Characterisation of $GABA_A$ receptors and cation-chloride cotransporters in the uterus and their role in pre-term labour

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Declaration

It is hereby declared that this thesis is the result of the author's original research. It has been composed by the author and has not been previously submitted for examination, which has led to the award of a degree or professional qualification.

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Abbreviations

APα: Allopregnanolone

BZD: Benzodiazepine

Ca^{2+}: Calcium

CAPs: Proteins associated with contractions

CCC: Cation chloride co-transporters

cDNA: Complementary deoxyribonucleic acid

Cl^-: Chloride ions

CNS: Central nervous system

CREB: cAMP- response element binding protein

DEPC-H_2O: Diethylpyrocarbonate treated water

DNA: Deoxyribonucleic acid

EC: Endometrial cancer

ECM: Extracellular matrix

ERK: Extracellular signal-regulated kinase cascade

GABA: γ **–** aminobutyric acid

$GABA_A$-R: γ – aminobutyric acid receptor

GABA-T: GABA transaminase

GAD: Glutamate decarboxylase

GABRP: $GABA_A$ receptor π subunit

MAPK: Mitogen-activated protein kinase cascade

mL: Millilitre

mM: Millimolar

MMPs: Matrix metalloproteinases

mRNA: Messenger Ribonucleic acid

ng/µl: Nanogram/microlitre

NKCC1: Na^+-K^+-Cl^- co-transporters 1

PI3K: Phosphoinositide 3- kinase

PKA: Cyclic AMP-dependent protein kinase

PKC: Protein kinase C

PCR: Polymerase chain reaction

pmol: Picomole

RNA: Ribonucleic acid

qRT-PCR: Quantitative reverse transcription polymerase chain reaction

T_M: Melting temperature

TM: Transmembrane

Scr: Nonreceptor tyrosine kinase

UV: Ultra violet

V/V: Volume/Volume

V: Volts

VGCC: Voltage gated calcium channels

W/V: Weight/Volume

α: Alpha

β: Beta

δ: Delta

ε: Epsilon

γ: Gamma

θ: Theta

µl: Microlitre

π: Pi

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Abstract

$GABA_A$ receptor ($GABA_AR$) function may be inhibitory or excitatory in the brain depending on the chloride electrochemical gradient over the neuronal membrane. This gradient is tightly regulated by cation-chloride transporter (CCC) expression.

Premature birth (PTB) occurs in 5-18% of pregnancies, however the mechanism behind PTB remains unknown. The progesterone metabolite, allopregnanolone (APα) inhibits uterine contractions and $GABA_AR$ sensitivity to APα is increased by presence of the π subunit. $GABA_AR$ π subunit expression and the presence of APα may therefore be contributing factors in premature birth.

The hormones that regulate pregnancy have also been shown to regulate endometrial cancer. Analysis of $GABA_ARs$ and CCCs expression patterns in endometrial cancer samples may indicate changes during pregnancy, contributing to premature birth.

This study aimed to provide the first complete quantitative analysis of $GABA_AR$ and CCC gene expression in the uterus, and endometrial cancer and to develop an appropriate *in vitro* model to investigate the effects of APα on uterine cell function to further understand the functional role of $GABA_ARs$ and CCCs in the uterus.

Gene expression was analysed in healthy human uterus, endometrial cancer and APα treated COLO684 cells using qRT-PCR. COLO684 cells were used as an *in vitro* uterine model and treated with physiologically-relevant concentrations of APα. Protein expression was assessed by immunoblotting.

$GABA_AR$ subunits and CCCs changed in expression when comparing healthy uterus to endometrial cancer data. The data from APα treated COLO684 cells indicated that the $GABA_AR$ π subunit was not expressed but other $GABA_AR$ subunits were. These results support the hypothesis that $GABA_ARs$ and CCCs are present and possibly functional in the uterus, and that $GABA_AR$ subunit expression may be influenced by APα, which subsequently may indicate a role in preterm birth.

CHAPTER 1. Introduction

1.1 γ-aminobutyric acid (GABA)

γ-aminobutyric acid (GABA) is an inhibitory amino acid neurotransmitter in the central nervous system (CNS). The synthesis of GABA occurs by decarboxylation of glutamate by glutamate decarboxylase (GAD), which relies on the cofactor pyridoxal 5'- phosphate (pyridoxal-P) for activity (Abdul *et al.*, 2008, Wu *et al.,* 2014). GABA is released from the pre-synaptic nerve terminal and binds to the $GABA_A$ receptor ($GABA_AR$) located at the postsynaptic membrane; any remaining unbound GABA is taken back up into the neurons by the Na^+- and Cl^--dependent GABA transporter. GABA is then metabolised to succinic semialdehyde (SSA) by GABA transaminase (GABA-T) (Li and Xu, 2008). GAD67 and GAD65 (human isoforms of GAD) have been identified in the oviduct, indicating that GABA is produced by the reproductive system, supporting the hypothesis of the study that GABA and GABA receptors may have a regulatory role in uterine contractions (Fujii and Mellon, 2001).

1.2 GABA receptor structure and function

$GABA_A$ receptors ($GABA_ARs$) are pentameric channels composed of a variety of different subunits with receptor subtypes displaying diverse pharmacological and physiological properties (Bracamontes and Steinbach, 2008; Harvey *et al.*, 1993). In mammals, there are 16 $GABA_AR$ subunits α1−6, β1−3, γ1−3, δ, ε, θ and π, which are classified depending on sequence homology (Bracamontes and Steinbach, 2008; Harvey *et al.*, 1993). The subunit transcripts can also undergo alternative splicing, which further increases diversity between receptor subtypes (Mehta and Ticku, 1999; Zafrakas *et al.*, 2006). Each subunit is composed of an extracellular NH_2 terminal domain (~200 amino acids) with four putative membrane-spanning domains, referred to as M1-M4 with a large, variable, cytoplasmic domain between M3 and M4 (Figure 1.1; Connor *et al*., 1998).

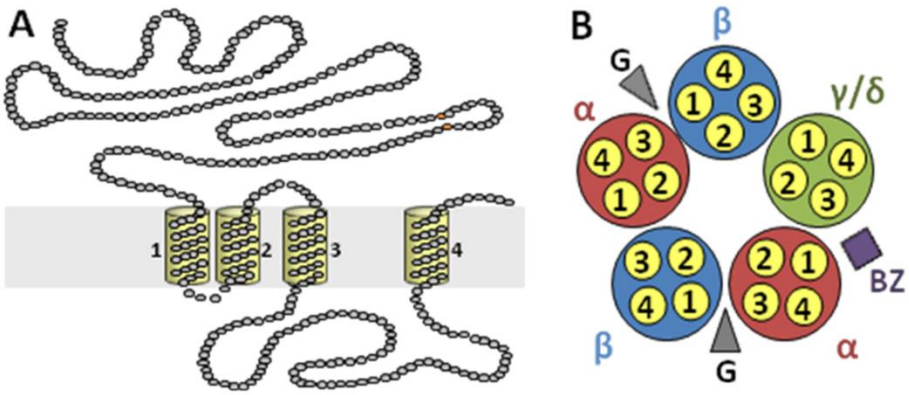

Figure 1.1 Schematic diagram of the $GABA_A$ subunit and receptor. The $GABA_A$ receptor subunit structure comprises an extracellular hydrophilic N-terminal, which connects to the M1 transmembrane α-helixes (labelled 1-4). There is a large intracellular loop between M3 and M4. The M2 domains of each subunits form a ligand-gated ion (Cl^-) channel lining. (B) The purple square (BZs) represents benzodiazepines binding site to the γ/δ. The GABA binding sites are represented by the grey triangle G on the $GABA_AR$ lie at the αβ interfaces. The image is extracted from (Gurba, 2010).

The α and β subunits are essential for a normal functioning $GABA_AR$; most receptors are typically composed of two α subunits and two β subunits. The α and β subunits can form functional receptors alone, however, more commonly, there is an additional γ subunit. In fact, the γ subunit is required to confer sensitivity to benzodiazepines (Connor *et al*., 1998). The γ subunit can be substituted by other subunits, including δ, ε or π, however, this is less common (Connor *et al*., 1998, Harvey *et al.*, 1993, Olson and Seighart, 2009). The π subunit has been shown to assemble with α, β and γ subunits into a functional $GABA_AR$ *in vitro* (Neelands and Macdonald, 1999), although there is still more evidence needed for the existence *in vivo* of this assembly. The most abundant $GABA_AR$ subtypes found in the CNS are α, β, γ complexes, however, it should be noted that despite decades of research, only 11 receptor subtypes have been fully confirmed (Olsen and Sieghart, 2009). The expression of the different subunits not only dictates the receptor properties, but also the receptor localisation (Harvey *et al.*, 1993). The majority of the work focusses on $GABA_ARs$

in the brain; there is far less is known about the receptor subtypes outwith the brain. It is noteworthy that the π subunit is not present in the brain, but is found in the uterus. The benefit of having a receptor subtype not found in the brain is that it may be targeted with drugs without such severe side effects.

1.3 $GABA_AR$s role in development central nervous system

$GABA_AR$s are ligand-gated Cl^- channels, with a primarily inhibitory function in the adult CNS. Their main role is regulation of neuronal excitability, elicited by glutamate. Opening of the central chloride channel occurs when two molecules of GABA simultaneously bind to the $GABA_AR$, causing a change in conformation, which in turn opens the channel. Once the channel is open, the Cl^- ions influx/efflux into or out of the cells depending upon the Cl^- electrochemical gradient, thereby causing hyperpolarisation (chloride influx) or depolarisation (chloride efflux) of the cell (Owens *et al*., 1996).

The receptors themselves are neither inhibitory nor excitatory, their function is dependent on the chloride electrochemical gradient, which influences the direction of chloride flux (Borodinsky *et al.,* 2003, Ganguly *et al.,* 2001). This is determined by expression levels of CCCs. During early development, the intracellular concentration of chloride is higher than the extracellular concentration, therefore, activation of $GABA_AR$s causes excitation (Ben-Ari, 2002). In the mature CNS (>3 weeks in mammals) the intracellular chloride concentration is much lower and activation of receptors elicits hyperpolarisation, an inhibitory response (Ben-Ari, 2002). This change in activity is referred to as the GABA or chloride switch (Figure 1.2). In addition to the Cl^- gradient changes, the expression of post-synaptic $GABA_AR$ subtype also undergo changes by replacing α3 containing $GABA_AR$s with α1 containing $GABA_AR$s in mature synapses. This is also dependent on the Cl^- gradient (Succol *et al*, 2012). Therefore, the identification of the $GABA_AR$s and the CCCs expressed in the uterus could give a possible indication of the function of the $GABA_AR$ as well as the possibility of a GABA or chloride switch.

The intracellular concentration of Cl^- is dependent on the expression of an importer or by the delayed expression of an exporter. Na^+– K^+– $2Cl^–$ co-transporters (NKCC1-2), which typically raise intracellular $Cl^–$ concentrations, and the K^+–$Cl^–$ co-transporters (KCC1-4), which normally lower intracellular $Cl^–$ concentrations. In the brain, NKCC1 accumulates intracellular Cl^- and is highly expressed during early development and is responsible for high intracellular Cl^- concentrations (Succol *et al*., 2012). At this stage, when GABA binds to the $GABA_AR$, the Cl^- channel opens leading to Cl^- efflux, which depolarises the cell and, in turn, activates voltage-dependent calcium channels resulting in an increase of Ca^{2+} currents into the neuron thus causing further excitatory depolarisation (Figure 1.2) (Ben-Ari, 2002). However, the expression of the Cl^- exporter, KCC2 later in development has been shown to have a key role in the GABA switch from excitatory to inhibitory (Ben-Ari, 2002). It is hypothesised that KCC2 expression is self-limiting. GABA itself promotes the shift from excitation to inhibition (Ben-Ari, 2002). When GABA binds to the $GABA_ARs$, the Cl^- channel opens leading to Cl^- influx, which in turn hyperpolarises the cell thus inhibiting voltage-dependent calcium channels resulting in a decrease of Ca^{2+} currents in to the neuron (Figure 1.2). The action of GABA has switched from excitatory to inhibitory (Ben-Ari, 2002).

Understanding the possible expression of $GABA_ARs$, NKCCs, and KCCs in the uterus may help to determine if $GABA_ARs$ are localised there and if they are excitiory or inhibitory in function. Functional $GABA_ARs$ within the uterus may therefore have a role in the regulation of myometrial contraction.

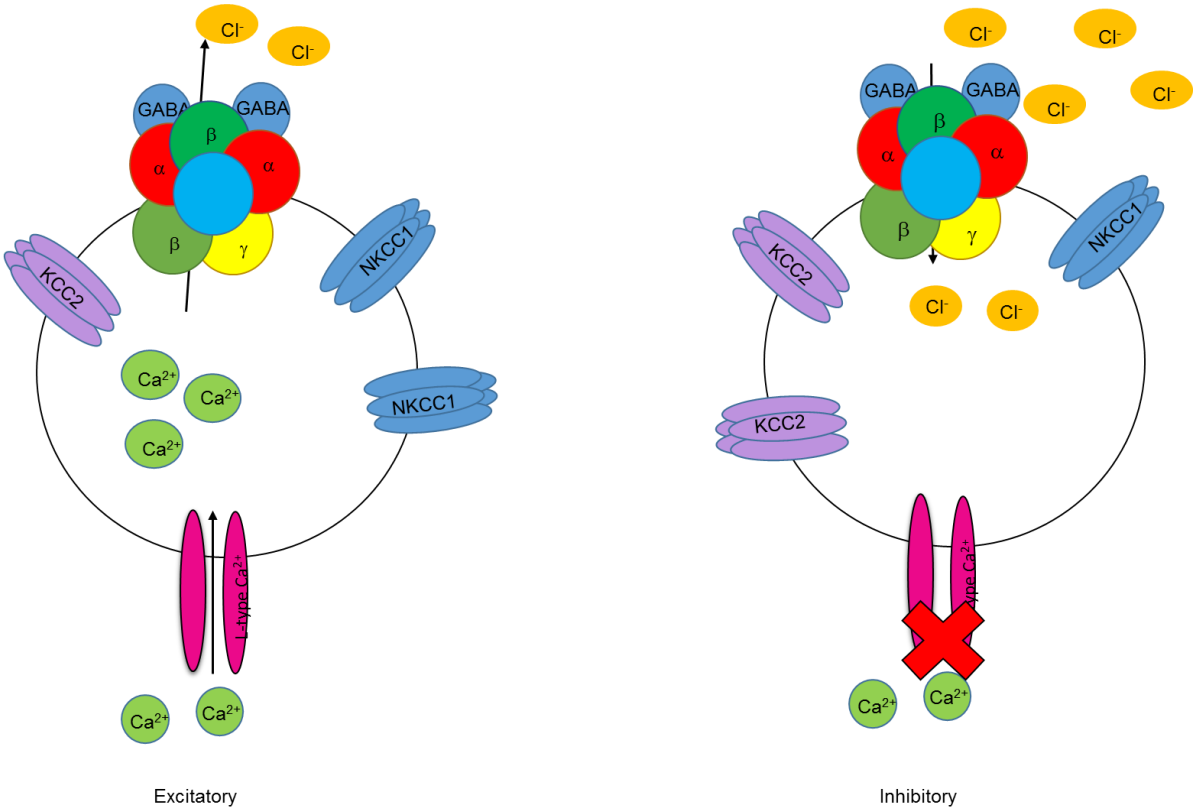

Figure 1.2 The change in the action of GABA during early development of the CNS. The GABA switch is postulated due to the activity of the sodium, potassium coupled chloride co-transporter (NKCC1), which imports chloride into the cells. In developing neurons (left-hand side) there is high expression of NKCC1 transporters and low expression of potassium chloride co-transporters (KCC) type 2 (KCC2), which export chloride from cells. As development of the CNS progresses (right-hand side), NKCC1 expression is reduced and increased expression of KCC2 is seen, thereby changing the intracellular chloride concentration from high to low (Li and Xu, 2008). In line with the changes in CCC expression, GABA changes from eliciting a depolarising (excitatory) response in immature neurons to a hyperpolarising (inhibitory) response in mature neurons.

1.4 Cation chloride co-transporters

The movement of ions across the membrane is controlled by channels and transporters. Cation chloride co-transporters (CCCs) are important in regulating the intracellular Cl^- gradient. The CCC family is made up of nine members, which are encoded in the SLC12 gene family (*Slc12a1-9*) (Blaesse *et al*., 2009, Vardi *et al*., 2000). The CCC proteins are plasma membrane ion transporters, glycoproteins with a molecular weight range of 120-200 kDa (Blaesse *et al*., 2009). CCC functions are divided into two groups: Na^+– K^+–$2Cl^–$ co-transporters (NKCCs) which has two members; NKCC1 and NKCC2, and K^+– $Cl^–$ co-transporters which has four members, KCC1-4 (Blaesse *et al*., 2009, Chen *et al*., 2010, Vardi *et al*., 2000).

NKCC1 and KCC2 are the isoforms predominantly found in the CNS and are therefore important in controlling the neuronal Cl^- intracellular concentration (Rivera *et al*., 2005). NKCC1 accumulates Cl^- in the neuron, whereas KCC2 extrudes Cl^- from the neuron (Delpire, 2000; Succol *et al*., 2012). The expression of these two CCCs relates directly to the function of $GABA_A$Rs (Succol *et al*., 2012). The expression of NKCC1 increases the depolarisation of the neuron, whereas the expression of KCC2 increases the hyperpolarisation of the neuron when the $GABA_A$Rs are activated (Figure 1.2) (Rivera *et al*., 2005).

NKCC1 expression is found in neurons in the CNS, and also in the periphery (Blaesse *et al*., 2009). The other CCCs have been identified out with the CNS. NKCC2 is abundantly expressed in the kidneys, and is a drug target for the diuretic, bumetanide (Blaesse *et al*., 2009). The transport of K^+ and Cl^- has a significant role in the homoeostasis of epithelial cells: KCC1, KCC3 and KCC4 transporters occurs in response to cell swelling and change in pH. The KCC isoforms are co-expressed together; KCC1 is co-expressed with KCC3, while KCC2 is co-expressed with KCC4. Post-transcriptional modifications such as phosphorylation are essential for the activation of the KCCs (Blaesse *et al*., 2009, Chen *et al*., 2010).

The relationship between CCCs and $GABA_AR$s is important to note as the expression of CCCs influences function of the $GABA_AR$, whether it be excitatory or inhibitory. Research by Succol et al. (2012) found that the post synaptic $GABA_AR$ subtypes change depending on whether the neuron is immature or mature. The change in subtype has been shown to be due to the Cl^- gradient and the expression of KCC2. The work herein is focused on peripheral $GABA_AR$ and CCC expression. Identification of specific $GABA_AR$ subunits and CCCs expressed in the uterus, will help provide the first information towards potential receptor subtypes and their role in the uterus.

1.5 GABA out with the CNS

$GABA_AR$s are abundant in mammalian brains, but have also been identified in other regions outside the CNS (refer to table 1.1), including: uterus, placenta, peripheral nervous system, lungs, liver, ovary, small intestine, testis (Akinic and Schofield, 1999), pancreas (Johnson and Haun, 2005), endocrine cells of the pituitary, adrenal, and islets of Langerhans (Hedblom and Kirkness, 1997), and the palate (Ymer *et al.*, 1989). Within endocrine cells, $GABA_AR$s are involved in the regulation of hormone secretion (Akinic and Schofield, 1999, Hedblom and Kirkness, 1997, Johnson and Haun, 2005, Ymer *et al.*, 1989).

Table 1.1. Expression of $GABA_AR$ subunits outside the CNS. A survey of the literature was completed to identify the expression of $GABA_AR$ subunits in peripheral tissues. Documented gene expression is represented by +.

Subunits	Ovary	Placenta	Small Intestine	Testis	Uterus	Adrenal medulla	Adrenal cortex	Pancreas
α1			+		+	+	+	
α2			+		+		+	+
α3			+				+	
α5	+			+	+			
α6		+			+			
β1						+		
β2						+		
β3			+		+	+		+
γ1					+		+	+
γ2			+			+		
γ3			+		+			
π					+			
ε			+		+			

The presence of $GABA_ARs$ in the periphery is important to note as it indicates that $GABA_ARs$ may not only be functional in the CNS. The identification of $GABA_ARs$ in the uterus supports the hypothesis that these may have a functional role in uterine contractions. This study aimed to characterise the presence of potential $GABA_AR$ subtypes in the uterus (by investigating $GABA_AR$ subunit gene expression) while also helping to determine the function during the gestational period. This would help provide a novel drug target for modulation of myometrial contractions in premature birth.

1.6 The mechanism behind labour

1.6.1 Uterine contraction

The smooth muscle layer of the uterus, the myometrium, is active throughout a women's life, not just during labour (Pehlivanoglu *et al*., 2013). Uterine myometrium serves by contracting at the right time with exactly the desired force during labour and remaining relaxed during the period of pregnancy

(Pehlivanoglu *et al*., 2013). Some of the unwanted results of myometrial dysfunction are untimed contractions leading to preterm delivery, or stronger than necessary contractions causing foetal distress, hypoxia and even death of the foetus (Pehlivanoglu *et al*., 2013).

Uterine contractions in pregnant women transforms from a silent, non-contracting state to an actively contracting organ for a successful delivery (Pehlivanoglu *et al*., 2013). This is achieved by morphological changes and adaptations under the effect of elevated oestrogen and progesterone and the balance between these two hormones (Pehlivanoglu *et al*., 2013). Uterine contractions are predominantly regulated by intracellular calcium concentration [Ca^{+2}] (Pehlivanoglu *et al*., 2013). Irrespective of the triggering stimulus, [Ca^{+2}] elevation is essential for contraction, which may either enter from the extracellular fluid into the cell across the surface membrane through voltage-gated L-type Ca^{+2} channels and/or be released from intracellular stores in the sarcoplasmic reticulum (SR) (Pehlivanoglu *et al*., 2013).

During pregnancy the uterus is relaxed and the cervix remains closed and rigid (Garfield and Maner, 2007). However at term, the cervix softens and dilates while the uterus becomes contracted. Contractions occur in the myometrial cells and the depolarisation of the uterus muscles is due to inward current Na^{+} ions (Garfield and Maner, 2007). This leads to activation of voltage gated L- type channels which causes increased intracellular concentration of Ca^{2+}. Myosin light chain kinase is then activated by Ca^{2+}-calmodulin (Garfield and Maner, 2007). The activation results in phosphorylation of myosin and contraction of the uterus. Once contraction occurs, the uterus relaxes and this is a direct result of the extraction of intracellular Ca^{2+} by the Na-Ca exchanger and Ca^{2+}-ATPase (Garfield and Maner, 2007). The sarcoplasmic reticulum is also involved in the removal of intracellular Ca^{2+} resulting in hyperpolarisation of the cells causing relaxation of the uterus (Garfield and Maner, 2007).

Extracellular signal regulated kinase (ERK) plays a role in the regulation of uterine contractions (Xiao *et al.*, 2002). The activation of the ERK pathway is dependent on dual phosphorylation of Ser and Thr by mitogen activated/ extracellular-regulated kinase (MEK) (Xiao *et al.*, 2002). Pregnancy has been shown to

increase the ERK signalling in a1-adrenoceptor-mediated contractions (Xiao *et al.*, 2002). The identification of the ERK pathway in the uterus and the active role in contractions supports the hypothesis that $GABA_AR$s may be involved in the uterine contraction via activation of the ERK pathway (see Figure 1.4).

Uterine contractions occur in the smooth muscle of the myometrium. When stimulated too early during the gestation period, the cells become depolarised, which can lead to preterm labour. As previously mentioned, the $GABA_AR$s function is dependent on the Cl^- intracellular concentration. We hypothesise that the $GABA_AR$s in the uterus may therefore have a role in the regulation of myometrial contraction. Identification of the characteristics of the $GABA_AR$s in the uterus may show potential for a new drug to help prevent premature birth.

1.7 Premature birth

Birth before the 37th week of gestation is defined as premature birth (PTB). It occurs in 5-18% of births worldwide (Liong *et al*., 2013) and is the leading cause of neonatal death or disabilities, such as respiratory disorders, blindness and deafness (Henge *et al*., 2014, Challis *et al*, 2000,). The precise cause of PTB remains unknown, however, it has been proposed that approximately 30% of PTB is due to underlying infections (Challis *et al*., 2002). Premature rupture of the foetal membranes also occurs in 1 in 3 cases (Sundrani *et al.,* 2012). To date, there is still no useful biomarker available to predict women with preterm premature rupture of membranes and PTB (Liong *et al*., 2013). Currently, two diagnostic tests that are used to predict women at risk of PTB. However, these diagnostic tests have limitations as they rely on the abnormal presence of foetal fibronectin or phosphorylated insulin-like growth factor binding protein 1 in the cervicovaginal fluid (Liong *et al*., 2013). The foetal fibronectin (fFN) test is the less accurate of the two as the test has been known to give false positives due to unprotected sexual intercourse (Liong *et al*., 2013). In both tests the biomarkers give low positive values in predicting premature birth. Therefore, the use of these tests has had little impact on the premature birth rates (Liong *et al*., 2013).

1.7.1 Therapeutic intervention in premature birth

The main treatment available to women with previous premature births is the administration of 17α-hydroxyprogesterone caproate. The drug relaxes the uterus by inhibiting contractions and acts by binding to progesterone receptors or glucocorticoid receptors (Hines *et al*., 2013). However, 17α-hydroxyprogesterone caproate binds to these receptors with a lower affinity than progesterone and glucocorticoids (Hines *et al*., 2013). Therefore, the drug may not function fully; however, the exact mechanism of action of 17α-hydroxyprogesterone caproate is still poorly understood (Hines *et al*., 2013). Muscimol (a $GABA_AR$ agonist) is another drug administered to inhibit uterine contractibility (Putnam *et al.*, 1991). Other interventions to prevent preterm labour include cervical stitching and cervical pessary (Abdel-Aleem *et al*., 2013; Alfirevic *et al*., 2013). However, these treatments are not fully effective and have limitations.

Due to the lack of information surrounding the mechanisms of premature birth (PTB), the identification of potential biomarkers or treatments remains challenging. A clear understanding of the role of $GABA_ARs$ (and CCCs) in preterm labour may offer a novel perspective on this under-researched area. It is important to understand the mechanism of preterm labour not only due to the effects on the infant but also burden on health care around the world.

1.8 Pregnancy regulation and hormone production

The normal human gestation period is approximately nine months (40 weeks). In the last six decades the endocrine system has been shown to play a functional role in regulation of the myometrium during gestation period (Hill *et al.*, 2010). The production of steroid hormones varies at different stages of the pregnancy, however the main role of steroid hormones during gestation are involved in regulation of uterine contractions (Hill *et al.*, 2010).

The progesterone metabolite of particular interest; allopregnanole (5α derivate), has a role in the regulation of pregnancy by binding to $GABA_ARs$ (Byrns, 2014;

Hill *et al.,* 2001; Figure 1.5). The enzyme 5α reductase type 1 catalyses the conversion of progesterone to 5 α dihydroprogesterone (5α DHP) the precursor for the formation of allopregnanole (3α-hyroxy 5α−pregnan 20 one (APα)) (Figure 1.3) (Byrns, 2014; Hill *et al,* 2001). The activation of chloride channels, such as $GABA_A$Rs and CCCs, by steroid hormones or GABA, could cause chloride efflux causing the cells to become depolarised and therefore increase contractions. This study aimed to look at the changes in gene expression when subjected to APα.

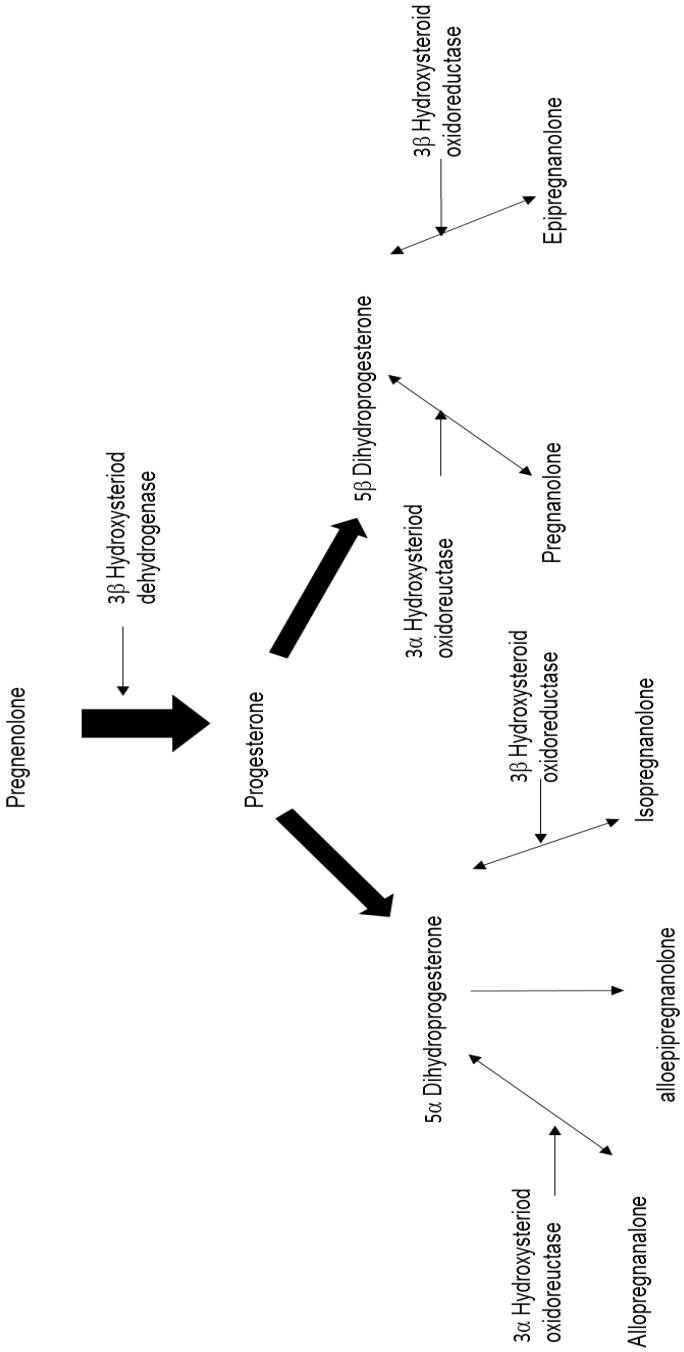

Figure 1.3 Steroid hormone synthesis from the precursor pregnenolone. Enzymes involved in producing progesterone and the metabolites APα and pregnanolone. Adaptation from Byrns (2014), Hill et al. (2001) and Parizek et al. (2005).

1.9 Allopregnanole in pregnancy

APα is a neurosteriod found in the CNS (Wang *et al.*, 2005). APα binds with high affinity to the $GABA_A$Rs (Wang *et al.*, 2005). In mature neurons, APα is an allosteric modulator, which binds to $GABA_A$Rs resulting in increased chloride influx, causing hyperpolarisation which in turn decreases neuronal excitability (Wang *et al.*, 2005). However, in immature neurons the action of APα has the opposite effect. The exact binding site or sites for APα is still unknown.

The main source of APα in women is the corpus luteum and the adrenal cortex (Ottander *et al.*, 2005; Timby *et al.*, 2006). During the menstrual cycle, serum APα concentrations are approximately 0.5 nmole/L, but rise in the mid luteal phase to 4-5 nmole/L (Luisi *et al.*, 2000). However, during pregnancy, fetoplacental synthesis causes maternal APα serum levels to rise more than 10 times the maximum menstrual cycle levels (Beverley *et al.*, 2001; Parizek *et al.*, 2005), while falling to 20-30 nmol/L at the time of labour (Hill *et al.*, 2001) and dropping to 2 nmol/L after delivery.

1.9.1 Uterine $GABA_A$Rs and the potential role of APα

Of particular interest is the robust expression of $GABA_A$ receptor subunit genes in mammalian uterus (Fujii and Mellon., 2001, Hedblom and Kirkness, 1997, Putnam *et al.*, 1991). Several subunits (α1, α2, β3, δ and π) were identified in rat uteri by end-point polymerase chain reaction (PCR) (Fujii and Mellon., 2001). Interestingly, the expression of these different subunits fluctuated over the gestational period (Fujii and Mellon, 2001), although the analysis completed was only semi-quantitative at best. The proposed function of the receptors is suggested to be the inhibition of uterine contraction via binding of the progesterone metabolite APα, (Fujii and Mellon, 2001, Hedblom and Kirkness, 1997).

APα is a much more potent inhibitor of uterine contractions than its precursor progesterone, and acts by binding specifically to $GABA_A$Rs. Increased sensitivity

to APα was found in $GABA_AR$s that contained α1 and π subunits when compared to $GABA_AR$s comprising α2 and δ subunits (Fujii and Mellon, 2001) however, other studies have found δ subunits to increase $GABA_AR$s sensitivity to APα. however, previous research is incomplete as it only analyses a small subset of receptor subunits and the assembly of a native uterine $GABA_AR$s has not been fully elucidated. There is a particular paucity of information relating to the π subunit (as it is not expressed in mammalian brain). However, due to the strong expression in uterus, the ability to bind APα, and the unique pharmacological properties it may confer to the receptor, it is an interesting area of investigation in the field of pregnancy and preterm labour.

1.9.2 Sulphated APα hormones in pregnancy and their role on $GABA_AR$s

During pregnancy, APα becomes sulphated (Park-Chung *et al.*, 1999). The addition of the sulphate group at the 3 alpha hydroxyl group of the APα, changes the modulation of $GABA_AR$s from positive to negative (Beverley *et al.*, 2001; Parizek *et al.*, 2005; Park-Chung *et al.*, 1999). The identification of sulphated forms of the steroid hormones and the effect they have on $GABA_AR$s is important to note as this may help to indicate the function $GABA_AR$s have during pregnancy and labour. This was beyond the scope of the current study and requires further investigation into the possible change in $GABA_AR$s function.

1.10 The link between endometrial cancer and pregnancy

Evidence in endometrial cancer indicates that steroid hormones are involved in the progression of the cancer, with high levels of estrogen and insufficient levels of progesterone being detected (Julie Kim and Chapman-Davis, 2010). The changes in estrogen and progesterone levels in endometrial cancer are similar to those found at the time of labour as progesterone falls and estrogen levels rise (Kim and Chapman-Davis, 2010). In this study we were unable to obtain human pregnancy samples, however we used human endometrial cancer samples to help determine if the $GABA_AR$ subunits and CCCs change expression pattern

depending on the change in physiological conditions when compared to healthy human uterus.

On a side note to the study the current literature to date shows that $GABA_AR$s are involved in cancer proliferation and cell invasion, however the $GABA_AR$ subunits have never been identified in endometrial cancer. We also took this opportunity to identify the possible $GABA_AR$ subunits expression in endometrial cancer as the exact characterisation and function is still unknown.

1.11 Cancer

Cancer is characterised by the ability of cells to sustain chronic proliferation. Normal tissues and organs carefully control the production and secretion of growth promoting signals, which contribute to cell growth and division (Hanahan and Weinberg, 2011). However, cancer cells do not regulate the growth promoting signals which bind to cell receptors (Hanahan and Weinberg, 2011). These signals activate signaling cascades, which result in cell proliferation and uncontrolled growth (Hanahan and Weinberg, 2011).

It is predicted that there will be 22.2 million new cases of cancer by 2030 (Lee *et al.*, 2014). The five most frequently occurring cancers are lung, breast, colorectum, stomach and prostate (Lee *et al.*, 2014). Cancer represents a broad group of pathophysiology, typically occurring with uncontrolled multiplication of cells, this forms the primary tumour. Metastasis (secondary tumorigenesis) is, however, independent of the initial proliferative activity (Lee *et al.*, 2014). The overall progression of cancers is strongly based on epigenetic, chemical and physical factors (Lee *et al.*, 2014).

Inflammation triggers cell division and damage to DNA as a result of the increased production of free radicals (Hanahan and Weinberg, 2011, Mondugno *et al.*, 2005). These are thought to introduce somatic mutations, which have been identified in the P13K/Akt and Erk/MAPK signaling pathways (Hanahan and

Weinberg, 2011, Mondugno *et al.*, 2005). Cancers have been associated with increased replication errors and ineffective DNA repair of key regulatory genes, especially tumour suppressor genes. Inflammatory cytokines induce a variety of inflammatory enzymes, which contribute to the formation of prostaglandin which stimulates factors that have characteristics of activating cell migration and proliferation (Modugno *et al.*, 2005).

1.11.1 Metastases

The life-threatening aspect of cancer is metastasis. This is defined by the tumour cells detaching from the primary tumour, invading the stromal tissue, entering the bloodstream or lymphatic system and migrating around the body (Lee *et al*, 2014). The cancer cells, which are able to survive, either remain dormant or form secondary tumours in the targeted organs such as liver, brain, lung and bones (Lee *et al*, 2014). The development of metastasis is dependent on the interactions between cancer cells and stroma (Lee *et al.*, 2014).

Cancers that metastasise are shown to have poor prognosis. This has been shown in liver and pancreas cancer due to a change in GABA signaling, which dysregulates the MAPK pathway (Young and Bordey 2009)**.** The regulation of the cell cycle through GABA and glutamate signaling may contribute to cancer survival (Neman *et al.*, 2013). A study by Miao et al. (2010) found that treatment with propofol, a commonly used intravenous anaesthetic agent, can directly activate $GABA_AR$ channels. The study concluded that propofol may inhibit cancer cell invasions due to the decrease in MMP production as a result of $GABA_AR$ pathways MAPKs and ERK1/2 deactivation.

1.12 Endometrial Cancer

Endometrial cancer (EC) is a gynaecological malignancy, the sixth most commonly diagnosed cancer. There are approximately 288,000 new cases and 50,327 deaths annually (Subramaniam *et al.*, 2013). Incidence and mortality rates of EC are rising in developing counties and are predicted to rise further with

ageing and obesity (Subramaniam *et al.*, 2013). Between 20 % and 25% of cases are diagnosed pre-menopause, of which 5% are before the age of 40 (Yamazawa *et al.*, 2007). Cancer that occurs at a younger age is related to estrogen exposure through hormone disorders (Yamazawa *et al.*, 2007).

Uterine cancer has two main types: uterine sarcomas, which usually arise from the myometrium (smooth muscle) or endometrial stromal sarcomas and leiomyosarcomas which arise from the supporting connective tissue of the uterus (Yamazawa *et al.*, 2007).

Endometrial carcinomas originate in the cells of the endometrium (inner lining) and these are the most common types of uterine cancer. Most endometrial cancers are known to be adenocarcinomas, malignant tumours formed from the epithelial tissue glandular structures (Sadeghi and Taylor, 2010). The COL684 cell line are human uterus adenocarcinoma, and were used in this study to analyse the change in $GABA_AR$ and CCC gene expression in the presence of APα, and various $GABA_AR$ and CCC agonists and antagonists.

1.13 GABA and Endometrial Cancer

GABA and $GABA_ARs$ have been identified in a variety of cancers, including prostate, breast, colon, gastric, ovarian and pancreatic cancers (Abdul *et al.,* 2008, Roberts *et al.*, 2009 Wu *et al*., 2014, Young and Bordey, 2009, Zhang *et al.*, 2013). In these cancers, dysregulation in the GABAergic system occurs resulting in increased GAD enzyme and GABA, which, in turn increases cell proliferation. This occurs as a result of activation of the Erk1/2 MAP kinase and PI3K, signaling pathways (Figure 1.5) (Abdul *et al.,* 2008, Roberts *et al.*, 2009 Wu *et al* 2014., Young and Bordey, 2009, Zhang *et al.*,2013).

The $GABA_AR$ π subunit gene has also been identified in uterine tissue, (Fujii and Mellon, 2001; Symmans *et al.*, 2005). The APα levels are also thought to be associated with π subunit expression (Symmans *et al.*, 2005). APα in the brain

binds to the $GABA_AR$ which triggers expression of genes that support the cell cycle (Wang *et al.*, 2005) as well as activating the ERK/MAP kinase (MAPK) pathway, although this however has still to be identified in EC (Wang *et al.*, 2005)**.** EC treatment is still limited, and the standard therapy used today for EC is a full hysterectomy as well as the removal of the ovaries. These therapies are invasive and not ideal if the patient is young, though hormonal therapy has been used in such cases to avoid surgery (Senna *et al.*, 2005: Yamazawa *et al.*, 2007). However, there are still concerns as to which therapy is the optimal one to use in EC.

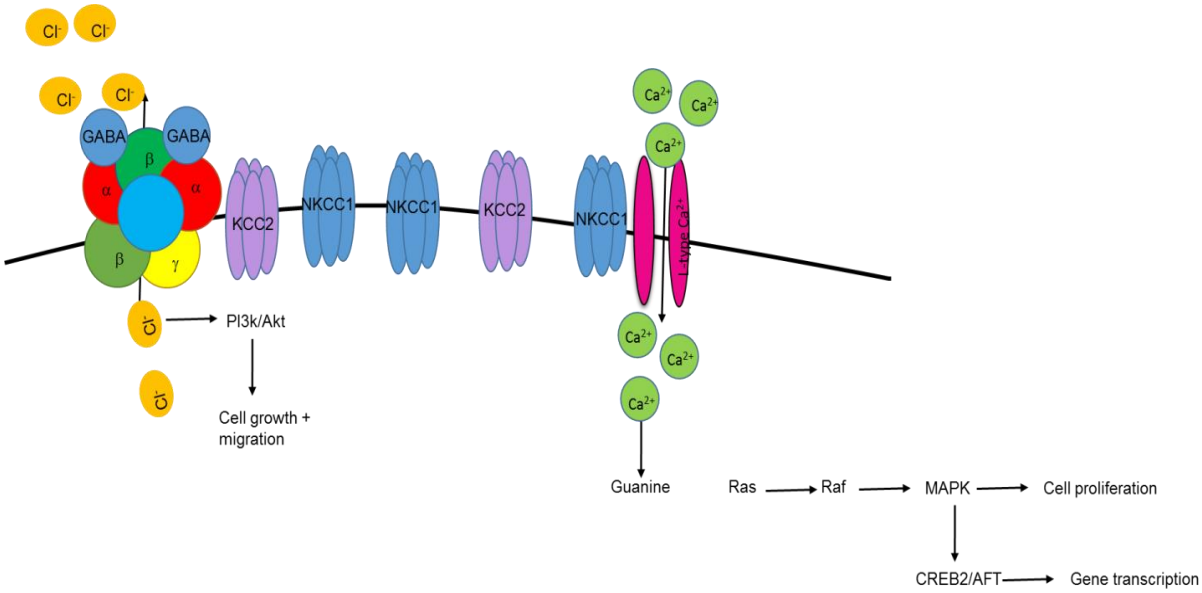

Figure 1.5 MAPK and PI3K pathways in cancer cells. MAPK and PI3K pathways have shown to contribute to cancer cell proliferation. The pathways are also involved in the regulation of gene expression and migration. The pathways are triggered by excitatory GABAergic activity, which leads to the influx of Ca^{2+} and the activation of the small guanine nucleotide binding to Ras. This activates Raf followed by the mitogen – activated ERK- activating kinase (MEK) refer to Figure 1.5 (Bell–Horner *et al.*, 2006).

The PI3k/Akt signaling pathway is activated in parallel to the ERK/MAP kinase (MAPK) pathway (Davies and Samuels 2010, Xie *et al.*, 2004). The P13K /Akt pathway has also been associated with cell proliferation and cell growth along with migration (Figure 1.5); these pathways have been identified in several cancers (Jiang and Liu, 2009). The activation of the PI3k/Akt signaling and ERK/MAP kinase (MAPK) pathway may be due to the $GABA_A$Rs activation, supporting the idea that these pathways and $GABA_A$R may be involved in uterine cancer. While the $GABA_A$Rs may change from an inhibitory role to excitatory role depending on the intracellular Cl^- concentration.

$GABA_A$Rs have been previously identified in uterine cancer. Reverse transcriptase PCR has identified moderate expression of the α3 subunit in endometrial cancer (Liu *et al.*, 2009). The $GABA_A$R is believed to activate the PI3K/Akt and ERK/MAPK signaling pathways (Subramaniam *et al.*, 2013). These pathways are involved in regulating the $GABA_A$R, gene transcription as well as cell proliferation and cell survival (Subramaniam *et al.*, 2013). The pathways can also be activated by the $GABA_A$R which are activated by the phosphorylation of the α subunit of the receptor (Subramaniam *et al.*, 2013). Identification of these pathways show promise in identifying $GABA_A$R in EC.

1.14 CCCs and cancer

The role of CCCs has become of interest in terms of tumour invasion, cell proliferation and migration. Cancer cells have shown to have more active proliferation, migration as well as mitosis (Hanahan and Weinberg., 2011). Up-regulation of KCC1, KCC3 and KCC4 gene expression occurs during cervical carcinogenesis (Chen *et al*., 2010). Studies have identified a relationship between insulin like growth factor 1 (IGF-1) and KCC activity in cervical, ovarian and breast cancer (Chen *et al*., 2010). The KCC activity is linked to the phosphophatidylinostiol 3-kinase (PI3K) and extracellular signal–regulated kinase-1/2 (Erk1/2) MAP kinase signaling pathways (Chen *et al*., 2010). The increased KCC3 and KCC4 expression in these cancers have a physiological role in cell proliferation, invasion and metastasis (Chen *et al*., 2010). The expression

of KCC in cervical cancer cells gives rise to the hypothesis that KCC are present in the uterus and in endometrial cancer. This however, requires further study. The link between CCCs and $GABA_A$ receptors has not been well researched in cancer.

1.15 $GABA_AR$ Therapeutics

1.15.1 Traditional $GABA_AR$ therapeutics

$GABA_ARs$ are drug targets for several pharmacologically important drugs. The assembly of the different subunits determines the pharmacology of the individual receptor (Olsen and Sieghart, 2009). The configuration of the $GABA_AR$ results in five binding sites available on the receptor surface: two binding sites are present at α, β region, which form the GABA binding site (Figure 1.1; Olsen and Sieghart, 2009). The binding site present at the α/γ subunit interface on the receptor is bound by benzodiazepines (Figure 1.1). Benzodiazepines are a class of drug clinically used as muscle relaxants, anxioytics, hypnotic agents and anticonvulsants (Weizman *et al*., 1997). The sensitivity to different benzodiazepines is dependent upon the type of α and γ subunits present within the receptor (Olsen and Sieghart, 2009). There are different forms of benzodiazepines, such as diazepam and flunitrazepam, and these drugs have been shown to activate $GABA_AR$ subtypes, α1βγ2, α2βγ2, α3βγ2 or α5βγ2, but show no activity with the subtypes, which contain α4βγ2 or α6βγ2 (Olsen and Sieghart, 2009). Imidazobenzodiazepines, flumazenil, or Ro15-4513 interact with the receptor subtypes, which contain α4βγ2 or α6βγ2. When benzodiazepines bind to $GABA_ARs$ they produce a sedative effect. Other positive and negative allosteric drugs such as barbiturates, neuroactive steroids, propofol and picrotoxin have shown to act through $GABA_ARs$ and their sensitivity and activity is dependent on the $GABA_AR$ subtype (Olsen and Sieghart, 2009; Watanabe *et al.,* 2000 and Wu *et al*., 2014). These drugs are used to treat many anxiety disorders, however, the properties of these agents may help to prevent premature birth due to their ability to allow muscle relaxation (Weizman et al., 1997).

Neelands and Macdonald (1999) found that $GABA_AR$ subtype expressing the $\alpha5\beta3\gamma3\pi$ configuration formed a functional $GABA_AR$ with different pharmacological properties from those $GABA_AR$ expressing $\alpha5\beta3\gamma3$ and $\alpha5\beta3$. The $GABA_AR$ expressing $\alpha5\beta3\gamma3\pi$ showed no sensitivity to benzodiazepines however did have increased sensitivity to loreclezole an anticonvulsant drug. Lareclezole may therefore have the potential to help prevent premature births depending on the $GABA_AR$ expressed. The change in pharmacological properties of the $GABA_AR$ in which π is expressed helps support the hypothesis of the study that characterising non-classical $GABA_AR$, π-containing GABA conduction in the periphery could provide a novel drug target for modulation of myometrial contractions.

1.16 $GABA_AR$ therapeutics and cancer

Patients who suffer from cancer pain are provided with a wide range of pharmacological therapies, which include non-opioid analgesics, acetaminophen and non-steroidal anti-inflammatory drugs (Lee *et al.*, 2014). When looking at treatment for cancer pain, Benzodiazepines, such as Valium, enhance GABAergic signaling. Gabapentin is known as a GABA mimetic which is commonly prescribed to cancer patients to reduce neuropathic and postoperative pain. Gabapentin acts mainly on the $\alpha2\delta$ subunit configuration of the $GABA_ARs$ (Lee *et al.*, 2014).

GABA and $GABA_ARs$ are therapeutic targets in cancer pain patients, however research has shown that some tumour characteristics, such as uncontrolled proliferation, can be induced by GABA, and this is apparent in a variety of cancers (Lee *et al.*, 2014).

The $GABA_AR$ pathways such as MAPK pathways have shown promising therapeutic targets inhibiting the pathway to prevent tumour growth (Serra *et al*., 2013). PI3K inhibitors reduce cell proliferation (Serra *et al*., 2013), however further study is needed to identify the least toxic combinations for inhibiting MAPK

pathways. MEK-PI3K inhibitors have also shown promising effects on certain tumours (Serra *et al.*, 2013).

$GABA_AR$ agonists and antagonists have been tested in cancer such as colon, lung and pancreatic carcinoma cells however only propofol had an effect on the different colon cancer cells as it may inhibit cancer cell invasion (Miao *et al*., 2010) while also decreasing the production of MMPs production due to the deactivation of the $GABA_AR$ MAPKs pathway. Although there is limited research on effect of $GABA_AR$ drugs in cancer, propofol shows potential and may be developed into a specific cancer anesthetic especially in uterine cancer if $GABA_ARs$ are identified (Miao *et al*., 2010).

1.17 Hypothesis: The potential role of $GABA_ARs$ in prevention and treatment of pre-term labour

Uterine contractions occur in the smooth muscles of the myometrium and are regulated by the balance between the two steroid hormones estrogen and progesterone. The intracellular calcium concentration [Ca^{+2}] also has an effect on regulating uterine contractions. When the myometrium is stimulated too early during the gestation period, the cells become depolarised, which can lead to preterm labour.

$GABA_A$ receptors have been shown to have an inhibitory response during gestation, however in preterm labour, we hypothesise that a change in $GABA_AR$ subtype and function in the uterus may play a role in induction of preterm contraction. To determine the actual subtype of $GABA_ARs$ and CCCs in the uterus, healthy and endometrial cancer samples were used, this was due to two reason: 1) the similar physlogical condtions found in both pregnantant and miligant uteri. 2) many studies of $GABA_ARs$ in the periphery have been done in cancer samples. Functional $GABA_ARs$ within the uterus may therefore have a role in the regulation of myometrial contraction. Due to the wide range of possible $GABA_A$ receptor subtypes within the CNS, characterising non-classical, π-

containing GABA conduction in the periphery could provide a novel drug target for modulation of myometrial contractions.

1.18 Aims of research study

Identification of potential $GABA_AR$ subtypes and characterisation of GABAergic and CCC receptor expression in mammalian uterus will further understanding of the functional role of $GABA_ARs$ in the uterus. This study aimed to provide the first complete analysis of $GABA_AR$ and CCC gene expression in uterus, fully quantify gene expression changes during gestation and develop an appropriate *in vitro* model to analyse the effect of allopregnanole in uterine cells. This novel information forms a strong basis to conduct further research into the functionality of uterine $GABA_ARs$ and their role in pre-term labour. As a complementary side-line to the work, the expression of $GABA_ARs$ and CCCs in uterine cancer was conducted, providing further evidence of the existence, and importance, of $GABA_A$ receptors in mammalian uterus.

The individual aims of this project were:

1) To analyse $GABA_AR$ and CCC gene expression at RNA and protein level in human healthy uterus and human endometrial cancer samples using quantitative polymerase chain reaction (qPCR) and immunoblotting.
2) To analyse $GABA_AR$ and CCC gene expression in non-pregnant vs. pregnant uterine tissue from sheep.
3) To quantitatively analyse selected $GABA_AR$ subunit genes and CCC genes in mouse uteri at nine different time points during gestation.
4) Development of an *in vitro* model using an appropriate cell line (COLO684) to analyse $GABA_AR$ and CCCs expression and functionality in the presence of allopregnanalone, and various $GABA_AR$ and CCC agonists and antagonists.

CHAPTER 2. Materials and Methods

2.1 Diethyl pyrocarbonate (DEPC)-treated water preparation

DEPC-treated water was prepared to remove DNase and RNase activity from dH_2O in preparation for gene analysis. The DEPC-treated water contained a final concentration 0.001% (V/V) DEPC in a total volume of 1L. Once prepared, the solution was incubated for 24 h at room temperature and autoclaved at 121^{o}C to inactivate the DEPC. Unless specified, all work involving RNA used water treated in this way.

2.2 COLO684 cell culture

The COL684 cell line (human uterus adenocarcinoma; HPA culture collections, Salisbury, UK) was available at the laboratory at passage 2. COLO684 cells were grown in suspension in Roswell Park Memorial Institute (RPMI) 1640 media without L-glutamine (GE, healthcare, UK), supplemented with 10% (v/v) foetal bovine serum (FBS), 1% (v/v) penicillin streptomycin and 2 mM L- glutamine (Life technologies^{TM}, Paisley, UK), and incubated in an atmosphere of 5% CO_2, at 37°C.

Cells were counted by diluting 1:2 with Trypan blue (Sigma-Aldrich, Poole, UK), where 10 µL was added to a haemocytometer and the cells were counted using the central square representing 1x 10^4 cell/ mL. Once the cells had reached 3-9 x 10^6 cell/ mL, they were passaged at a ratio of 1:10. Cells were centrifuged at 110 *x g* for 5 minutes at room temperature, the old media was removed and the cells re-suspended in fresh media. COLO684 cells were seeded in a T75 flask in a 1:10 dilution of fresh RPMI every 48 h.

Cell freezing solution was prepared with a 1:5 dilution of FBS and dimethyl sulfoxide (DMSO). COLO684 cell stocks was prepared by freezing 500 µL of cell suspension with 1 mL freezing solution and 1 mL media, cells were transferred to cyrotubes and placed in a Mr. Frosty freezing container with isopropanol (Nalgene®, UK) at -80^{o}C before being transferred to liquid nitrogen storage.

COLO684 cell were revived by adding 1 mL media to the cells before transferring to a falcon tube. The cells were centrifuged at 110 *x g* for 5 minutes at room temperature, the old media was removed and the cells resuspended in fresh media.

2.3 Treating COLO684 cells with allopregnanalone.

An *in vitro* model using uterine cells treated with allopregnanalone (APα) was established to determine the effects on $GABA_AR$ subunit and CCC gene expression. For the preliminary assay, cells were counted as described in section 2.2. The COLO684 cells were seeded at 1 x 10^6 cell/ mL in a T25 flask in 5 mL RPMI 1640 without L-glutamine and grown for 24 h at 37°C. After 24 h, cells were treated with a range of concentrations of APα, 0-150 nM (Tocris Bioscience, UK). APα was prepared to a final concentration of 10 mM in dimethyl sulfoxide (DMSO). Intermediate APα stocks were prepared at 0-150 µM in DMSO and 5 µL of APα intermediate stock was added to cells in 5 mL of media to give a 1:1000 dilution and a final contraction of 0-150 nM APα. Cells were also treated with DMSO only to a final concentration of 0.01% (V/V) as a vehicle control. COLO684 cells were treated for 24 h and re-dosed at 48 h and 72 h. After each exposure, the cells were harvested as previously described in section 2.3. Samples were stored at -80°C until further analysis.

2.3.1 Analysing cell viability by Alamar blue.

Cell viability was analysed by Alamar assay to determine the effects of the range of allopregnanalone concentrations and the vehicle control DMSO on COLO684 cells. The cells were counted as described in section 2.2, seeded at a density of 5x 10^2 cell/ mL per well in a 96-well plate in a volume of 200 µL media and incubated for 1 h at 37°C. The cells were treated in triplicate with a range of concentrations 0-100 nM of APα (Tocris Bioscience, UK). APα stocks were prepared at 0, 50 nM, 500 nM and 1 µM in media, 20 µL of APα intermediate stock were added to cells in 200 µL of media to give a 1:10 dilution and a final contraction of 0-100 nM APα. Cells were also treated with DMSO only to a final

concentration of 0.01% (V/V) as drug vehicle control. Alamar blue ® (Invitrogen, UK) was added 24 h before the plate reading, 10 µL of Alamar blue ® was added to each treatment in triplicate except for the media only control. COLO684 cells were treated for 24 h, 48 h and 72 h. The data was acquired on a 96 plate reader LT-50000MS ELISA READER (Thermo scientific, Waltham Massachusetts, USA), the blank reading at 600 nm wavelength was subtracted from all readings and the data was expressed as a percentage of the untreated control.

2.4 Harvesting COLO684 cells

COLO684 cells were harvested by transferring the cells from the plate into a sterile microfuge tube then centrifuged at 110 *x g* for 5 minutes at room temperature. The media was discarded and the cell pellet was washed by resuspension in 1 mL phosphate- buffered saline (PBS) and transferred into a sterile 1.5 mL microfuge tube. The suspension was centrifuged for 5 minutes at 800 x *g*, at 4^{o}C, the PBS was removed and the pellet left to dry and stored at -80^{o}C for subsequent RNA or protein extraction.

2.5 RNA extraction

RNA was extracted from COLO684 cells in preparation for complementary (c)DNA synthesis and quantitative polymerase chain reaction (qRT-PCR) analysis. After the cells were harvested, 1 mL of TRI Reagent® solution (Ambion™, UK) was added to the cell pellet, and the lysate passed several times through the pipette tip. The samples were incubated for 5 minutes at room temperature before 0.2 mL of chloroform was added per 1 mL of TRI Reagent® solution and mixed by inversion for 15 seconds. Samples were incubated at room temperature for 3 minutes followed by centrifugation at 12000 x *g* for 15 minutes at 4^{o}C. The aqueous phase was removed and transferred to a sterile microfuge tube and the RNA was precipitated from solution by adding 0.5 mL cold isopropyl alcohol. Samples were incubated for a further 30 minutes on ice before they were centrifuged at 12000 x *g* for 10 minutes at 4^{o}C. The supernatant was removed and the RNA pellet washed with 1 mL 75% (v/v) ethanol and vortexed. Pellets

were centrifuged at 7000 x *g* for 5 minutes at 4ºC, then left to air dry. The RNA pellet was resuspended in 20 µL of DEPC-treated water and stored at -80ºC.

2.5.1 Commercial RNA samples

Commercially-available matched RNA from healthy human uterus and human endometrial cancer were obtained from AMSBio®, Abingdon, UK. Healthy human brain RNA was also obtained from (AMSBio®, Abingdon, UK) and was from a 65-year-old female. Pregnant whole mouse uterus RNA was obtained from AMSBio, (Abingdon, UK) and stored at -80ºC until use. The pregnant mouse RNA was extracted from nine different gestation period time points (E10-E18), which correspond to the Theiler stages 16-26 of the embryo.

2.5.2 Analysis of RNA integrity by agarose gel electrophoresis

RNA integrity was analysed on 1% (w/v) agarose gel in (Tris- acetate- EDTA) TAE buffer (0.01 M Ethylenediaminetetraacetic acid (EDTA), 0.4 M Tri acetate EDTA-Trizma and Acetric acid). Once heated, 4 µL of Safeview (NBS Biologicals Ltd, UK) was added. The gel was placed in the Bio-rad mini-sub cell gt® tanks (Bio-Rad, California, USA). RNA samples were diluted 1:15 with DEPC-treated water to a final concentration of 90 nM/ µL and loading buffer (4x LSB (20% (w/v) glycerol, 200 mM Tris pH 6.8, 4% (w/v) SDS, 10 mM EDTA, 1% (w/v) bromophenol blue supplemented with 10% (v/v) β-mercaptoethanol) was added, and 15 µL of diluted RNA was added to each well. Samples were electrophoresed at 80 V for 1 h in TAE buffer. The gel was then analysed using the Biorad molecular imager Chemidoc™ xrs+ (California, USA) and an image was taken with Image lab™ software. The hyperladder™ (Bioline, UK) was used to indicate the size, all RNA underwent bioanalyser analysis.

2.6 Bioanalyser analysis

The quality and quantity of each RNA sample was further analysed by use of the Agilent Bioanalyzer (Agilent Technologies Ltd, UK). The gel matrix was prepared using the provided gel matrix (Agilent RNA 6000 Nano Kit, Catalogue number

5067-1511), which was filtered by centrifugation for 10 minutes at 1500 x *g*, 65 µL was aliquoted into RNase-free microfuge tubes. Following this, 1 µL of RNA dye concentrate (Agilent, Catalogue number 5067-1511) was added to the 65 µL filtered gel matrix and vortexed. The gel-dye mix was centrifuged for 10 minutes at 13000 x *g* at room temperature. A RNA 6000 Nano chip was placed on the priming station and 9 µL of gel-dye mix was placed into allocated wells. In every well, 5 µL of RNA marker (Agilent, Catalogue number 5067-1511) was added. For sample analysis, 1 µL of diluted RNA ranging from 20 ng to 500 ng was added to the wells. Any unused wells were filled with 1 µL of RNA marker. The chip was placed on the IKA vortex (Agilent Technologies Ltd, UK) for 1 minute at 534 RCF and analysed on the Agilent 2100 Bioanalyser. Only RNA samples with an RNA integrity number (RIN) of above 7.5 were used for further gene expression analyses (Bustin *et al*., 2009).

2.7 DNase treatment of pregnant mouse uterus RNA samples

The commercial mouse uterine RNA samples showed evidence of DNA contamination, subsequently, they were subjected to DNase treatment. The DNase digestion reaction mix contained 2 µg each RNA, in 1x final concentration of RQ1 reaction buffer, 1 unit/µg RNA RQ1 RNase-Free DNase (Promega) and DEPC-treated water in a final volume of 10 µL. Samples were incubated for 30 minutes at 37^{o}C. The reaction was terminated by the addition of 1 µL of DQ1 DNase stop solution. Once the stop solution was added the samples were then incubated at 65^{o}C for 10 minutes to inactivate the DNase, and stored at -80^{0}C for future use. Prior to storage, RNAs were reanalyzed using the Bioanalyzer to verify removal of DNA contaminants.

2.8 Complementary (c)DNA synthesis

Reverse transcription was used to convert RNA extracted from the treated COLO 684 cells and commercial RNA into cDNA to allow amplification during quantitative polymerase chain reaction (qRT-PCR). Firstly, 20 µL of reverse transcription premix 2,oligonucleotides used were random Nonamer and OligoDT

mix (PrimerDesign™, Southampton, UK), was dispensed into thin-walled reaction tubes and 2 µg RNA was added. The samples were placed into a thermocycler set at 42ºC for 20 minutes followed by 10 minutes of incubation at 72ºC. cDNA samples were diluted with DEPC-treated water to 5 ng/µL and stored at -20ºC. A non-reverse transcriptase (Non RT) control was prepared for each cDNA using the Precision Nanoscript™2 reverse transcription kit (PrimerDesign™,Southampton, UK).

2.9 Oligonucleotides used in this study

Specific qRT-PCR oligonucleotides were designed to amplify cDNAs encoding $GABA_A$Rs subunit transcripts, namely, $\alpha 1-6$, β 1-3, γ 1-3, δ, ε, θ and π $\alpha\nu\delta$ the chloride cotransporters (NKCC1-2 and KCC1-4) from both human and mouse. The majority of oligonucleotides were designed previously by other researchers in the lab. The sequences of oligonucleotides targeting human CCC transcripts are given in Table 2.1 and oligonucleotides to amplify $GABA_A$R subunit transcripts in Table 2.2. The mouse CCC transcript oligonucleotides are shown in Table 2.3 and oligonucleotides towards $GABA_A$Rs subunits in table 2.4. Housekeeping gene oligonucleotides were purchased commercially (GeNorm, PrimerDesign™, UK). Details of the oligonucleotides used in this study can be found in Table 2.3, 2.4, 2.5 and 2.6. Each oligonucleotide was designed to cross exon boundaries, either in the oligonucleotide sequence or in the amplicon, to prevent amplification of genomic DNA. Oligonucleotides were designed with a melting temperature (Tm) of 58-61ºC and a GC content of 40-60%. All oligonucleotides were subjected to Basic Local Alignment Search Tool (BLAST) analyses to ensure specificity (Nation Center for Biotechnology information, https://www.ncbi.nlm.nih.gov).

Oligonucleotides were synthesised by Eurofins MWG Biotech (Germany) and resuspended in the appropriate volume of DEPC-treated water outlined by the manufacturer to give a final concentration of 100 pmol/ µL. All oligonucleotides were stored at -20ºC until use. Oligonucleotides were validated by BLAST

analysis along with analysis of the Melt curves to ensure one amplicon and the use of a negative control.

Table 2.1. Oligonucleotides sequences towards human cation chloride co-transporter (CCC) transcripts. Forward and complementary oligonucleotide sequences, amplicon size, melting temperature (Tm) and relevant NCBI accession number are shown.

Target	Sequence 5' to 3'	Amplicon (bp)	Tm (°C)	Accession number
NKCC1	F- TTATTAAAGGAACATTCAAGCACAG	92	69.5	NM_001046
	R- GCCATGTAGAGAGCACTAGAC		55.9	
NKCC2	F- GTTATAGTCAGAATCAGCCAAGGA	130	57	NM_000338
	R- TTTCCTCTTCACACTCATTATCTTTG		57	
KCC1	F- AATGAAGTCATTGTCACGCGC	207	57	NM_020708
	R- GCCACAAGATGACACTGGG		58	
KCC2	F- AGCGTTCCCAGATCCTCAAA	177	57	NM_001134771
	R- GCGTGTTGGCTGGATTCTT		56.5	
KCC3	F-TGTTCCCTTTGCTTGCTTCA	115	56.2	NM_133647
	R- GGAGTTGTAAATAATGCTTCTACCA		56.3	
KCC4	F- TACGGAAGTTTCTAGGCCCG	165	58	NM_00658.2
	R- TCACTGGCTTCTTGTGACCTG		59	

Table 2.2. Oligonucleotides sequences towards human $GABA_AR$ subunit transcripts and reference genes. Forward and complementary oligonucleotide sequences, amplicon size, melting temperature (Tm) and relevant NCBI accession number are shown.

Target	Sequence 5' to 3'	Amplicon (bp)	Tm (°C)	Accession number
GABRA1	F- ATCTTCAGCAAAGGAGCACG	243	56	NM_000806.5
	R- AAAGACAGTCAGACAGACCTG		57	
GABRA2	F- CTGATTCAGTACAGGTTGCTCC	94	59	NM_000807.2
	R- ACTGGATTTAATTGTCTCCTTTCCG		59	
GABRA3	F- TTGGGAAGGCAAGAAGGTGC	130	55	NM_000808
	R- AGTGTCCTTGGCCAGGTTG		58	
GABRA4	F- TGCGGAGTGTCCCATGAGA	98	58	NM_000809.3
	R- CTAAGCTGGAAGACTCCTTCG		59	
GABRA5	F- TGAAATTTGGCAGCTATGCGTAC	168	58	NM_000810
	R- TGTATTCGCCTGTGCTGGTG		58	
GABRA6	F-CAGGACATAATCTAAGACCSCAAC	127	56.8	NM_000811
	R- TGCCTCAAGTCAGTAATCCAATAG		57.3	
GABRB1	F- AGGATATGACATTCGCTTGCGG	136	59	NM_000812.3
	R- AGACTGCTGGAAATACATGGTGAG		60	
GABRB2	F- TTAAAGAGACGGTGGATAGACTC	98	58	NM_021911.2
	R- AATGTCAATGTTCATCCCCACAG		58	
GABRB3	F- CATATTTCATAAGCACTCCAACTACT	110	57.4	NM_000814.5
	R- TTGCCTCAGAGAACGGTCAT		57	
GABRG1	F- CATGGATGAACATTCCTGTCCAC	183	60	NM_173536.3
	R- TAATCCCCAGAGATCGTGTGAG		59	
GABRG2	F- ACAGGAAGCTCAGTCTACTCG	116	59	NM_198904.2
	R- TCATAGTCATCATCAGATTTCTGGC		59	
GABRG3	F- TCCTCAAGATGGATTCCTGAGC	153	59	NM_033223.4
	R- ATCCAGACACTCATAGACACAGG		60	

Table 2.2.continued:

Target	Sequence 5' to 3'	Amplicon (bp)	Tm (°C)	Accession number
GABRP	F-TGCATTGGAGTGACGACCG	175	59	NM_014211
	R- GCTGTAAGGAACTGTAGTGAGC		59	
GABRD	F- CGACTACAGGAAGAAGCAGAAG	92	57	NM_000815.4
	R- AGAGGGAGAAGAGGACAATGG		56.8	
GABRE	F-TTCTGAATGGCAATGTGGTGAGCC	179	59	NM_004961.3
	R-TTGATACGAGGATGGCGGAGTTTAG		60	
GABRQ	F-TGGTAGAGAGCTATGGTTACACG	129	59	NM_018558.3
	R-TAGACATACTCCAGCAAGGACAG		59	
EIF2B1	F- TATACGTCACAGAGTCACAGCC	106	59	NM_001414.3
	R- TAGCCGACAGCAGCATCTAGC		61	

Table 2.3. Oligonucleotides sequences towards mouse chloride co-transporter (CCC) transcripts. Forward and complementary oligonucleotide sequences, amplicon size, melting temperature (Tm) and relevant NCBI accession number are shown.

Target	Sequence 5' to 3'	Amplicon (bp)	Tm (°C)	Accession number
NKCC1	F- TGTGGGTTCGTGTGTTGTTCGG	144	61	NM_009194.3
	R- ATTAGCCCGTAAGAACAAGTATTGC		59	
NKCC2	F-TTCATTGGAACTGTCATCCCATCC	128	60	NM_183354.2
	R- AAGACCGAGAAGAAGCCTTCCC		61	
KCC1	F-TCATCTGCTGCTGCTGTACCC	156	61	NM_001253804.1
	R- TCGTCCCCAGGTAGAAGCAC		60	
KCC2	F- TGCTGGCTTATCTCTTCCCAG	145	59	NM_020333.2
	R- TACTTGACACCCACAAAGACAACG		60	
KCC3	F- CTGGCCTGTGTAATTGTGTCG	154	59	NM_133648.2
	R- TTGTCAACCTCCTTGGTCTTAGAG		60	
KCC4	F- GTCAAATATGTCAACAAGCTGGCAC	163	60	NM_011390.2
	R- CACAGGTATCAAAGTTGCGATTTGC		60	

Table 2.4. Oligonucleotides sequences towards mouse $GABA_AR$ subunit genes of interest and housekeepers. Forward and complementary oligonucleotide sequences, amplicon size, melting temperature (Tm) and relevant accession number are shown.

Target	Sequence 5' to 3'	Amplicon (bp)	Tm (°C)	Accession number
GABRA1	F- AAGAAGCTATGGACAGCCCTCC	181	61	NM_010250.5
	R- ATCGTGGTCTGAAACTGGTCCG		61	
GABRA2	F- ATGCCCACTGAAATTTGGAAGCTAC	88	60	NM_008066.3
	R- AACCTGAACGGAGTCAGAAGC		59	
GABRA3	F- TAGCCAAGGGGAATCAAGACG	91	59	NM_008067.4
	R- AGGAATATCTGGGGCATGCTTGG		52	
GABRA4	F- AAGGACGAGAAATTGTGCCCG	162	59	NM_010251.2
	R- TTCCATTTCAACATCAGAAACGGGC		60	
GABRA5	F-AGAGCGAATCACGCAGGTGC	162	60	NM_176942.4
	R- AGGTTGTTGAGAGGGAGGCG		60	
GABRA6	F- CAGGACATAATCTAAGACCSCAAC	127	56.8	NM_000811
	R- TGCCTCAAGTCAGTAATCCAATAG		57.3	
GABRB1	F- TCTATGGACTACGGATCACAACC	132	60	NM_008069.4
	R-TCCAGTAAAATTCGATGTCATCCGTG		59	
GABRB2	F- GCTTTGTCTTCGTCTTTATGGCC	146	60	NM_008070.3
	R- CATCTTGTTGACATCCAGGCG		59	
GABRB3	F- ATCGGGTACTTCATTCTTCAGACG	125	60	NM_008071.3
	R- AGCACGGTGGTAATCCCAAGG		61	
GABRG1	F- CATGGATGAACATTCCTGTCCAC	183	60	NM_173536.3
	R- TAATCCCCAGAGATCGTGTGAG		59	
GABRG2	F- ACAGGAAGCTCAGTCTACTCG	116	59	NM_198904.2
	R-TCATAGTCATCATCAGATTTCTGGC		59	
GABRG3	F- TCCTCAAGATGGATTCCTGAGC	153	59	NM_033223.4
	R- ATCCAGACACTCATAGACACAGG		59	

Table 2.4. Continued:

Target	Sequence 5' to 3'	Amplicon (bp)	Tm (°C)	Accession number
GABRP	F-TTCTGGAAACCTATGTCCCTTCC	100	61	NM_146017.3
	R- ACTCCAATGCAGGTTCTCGCAG		61	
YWHAZ	F- ATCCTGAACTCCCCAGGAAAGC	178	61	NM_011740.3
	R- CTGCTTCTGCTTCATCTCCTTGG		61	
TOP1	F- ATGGTAAAGTTATGAAGCTGAGTCC	196	59	NM_009408.2
	R- TACTGGCTCATCTGTGTAAAGTCG		60	
CYC1	F- TTGGACCACACCAGCATTCG	125	58	NM_025567.2
	R- TTGGCTTCTTCCTCCGTGTAG		59	

2.10 Quantitative polymerase chain reaction (qPCR)

qPCR was employed to profile $GABA_AR$ subunit and CCC gene expression in healthy human uterus, endometrial cancer, pregnant mouse uterine tissues and COLO684 cells.

The PrimerDesign™ PrecisionPLUS™ Master mix gene detection kit was used. Each reaction contained, a final concentration of 6 pmol forward and reverse oligonucleotide, 25 ng cDNA template, DEPC-treated water and 10 µL precisionPLUS/ PrecisionFast to give final volume of 20 µL. Positive controls to test the oligonucleotide specificity and reaction conditions included human or mouse/rat brain cDNA. Each reaction was completed in triplicate wells alongside a non-RT and no-template negative control. The samples were prepared on BrightWhite 96 well plates (PrimerDesign™, UK) and sealed with MicroAmp™ optical adhesive film (Applied Biosystems,UK) . Amplification and quantification was carried out on the Step One Plus™ (Applied Biosystems,UK), thermocycler conditions were set at initial denaturation 95°C for 10 minutes followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. The one melt curve cycle was set to 95°C for 15 seconds 60°C for seconds followed by a further 95°C for 15 seconds.

After each reaction, a melt curve was conducted at 60^{o}C increasing to 95^{o}C with 0.3^{o}C increment with a final 15 second hold step to verify oligonucleotide specificity and to confirm amplification of a single product of the amplified transcript. The qPCRs were all analysed on Step One Plus Applied Biosystems machine (Foster City, United states).

Before analysis of the genes of interest, a GeNorm experiment was completed to identify suitable reference genes for the individual experiments. The genes involved in this analysis were: BM2, YWHAZ, EFI4A2, TOP1, BIRF2, CYC1, SDHA, UBC, ATP5, EIFRAZ, RPLIZA, 18S, GAPDH, (available on the Primer Design website) EIF2B1 was also designed as a uterus specific reference gene (Almeida *et al*., 2014). The oligonucleotides were resuspended in 220 µL following the primer design protocol. These samples were prepared in the same way as The PrimerDesign™ PrecisionPLUS^{TM} Master mix.

The qRT-PCR data was analysed by using the $2^{\Delta\Delta}$ method (Pfaffl, 2004) and data was analysed using SPSS for statistical significance using a three way repeated measures ANOVA and Tukey *post hoc* analysis (Livak and Schmittgen, 2001).

2.11 Analysis of protein expression in COLO684 cells

Following cell harvest, total protein was extracted from APα-treated COLO684 cells in preparation for immunoblotting analysis of $GABA_AR$ π subunit and CCC protein expression. The extraction buffer comprised (50 mM Tris pH8, 150 mM NaCl, 1 mM EDTA, 1% (w/v) Triton X and 1% (v/v)) halt protease inhibitor cocktail (Thermo Scientific, UK). The volume of the cell pellet was estimated and approximately 3x the pellet volume of ice cold lysis buffer was added to each pellet. The pellet was thoroughly resuspended by pipetting and lysed by incubating on ice for 30 minutes. Lysate was clarified by centrifugation at 16000 x *g* for 5 minutes at 4^{o}C in benchtop centrifuge. The supernatant was transferred into sterile 1.5 ml microfuge tubes and stored at -80^{o}C until further analysis.

2.11.1 Protein quantification

A Bradford assay was carried out to determine the extracted protein concentration from the treated COLO684 cells. Protein samples (1 µL) were diluted 1:5 in dH_2O and mixed with 200 µL of Bradford reagent (100 mg/ L Coomassie blue G250, 95% (v/v) ethanol, 85% (w/v) H_3PO_4) and added in triplicate to a 96-well plate. Stock bovine serum albumin (BSA) was prepared in dH_2O to a final concentration of 10 mg/ mL BSA standards were prepared by diluting the stock in dH_2O to final concentrations of 0.15, 0.25, 0.5, 0.75, 1.0 and 1.5 mg/ml and 1 µL of each standard was added per well, in triplicate. The plate was incubated at room temperature for 5 minutes. The absorbance at 595 nm was measured using the LT-50000MS ELISA READER (Thermo scientific, Waltham Massachusetts, USA) and the protein concentration was calculated from the standard curve generated from the BSA concentration standards.

2.11.2 Sample preparation

Protein samples were prepared to a final concentration of 1 µg/ µL in 4 x loading sample buffer (20% (v/v) glycerol, 200 mM Tris pH 6.8, 4% (w/v) sodium dodecyl sulfate (SDS), 10 mM EDTA bromophenol blue, 100 µL β-mercaptoethanol). Protein samples were heated at 95^{o}C using a heat block for 5 minutes then cooled on ice before being loaded onto the sodium dodecyl sulphate - polyacrylamide gel (SDS-PAGE).

2.11.3 Sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed following the method of Laemmli (1970) using the Biorad mini-PROTEAN® Tetra system (Bio Rad, UK). The resolving gel consisted of 12% (w/v) acrylamide, 375 mM Tris pH 8.8, 0.1% (w/v) SDS, dH_2O and 0.08% (w/v) ammonium peroxidosulphate. The stacking gel consisted of 5.1% (w/v) acrylamide, 0.125 M Tris pH 6.8, 0.1% (w/v) SDS, dH_2O and 0.12% (w/v) ammonium peroxidosulphate. The gels were polymerised by the addition of 5 µL N,N,N',N',-tetranethyl-ethylenediamine (TEMED) to the resolving gel and 10 µL

added to the stacking gel. Samples were electrophoresed at 185 V for 50 minutes in running buffer (25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS, pH 8.3), and compared to the PageRule™ Prestained protein ladder (Thermo Scientific USA),

2.11.4 Immunoblotting

Immunoblotting analysis was carried out to analyse expression of the $GABA_AR$ π-subunit and the CCCs at protein level in response to allopregnanalone treatment. Following SDS-PAGE, proteins were transferred onto nitro-cellulose membrane (GE healthcare, life sciences, Amersham, UK) by electrophoresis using the BioRad mini-PROTEAN® Tetra system. The electrophoresis was conducted in transfer buffer (25 mM Tris, 192 mM glycine, 20 % methanol) with an ice pack to prevent overheating, at 100 V for 1 hour, following the method of Towbin et al., (1979).

In order to check equal loading and even transfer, membranes were stained with India ink. Ink stain was prepared with a 1:2 dilution of Indian ink in dH_2O and membranes were stained at room temperature for 10 minutes with gentle shaking (50 rpm). The membrane was then washed with dH_2O and blocked with 5% (w/v) dried milk powder in phosphate buffered saline Tween 20 (PBS-T, 0.05M phosphate buffer, 0.0135 M potassium chloride, 0.685 M sodium chloride, 1 mL Tween 20) for 1 hour at room temperature with gentle shaking (50 rpm). After blocking, the primary antibody (π rabbit polycolonal ab26055 (Abcam, UK), β actin (C-11) goat polyclonal (Santa Cruz Biotechnology,INC, Germany), NKCC1 (C-14: c-21547) goat polyclonal (Santa Cruz Biotechnology,INC, Germany), KCC3 rabbit polyclonal (Abgent, INC, San Diego, USA) and KCC4 rabbit polyclonal (Abgent, INC, San Diego, USA) were diluted 1:1000 and 1:200 respectively in 5% (w/v) Marvel milk powder, added to the membrane and incubated overnight at 4^{o}C. Primary antibody was removed and membrane was washed with 3 x 5 minute washes in PBS-T, secondary antibody (Goat anti rabbit and donkey anti goat, Odyssey, LI-COR® Biosciences, UK) were diluted in 1:10000 in PBS-T plus 0.01% (w/v) SDS, was incubated with the membrane for 45 minutes at room temperature while protected from light. After another 3 x 5-

minute washes in PBS-T the membrane was analysed on the Odyssey® Li-cor (Cambridge, UK) using the programme Image studio version 2.0.

2.12 Statistical analyses

ANOVA and Tukey *post hoc* statistical analysis were performed on quantified data, and $P<0.05$ was set as the level of significance.

Chapter 3. Results

3.1 Analysis of $GABA_AR$ gene expression in healthy human uterus

The main aim of this study was to identify the potential presence of the $GABA_ARs$ and CCCs in healthy human uterus by investigating gene expression. The expression was initially analysed using qRT-PCR. Current research suggests that the $GABA_A$ receptor π subunit is highly expressed in the uterus (Fujii and Mellon, 2001) and in order to form a functional receptor, other subunits must also associate with the π subunit. This study wanted to examine whether the $GABA_A$ receptor π subunit potentially forms a $GABA_AR$ in the uterus and subsequently, if there may be different $GABA_ARs$ subtype present in the uterus compared to the CNS (which does not express π-containing receptors).

For the first time, this study quantified complete $GABA_AR$ subunit expression in healthy human uterus (n=2, refer to Table 3.1). The first sample of healthy uterus was analysed and the data showed moderate expression of the $GABA_AR$ α2 and α3 subunits, weak expression of α4 and α5 subunits and interestingly, α1 and α6 subunit transcripts were not detected. When analysing beta subunit gene expression, the β1 and β3 subunit genes were well expressed; although the β2 subunit was detected, this was at a much lower level (Table 3.1). The $GABA_AR$ gamma subunit genes also displayed varying levels of expression in human uterus with the γ1 subunit having higher expression when compared to the γ2 and γ3 subunit genes. The study indicated robust expression of the π subunit in the healthy human uterus.

After the initial findings, the second sample of healthy human uterus was analysed for confirmation of $GABA_AR$ subunit expression. The data from the second sample of healthy uterus showed similar findings, with the alpha subunit genes, moderate expression of the α2 and α3 subunit genes and weak expression of the α4 and α5 subunits. Again, the α1 and α6 subunit transcripts were not detected (Table 3.1). The data does however differ when comparing the beta and gamma subunits. β1 and β2 were undetected and β3 showed weak expression. The γ subunits data appeared to show low levels of γ3 whilst γ1 and γ2 were undetected. The different isoforms differ with the expression level and

pattern between the two uterine samples however, interestingly, both samples showed high expression of the π subunit gene (refer to Table 3.1). Expression of the $GABA_A$ δ subunit gene was also analysed in healthy uterus, however it was not detected; ε and θ were not detected. The primary data shows that the $GABA_AR$ subunit genes are expressed in the uterus. The combination of receptor subunits expressed differs from that of the CNS (Bracamontes and Steinbach, 2008) with π being highly expressed, potentially indicating different $GABA_AR$ receptor subtypes may be present in the uterus.

3.2 Analysis of $GABA_AR$ gene expression in human endometrial cancer

As $GABA_AR$ subunit gene expression was identified in healthy human uterine tissue, the study then analysed the possible changes in gene expression of $GABA_AR$ subunits in endometrial cancer using qRT-PCR. The two case studies were used for confirmation of gene expression. Within both of the endometrial cancer samples the results showed variation in gene expression (refer to Table 3.1). Within both endometrial cancer samples the $GABA_AR$ α subunits were varied with the exception of α2 which was expressed in both endometrial cancers as well as human healthy uterus. α1 and α6 were not detected in endometrial cancer or in human healthy uterus. $GABA_AR$ subunits α4, α5 were detected in healthy uterus but not both of the endometrial cancer samples (Refer to Table 3.1). Interestingly α3 expression appear to vary, the expression is noted in healthy human uterus and in the first endometrial cancer sample, however the expression was not detected in the second sample of endometrial cancer disagreeing with the Liu *et al*. (2009) study. When analysing the β subunit genes the data shows expression of β2 and β3 in both endometrial samples, however in sample 2 β2 was not expressed in healthy uterus. $GABA_AR$ subunit β1 expression was not detected in both endometrial cancer but was detected in sample 1 healthy human uterus although, not in sample 2 of healthy human uterus. The most variation between the $GABA_AR$ subunits was detected within the γ subunits, in sample 1 of healthy human uterus all γ subunits were expressed when compared to endometrial cancer only γ3 was expressed. However in sample 2 healthy human uterus only γ3 was expressed, γ1 and γ2 were undetected, the expression patter changed in sample 2 of endometrial cancer as

the only γ subunit not detected was γ3. The π subunit gene was expressed in human healthy uterus as well as uterine cancer. In human brain the π subunit is not expressed, but is documented to be expressed in smooth muscle tissue (Fujii and Mellon, 2001). The data herein correlates with these findings. The δ, ε and θ subunit genes were also studied however, no expression was detected in both endometrial cancers.

In summary, when comparing both endometrial cancer samples, there was a variation in $GABA_AR$ subunit gene expression patterns. In the endometrial cancer samples the consistent $GABA_ARs$ were α2, β2 and β3 and π (refer to Table 3.2). The data indicate the presence of $GABA_ARs$ in uterine cancer, the subunit gene expression was different to that of healthy uterus, indicating a possible change in expression, which may play a role in endometrial cancer. The differences in subunit gene expression between the two cancer samples may be due to the natural heterogeneity of cancer between different patients (Hanahan and Weinberg, 2011).

Table 3.1. Analysis of expression of selected GABAAR subunit genes in two healthy human uterus and matched endometrial cancer samples Expression of $GABA_AR$ subunit genes was assessed by qRT-PCR and normalised to expression of the housekeeper gene, EIF2B1. Relative levels of expression in healthy uterus are represented by +: low; ++: relative; +++: strong; ND: not detected. Arrows represent an increase (↑) or decrease (↓) in gene expression in uterine cancer relative to healthy uterus.

$GABA_AR$ subunit	Gene expression (Sample one)		Gene expression (Sample two)	
	Healthy	Cancer	Healthy	Cancer
α1	ND	↓	ND	↓
α2	++	↑	++	↑
α3	++	↑	++	↓
α4	+	↓	+	↓
α5	+	↓	+	↓
α6	ND	↓	ND	↓
β1	++	↓	ND	↓
β2	+	↑	ND	↑
β3	++	↑	+	↑
γ1	++	↓	ND	↑
γ2	+	↓	ND	↑
γ3	+	↑	+	↓
π	+++	↑	+++	↑
δ	ND	↓	ND	↓
ε	ND	↓	ND	↓
θ	ND	↓	ND	↓

3.3 Analysis of CCCs gene expression in healthy human uterus

Current studies show that the expression of chloride co-transporter (CCC) have a direct effect on $GABA_A$ R function (Ben-Ari, 2002). Therefore, gene expression of the CCC family members was also investigated in healthy uterus using qRT-PCR, the two healthy human uterus samples analysed in this study showed the same CCC gene expression profiles. The NKCC1 transcript had the highest expression in both samples of heathy uterus (refer to Table 3.2) and KCC4 gene expression was moderate when compared to NKCC1. The other CCCs, KCC1 and KCC3 both had weak expression (refer to Table 3.2). In both samples, transcripts encoding NKCC2 or KCC2 were not detected; this was anticipated as both are tissue specific to the brain and kidney respectively (Blaesse *et al*., 2009; Delpire, 2000).

3.4 Analysis of CCC gene expression in endometrial cancer

Once CCC gene expression had been established in healthy tissue, further analysis was undertaken to investigate the possible changes in gene expression in endometrial cancer. The CCC expression in both batches of endometrial cancer had the same expression profile (refer to Table 3.2), although interestingly, the expression pattern does not differ from healthy uterus. NKCC1, KCC1, KCC3 and KCC4 were all expressed in healthy and in both endometrial cancer samples.

This data show that selected CCC genes were expressed in human uterus in both healthy and cancerous samples. Overall there appeared to be an increase in CCC expression in endometrial cancer. The expression of CCCs in the uterus, which is different to that in the CNS, supports the hypothesis that the CCC gene expression could influence the function of the $GABA_A$Rs in the uterus. The correlation in change in gene expression between $GABA_A$Rs and CCC has been shown in the developing CNS.

Table 3.2. Analysis of expression of selected CCCs genes in two healthy human uterus and matched endometrial cancer samples Expression of CCC targets was assessed by qRT-PCR and normalised to expression of the housekeeper gene, EIF2B1. Relative levels of expression in healthy uterus are represented by +: low; ++: relative; +++: strong; ND: not detected. Arrows represent an increase (↑) or decrease (↓) in gene expression in uterine cancer relative to healthy uterus.

CCC Target	Gene expression (Sample one)		Gene expression (Sample two)	
	Healthy	Cancer	Healthy	Cancer
NKCC1	+++	↑	+++	↑
NKCC2	ND	↓	ND	↓
KCC1	+	↑	+	↑
KCC2	ND	↓	ND	↓
KCC3	+	↑	+	↑
KCC4	++	↑	++	↑

3.5 Protein expression of the $GABA_AR$ π subunit in healthy human uterus and endometrial cancer

Once robust $GABA_AR$ π subunit gene expression was found in healthy and endometrial cancer, the next stage was to identify whether it was possible to detect the π subunit at the protein level. Immunoblotting was performed to analyse the protein expression in healthy uterus and endometrial cancer. The immunoblot results were compared to β–actin. Expression of the $GABA_AR$ π subunit polypeptide in healthy uterine tissue was not detectable as no band could be observed at 50 kDa; the molecular weight of the π protein. Whilst in endometrial cancer, π subunit protein expression was apparent with a high intensity band appearing at 50 kDa (refer to Figure 3.1). Interestingly, this did not correlate with the mRNA data, which indicated π expression in both healthy and endometrial cancer samples.

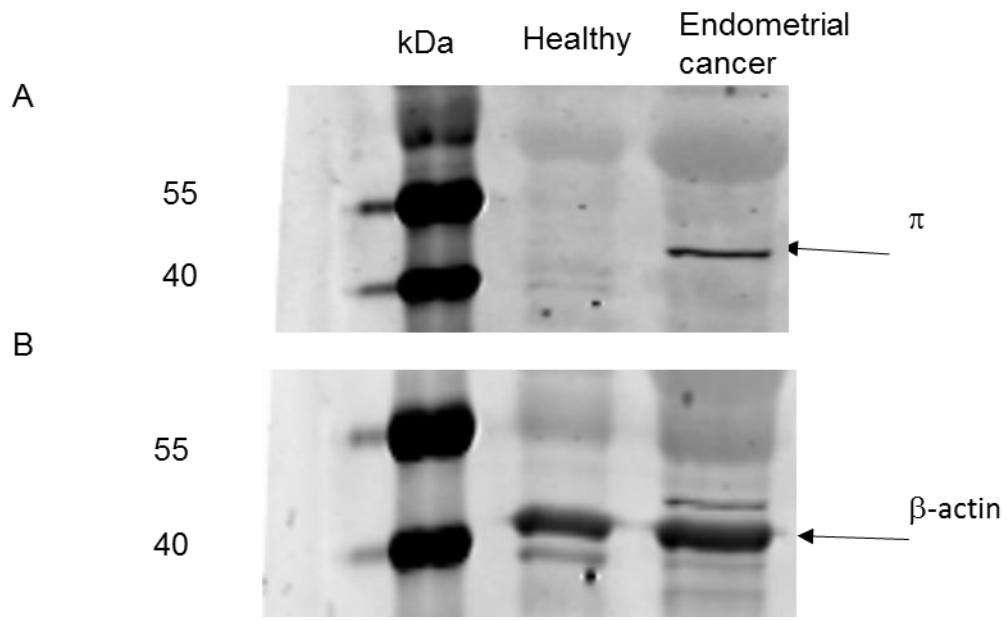

Figure 3.1 Protein expression of the $GABA_AR$ π subunit in human healthy uterus and endometrial cancer. A: Immunoblot showing π subunit polypeptide expression human endometrial cancer. **B**: 12% Immunoblot of the control β-actin.

3.6 Alamar blue toxicity assay for allopregnanalone (APα) treatment on COL684 cells

The research to date has shown APα can alter $GABA_AR$ subunit expression and only receptors comprising the π subunit are sensitive to APα (Fujii and Mellon., 2001, Hedblom and Kirkness, 1997). This work identified the presence of $GABA_AR$ subunits, notably the π subunit, and CCCs in the uterus, therefore, the next step was to investigate the effect of APα in the uterus. An *in vitro* model was developed using COLO684 cells to analyse the effect of APα on gene and protein expression of $GABA_AR$ subunits and CCCs. Initially, an Alamar Blue assay was performed to determine if the concentrations and the exposure times of the vehicle DMSO and APα were toxic to the cells (refer to Figure 3.2). Statistical analysis was performed and no statistical significance between the different concentrations and exposure time were observed. This therefore showed that exposure to APα was not toxic to COLO684 cells.

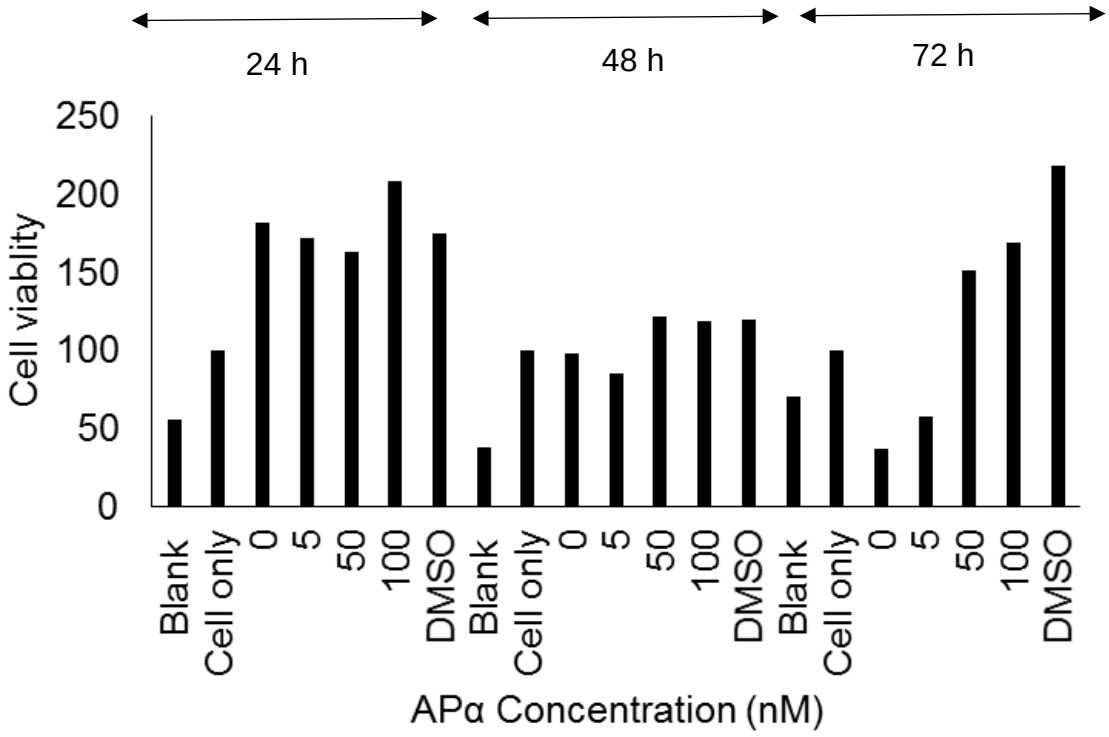

Figure 3.2 Analysis of potential APα toxicity via Alamar blue. Cells were incubated with 5 nM, 50 nM and 100 nM concentrations of APα for 24, 48 and 72 hours. Absorbance was read at 595 nm wavelength and normalized to 600 nm. Untreated cells were taken to be 100 % viable and data was expressed as a percentage of untreated cells; n=3

3.7 The effects of APα treatment on π expression in COLO684 cells

APα is a metabolite of progesterone, which, has an important functional role in inhibiting uterine contractions during the gestational period. During the gestation period, APα fluctuates depending on the stage of gestation (Hill *et al.*, 2001). The study aimed to analyse the effect of physiological concentrations of APα on the π subunit protein level, as several studies have indicated the π subunit affects the sensitivity of $GABA_AR$s to APα during pregnancy (Fujii and Mellon, 2001). In particular, AP levels found in the uterus of premenstrual women (2 nM), menstruation cycle (5 nM), third trimester (75 nM, and 150 nM), pre labour (50 nM) and labour (25 nM) were examined Beverley *et al.*, 2001; Luisi *et al*, 2000; Hill *et al.*, 2001and Parizek *et al.*, 2005).

The initial assay was developed by treating COLO684 cells with APα concentrations of 2 nM, 5 nM, 25 nM, 50 nM, 75 nM, 100 nM, 150 nM and 200 nM. Immunoblotting was then performed to analyse π subunit protein expression. The initial assay data showed that $GABA_AR$ π subunit was expressed at low levels in COLO684 cells as faint bands were identified at 50 kDa (refer to Figure 3.3 A). π subunit expression was highest at 25 nM APα, (refer to Figure 3.3 A and B). π subunit expression at 5 nM and 75 nM was moderately increased; at 50 nM and 2 nM there was weak expression of the π protein. However, this data may not be fully representative of expression as the assay exposure time was 24 h, and the $GABA_AR$ subunit polypeptide half-life is reported to be 25 h (Lyons *et al*., 2000).

After the primary assay was conducted, alterations were made to increase the exposure time to 72 h to account for the $GABA_AR$s protein half-life. The APα concentrations 2 nM and 200 nM were removed to increase accuracy of the assay as 200 nM is not a true representation of a physiological concentration (Parizek *et al.*, 2005). COLO684 cells were treated with APα concentrations of 5 nM, 25 nM, 50 nM, 75 nM, and 150 nM. The immunoblot appeared to have faint bands at 50 kDa running across the range of APα concentrations 0 nM, 5 nM, 25 nM, 50 nM, 75 nM, and 150 nM (refer to Figure 3.3 C and D). Unspecific binding

of the π antibody,also occurred in the secondary assay. These bands can be noted in all samples at approximately 80 kDa. The data taken from both assays shows that π subunit is expressed in the COLO684 cells but appears unaffected by exposure to APα at physiologically-relevant concentrations.

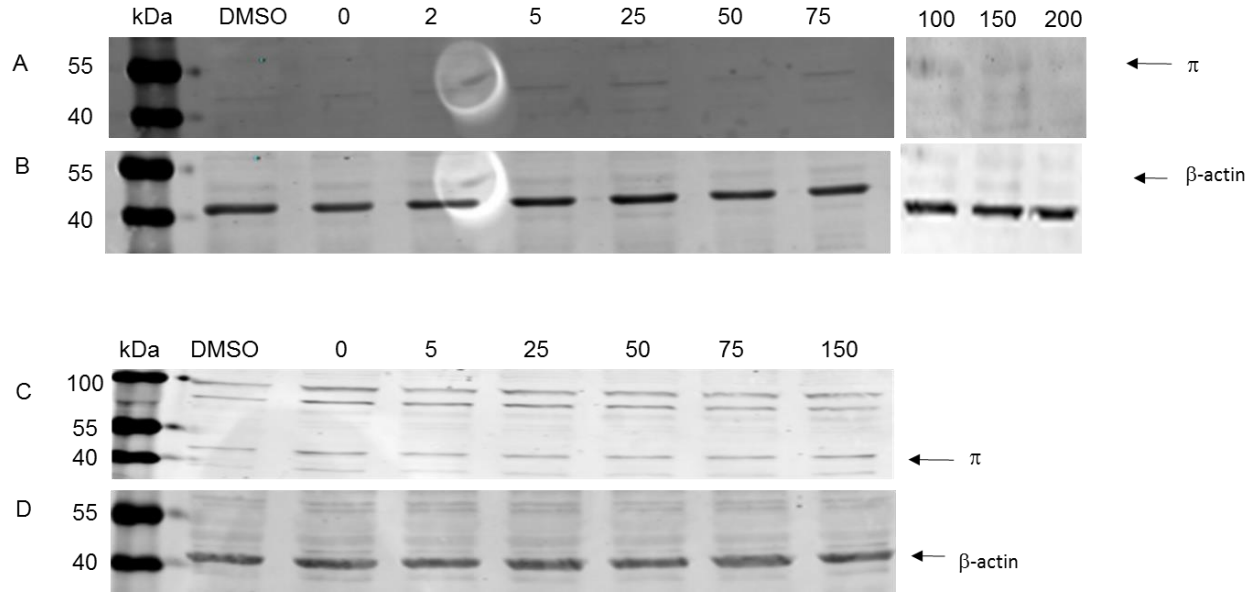

Figure 3.3 Analysis of the effect of APα on GABA$_A$ receptor π subunit expression. A: π expression in COLO684 cells when treated with APα 0- 200 nM for 48 h, 30 ng loaded on a 12% gel. **B:** Control β-actin 30 ng loaded to a 12% gel. **C:** π expression in COLO684 cells when treated with APα 0- 150 nM for 72 h, 30 ng loaded on a 8% gel D) Control β-actin 30 ng loaded to a 12% gel.

3.8 APα affect on GABA$_A$R subunit gene expression

After the primary and secondary assays were conducted as described in section 2.5, alterations were made to increase the APα exposure time to 48 h to account for the protein half-life. COLO684 cells were treated with 5 nM, 50 nM and 100 nM APα. The range of APα concentration was to account for varying reports physiological concentrations, while also accounting for the changes in data obtained from the primary and secondary assays (data shown in Figure 3.3 A,B,C and D).

The $GABA_AR$ subunits analysed in COLO684 cells treated with 5 nM, 50 nM and 100 nM APα for 48 h, were α1, α2, α5, β2, β3, γ3. π and δ. α1, α2, β2, γ3 and π were undetectable in COLO684 cells (refer to Table 3.3). These results are contradictory, as the π subunit increases receptor sensitivity to APα. The α1 and α2 subunits have also been reported to change in expression when exposed to APα (Brussaard *et al.,* 1997).

The $GABA_AR$ α5 subunit showed a decrease in expression at 5 nM, however the highest expression of α5 transcript appeared to occur at 50 nM APα and 100 nM APα with both showing a 6-fold increase (refer to Figure 3.4 A). The δ subunit was not detected in healthy or cancerous uterine samples, but it was detected in COLO684 cells. Following 48 h exposure to APα, the data shows lowest expression occurring at the 100 nM, and the highest expression occurring at 50 nM showing a 4-fold increase (refer to Figure 3.4 B). The β3 subunit was also identified in the COLO684 cells (refer to Figure 3.4 C), which correlated with robust expression in the human cancerous samples. The expression profile in response to APα treatment was similar to both α5 and δ.The highest expression of β3 subunit was at 50 nM APα showing a 3-fold increase, the lowest expression was noted at 5 nM APα.

ANOVA and Tukey *post hoc* statistical analysis tests showed no significant difference in treatments between DMSO, 5 nM, 50 nM and 100 nM APα. The primary data indicate that APα does not have an effect on the α5, δ and β3 gene expression.

Table 3.3 Gene expression of the selected $GABA_AR$ subunits in APα treated COLO684 Cells. Expression of the different $GABA_AR$ subunits (n=3) was assessed by qRT-PCR and normalised to expression of the GAPDH and ATP5B reference genes. Expression represented by +: expression of $GABA_AR$ subunits; ND: not detected and NA: not assessed

$GABA_AR$ subunits	Expression in treated APα COLO684 Cells N3
α1	ND
α2	ND
α5	+
α6	+
β2	ND
β3	+
γ3	ND
π	ND
δ	+
ε	NS
θ	NS

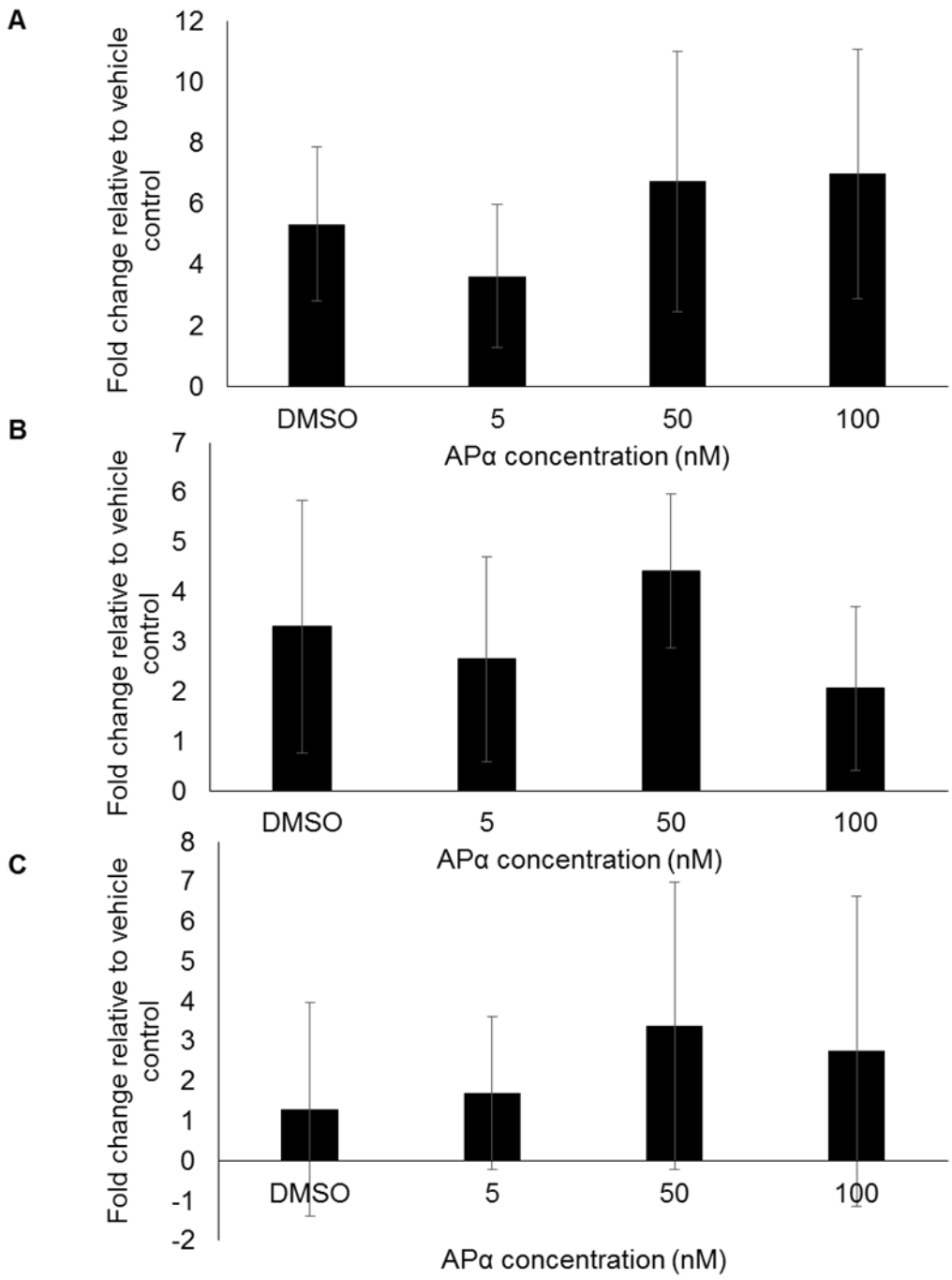

Figure 3.4 Change in GABA$_A$R α5, δ and β3 subunit gene expression in COLO684 cells treated with different concentrations of APα. GABA$_A$R subunit gene expression was assessed in COLO684 cells treated with 5, 50 and 100 nM APα for 48 h via qRT-PCR and normalised to the GAPDH and ATP5B reference genes. Data was expressed as a fold change in gene expression relative to the vehicle control (DMSO). **A**: α5 subunit gene; **B**: δ subunit gene; **C**: β3 subunit gene.

3.9 APα effect on CCCs gene expression

This study also looked at the possible change in expression of the CCCs to help identify the first evidence of a potential GABA switch/change in chloride gradient in uterine cells (Ganguly *et al*., 2001) at different APα concentrations relevant to the gestation period. COLO684 cells were treated with 5 nM, 50 nM and 100 nM APα for 48 h and analysed using qRT-PCR.

NKCC1 (refer to Figure 3.5 A) showed a decrease in expression at 5 nM, however the highest expression of NKCC1 transcript appeared to occur at 50 nM APα and 100 nM APα with both showing a 4-fold increase. KCC1 data collected (refer to Figure 3.5 B) showed no change in KCC1 gene expression at 5 nM APα with a slight increase in expression occurring at 50 nM APα. However KCC1 expression was noted to decrease at 100 nM. The results show KCC1 is expressed in COLO684 cells at low levels, and when APα is added gene expression is reduced, although these changes were not significant. KCC3 data shows varying expression when treated with APα,(refer to Figure 3.5 C).The highest expression appear at 5 nM with a 3 fold change. High expression was noted at 100 APα, with the lowest expression of KCC3 occurring at 5 nM. KCC4 expression (refer to figure 3.5 D) was shown to be highly expressed at 100 nM with a 4 fold change, the lowest expression appears at 5 nM, however the DMSO shows high expression of KCC4 so is hard to determine whether it is a APα or drug vehicle causing the effect.

ANOVA and Tukey *post hoc* statistical analysis tests showed no significant difference in treatments between DMSO, 5 nM, 50 nM and 100 nM APα. The primary data indicate that APα does not have an effect on NKCC1, KCC1, KCC3 and KCC4 gene expression.

These initial results show that many of the $GABA_A$ receptor subunit genes and the CCC genes are expressed in uterine cells and that the expression of a number of these genes is not affected by APα at physiologically-relevant concentrations. This provided the first evidence that during gestation, there is the potential for $GABA_A$ receptor and CCC gene expression to be regulated in response to other steroid hormones.

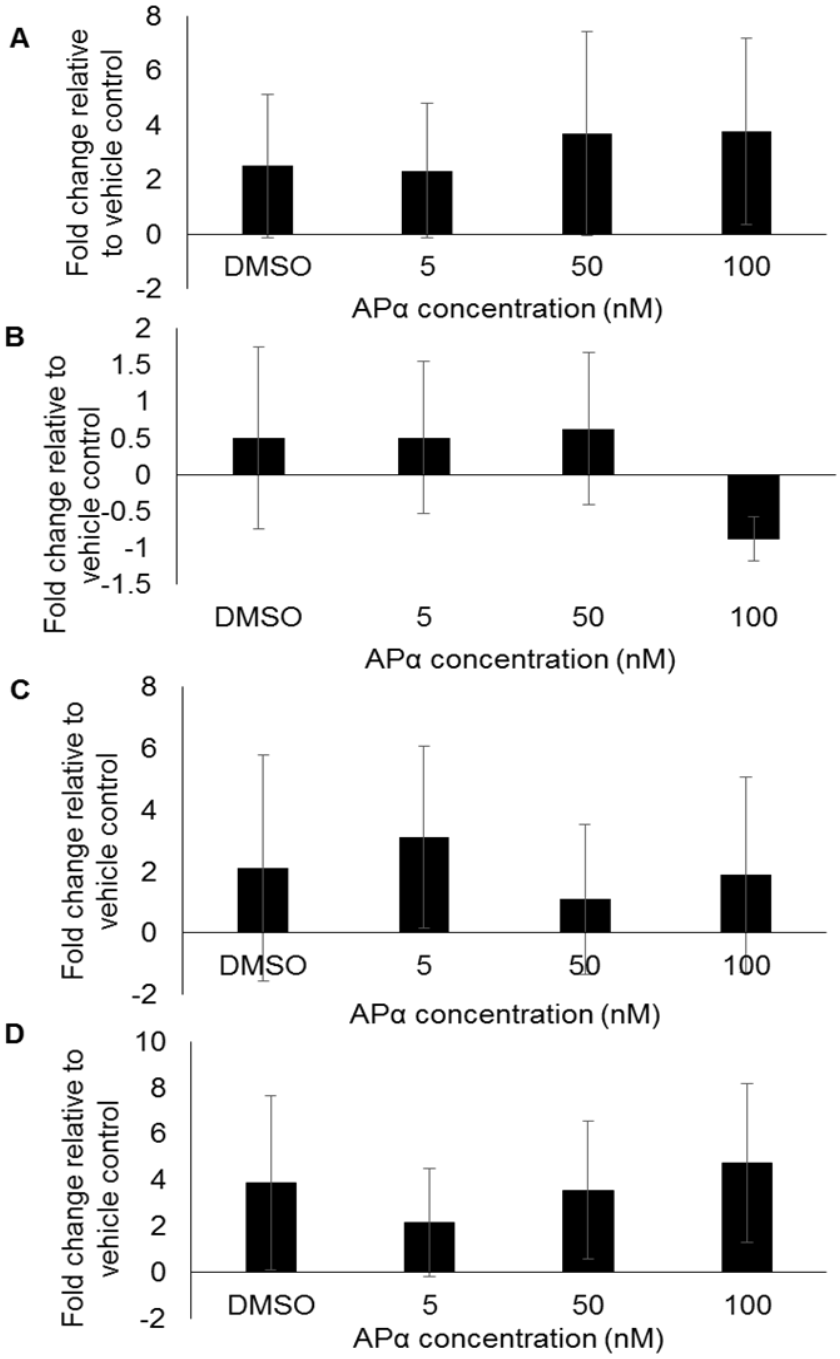

Figure 3.5 Change in CCC gene expression in COLO684 cells treated with APα. CCC expression was assessed by qRT-PCR in COLO684 cells treated with 5 nM, 50 nM and 100 nM APα for 24h, 48 h and 72 h. Expression was normalised to GAPDH and ATP5B reference genes. Data was expressed as a fold change in gene expression relative to untreated control. **A**: NKCC1; **B**: KCC1; **C**: KCC3 **D**: KCC4.

3.10 APα effect on $GABA_AR$ subunit protein, and CCCs protein expression

Once the $GABA_AR$ and CCC gene expression was analysed by qRT-PCR the next stage of the study was to analyse the change in π protein level in APα treated COLO684 cells by immunoblotting. There was variation noted between the triplicate treated COLO684 cells with APα (refer to Figure 3.6 A). The first immunoblot illustrates π protein is present in untreated COLO684 cells. π protein bands were also detected in the presence of 5 nM, 50 nM and 100 nM APα as well as in the DMSO control and the positive control of endometrial cancer. The bands are, however, located at 100 kDa rather than 50 kDa indicated in the antibody datasheet. In the second blot (refer to Figure 3.6 B) faint bands of π expression are detected at 5 nM, 50 nM and 100 nM APα as well as the untreated control, DMSO and endometrial cancer. These bands are detected at the correct size of 50 kDa, however unspecific binding can also be noted in all lanes at the 42 kDa mark. The third blot (refer to Figure 3.6 C) shows π expression in endometrial cancer, however no other signs of the π protein at any of the times or APα concentrations tested. These results show that unfortunately, the π protein data was unreliable due to varying results with the immunoblots. Immunoblotting with actin, indicates equal loading of the protein across the membrane (refer to Figure 3.6 D).

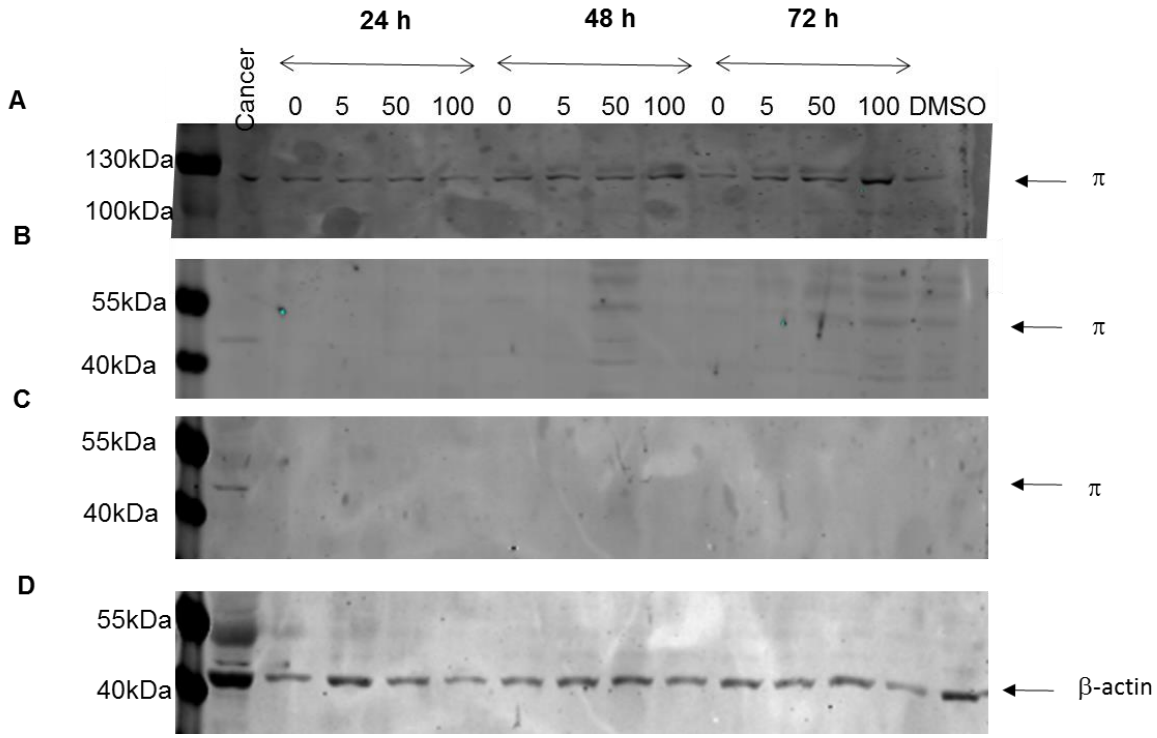

Figure 3.6 Analysis of the effect of APα on π protein expression in COLO684 cells. COLO684 cells were treated with APα 0-100 nM for 24 h, 48 h and 72 h and π protein expression was analysed by immunoblotting. Thirty micrograms of lysate were resolved via 8% SDS-PAGE gel and transferred to nitrocellulose. Three individual replicate experiments are shown (**A**-**C**). β-actin immunoblot is shown in **D**.

3.11 APα affect on CCCs protein expression

The CCC protein expression was analysed using immunoblotting to identify any possible changes in protein expression when treated with APα. The first CCC member to be analysed was NKCC1, which was expected to have a molecular weight of 130 kDa to 180 kDa (Santa Cruz Biotechnology, INC, Germany). The initial 72 h exposure time showed expression of NKCC1 with a band detected at 130 kDa however, there appeared to be no induction of NKCC1 with 5 nM, 25 nM, 50 nM, 75 nM, or 150 nM APα (refer to Figure 3.7 panel 1 A,B). In the second replicate blot, low expression of NKCC1 was observed with a slight induction at 72 h 5 nM and 50 nM APα (refer to Figure 3.7 panel 2 A,B). The results show that NKCC1 protein expression may be affected by APα.

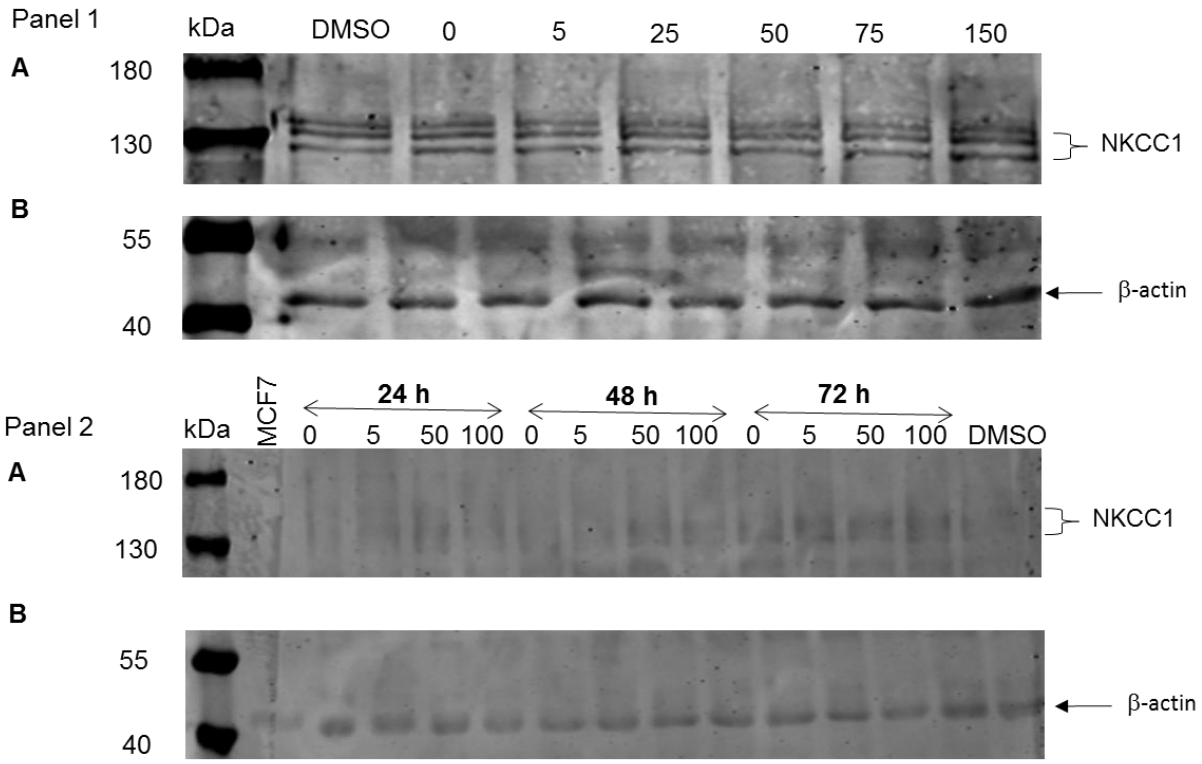

Figure 3.7 Analysis of the effect of APα on NKCC1 expression. NKCC1 expression was analysed in COLO684 cells treated with APα 0 - 150 nM for 72 h. Thirty micrograms of lysate were resolved via 8% SDS-PAGE gel and transferred to nitrocellulose. Membranes were probed with antibodies towards NKCC1 (panel 1 and 2, **A**) or β actin (panel 1 and 2, **B**) indicated by arrows.

3.11.1 APα effect on KCC3 protein expression

KCC3 protein expression in COLO684 cells was also analysed. KCC3 has an expected molecular weight of 127 kDa and the results indicated induction of KCC3 protein expression at 24 h in the presence of 5 nM, 50 nM and 100 nM APα. KCC3 was also induced at 72 h in the presence of 5 nM, 50 nM, 100 nM APα (Figure 3.8 A) however induction was also observed when COLO684 cells were treated with DMSO (refer to Figure 3.8 A).There is also no sign of induction during the 48 h exposure time (Figure 3.8 A). The data indicates a change in KCC3 protein expression with longer exposure to APα increasing the protein expression. Although the antibody used does show unspecific binding to the membrane. The non-specific binding occurs at 150 kDa, 129 kDa and 75 kDa and the intensity of the unspecific bands does not appear to change depending on the APα concentration or the exposure time.

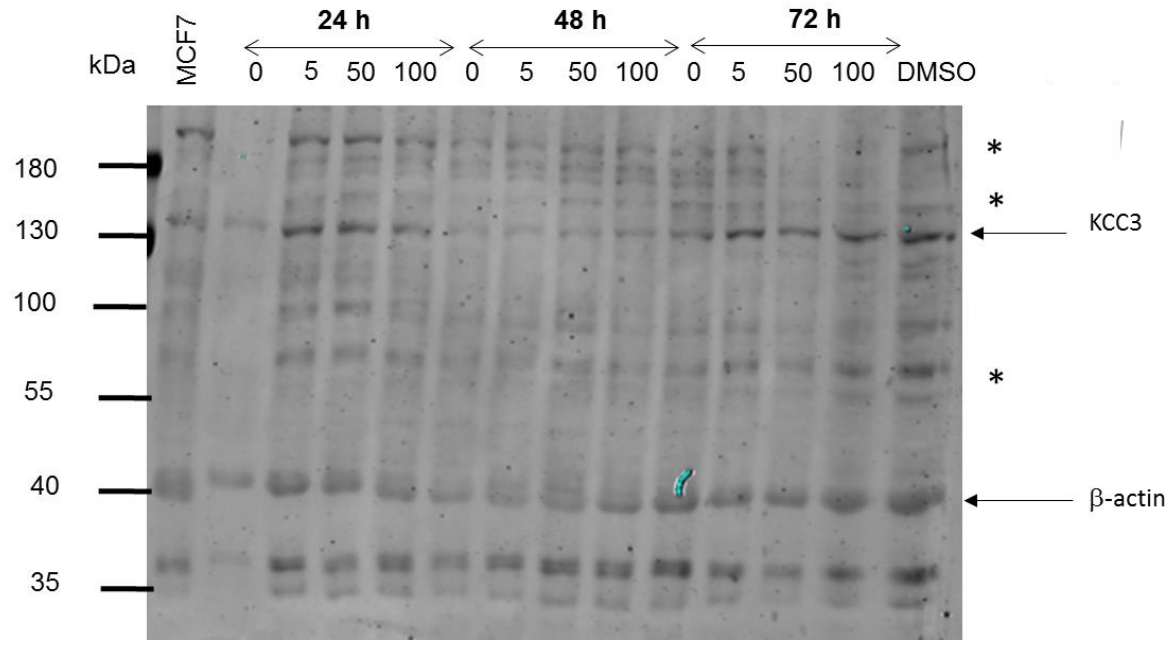

Figure 3.8 Analysis of the effect of APα on KCC3 expression. KCC3 expression was analysed in COLO684 cells treated with 0-100 nM APα for 24 h, 48 h and 72 h. 30 µg was loaded on a 8% SDS-PAGE and transferred to nitrocellulose. Membranes were probed with antibodies towards KCC3 or β actin indicated by arrows.

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3.11.2 APα effect on KCC4 protein expression.

KCC4 has an expected molecular weight of 100 kDa and a faint band representing KCC4 was detected (Figure 3.9 A). There appeared to be induction occurring with 100 nM APα exposure for 72 h (Figure 3.9 A). The antibody used shows unspecific binding at 180 kDa, 130 kDa and 75 kDa. However, the intensity of the non-specific bands does not change depending on the APα concentration or the exposure time. The immunoblot data of the CCCs may show a change in CCC protein expression depending on the APα concentration in accordance with the exposure time.

The change in protein expression potentially show that $GABA_A$Rs have an excitatory or inhibitory effect during the gestational period however the exact function cannot be determined by the results of the study.

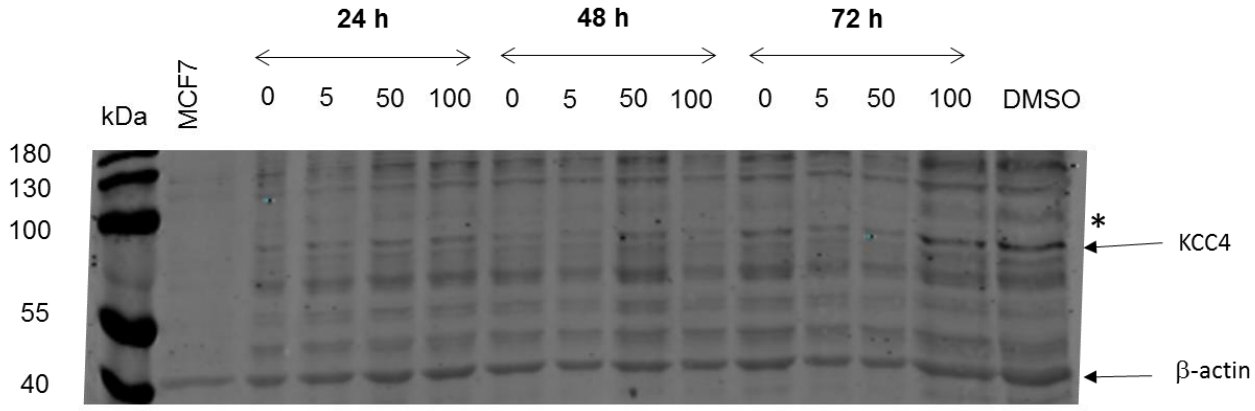


Figure 3.9 Analysis of the effect of APα on KCC4 expression. KCC4 expression was analysed in COLO684 cells treated with 0-100 nM APα for 24 h, 48 h and 72 h. Thirty µg was loaded on a 8% SDS-PAGE and transferred to nitrocellulose. Membranes were probed with antibodies towards KCC3 or β actin, indicated by arrows.

3.12 Analysis of π subunit expression in pregnant and non-pregnant sheep uterine samples

The identification of π-subunit expression in non-pregnant and pregnant sheep uterine samples was of particular interest in the current study, so π expression in human uterus, endometrial cancer, pregnant, non-pregnant samples and APα treated and untreated COLO684 cells were analysed by immunoblotting (Figure 3.10 A). The data clearly shows $GABA_AR$ π subunit was present in endometrial cancer but expression was not detected in healthy human uterus (Figure 3.10 A). In the sheep uterine samples, there was potentially an increase in π protein expression in pregnancy (Figure 3.10 A), but this data needs to be confirmed. π protein expression was downregulated in the 100 nM APα treated cells when compared to the untreated COLO684 cells (Figure 3.10 A). These preliminary results indicated that π protein is expressed in pregnancy which supports the study hypothesis that $GABA_A$ receptors containing the π subunit may have a potential role in premature labour.

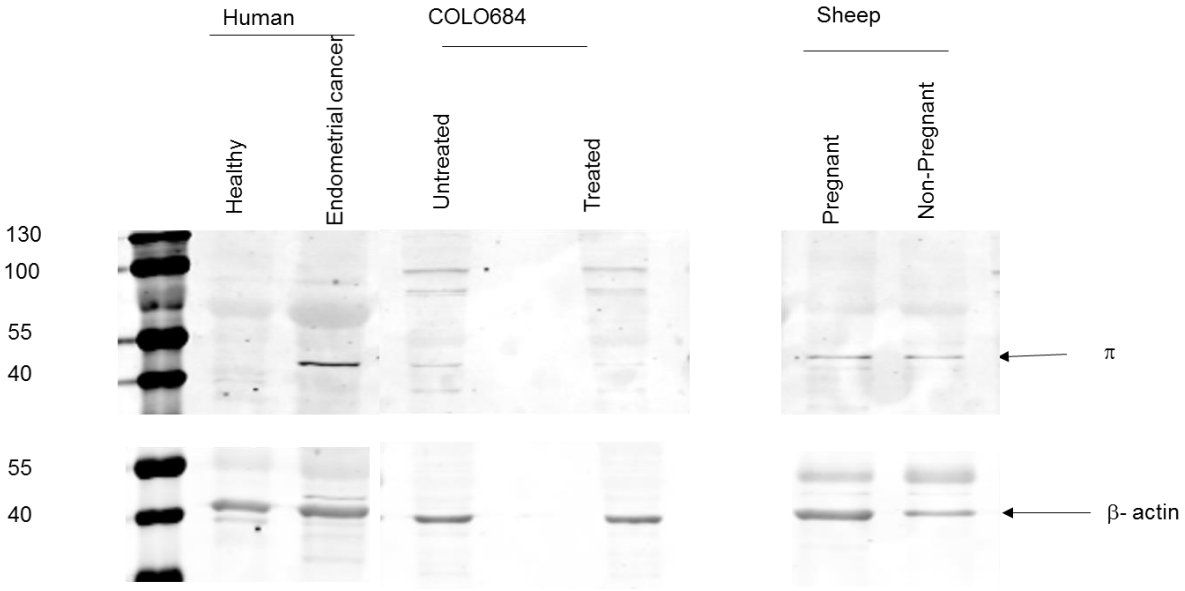


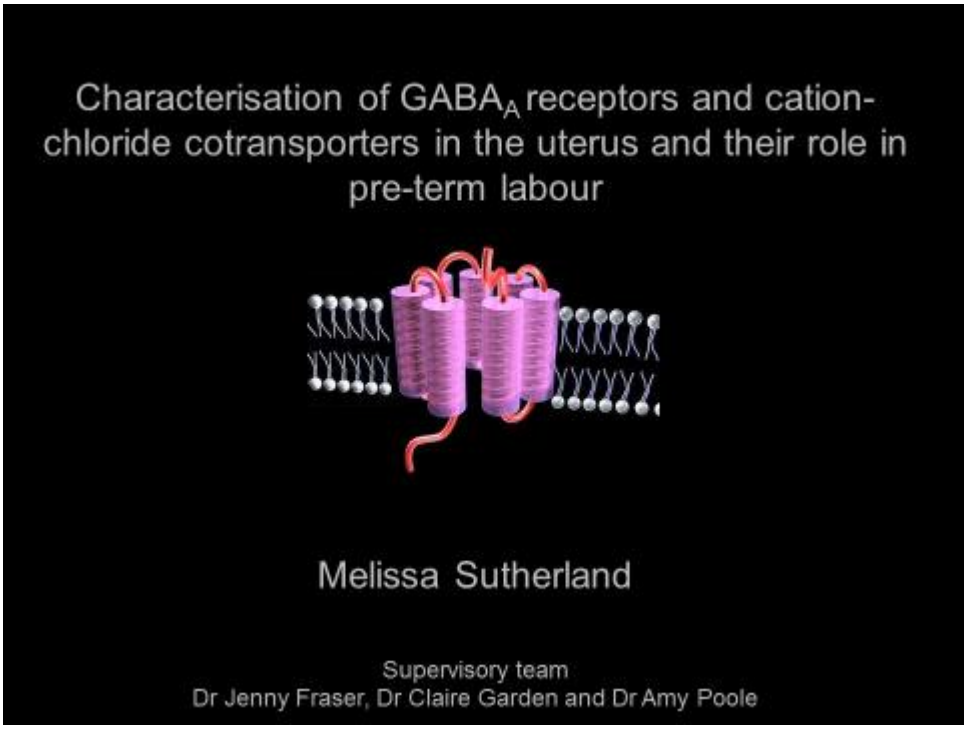
Figure 3.10 $GABA_AR$ π subunit protein expression in pregnant and non-pregnant sheep uterine samples. A) π subunit protein expression in healthy human uterus, endometrial cancer, pregnant, non-pregnant samples and APα treated and non-treated COLO684 cells. B) Immunoblot of the control β-actin.

3.13 Key findings in relation to the aims of the project

1) $GABA_AR$ subunit expression was found in human uterus. This is the first study to quantitatively analyse all the relevant subunits.
2) The subunits expressed in uterus were different to those normally expressed in the human brain. This is consistent with data in the literature about receptors found outside the CNS.
3) The subunit expression was different in human uterine cancer. $GABA_AR$ are well documented to be expressed in different cancers, but their role is unclear.
4) The π subunit, which is the one we are interested in as it confers APα sensitivity to the receptor is robustly expressed in uterus and uterine cancer.
5) This is the first study that has also looked at CCC expression, alongside $GABA_AR$ expression. Selected CCC genes are expressed in human uterus and the expression profile changes in cancer.
6) $GABA_AR$ π subunit protein is expressed in sheep pregnancy samples. This is consistent with data in the literature about π in pregnancy.
7) *In vitro* model was developed using COLO684 cell line to analyse $GABA_AR$ and CCCs expression in the presence of APα.
8) $GABA_AR$ subunits expression changes with APα. These results are consistent with the literature data.
9) Selected CCC expression changes with APα. T`his is the first study to quantitatively analyse all CCC and the effect with APα.

In this study we hypothesise that a lack, or a change in phenotype/ activity of $GABA_AR$ subtype might induce preterm contraction, to prove or disprove the hypothesis certain aims were set out. To identify the difference in $GABA_AR$ subunit and CCC expression in healthy human uterus and human endometrial cancer, pregnant and non pregnant sheep samples and treated APα COLO684 cell line were used. The results of the study show that $GABA_AR$ subunit and CCC are expressed in human uterus and endometrial cancer with the expression pattern of $GABA_AR$ subunit and CCC changing in endometrial cancer. This supports, but does not prove the hypothesis as it shows a possible change in the $GABA_AR$ subtype in the diseased sample which is exposed to the same hormone as the pregnant uterus. This supports the hypotheses that a change in phenotype/ activity might induce preterm contraction. The data gathered from the sheep samples show that the $GABA_AR$ π subunit was expressed in pregnant and non pregnant uteri, however no major changes could be confirmed. The data does however support the hypothesis by showing that $GABA_AR$ π subunit is expressed in pregnancy and a change in the expression of the $GABA_AR$ π subunit may induce preterm contraction. The COLO684 cells when treated with APα showed no expression of the $GABA_AR$ π subunit however other subunits were expressed. COLO684 cell line may not be expressing the $GABA_AR$ subunits in a similar fashion as a result of the cells becoming immortalised. This data does not confirm nor deny the study hypothesis but shows support, as it proves that potentially a different $GABA_AR$ subtype with a different function is present in the endometrium.

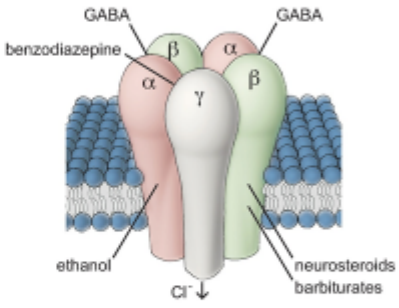
Chapter 4 Discussion



Research Question: Are GABA$_A$ receptors expressed in human uterus?

Aim: Characterise expression of GABA$_A$ receptor subunit genes in human uterus and human uterine cancer.

GABA$_A$R subunits	Expression in healthy uterus (Sample 1)	Expression in healthy uterus (Sample 2)
α1	ND	ND
α2	++	++
α3	++	++
α4	+	+
α5	+	+
α6	ND	ND
β1	++	ND
β2	+	ND
β3	++	+
γ1	++	ND
γ2	+	ND
γ3	+	+
π	+++	+++
δ	ND	ND
ε	ND	ND
θ	ND	ND



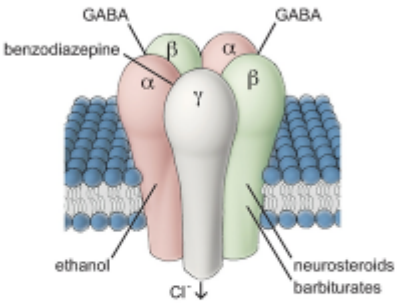
Brain: α1/2, β2, γ2

Periphery: ?

Research Question: Are $GABA_A$ receptors expressed in human uterus?

Aim: Characterise expression of $GABA_A$ receptor subunit genes in human uterus and human uterine cancer.

$GABA_AR$ subunits	Expression in healthy uterus (Sample 1)	Expression in healthy uterus (Sample 2)
α1	ND ↓	ND ↓
α2	++ ↑	++ ↑
α3	++ ↑	++ ↓
α4	+ ↓	+ ↓
α5	+ ↓	+ ↓
α6	ND ↓	ND ↓
β1	++ ↓	ND ↓
β2	+ ↑	ND ↑
β3	++ ↑	+ ↑
γ1	++ ↓	ND ↑
γ2	+ ↓	ND ↓
γ3	+ ↑	+ ↓
π	+++ ↑	+++ ↑
δ	ND ↓	ND ↓
ε	ND ↓	ND ↓
θ	ND ↓	ND ↓



Brain: α1/2, β2, γ2

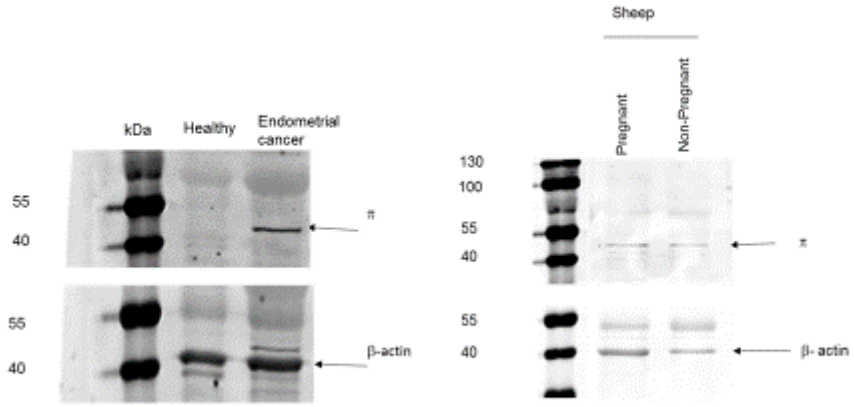
Periphery: ?

π subunit gene expression has been identified in the rat uterus, at the time of gestation, modulating the $GABA_A$ receptor sensitivity to allopregnanole

(Fujii and Mellon., 2001)

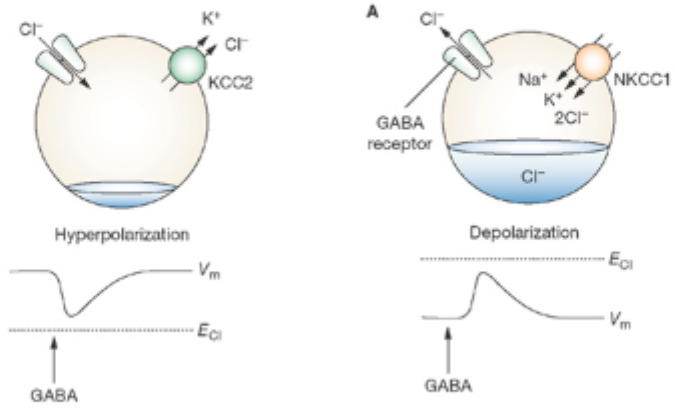
Research Question: Are $GABA_A$ receptors expressed in human uterus?

Aim: Characterise expression of $GABA_A$ receptor π subunit in uterus and uterine cancer.



Research Question: Could the $GABA_A$ receptors in the uterus be excitatory?

Aim: Characterise expression of Cation Chloride Cotransporter (CCC) genes in human uterus and human uterine cancer.



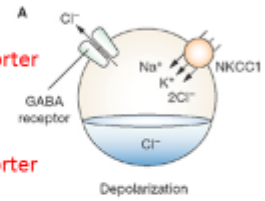
Typical GABA function

Atypical GABA function
Dependant on CCC expression

Research Question: Could the $GABA_A$ receptors in the uterus be excitatory?

Aim: Characterise expression of CCC genes in human uterus and human uterine cancer.

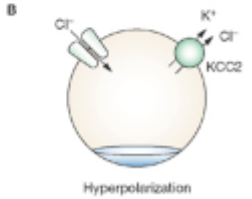
CCC genes	Expression in healthy human uterus (sample 1)	Expression in healthy human uterus (sample 2)	
NKCC1	+++	+++	Importer
NKCC2	ND	ND	
KCC1	+	+	Exporter
KCC2	ND	ND	
KCC3	+	+	
KCC4	++	++	



Contraction/ quiescence?

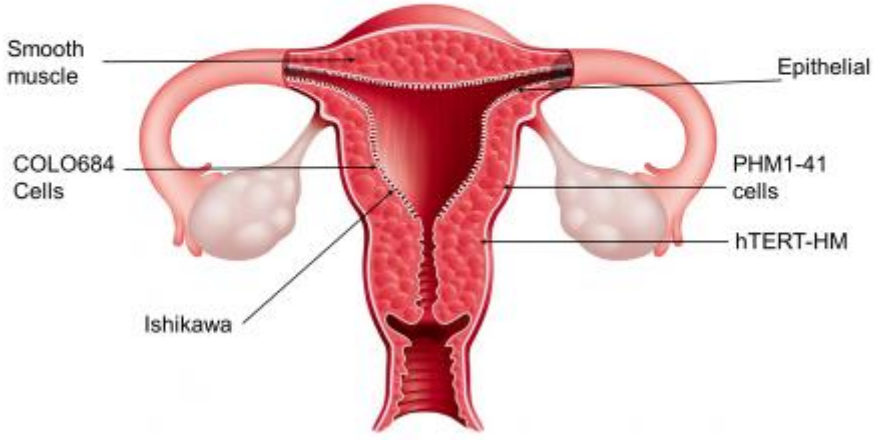
Endometrial cancer

CCC genes	Expression in healthy human uterus (sample 1)	Expression in healthy human uterus (sample 2)
NKCC1	+++ ↑	+++ ↑
NKCC2	ND ↓	ND ↓
KCC1	+ ↑	+ ↑
KCC2	ND ↓	ND ↓
KCC3	+ ↑	+ ↑
KCC4	++ ↑	++ ↑



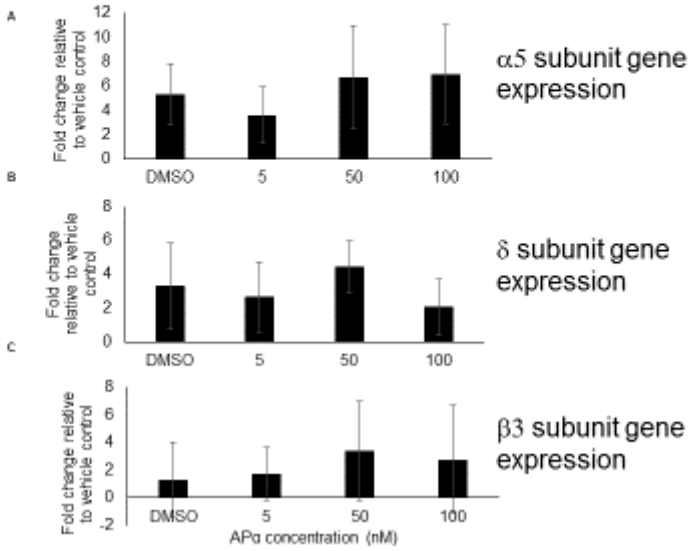
Research Question: Are $GABA_A$ receptors in uterus modulated by allopregnanolone?

Aim: Develop an *in vitro* model using uterine cells to analyse $GABA_A$ receptors and CCCs in response to treatment with allopregnanolone



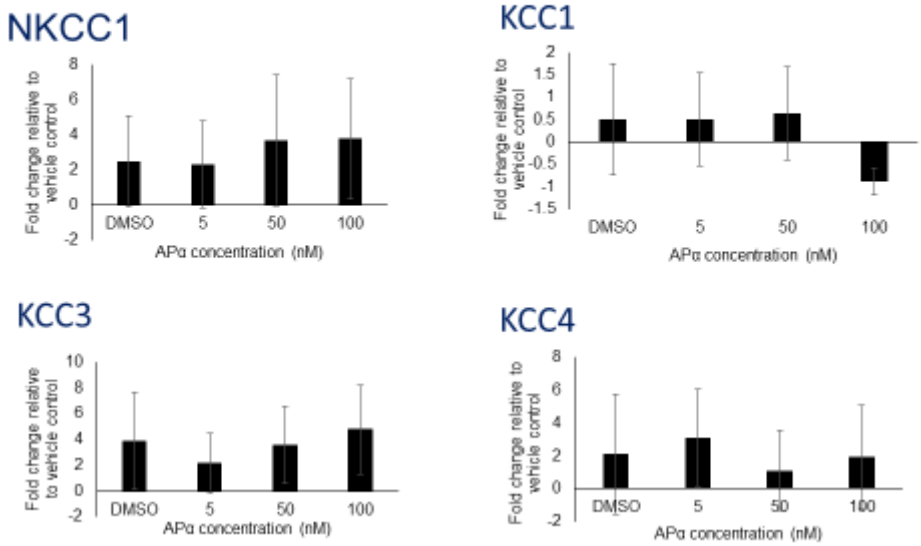
Research Question: Are $GABA_A$ receptors in uterus modulated by allopregnanolone?

Aim: Develop an *in vitro* model using uterine cells to analyse $GABA_A$ receptor and CCC gene expression in response to 48 h treatment with allopregnanolone



Research Question: Are CCCs in uterus modulated by allopregnanolone?

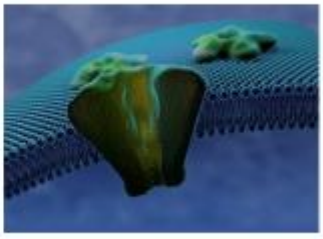
Aim: Develop an *in vitro* model using uterine cells to analyse $GABA_A$ receptor and CCC gene expression in response to 48 h treatment with allopregnanolone



Confounders

- Unknown cell composition of human uteri samples.
- Sheep samples very little information received about the gestation stage when the sample was taken and no information was received about the collection point in the cycle of the non pregnant sheep.
- $GABA_A$ receptor subunit and CCCs genes have spliced variants which were not analysed.
- COLO684 cell line does not express $GABA_A$ receptor π subunit.
- The exact effect allopregnenolone has on $GABA_A$ receptor subunit and CCCs genes is still unclear.

Conclusion



Primary data herein demonstrates that $GABA_A$ receptor subunit and CCC genes are expressed in the human uterus and uterine cancer, indicating functional receptors may be present.

However, further investigation is required to determine their function and precise location, to fully understood the potential role of $GABA_A$ receptors and CCCs in endometrium and pregnancy.

Future Work

- Development of an *in vitro* model using a more appropriate uterine cell line, which is know to express $GABA_AR$ π subunit.
  - This can then be used to look at whether π-subunit-containing GABAA receptors are expressed at the surface of the cells.
  - Co-precipitation studies could show potential receptor assemblies in uterine cells.
  - Pharmacological testing to investigate properties of the receptors.
  - RNAi/CRISPR techniques to ascertain importance of the π subunit in allopregnanolone binding.
  - Analysis of chloride movement *in vitro*.

- Reattempt to investigate $GABA_A$ receptor and CCC gene expression over the gestation period in mouse uterus.

- Employ an organ bath to analyse contraction in uterine tissue in response to allopregnanolone and $GABA_A$ receptor drugs.

Chapter 5 References

Abdel-Aleem, H., Shaaban, OM. and Abdel- Aleem, MA. (2013). Cervical pessary for preventing preterm birth. *The Cochrane Collaboration*. **5**: 1-26.

Abdul, M., Mccray, SD. and Hoosein, MN. (2008). Expression of gamma aminobutyric acid receptor (subtype A) in prostate cancer. *Acta Onclogica*. **47**: 1546-1550.

Alfirevic, Z., Owen, J., Carreras-Moratonas, E., Sharp, NA., Szychowskis, MJ. and Goya, M. (2013). Vaginal progesterone, cerclage or cervical pessary for preventing preterm birth in asymptomatic singleton pregnant women with a history of preterm birth and a sonographic short cervix. *Ultrasound in Obstetrics & Gynecology*.**41**: 146-151.

Almedida, AT., Quispe-Ricalde, A., Montes de Oca, F., Foronda, P. and Hernandez, MM. (2014). A high- throughput open–array qPCR gene panel to identify housekeeping genes suitable for myometrium and leiomyoma expression analysis. *Gynecoogic Oncology.***134**: 138-143.

Akinic, KM. and Schofield, RP.(1999). Widespread expression of $GABA_A$ receptor subunits in peripheral tissues. *Neuroscience Research*. **35**:145-153.

Azuma,H.,Iamoto,T.,Sakamoto,T.,Kiyama,S.,Ubai,T.,Shinohara,Y.,Maemura,K., Tsuji,M., Segawa,N.,Masuda,H., Takahara,K., Katsuoka,Y. and Watanabe,M (2003). γ-Aminobutyric acid as a promoting factor of cancer etastasis; Induction of matrix metalloproteinase production is potentially its underlying mechanism. *Cancer Research.* **63**: 8090-8096.

Bracamontes, RJ. and Steinbach,HJ. Multiple Modes for conferring surface expression of homomeric β1 GABAA receptors. *The Journal of Biological chemistry*. **283**. 26128-26136.

Ben-Ari, Y. (2002). Excitatory actions of GABA during development: the nature of the nurture. *Nature*. **4**: 728-739.

Bell-Horner, CL., Dohi, A., Nguyen, Q., Dillon, HG. and Singh, M. (2006).ERK/MAPK pathway regulates $GABA_A$ receptors. *Wiley Inter Science*: 1467-1475.

Beverley, E., Murphy, P., Steinberg, IS., Fen- Yun, H. and Allison, MC. (2001). Neuroactive ring A – Reduced metabolites of progesterone in human plasma during pregnancy: elevated levels of 5α- Dihydroprogesterone in depressed

patients during the latter half of pregnancy. *The Journal of Clinical Endocrinology & Metabolism*. **86**: 5981-5987.

Blaesse, P., Airaksine, SM., Rivera, C. and Kaila, K. (2009). Cation-Chloride cotransporters and Neuronal Function. *Cell Press.* **61**:820:838.

Borodinsky, NL., O'Leary, D., Neale, HJ., Vicini, S., Coso, OA. and Fiszman,LM. (2003). GABA- induced neurite outgrowth of cerebellar granule cells is mediated by $GABA_A$ receptor activation, calcium influx and CaMKII and erk1/2 pathways. *Journal of Neurochemistry*.**84**:1411-1420.

Bracamontes, RJ. and Steinbach, HJ. (2008) Multiple Modes for conferring surface expression of homomeric β1 $GABA_A$ receptors. *The Journal of Biological Chemistry*. **283**: 26128-26136.

Bruaard, BA., Kits, SK., Baker, ER., Willems, APW., Leyting-Vermulen, WJ., Voom, P., Smit, BA., Bicknell, JR. and Herbison, EA. (1997). Plasticity in Fast Synaptic Inhibition of Adult Oxytocin Neurons Caused by Switch in $GABA_A$ Receptor Subunit Expression. *Neuron*. **5**:1103-1114.

Bustin, AS., Benes, V., Garson, AG., Hellemans, J., Huggett, J., Kubista, M., Mueller, E., Nolan, T., Pfaffl, WM., Shipley, LG., Vandesompele, J. and Wittwer, TC. (2009). The MIQE guidelines: Minimum Information for publication of Quantitative Real-Time PCR experiments. *Clinical Chemistry.* **55**: 611-622.

Byrns, CM. (2014). Regulation of progesterone signaling during pregnancy: Implications for the use of progestins for the prevention of premature birth. *Journal of Steroid Biochemistry and Molecular Biology.***139**: 173-181.

Carta, M., Murru, L., Botta, P., Talani, G., Sechi, GP., Rio, PLD., Sanna, E. and Biggio, G. (2006). The muscle relaxant thiocolchicoside is an antagonist of $GABA_A$ receptor function in the central nervous system. *Neuropharmacology.* **51**: 805-815.

Challis, GRJ., Slobosa, MD., Alfaidy, N., Lye, JS., Gibb, W., Patel, AF., Whittle, LW. and Newnha, PJ. (2002). Prostaglandins and mechanisms of preterm birth. *Reproduction*. **124**: 1-17.

Chen, FY., Chou, YC., Ellory, CJ. and Shen, RM. (2010). The emerging role of KCl cotransport in tumour biology. *American Journal of Roentgenology*. **4**: 345-355.

Condon, J., Yin, S., Mayhew, B., Word, RA., Write, WE., Shay, JW. and Rainey, WE. (2002). Telomerase immortalization of human myometrial cells. *Biology of Reproduction.***67**: 506-514.

Connor, XJ., Boileau, JA. and Czajkowski. (1988). A $GABA_A$ Receptor α1 Subunit tagged with Green fluorescent protein requires a β subunit for functional surface expression. *The Journal of Biological Chemistry*.**273**: 28906-28911.

Cossette, P., Liu, L., Brisebois, K. (2000). Mutation of *GABRA1* in an autosomal dominant form of juvenile myoclonic epilepsy. *Nature Genetics.***31**: 184-189.

Davies , AA and Samueks, Y. (2010). Analysis of the genome to personalize therapy for melanoma. *National institutes of health*. **29**. 5545-5555.

Delpire, E. (2000). Cation- chloride cotransporters in neuronal communication. *News in Physiological Sciences.* **15**: 309-312.

Follesa, P., Biggio, F., Stefania, C., Gorini, G. and Biggio, G. (2004). Modulation of $GABA_A$ receptor gene expression by allopregnanolone and ethanol. *European Journal of Pharmacology.* **500**: 431-425.

Fujii, E. and Mellon, HS. (2000). Regulation of uterine γ-Aminobutyric Acid A Receptor Subunit Expression throughout Pregnancy. *Endocrinology*.**142**:1770-1777.

Ganguly, K., Schinder, FA., Wong, TS. and Poo, MM. (2001). GABA itself promotes the developmental switch of neuronal GABAergic responses from excitation to inhibition. *Cell.* **105**:521-532.

Garfield, ER. and Maner, LW. (2007). Physiology and electrical activity of uterine contractions. *Semin Cell Development Biology.***18**: 289-295.

Gurba, NK. (2012).Assembly and Heterogeneity of $GABA_A$ Receptors. *Candidate reviews.***2**: 25-32.

Hanahan, D and Weinberg, RA. (2011).Hallmarks of Cancer: The Next Generation. *Cell*.**144**. 646-674.

Harvey, JR., Kim, CH. and Darlison, MG. (1993). Molecular cloning reveals the existence of a fourth γ subunit of vertebrate brain GABA receptor. *Federation of European Biochemical Societies.***331**: 211-216.

Hedblom, E and Kirkness, F. (1997). A novel class of $GABA_A$ Receptor subunit in tissues of the reproductive system. *The Journal of Biological Chemistry*. **272**: 15346-15350.

Hellgren, C., Akerud, H., Stalkidou, A., Backstrom, T. and Sundstrom-poromaa, I. (2013). Low serum allopregnanolone is associated with symptoms of depression in late pregnancy. *Neuropsychobiolgy.***69**: 147-153.

Helguera, H., Eghbali, M., Sforza, D., Minosyn,Y, T., Toro, L. and Stefani, E. (2009). Changes in global gene expression in rat myometrium in transition from late pregnancy to parturition. *Physoil Genomics.***3:** 89-97.

Henge, JY., Pennell, EC., Chua ,NH., Perkins, EJ. and Lye, JS. (2014). Whole blood gene expression profile associated with spontaneous preterm birth in women with threatened preterm labour. *PLOS One*. **9**: 1-13.

Hill, M., Parizek, A., Cibula, D., Kancheva, R., Jirasek, EJ., Jirkovska, M., Velikova, M., Kubatova, J., Klimkiva, M., Paskova, A., Zizka, Z, Kancheva, L.,Kazihnitkova, H., Zamrazilova, L. and Starka, L. (2010). Steriod metabolome in fetal and maternal body fluids in human late pregnancy. *Journal of Steroid Biochemistry and Molecular Biology.***122:** 114-132*.*

Hill, M., bicikova, M., Parizek, A., Havlikova, H., Klak, J., Fajt, T., Meloun, M., Cibula, D., Cegan, A., Sulcova, J., Hampl, R. and Strka, L. (2001). Neroactive steroids, their precursors and polar conjugates during parturition and postpartum in maternal blood: 2. Time profiles of pregnanolone isomers. *Journal of Steroid Biochemistry and Molecular Biology.***78:** *51-57.*

Hines, M., Katherine, A., lyseng- Williamson, E. and Deeks, D. (2013). 17 a-Hydroxyprogesterone Caproate (Makena_). A Guide to Its Use in the Prevention of Preterm Birth. *Clinical Drug Investigation*. **33**: 223-227.

Hirose, S., Mitsudome, A., Okada, M. and Kaneko, S. (2005). Genetics of idiopathic epilepsies. *Epolepsia*.**46**: 38-43.

Jiang, HB. and Liu, ZL. (2009). PI3K/PTEN Signalling in Angiogenesis and Tumorigenesis. *National institutes of health*.**102**. 19-65.

Johnson, KS and Haun, RS. (2005). The Gamma-Aminobutyric Acid A Receptor π subunit is overexpressed in pancreatic adenocarcinomas. *Journal of the Pancreas*.**6**:136-142.

Julie kim, J and Chapman-Davis, E. (2010). Role of progesterone in Endometrial cancer. *Semin Reproduction Medicine.***28**:81-90.

Koksma, JJ., Keteren, VER., Rosahl, WT., Zwart, R., Smit, BA., Luddens, H. and Brussaard, BA. (2003). Oxytocin regulates neurosteroid modulation of $GABA_A$

receptors in supraoptic nucleus around parturition. *The Journal of Neuroscience.***23**: 788-797.

Lee, S., Dawson, J., Osman, G., Levitin, M., Guzel, MR. and Djamgoz, BAM. (2014). Management of cancer pain:I. Wider implications of orthodox analgesics.

LI,K and Xu,E.(2008). The role and the mechanism of γ-aminobutyric acid during central nervous system development. *Neuroscience Bulletin*.**24**:195-200

Liong, S., Quinzio, DM., Fleming, G., Permezel, M., Rice, EG. and Georgiou, MH. Prediction of spontaneous preterm labour in at risk pregnant women. (2013). *Reproduction.* **14**: 335-345.

Liong, S., Quinzio, DM., Heng, JY., Fleming, G., Permezel, M., Rice, EG. and Georgiou, MH. (2013). Proteomic analysis of human cervicovaginal fluid collected before preterm premature rupture of the foetal membranes. *Reproduction*. **145**: 1741-7899.

Liu, Y., LI, H.Y., Guo, JF., Wang, JJ., Sun, LR., Hu, YJ. and Li, CG. (2008). Gamma aminobutyric acid promotes human hepatocellular carcinoma growth through overexpressed gamma- aminobutyric acid A receptor α3 subunit. *World Journal of Gasteroenterology*.**14**:7175-782.

Liu,Y.,Guo, F.,Dai, M.,Wang, D., Tong, Y.,Huang,J., Hu, J. and Li, G. (2009). Gamma-aminobuyric acid A receptor Alpha 3 subunit is overexpressed in lung cancer. *Pathol Oncology Research*.**15**: 351-358.

Livak, JK. and Schmittgen, DT. (2001). Analysis of Relative gene expression data using Real-Time quantitative PCR and the $2^{-\Delta\Delta C}$T method. *Methods*. **25**: 402-408.

LI, K and Xu, E. (2008). The role and the mechanism of γ-aminobutyric acid during central nervous system development. *Neurosci Bull*.**24**.195-200.

Loh, WE. and Ball, D. (2000). Role of the GABAAβ2, GABAAα6, GABAAα1 and GABAAγ2 receptor subunit gene cluster in drugs responses and the development of alcohol dependence. *Neurochemistry International*.**37**: 413-423.

Luisi, S., Petraglia, f., Benedetto, C., Nappi, RE., Bernardi, F., Fadalti, M., Reis, FM., Luisi, M. and Genazzani, AR. (2000). Serum Allopregnanolone levels in pregnant women: changes during pregnancy, at delivery, and in hypertensive patients. *The Journal of Clinical Endocrinology and Metabolism*. **85**: 2429-2433.

Lyons, RH., Gibbs, TT. and Farb, HD. (2000). Turnover and down-regulation of GABA(A) receptor alpha1, beta2, and gamma1 subunit mRNAs by neurons in culture. *Journal of Neurochemistry*. **74**: 1041-8.

Maguire, J., Ferando, I., Simonsen, C. and Mody, I. (2009). Excitability changes Related to $GABA_A$ Receptor Plasticity during pregnancy. *The Journal of Neuroscience*. **29**: 9529-9601.

Martin, JL. and Chang, Q. (2012). Inhibitory synaptic regulation of motoneurons: A new target of disease mechanisms in amyotrophic lateral sclerosis. *Molecular Neurobiology.* **45**: 30-42.

Mehta, KA. and Ticku, KM. (1999). An update on GABAA receptors. *Brain research reviews*.**29**.196-217

Mesiano, S., Chan, EC., Fitter, JT., Kwek, K., Yeo, G. and Smith, R. (2002). Progesterone withdrawal and estrogen activation in human parturition are coordinated by progesterone receptor A expression in the myometrium. *The Journal of Clinical Endocrinology and Metabolism*. **87**: 2924-2930.

Miao, Y., Zhang, Y., Wan, H., Chen, L. and Wang, F. (2010). GABA-receptor agonist, propofol inhibit invasion of colon carcinoma cells. *Biomedicine and Pharcotherapy*.**64:** 583-588.

Mijovic, EJ., Zarkar, T., Ngelova, J. and Olson, MD. (1999). Prostaglandin endoperoxide H synthase mRNA expression in the human amnion and decidua during pregnancy and in the amnion at premature birth. *Molecular Human Reproduction*. **5**: 182-187.

Mondugno, F., Ness, BR., Chen, C. and Weiss, NS. (2005). Inflammation and Endometrial cancer: A Hypothesis. *Inflammation and Endometrial cancer*.**14**.2840-2844.

Monga, M., Ku, YC., Dodge, K. and Sanborn, MB. (1996). Oxytocin-stimulated responses in a pregnant human immortalized myometrial cell line. *Biology of Reproduction*. **55**: 427-432.

Mulley, JC., Scheffer, IE., Petrou, S. and Kaneko, S. (2003). Channelopathies as a genetic cause of epilepsy. *Current Opinion in Neurology.***16**: 171-176.

Neelands, RT. and Macdonald, LR. (1999). Incorporation of the π subunit into functional γ aminobutyric acidA receptor. *Molecular Pharmacology.***56**: 598-610.

Neman, J., Termini, J., Wilczynski, S., Vaidehi, N., Choy, C., Kowolik, MC., Li, Hubert., Hambrecht, CA., Roberts, E. and Jandial, R. (2013). Human breast cancer metastases to the brain display GABAergic properties in the neural niche. PNAS. **111**. 984-989.

Newland, CF. and Cull-Candy, SG. (1992). On the mechanism of action of picrotoxin on GABA receptor channels in dissociated sympathetic neurons of the rat. *Journal of Physiology*. **447**: 191-213.

Olsen, RW. and Sieghart, W. (2009). $GABA_A$ receptors: Subtypes provide diversity of function and pharmacology. *Neuropharmacology.***56**: 141-148.

Ottander, U., Poromaa, S. I., Bjurulf, E., Skytt ,A., Backstrom, T. and Olofsson, I. J. (2005). Allopregnanolone and pregnanolone are produced by the human corpus luteum. *Molecular and Cellular Endcrinology.***239**: 37-44.

Owens, FD., Boyce, HL., Davis, EBM. and Kriegstein, RA. (1996). Excitatory GABA responses in embryonic and neonatal cortical slices demonstrated by gramicidin perforated-patch recordings and calcium imaging. *The Journal of Neuroscience.***16**: 6414-6423.

Park- Chung, M., Malayev, A., Purdy, HR., Gibbs, TT. and Farb, HD. (1999). Sulphated and unsalfated steroids modulate γ- aminobutyric $acid_A$ receptor function through distinct sites. *Brain Research*. **830**: 72-87.

Parizek, A., Hill, M., Kancheva, R., Havlikova, H., Kancheva, L., Cindr, J., Paskova, A., Pouzar, V., Cerny, I., Drobohlav, P., Hajek, Z. and Starka, L. (2004). Neuroactive Pregnanolone Isomers during Pregnancy. *The Journal of Clinical Endocrinology & Metabolism*. **90**: 395-403.

Payne, JA., Rivera, C., Voipio, J. and Kaila, K. (2003). Cation- chloride cotransporters in neuronal communication, development and trauma. *Trends in Neurosciences*. **26**:199-206.

Pehlivanogul, B., Bayrak, S. and Dogan, M. (2013). A close look at the contraction and relaxation of the myometrium; the role of calcium. *Journal of the Turkish-German Gynecological Association.***4**: 230-234.

Pfaffl., WM. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Research*. **29**: 2002-2007.

Putnam, DC., Brann, WD., Kolbeck, CR. and Mahesh, BV. (1991). Inhibition of uterine contractility by progesterone and progesterone metabolites: mediation by progesterone and gamma amino butyric acid A receptor system. *Biology of Reproduction*. **45**: 266-272.

Rivera, C., Voipio, J. and Kaila, K. (2005). Two developmental switches in GABAergic signalling: the K^+ Cl^- cotransporter KCC2 and carbonic anhydrase CAVII. *The Journal of Physiology.***562**: 27-36.

Roberts, SS., Meendonca-torres, MC., Jensen, K., Francis, LG. and Vasko, V. (2009). GABA receptor Expression in Benign and Malignant Thyroid Tumours. *Pathological Oncology Research*.**15**: 645-650.

Subramaniam, SK., Tham,TS., Mohamed, Z., Woo,LY., Adenan, NAM. and Chung, I. (2013).Cancer-Associated Fibroblasts Promote Proliferation of Endometrial Cancer Cells. *Plos one*.**8**.1-16.

Sadeghi, H and Taylor, HS. (2010). HOXA10 regulates endometrial $GABA_A$ π receptor expression and membrane translocation. *The American Physiological Society*.**298**.889-893.

Sadeghi, PD., Chaven-Gautam, MP., Pisal, RH., Mehendale, SS. and Joshi, RS. (2012). Matrix Metalloproteinse-1 and -9 in Human Placenta during Spontaneous Vaginal Delivery and Caesarean Sectioning in Preterm Pregnancy. *PLOS One.***7**.

Sanna, E., Mostllino, CM., Busonero, F., Talani, G., Tranquilli, S., Mameli, M., Spiga, S., Follea, P. and Biggio, G. (2003). Changes in $GABA_A$ receptor gene expression associated with selective alterations in receptor function and pharmacology after ethanol withdrawal. *Journal of Neuroscience*.**23**:11711-11724.

Serra, V., Eichhorn, AJP., GarciaGarcia, C., Ibrahim, HY., Prudkin, L., Sanchez, G., Rodriguez, O., Anton, P., Parra, LJ., Marlow, S., Perez-Garcia, J., Prat, A., Arribas, J., Hahn, CW., Kim, YS. and Baselga, J. (2013). RSK3/4 mediate resistance to PI3K pathway inhibitors in breast cancer. *The Journal of Clinical Investigation*. **123**: 2551-2563.

Shimizu-Okabe, C., Okabe, A., Kilb, W., Sato, K., Luhmann, JH. and Fukuda, A. (2007). Changes in the expression of cation Cl^- cotransporters, NKCC1 and KCC2 during cortical malformation induced by neonatal freeze-lesion. *Neuroscience Research*. **59**: 288-295.

Succol, F., Fiumelli, H., Benfenati, F., Cancedda, L. and Barberis, A. (2012). Intracellular chloride concentration influences the $GABA_A$ Receptor subunit composition. *Nature.***1**: 1-9.

Symmans, FW., Fiterman, JD., Anderson, KS., Ayers, M., Rouzier, R., Dummire, V., Stec, J., Valero, V., Sneige, N., Albarracin, C., Wu, Y., Ross, SJ., Wager, P., Theriault, LR., Arun, B., Kueerer, H., Hess, RK., Zhang, W., Hortobagyi, NG. and Pusztai, L. (2005). A single-gene biomarker identifies breast cancers associated with immature cell type and short duration of prior breastfeeding.*Endocrine-Related Cancer.***12**. 1059-1069.

Takehara, A., Hosokawa, M., Eguchi, H., Ohigash, H., Ishikawa, O., Nakamura, Y. and Nakagawa,H. (2007). γ- Amimobutyric Acid (GABA) Stimulates Pancreatic Cancer Growth through Overexpressing $GABA_A$ Receptor π subunit. *Cancer Research*.**67**: 9704-9712.

Timby, E., Balgard, M., Nyberg, S., Spigset, O., Andersson, A., Proankiewicz-Asplnd, J., Purdy, HR., Zhu, D.,Backstrom, T. and Poromaa, SI. (2006). Pharmacokineic and behavioral effect of allopregnanolone in healthy women.*Psychopharmacology.***186**:414-424.

Turkmen, S. Backstrom, T., Wahlstrom, G., Andreen, L. and Johansson, MI. (2011). Tolerance to allopregnanolone with focus on the GABA-A receptor. *British Journal of Pharmacology.* **162:** 311-327.

Vardi, N., Zhang, LL., Payne, AJ. and Sterling, P. (2000). Evidence That Different Cation Chloride Cotransporters in Retinal Neurons Allow Opposite Responses to GABA. *Journal of Neuroscience*. **20**: 7657-7663.

Veiga, GAL., Milazzotto, MP., Nichi, M., Lucio, FC., Siva, LCG., Angrimani, RSD. and Vannucchi, CI. (2015). Gene expression of estrogen and oxytocin receptors in the uterus of pregnant and parturient bitches. *Journal of Medical and Biological Research.*

Wang, MJ., Johnston, BP., Ball, GB. and Brinton, DR. (2005). The neurosteriod allopregnanolone promotes proliferation of rodent and human neural progenitor cells and regulates cell cycle gene and protein expression. *The Journal of Neuroscience.***25**: 4706-4718.

Wallace, RH., Marini, C., Petrou, S. (2001). Mutant GABA(A) receptor gamma2-subunit in childhood absence epilepsy and febrile seizures. *Nature Genetics.* **28**: 49-52.

Watanable, M., Maemura, K., Kanbara, K., Tamayama, T. and Hayasaki, H. (2002). GABA and GABA receptors in the central nervous system and other organs. *International Review of Cytology*. **213**: 1-38.

Weizam, R., Dagan, E., Snyder, H. and Gaish, M. (1997). Impact of pregnancy and lactation of $GABA_A$ receptor and central-type and peripheral-type benzodiazepine receptors. *Brain Research***. 752**:307-314.

Wu, W., Yang, Q., Fung, MK., Humphreys, RM., Brame, SL., Cao, A., Fang, T. Y., Shih, TP., Kropp, PB. and Lin, KH. (2014). Linking γ aminobutyric acid A receptor to epidermal growth factor receptor pathways activation in human prostate cancer. *Molecular and Cellular Endocrinology*. **383**: 69-79.

Xiao, W. and Zhang, L. (2002). ERK MAP kinases regulate smooth muscle contractions in ovine uterine artery: effect of pregnancy. *American Physiological society.* **282**: 292-300.

Yamazawa, K., Hirai, M., Fujito, A., Nishi, H., Terauchi, F.,Ishikura, H.,Shozu, M. and Isaka, K. (2007). Fertility-preserving treatment with progestin and pathological criteria to predict responses in young women with endometrial cancer. *Human Reproduction*.**22**.1953-1958.

Young, ZS. and Bordey, A. (2009). GABA's control of stem and cancer cell proliferation in adult neural and peripheral niches. *National institutes of health*. **24**. 171-185.

Ymer, S., Schofied, RP., Draguhn, A., Werner, P., Kohler, M. and Seeburg, HP. (1989). $GABA_A$ receptor β subunit heterogeneity: functional expression of cloned cDNAs. *The EMBO Journal*.**8**: 1665-1670.

Young, ZS. and Bordey, A. (2009). GABA's control of stem and cancer cell proliferation in adult neural and peripheral niches. *National Institutes of Health*. **24**: 171-185.

Zafrakas, M., Chorovicer, M., Klaman, I., Kristiansen, G., Wild, JP., Heindrichs, U., Knuchel, R. and Dahl, E. (2006). Systematic characterisation of GABRP expression in sporadic breast cancer and normal breast tissue. *International Journal of Cancer.***118**: 1453-1459.

Zhang, X., Zhang, R., Zheng, Y., Shen, J., Xiao, D., Li, J., Shi, X., Huang, L., Tang, H., Lui, J., He, J. and Zhang, H. (2013).Expression of gamma-aminobutyric acid receptors on neoplastic growth and prediction of prognosis in non-small cell lung cancer. *Journal of Translational Medicine*.**11**.