaha *et al.* 2015), and recent evidence demonstrates COCs from small antral follicles in unstimulated mouse ovaries were held at meiotic arrest in the presence of CNP which improved their inherent developmental competence (Romero *et al.* 2016). Therefore, assuming that CNP-NPR2-CGMP is functional in small COCs, then EGFR-ERK1/2 may lack functionality in these COCs. Considering we propose CNP-cGMP activation of ERK1/2 is via the activation of a matrix metalloproteinases (MMP), which enable EGF-like peptides to bind to a functional EGFR, the lack of CNP-cGMP induced ERK1/2 activation could be the result of absent MMP or EGFR activity in underdeveloped COCs. Porcine COCs collected from small follicles exhibited little cumulus expansion in response to exogenous EGF-like peptide *in vitro*, and exhibit less EGFR protein compared to COCs from large follicles (Ritter *et al.* 2015). This would suggest a lack of EGFR functionality and/or activity in this system. Meanwhile, MMP expression is known to fluctuate during porcine follicle development with MMP-2 and MMP-9 expression markedly increased with increasing follicle size (Curry and Osteen 2001; Kim *et al.* 2014). Considering this evidence, because CNP and cGMP has no effect on the activity of ERK1/2 in porcine COCs from small follicles, it can hypothesized that either MMPs are insufficiently activated, or EGFR lacks the ability to respond to CNP-cGMP signals.

Further work is needed to determine how the follicle and its COC develop to induce these signalling changes. Examining these changes can pinpoint the functional machinery in COCs that is altered during follicle growth which would have important implications for improving IVM methods to better support development of oocytes from smaller, less competent follicles.

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CHAPTER 6:

CNP STIMULATES THE RESUMPTION OF MEIOSIS IN PORCINE OOCYTE IN VITRO MATURATION

6.1 Abstract

Overwhelming evidence implicates increased intra-oocyte concentrations of cGMP with inhibition of meiosis. However, research conducted in our laboratory and others suggests a possible stimulatory function of the cGMP/PKG pathway. Abattoir-derived gilt porcine ovaries were collected, antral follicles were aspirated, and oocytes collected and cultured in medium. Treatment with FSH increased cumulus expansion compared to control and to all other treatments, whilst COCs exposed to AREG and EREG significantly increased the percentage of oocytes resuming meiosis compared to control and FSH. C-type natriuretic peptide (CNP) treatment had no effect on cumulus expansion but significantly increased the resumption of meiosis in porcine oocytes (main effect). CNP only inhibited meiotic resumption of COCs cultured *in vitro* when in the presence of FSH and porcine follicular fluid (pFF).This data shows that CNP alone cannot inhibit meiotic resumption in porcine COCs and that IVM media additives, FSH and pFF, may alter the response of COCs to CNP.

6.2 Introduction

The mammalian oocytes begin meiosis during fetal life and oocytes arrest at the diplotene stage of the first meiotic prophase for a prolonged period of time. Follicular development is regulated by FSH and LH secreted by the pituitary and the pre-ovulatory surge of LH induces the resumption of meiosis. Alternatively, removing the immature oocyte and culturing them *in-vitro*, causes the oocyte to undergo the spontaneous resumption of meiosis, demonstrating the follicular environment has meiotic inhibitory factors (Pincus and Enzmann 1935; Edwards 1965; Tsafriri *et al.* 1982; Tsafriri and Pomerantz 1986). Recent research suggests this follicular oocyte meiotic inhibitor could be C-type natriuretic peptide (CNP) (Zhang *et al.* 2010; Zhang and Xia 2012). Natriuretic peptides are a family of genetically distinct but structurally related peptides; atrial (ANP), brain (BNP), CNP, dendroaspis (DNP) natriuretic peptides, and urodilatin (Brenner *et al.* 1990; Sudoh *et al.* 1990; Potter *et al.* 2006). CNP is encoded by natriuretic peptide precursor C gene *(NPPC)*, which has been discovered in the granulosa/cumulus cell compartments of the ovarian follicle (Zhang *et al.* 2010). Binding of CNP to its cognate receptor guanylate cyclase-B (known as natriuretic peptide receptor B or 2, NPR2), stimulates the production of the second messenger, cyclic guanosine monophosphate (cGMP). The physiological effects of cGMP are elicited through the activation of cGMP-dependant protein kinases, cyclic nucleotide-gated ion channels, and cGMP-regulated phosphodiesterases (PDE). Importantly, cGMP influences the cAMP/PKA system via the inhibition of cAMP-degrading PDEs like PDE3A (Norris *et al.* 2009; Vaccari *et al.* 2009). Meiotic arrest is regulated by high intra-oocyte cAMP concentrations through its generation by adenylate cyclases with the constitutive action of GPR3 and GPR12 via the Gs protein (Mehlmann *et al.* 2004; Hinckley *et al.* 2005), therefore cGMP inhibits meiotic resumption by inhibiting cAMP degradation sustaining high intra-oocyte concentrations (Richard *et al.* 2001; Norris *et al.* 2009; Vaccari *et al.* 2009). Crucial to this signalling is sufficient communication between the granulosa, cumulus cells, and the oocyte. This occurs by paracrine signalling through the follicular fluid and via gap junctions connecting granulosa/cumulus cells together and between the cumulus cells and the oocyte (Matzuk *et al.* 2002). The gap junction requirement involves connexin isoforms -43 and -37, which are both necessary for the maintenance of meiotic arrest by CNP in mice (Richard and Baltz 2014).

Overwhelming evidence implicates increased intra-oocyte concentrations of cGMP with inhibition of meiosis. However, research conducted in our laboratory and others suggests a possible stimulatory function of cGMP/PKG pathway. Research indicates cGMP has the ability

to overcome hypoxanthine-arrested meiosis to promote resumption of meiosis (Bu *et al.* 2004), furthermore, low-level stimulation of guanosine cyclases in bovine COCs also promote GVBD (Bilodeau-Goeseels 2007). Precisely how this occurs in mammalian COCs remains unclear however, such contrast in results may be the result of different culture systems and medium supplements. This study focuses on whether CNP affects the resumption of meiosis of porcine oocytes *in vitro* and whether such a response is affected by IVM additives known to impact COC function.

6.3 Materials and Methods

Unless otherwise specified, all chemicals used in the following protocols were purchased from Sigma (St. Louis, MO, USA).

6.3.1 Collection, Preparation and Culture of Oocytes

Porcine gilt ovaries were obtained from an abattoir and transported in warm (29 – 32°C) saline (0.9% wt/vol NaCl; Baxter Healthcare) supplemented with antibiotics (100 U/mL penicillin G and 0.1 mg/mL streptomycin sulphate) within 3-4 h of slaughter. Small antral follicles, 2-4 mm in size, were aspirated using a 20-gauge needle and constant suction (1 L/min) into vacutainer tubes. Cellular sediment was transferred to 100 mm Petri dishes and searched then cumulus-oocyte complexes (COCs) with ooplasms of uniform appearance and surrounded by >3 cumulus cell layers were collected and washed once in handling medium (Hepes buffered culture medium (H-TCM-199) (GIBCO® Life Technologies, Mulgrave, Australia) + 3 mg/ml low fatty acid BSA), and once in culture medium (bicarbonate-buffered tissue culture medium (B-TCM-199) + 3 mg/ml low fatty acid BSA). Groups of up to 10 COCs were transferred into pre-equilibrated 100 µl drops of maturation medium overlaid with mineral oil and incubated at 38.5°C with 6% CO_2 humidified air.

6.3.2 Cumulus Expansion Assessment

Cumulus expansion was examined at 24 h of IVM and assessed according to the Vanderhyden scoring system (Vanderhyden *et al.* 1990). Briefly, a score of 0 indicates no detectable response; +1 indicates minimum response, with cells in the peripheral two layers beginning to expand; +2 indicates expansion extending inwards to several layers; +3 indicates expansion of all layers of cumulus except the corona radiata cells; and +4 indicates expansion of the entire cumulus including corona radiata cells.

6.3.3 Oocyte Meiotic Assessment

COC were cultured for 24 h then were mechanically denuded by repeated pipetting and fixed in 4% paraformaldehyde overnight at 4°C. Fixed oocytes were then incubated in permeabalisation solution (0.5% Triton X-100) for 15 – 30 min, followed by incubation in 3 µM 4',6-diamidino-2-phenylindole (DAPI) solution in darkness for 15 min. Oocytes were washed in 0.01% (w/v) BSA in phosphate buffered saline (PBS), and mounted on a slide with glycerol and antifade (Prolong, Invitrogen, Carlsbad, California) (3:1) and chromosome configurations were assessed using a Nikon Eclipse TE2000-E Microscope. Results were represented as

total germinal vesicle break down (GVBD) and encompass all oocytes that had resumed meiosis.

6.3.4 Western Blot Immunodetection

COCs were collected as described above and suspended in RIPA buffer (10 mM Tris, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100) containing phosphatase (Roche, Penzberg, Germany) and protease inhibitor cocktails, snap frozen in liquid nitrogen and stored at −80°C. Samples were mixed with loading buffer containing 100 mM dithiothreitol, heated at 100°C for 5 min, and loaded onto a 7.5% (separating) and 4% (stacking) SDS–polyacrylamide gel for electrophoresis. Proteins were transferred to Hybond-ECL membranes (GE Healthcare, Waukesha, USA), and the membrane was cut horizontally below 75 kDa marker and the upper section was discarded. The remaining membrane was blocked with 2% blocking reagent (supplied in an ECL Advance kit; GE Healthcare) diluted in Tris-buffered saline containing 0.1% (v/v) Tween 20. The membrane was incubated with two antibodies (See Chapter 4 - Table 1) simultaneously, pERK and ERK, pCREB and CREB, and ADAM17 and βACTIN at 4°C for 22-26 h, followed by incubation with goat anti-mouse IgG IRDye™ 680 (Licor Bioscience, Lincoln, NE) diluted 1:50000 and goat anti-rabbit IgG IRDye™ 800 (Licor Bioscience) for 1h at room temperature (antibody dilutions see Chapter 4 - Table 1). Membranes were washed with TBST, then scanned and quantified using Odyssey infrared imaging system (Licor Bioscience). Band intensities for pERK1/2 were normalised to ERK1/2 and standardised relative to no treatment controls.

6.3.5 Statistical Analyses

In all experiments, differences between treatments were analysed by one-way ANOVA followed by Tukey post hoc comparison.

6.4 Results

6.4.1 Effect of CNP on the ability of FSH, AREG, and EREG to induce cumulus expansion and the resumption of meiosis in COCs from small follicles

In vitro cultured porcine COCs from small follicles exposed to exogenous FSH significantly ($P<0.05$) increased cumulus expansion compared to all other treatments, however, FSH had no effect on the spontaneous resumption of meiosis with a similar percentage of oocytes at the GVBD stage compared to control (Figure 6.1). AREG and EREG treatments significantly ($P<0.05$) increased the percentage of oocytes resuming meiosis compared to control and FSH. Compared to control, AREG increased cumulus expansion, whilst EREG only increased cumulus expansion in the presence of CNP. Exposure of COCs to CNP across all treatments significantly increased the resumption of meiosis after 24 h of culture (2-way ANOVA main effect; $P<0.05$), however had no significant effect on cumulus expansion (2-way ANOVA main effect; $P>0.05$).

6.4.2 Effect of CNP on the ability of FSH, AREG, and EREG to induce cumulus expansion and the resumption of meiosis in COCs from large follicles

In vitro cultured porcine COCs from large follicles exposed to exogenous FSH significantly ($P<0.05$) increased the cumulus expansion index compared to all other treatments, however, FSH had no effect on the spontaneous resumption of meiosis with a similar percentage of oocytes at the GVBD stage compared to control (Figure 6.2). Only in the presence of CNP did AREG and EREG treatments significantly ($P<0.05$) increase the percentage of oocytes resuming meiosis compared to control and FSH. AREG and EREG with or without CNP increased cumulus expansion compared to control, with EREG+CNP inducing a significantly higher degree of cumulus expansion compared to AREG+CNP. Exposure of COCs to CNP across all treatments significantly increased the resumption of meiosis after 24 h of culture (2-way ANOVA main effect; $P<0.05$), however had no significant effect on cumulus expansion (2-way ANOVA main effect; $P>0.05$, Figure 2).

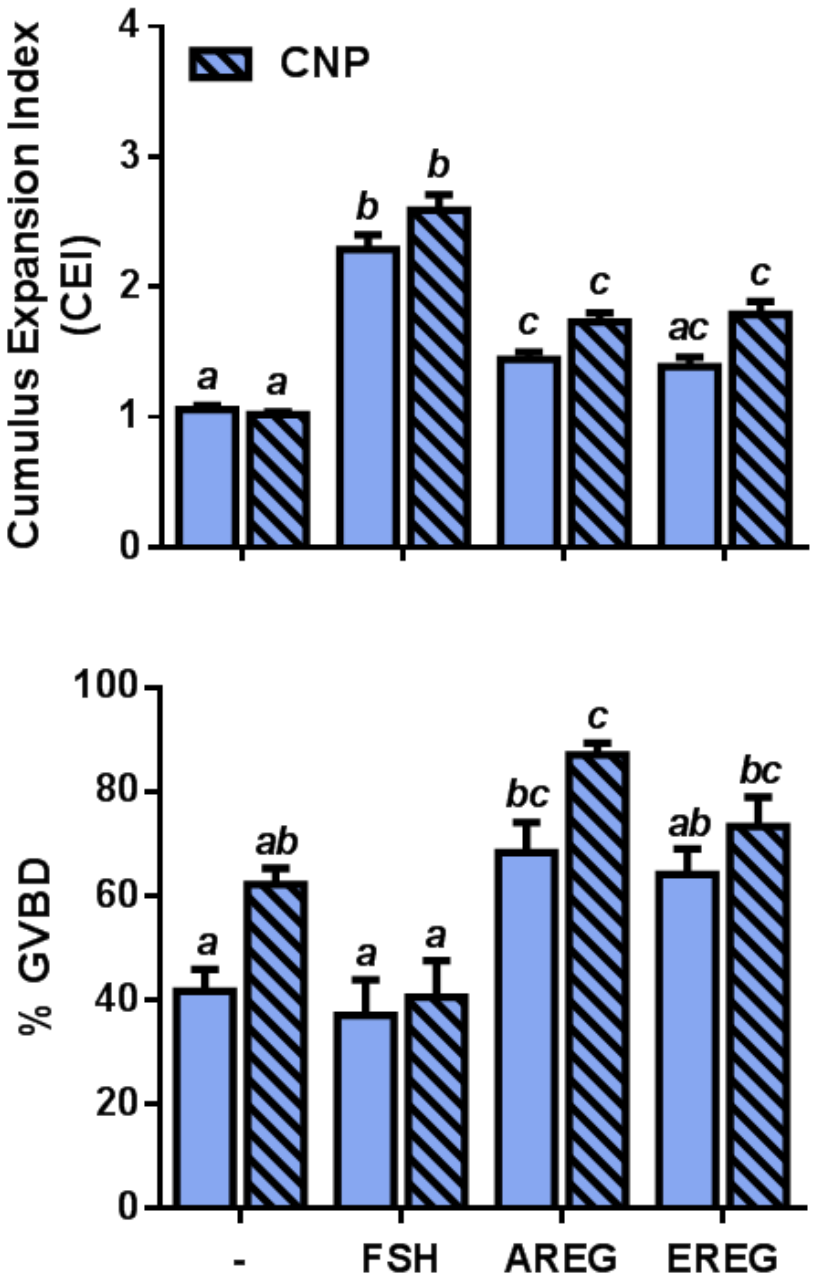

Figure 6.1 - Effect of IVM additives on cumulus expansion and the resumption of oocyte meiosis from COCs collected from small follicles. Cumulus expansion index (top) and total GVBD (bottom) of porcine COCs after 24 h culture in control medium or FSH (50 mIU/mL), AREG (100 ng/mL), or EREG (100 ng/mL), in the presence or absence of CNP (Black striped columns, 100 nM). Columns represent mean ± SEM of 40 – 90 COCs/treatment from 4 replicate experiments, means within a graph without a common superscript differed (^{abcd,} $P<0.05$).

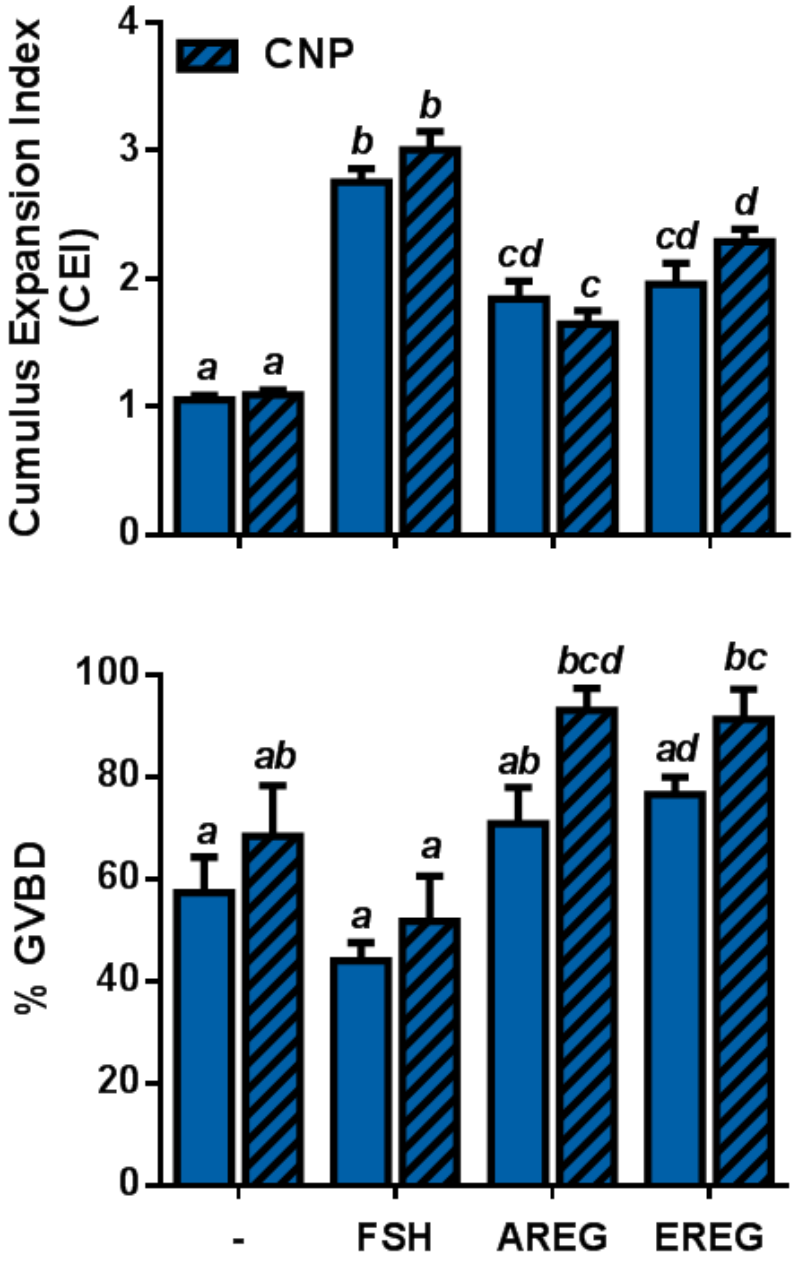

Figure 6.2 - Effect of IVM additives on cumulus expansion and the resumption of oocyte meiosis from COCs collected from large follicles. Cumulus expansion index (top) and total GVBD (bottom) of porcine COCs after 24 h culture in control medium or FSH (50 mIU/mL), AREG (100 ng/mL), or EREG (100 ng/mL), in the presence or absence of CNP (Black striped columns, 100 nM). Columns represent mean ± SEM of 20 – 60 COCs/treatment from 4 replicate experiments, means within a graph without a common superscript differed (^{abcd,} $P<0.05$).

6.4.3 Effect of FSH, porcine follicular fluid, and CNP on cumulus expansion and the resumption of meiosis in COCs from large follicles

In vitro cultured porcine COCs from large follicles exposed to exogenous FSH significantly increased the cumulus expansion index compared to control, with the presence of pFF generating an additive effect further increasing ($P<0.05$) the cumulus expansion index (Figure 6.3). Neither FSH, pFF, nor CNP alone had any effect on the spontaneous resumption of meiosis with similar percentages of oocytes at the GVBD stage compared to control (Figure 6.3). However, FSH+CNP led to significantly less oocytes resuming meiosis compared to FSH+pFF. ($P<0.05$, Figure 6.3).

6.4.4 Effect of FSH, porcine follicular fluid, and CNP on the phosphorylation of ERK1/2 in COCs from large follicles

The phosphorylation of ERK1/2 significantly ($P<0.05$) increased in COCs collected from large follicles in response to either FSH, pFF, CNP, or any combination of the three compared to control (Figure 6.4). The highest increase observed was in response to FSH+CNP at 2.9±0.2-fold increase from control (Figure 6.4).

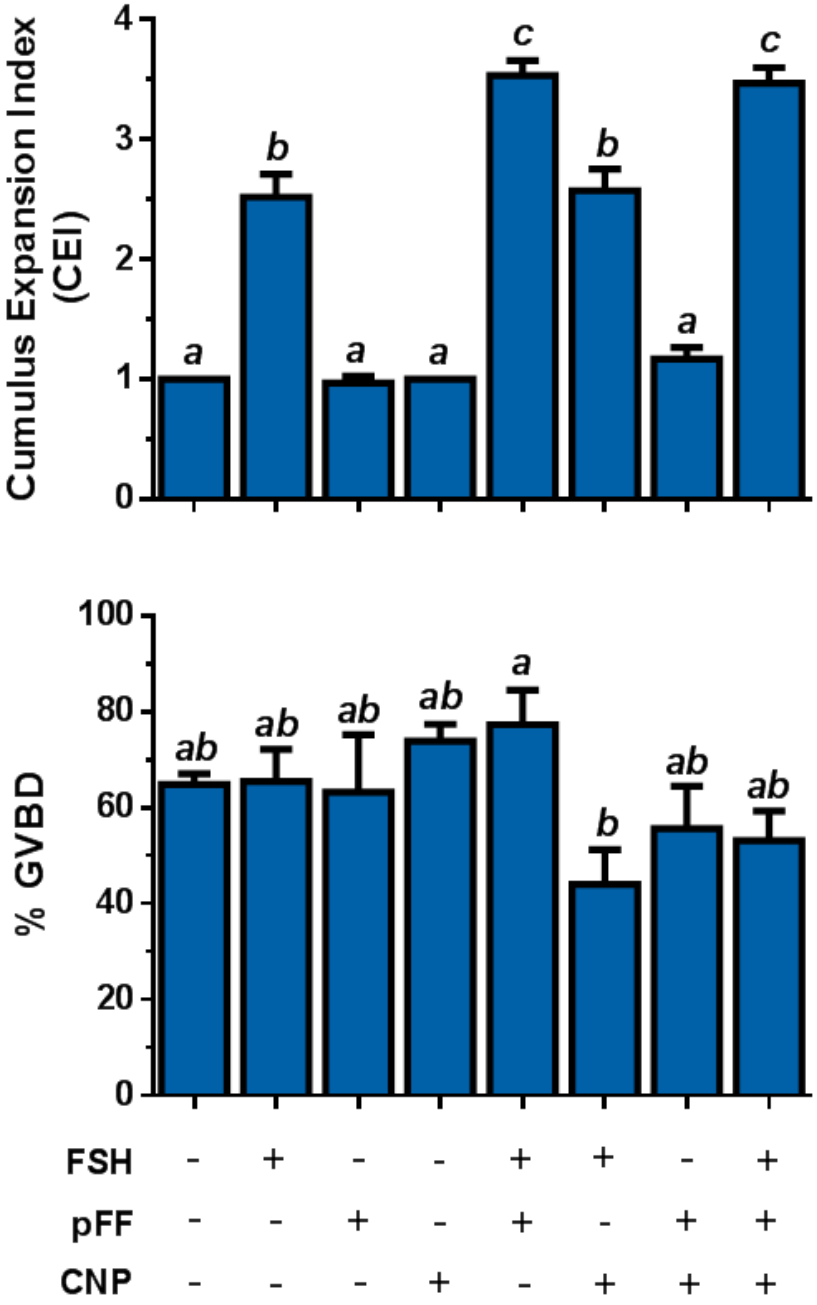

Figure 6.3 – Effect of IVM additives on cumulus expansion and the resumption of oocyte meiosis from COCs collected from large follicles. Cumulus expansion index (top) and total GVBD (bottom) of porcine COCs after 24 h culture in control medium or FSH (50 mIU/mL), or porcine follicular fluid (pFF 10%) or CNP (100 nM). Columns represent mean ± SEM of 20 – 60 COCs/treatment from 4 replicate experiments, means within a graph without a common superscript differed (^{abc,} $P<0.05$).

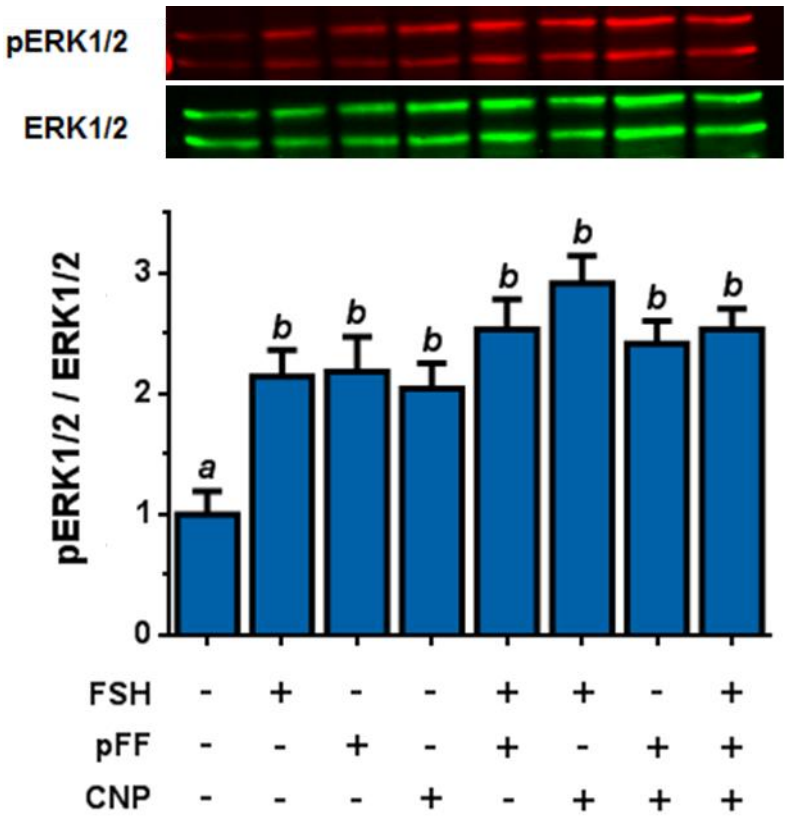

Figure 6.4 - Western blot analysis of the phosphorylation of ERK1/2 relative to total ERK1/2 protein in porcine COCs collected from large follicles after 2 h culture in control medium or FSH (50 mIU/mL), porcine follicular fluid (pFF 10%), or CNP (100 nM). Treatments are presented as fold-change from untreated control. Columns represent mean ± SEM of 4 replicates with 50 COCs/treatment/replicate, means within a graph without a common superscript differed (^{ab,} $P<0.05$).

6.5 Discussion

To investigate the role of CNP on porcine oocyte meiosis from different sized antral follicles, the present study evaluated the effects of CNP and meiotic inducers on the resumption of meiosis in porcine IVM. The effects of CNP on cumulus expansion and ERK1/2 phosphorylation were also evaluated. Our main findings were as follows; 1) CNP alone does not inhibit the spontaneous resumption of meiosis in porcine oocytes from small or large follicles, 2) CNP promotes meiotic resumption in porcine oocytes, 3) CNP can inhibit meiosis when oocytes are co-treated with FSH and pFF. The present study indicates that common additives to IVM media alter the roles of CNP in the porcine COC.

Previous reports demonstrate an inhibitory role of CNP during oocyte maturation (Tsafriri and Pomerantz 1986; Zhang *et al.* 2010). CNP and its receptor NPR2 play the role of the oocyte maturation inhibitor (OMI) in many species (Zhang *et al.* 2010; Egbert *et al.* 2014; Franciosi *et al.* 2014; Hiradate *et al.* 2014; Santiquet *et al.* 2014; Zhang *et al.* 2014a; Zhang *et al.* 2015a; Zhong *et al.* 2015), by promoting the production of cGMP in cumulus cells. Elevated somatic cell cGMP concentration leads to cGMP diffusion to the oocyte where it inhibits the cAMP degrading PDE3A, resulting in high meiotic inhibiting levels of cAMP. In bovine, CNP can inhibit meiotic resumption during *in vitro* culture at 8 h and maintain gap junction communication in bovine COCs (Franciosi *et al.* 2014). In contrast, Cesaro *et al.* (Cesaro *et al.* 2015) and Bilodeau-Goeseels (Bilodeau-Goeseels 2007) demonstrate none of the natriuretic peptides inhibit the spontaneous resumption of meiosis in bovine IVM. However, in the presence of 100 µM forskolin, an inhibitor of spontaneous meiotic resumption, ANP, BNP, and CNP could all promote the resumption of meiosis in bovine IVM oocytes (Cesaro *et al.* 2015). In the current study, CNP alone did not affect meiotic resumption or cumulus expansion of these oocytes compared to controls. However, treatments with CNP including with or without FSH, AREG, or EREG, induced oocyte meiotic resumption (main effect). This demonstrates that the CNP/cGMP pathway aids the meiosis promoting pathways of FSH, AREG, and EREG. In fact, EREG in small follicles and AREG and EREG in large follicles, only promote meiotic resumption in the presence of CNP in porcine oocytes. This is consistent with other reports indicating ANP induced meiotic resumption by stimulating cGMP accumulation and activating cAMP-phosphodiesterase in *Xenopus* (Sandberg *et al.* 1993) and hamster oocytes (Hubbard and Price 1988). Considering our results show a potential stimulatory role of CNP on porcine oocyte maturation, this deviates from most current literature (Zhang *et al.*

2005b; Hiradate *et al.* 2014; Santiquet *et al.* 2014; Blaha *et al.* 2015; Zhang *et al.* 2015b), and is likely the result of different IVM conditions and additives.

In porcine IVM follicular fluid is a common additive to base media systems, including the previously mentioned reports (Hiradate *et al.* 2014; Santiquet *et al.* 2014), because it is known to increase blastocyst development (Nascimento *et al.* 2010). Porcine COCs cultured in the presence of pFF *in vitro* is known to promote gap junction communication between cumulus cells (Santiquet *et al.* 2012), increased cumulus expansion and enhance meiotic maturation (Grupen and Armstrong 2010; Ducolomb *et al.* 2013). Follicular fluid contains a high concentration of CNP, consistent with pFF being the original biological source of the classic OMI experiments (Tsafriri and Pomerantz 1986). In this report, the addition of 10% (v/v) pFF did not effect the percentage of oocytes resuming meiosis compared to untreated controls. However, pFF addition with FSH significantly increased the percentage of oocytes resuming meiosis compared to COCs cultured in FSH and CNP. Furthermore COCs cultured in pFF lead to increased phosphorylation of ERK1/2 after 2 h. Together these results suggest that the actions of CNP in oocyte maturation could be altered by the presence of pFF during IVM. This could be the case with other common media additives like fetal calf serum (FCS), as used in Blaha *et al.* however this was not investigated during this study (Blaha *et al.* 2015).

In summary, CNP stimulated the resumption of meiosis in porcine oocytes from both small and large antral follicles across multiple IVM treatments. However, CNP significantly inhibited the resumption of meiosis when combined with FSH. Increased phosphorylation of ERK1/2 occurred after 2 h in response to CNP, pFF, and FSH. This work demonstrates that CNP addition to porcine IVM systems could produce different effects depending on the IVM medium or additives to base culture systems. Although these additives are used to promote oocyte developmental competence, they may alter or mask underlying effects of CNP on oocytes. Hence, the future application of CNP to IVM systems in reproductive medicine (Romero *et al.* 2016) will likely be impacted by the composition of IVM media and the choice of other IVM supplements such as FSH.

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CHAPTER 7:

DISCUSSION AND CONCLUSION

In mammals, the ability for oocytes to resume meiosis and develop *in vitro* has been known since the 1930s (Pincus and Enzmann 1935). *In vitro* maturation has since been adopted to study the final phases of oogenesis in an artificial, but highly controlled environment. Over several decades, great advances in understanding the underlying fundamental biological processes which drive fully grown immature oocytes into mature, fertilisable gametes have been elucidated, however, many aspects remain poorly understood. Reproductive biologists understand the initial resumption of meiosis during fetal life and meiotic arrest throughout oogenesis is central to achieving oocyte developmental competence. This has coincided with new knowledge that oocytes are able to regulate the function of the somatic compartment of the follicle, facilitating cell differentiation and driving follicle growth. These advances in knowledge have led to the development of more sophisticated IVM systems (Coticchio *et al.* 2015; Fadini *et al.* 2015) and IVM methods utilising natriuretic peptides, have led to improvements in oocyte quality (Romero *et al.* 2016). Advancing our understanding of the process of oocyte maturation, we can provide better opportunities for the application of IVM in assisted reproductive technology.

Natriuretic peptides are physiological regulators of oocyte meiosis, existing within ovarian follicles and are being exploited to reveal the mechanisms regulating meiosis. To date, assessment of natriuretic peptides have been examined during culture of COCs from many mammals including mice (Zhang *et al.* 2010; Zhang *et al.* 2011; Wigglesworth *et al.* 2013; Richard and Baltz 2014; Zhang *et al.* 2014b; Zhang *et al.* 2015b), rats (Tornell *et al.* 1990b), pigs (Zhang *et al.* 2005a; Zhang *et al.* 2005b; Hiradate *et al.* 2014; Santiquet *et al.* 2014), cows (Bilodeau-Goeseels 2007; Franciosi *et al.* 2014; Cesaro *et al.* 2015), goats (Zhang *et al.* 2015a), horses (Mugnier *et al.* 2009), and cats (Zhong *et al.* 2015). Mouse models have dominated this research, elucidating the functions of CNP and NPR2 signalling within the COC and what factors, both maternal and oocyte derived, regulate their activity. The expression, regulation, and roles of natriuretic peptides in sheep are less known. The research in this thesis demonstrates that sheep have altered patterns of natriuretic peptide receptors expression within different follicle cell compartments. In addition, I described that NPR2 is likely to be the major cGMP-generating guanylate cyclase in ovine cumulus cells based on the hypothesis that ANP and BNP selectively bind to NPR1 (Potter *et al.* 2006), and that they had no effect on the resumption of sheep oocyte meiosis when added to IVM culture. In contrast, CNP selectively bound to NPR2 to inhibit the resumption of meiosis *in vitro* at low concentrations, but not at higher concentrations in ovine COCs. Current CNP meiosis inhibiting hypotheses suggest that CNP binds to NPR2 in the cumulus cells to generate

cGMP, which is transported to the oocyte, where cGMP inhibits the cAMP degrading PDE3A to inhibit cAMP breakdown, thereby maintaining intra-oocyte-meiosis-inhibiting cAMP levels (Zhang *et al.* 2010; Zhang and Xia 2012). This existing model is consistent with our ovine results at low CNP concentrations. CNP had no effect at higher concentrations. In contrast to other species, increasing OSFs during IVM resulted in reduced expression of *NPR2* in sheep cumulus cells whilst increasing *NPR1*. This could be a significant species difference, or the result of different methods of cumulus cell isolation and culture (denuded cumulus cell cultures in this study vs. oocytectomised cumulus complexes (Zhang *et al.* 2010; Wigglesworth *et al.* 2013)) and could be the topic of further investigation. Whether OSFs and IGF-1 increase *NPR1* expression in CCs of sheep COCs to levels that would elicit a response to ANP or BNP *in vitro,* would be interesting to explore in future experiments.

Indisputably in mouse models, the binding of CNP to NPR2 initiates the production of cumulus cell cGMP, which is transported to the oocyte where it suppresses the cAMP-degrading PDE3A. This results in the inhibition of cAMP degradation, leading to MPF suppression and delayed resumption of meiosis. However, whether the CNP-NPR2-cGMP axis regulates other mechanisms involved in oocyte maturation like ERK1/2 activation (Zhang *et al.* 2005b; Blaha *et al.* 2015) and gap junction signalling (Franciosi *et al.* 2014; Santiquet *et al.* 2014) are debated and unresolved. The results from this body of work, demonstrates that both CNP and cGMP can activate ERK1/2 in a defined medium during controlled IVM of porcine COCS from large follicles. I hypothesise that this is independent of cAMP as PKA inhibition had no effect on CNP/cGMP-ERK1/2 activation and CREB activation remained unchanged in response to CNP/cGMP treatment. I further propose that this is dependent on MMP activation, yet this raises a further question; how does cGMP activate matrix metalloproteinases? Historically, cGMP has three modes of actions; the cGMP-dependant protein kinases (PKG), cGMP regulated phosphodiesterases (PDE), and cyclic nucleotide-gated ion channels (CNG) (Potter *et al.* 2006; Azevedo *et al.* 2014) (see Figure 7.1). This thesis investigated in some detail the CNP-cGMP-PKG axis. The results revealed that inhibition of PKG did not prevent CNP/cGMP-induced activation of ERK1/2, thus PKG is unlikely its mode of action. In ovarian follicles, cGMP will either inhibit or activate the PDE to hydrolyse cAMP and cGMP, thereby decreasing concentrations in the follicle and cells (Potter *et al.* 2006). cGMP can inhibit PDE3A, thereby preventing the degradation of cAMP, yet cAMP-dependent protein kinase (PKA) inhibition had no effect on CNP/cGMP ability to increase the phosphorylation of ERK1/2. Theoretically, cGMP could activate CNGs in the COC, however, little is known about their expression and function in the ovarian follicle; if present, CNGs would facilitate the transfer of Na^+, K^+, Ca^{2+}

and Mg^{2+} ions across membranes. Our data and evidence from other models (Marcet-Palacios *et al.* 2003; Chanthaphavong *et al.* 2012) supports the notion that cGMP activates ERK1/2 through the activation of MMPs directly, via a novel cGMP action or indirectly through the activation of an unknown cascade involving CNGs. Further research in the future is required to determine exactly how the CNP/cGMP signalling cascade leads to ERK1/2 phosphorylation.

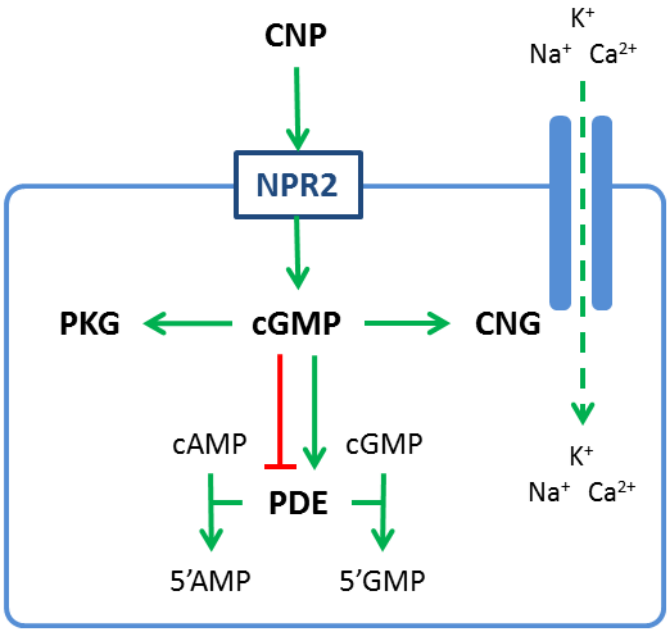

Figure 7.1 – The effectors of CNP induced cGMP production and traditional cGMP intercellular actions. CNP binds to NPR2 to stimulate the production of cGMP. cGMP has three established pathways of action; 1) Activation of cGMP-dependant protein kinases (PKG), 2) activation or inhibition of cGMP-regulated PDEs which induce the breakdown of cAMP or cGMP, and 3) activation of cyclic nucleotide gated ion channels, facilitating the exchange of ions like K^+, Na^+, Ca^{2+}.

Complex molecular signalling mechanisms drive gonadotropin-induced oocyte maturation. The collection of COCs from different size antral follicles represents a useful tool to evaluate the progressive differentiation of the molecular pathways controlling meiotic maturation and cumulus expansion. Understanding the development of oocytes with progressive follicular size is clinically important as clinical IVM is typically performed using oocytes from antral follicles that are still growing. When porcine COCs were sourced from small antral follicles, CNP and cGMP were ineffective at activating ERK1/2 when cultured *in vitro*. This suggests that COCs from small follicles lack the machinery to elicit these actions or that they contain some inhibitory molecules required to override this action. Previous work (Sugimura *et al.* 2015) and other observations suggest that an increased supply of endocrine hormones, like E_2 (Bagg *et al.* 2007), increased endogenous cAMP supply (Bagg *et al.* 2009), and OSF signalling (Sugimura *et al.* 2015), could suggest that as the follicle grows, increased E_2 and OSF signals result in improved expression and activity of CNP and NPR2, promoting cGMP production. However, CNP and cGMP systems are present in small antral follicles, and are responsible for the maintenance of oocyte meiotic arrest whilst the follicle matures. We speculate that CNP-cGMP activation of ERK1/2 in large follicles occurs via MMP activation of EGFp (Chapter 4). An inherent immaturity of COCs from small antral follicles is their poor capacity to translate *EGFR* mRNA into protein leading to poor cumulus EGFR functionality (Sugimura *et al.* 2015). Hence, even if MMPs are sufficiently activated by CNP-cGMP signals, the EGFR lacks the ability to respond to ligand activation. Therefore, I propose as the follicle grows, higher cAMP levels promote pro-EGFp expression, which in turn is cleaved and activated by a CNP-cGMP-stimulated MMP. With increased follicle size, increased OSF signalling (Sugimura *et al.* 2015) induces EGFR functionality (Ritter *et al.* 2015), the cleaved EGFps bind to EGFR, promoting downstream phosphorylation of ERK1/2 inducing the breakdown of gap junctions (see Figure 7.2).

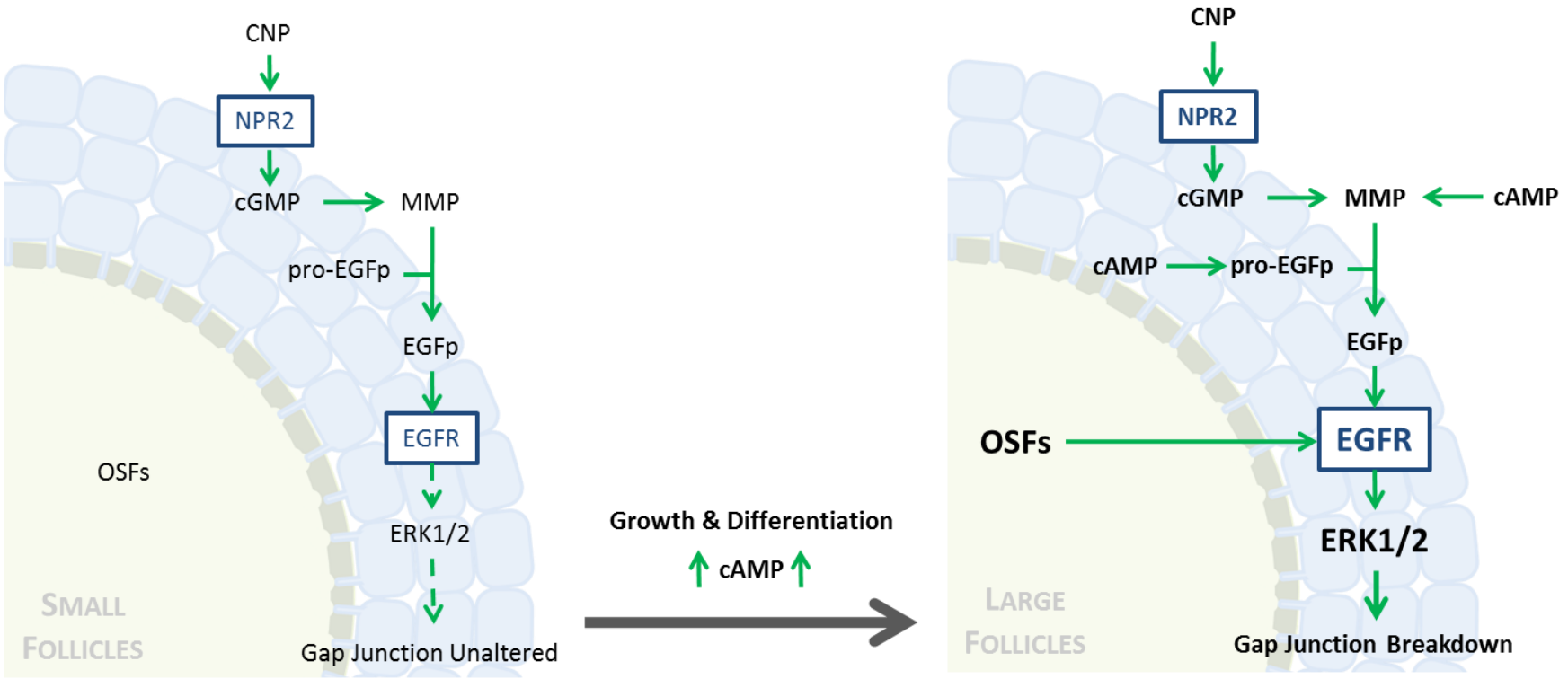

Figure 7.2 - Hypothetical mechanism of ERK1/2 activation in porcine COCs as they develop from small to large antral follicles

As the follicle grows and differentiates, increased cAMP supply promotes the expression of pro-EGFps and MMPs. CNP binds to NPR2, increases cGMP production which activates MMP, which in turn cleaves pro-EGFps to active EGFp ligands. Increased OSF signalling from oocytes increases EGFR responsiveness and significantly improves EGFp-EGFR binding compared to COCs from small follicles, substantially increasing ERK1/2 activation. ERK1/2 activation in large follicles eventually leads to the breakdown of gap junction communication (GJC) whilst basal ERK1/2 activity in small follicles leaves GJC unaltered.

In this study we used a simple chemically defined medium in order to elucidate the biology underpinning oocyte behaviour *in vitro*. Commonly, IVM culture media are supplemented with FF, FCS, and hormones to support maturation, cumulus expansion, fertilisation, and embryo development (Kumar *et al.* 2015). In porcine IVM models supplemented with pFF, FSH, and FCS, oocyte nuclear maturation is inhibited by the addition of CNP to IVM medium (Hiradate *et al.* 2014; Santiquet *et al.* 2014). In contrast, I demonstrated that CNP did not inhibit oocyte nuclear maturation and may promote nuclear resumption. Considering I also demonstrate that CNP/cGMP can promote early ERK1/2 phosphorylation and the breakdown of GJC in the same IVM system, both of which occur prior to the resumption of meiosis, this result is plausible. This discrepancy with the two publications (Hiradate *et al.* 2014; Santiquet *et al.* 2014) could be the result of media additives, specifically FSH and pFF. We demonstrate FSH and pFF stimulate early ERK1/2 phosphorylation equal to that of CNP. Furthermore, FSH and pFF resulted in the highest proportion of oocytes resuming meiosis in our model and similarly to current literature (Hiradate *et al.* 2014; Santiquet *et al.* 2014), FSH + pFF had significantly more oocytes resume meiosis compared to FSH+CNP treatment. It would take further investigation to determine the mechanism, but I hypothesise that the FSH increases cAMP levels and pFF is a rich source of the factor, originally called oocyte maturation inhibitor (OMI), which is likely to be CNP (Tsafriri and Pomerantz 1986). Collectively, this would allow CNP to maintain high intra-oocyte cAMP concentrations through increased cyclic nucleotide production and PDE inhibition, in the presence of medium containing FSH and pFF.

In summary, I have shown that *NPR2* is the predominant cGMP-generating natriuretic peptide receptor in cumulus cells, and is likely responsible for the majority of cGMP produced in the sheep ovarian follicle. OSFs and IGF-1 may inhibit *NPR2* expression in cumulus cells whilst promoting *NPR1* expression. Furthermore, I demonstrated CNP has the ability to inhibit the resumption of meiosis in ovine COCs *in vitro*, however is dose dependant. Using pig COCs, I determined CNP and cGMP have the ability to activate ERK1/2 in COCs from large follicles cultured *in vitro*. This action was independent of cAMP and CREB activation. However, it required functional MMPs and EGFR without altering the expression of the EGF-like peptides; AREG, EREG, or EGFR. CNP and cGMP also induced the breakdown of gap junctions in COCs from large follicles. In pig COCs from small follicles, CNP and cGMP had no effect of ERK1/2 activation or gap junction communication. In COCs collected from both small and large follicles, CNP promoted meiotic resumption of oocytes cultured *in vitro*. However, when cultured in the presence of a highly stimulated system (FSH+pFF), CNP could inhibit the meiotic resumption of oocytes *in vitro*. I further show that CNP stimulates ERK1/2 activation

to levels comparable to FSH and pFF, which therefore could explain why my results may vary from current published research.

It should be noted, that this body of work examines the COC as a whole when describing the phosphorylation of proteins. Although the ERK1/2 is present in porcine oocytes, the rapid activation of ERK1/2 by CNP and cGMP and was dependent on EGFR and MMP activity, both of which are absent from the oocyte. Therefore, although we cannot prove this is exclusively a cumulus cell response, it's improbable to be considerably affected by altered oocyte ERK1/2.

The findings in this thesis illustrate a major difference between cellular signalling between porcine COCs from small follicles, compared to COCs collected from larger follicle and therefore have importance to research into ovarian follicle development. Research reports the presence of CNP and NPR2 in ovarian follicles from early stages of development (Huang *et al.* 1996; Jankowski *et al.* 1997; Gutkowska *et al.* 1999) and concentrations of cGMP remain high in ovarian follicles until the LH surge. In fact, follicle development can be promoted by CNP and cGMP *in vivo* and potentially substitute FSH in stimulating *in vivo* follicle growth (McGee *et al.* 1997a; Sato *et al.* 2012). Research shows ERK activity during follicle growth is critical to the survival of granulosa cells (Shiota *et al.* 2003), and that a range of MMPs are expressed throughout ovarian follicular development, and MMPs promote follicle growth and remodelling (Smith *et al.* 1999; Goldman and Shalev 2004; Kim *et al.* 2014). Research from Sato *et al*. suggests CNP becomes a key component in the pre-antral to antral follicle transition (Sato *et al.* 2012). Therefore, as cumulus cells gain EGFR functionality during follicle development, CNP/cGMP pathways could promote follicle remodelling by activating MMPs and promoting granulosa cell proliferation and survival by activation of ERK1/2. Overall, this thesis contributes further insight into natriuretic peptide regulation of the ovarian follicle and COC. My research supports the notion that there may be more to the CNP/cGMP pathway than simply the inhibition of meiosis; it may promote oocyte development by propagating cumulus cell signals.

Modern IVM technologies have recognised the potential of natriuretic peptides and are now utilising them to enhance the efficiency of oocyte IVM (Romero *et al.* 2011; Wei *et al.* 2015; Zhang *et al.* 2015a; Romero *et al.* 2016; Zhang *et al.* 2016). Conventional IVF programs involve ovarian hyperstimulation with exogenous gonadotrophins to generate multiple growing antral follicles containing mature oocytes for insemination. Despite the wide use and the significant success of these methods, ovarian hyperstimulation has disadvantages, including increased risk of ovarian hyperstimulation syndrome (OHSS), which is particularly prevalent

in polycystic ovarian syndrome (PCOS) patients (Edwards 2007). Other factors such as increased embryo aneuploidy (Baart *et al.* 2007), the poor responsiveness of some patients to gonadotrophin hormone treatment, and the substantial costs associated with gonadotrophins to the patients and to the Australian Pharmaceutical Benefit Scheme, limit hyperstimulation suitability; a viable alternative would be beneficial. Although IVM alleviates the drawbacks identified with conventional IVF and is available to the ART sector, it is clinically underutilised because IVM leads to lower fertilisation, blastocyst, and live birth rates compared to conventional IVF (human (Child *et al.* 2002), cow (Sirard and Blondin 1996; Albuz *et al.* 2010), sheep (Thompson *et al.* 1995), and mouse (Eppig *et al.* 2009)). Recent research demonstrates pre-treatment in the presence of CNP improves blastocyst development in cows (Zhang *et al.* 2016), goats (Zhang *et al.* 2015a), and mice (Wei *et al.* 2015; Romero *et al.* 2016). Furthermore, treatment of immature oocytes with CNP preserve oocyte-cumulus cell interactions (Romero *et al.* 2016), increases the proportion of mature oocytes with normal spindle morphology (Wei *et al.* 2015), and result in improved developmental competence in mice (Wei *et al.* 2015; Romero *et al.* 2016). Novel research into CNP mechanisms and modernisation of IVM methods to utilise CNP as a pivotal factor, could lead to IVM becoming an equally effective, safer alternative to conventional IVF.

Reproductive biology is yet to provide a method of *in vitro* maturation of oocytes that can be universally applied to all species. Oocytes from different species appear to have subtle differences in their signalling cascades during the process of attaining developmental competence. Our knowledge of the molecular mechanisms which regulate oocyte maturation has grown significantly over the past few years. It is in the interest of researchers, ART specialists, and the wider community to investigate new pathways and mechanisms, in order to discover methods of improving oocyte developmental competence for IVM, in order to further develop this less invasive and cheaper form of infertility treatment for women.

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