CORTICOTROPIN-RELEASING HORMONE RECEPTOR SUBTYPE 1 AND SUBTYPE 2 mRNA EXPRESSION AND PROTEIN LOCALIZATION IN THE MYOMETRIUM IN PREGNANCY

by

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A thesis submitted in conformity with the requirements for the degree of Masters of Science
Graduate Department of Physiology
University of Toronto

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0-612-34091-0
Corticotropic-Releasing Hormone Receptor Subtype 1 And Subtype 2 mRNA

Expression And Protein Localization In The Myometrium In Pregnancy

Masters of Science 1998

Miata Yvette Stevens

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ABSTRACT

The human placenta secretes increasing concentrations of corticotropin-releasing hormone (CRH) in late pregnancy and in labour. CRH has been implicated in the regulation of myometrial contractility. We hypothesized that CRH receptors, CRH-R1 and CRH-R2, mRNA in the myometrium would be upregulated in labour. Myometrial samples were collected from nonpregnant, pregnant and laboring patients from the upper and lower uterine segment. CRH-R1 mRNA and protein were downregulated with pregnancy and significantly upregulated at labour. This rise appeared to be exclusive to the lower segment. CRH-R2 mRNA did not change.

Rats have been used extensively to study the regulation of CRH receptors. We examined CRH receptor mRNA in rat myometrium. CRH-R1 mRNA was undetectable. CRH-R2 mRNA was significantly increased at labour concomitant with a rise in connexin 43 mRNA, a gap junction protein associated with labour. In conclusion, at the time of labour CRH-R1 mRNA is upregulated in the human but in the rat CRH-R2 mRNA is upregulated.
Dedicated to my mother, my inspiration.

*Let earth and sky be your yardstick and eternity your measurement. There is no height that you cannot accomplish by using the active intelligence of your mind.*

The Hon. Marcus Mosaih Garvey
ACKNOWLEDGMENTS

I am forever indebted to my supervisor Dr. John Challis for giving me the opportunity to experience research. Research, with all its highs and lows. I thank John for his guidance, his support and his understanding throughout the time I have known him.

I would like to thank my Advisory Committee members Dr. Steve Lye, Dr. Steve Matthews and Dr Neil MacLusky for their helpful cooperation throughout my degree and for their invaluable assistance in editing and revising this thesis.

I thank all my lab members for the fond memories of early morning meetings and late night research always interlaced with good partying techniques. I would particularly like to thank Dr. Mhozyra Fraser, Dr. Alison Holloway, Dr. Wendy Whittle, Dr. Vicky Clifton, Debbie Sloboda and Fal Patel for their constant source of technical and emotional support. A very special thanks to the “Lye Lab” especially to Dr. Isabella Caniggia, Dr. Daniel MacPhee, Dr. Ryan Ou and Lindsay McWhirter for their readiness to teach me and help me with invaluable techniques.

Special thanks to Boris, Rebecca and Alex. Your love, your support and the wonderful strong bond we share makes me remember, every day, how blessed I am to have siblings. Extreme gratitude to my “twinie”, Yvonne, completing a Masters of Science concomitantly with you has made us share laughter, tears and a world of understanding. My heartfelt thanks to my Mom who taught me everything I need to know about being a powerful, intelligent and motivated woman. Infinite love and thanks to Toke Petersen- my love, my heart, my best friend. You make life, love and success worth living for.
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<tbody>
<tr>
<td>ACTH</td>
<td>adrenocorticotropic hormone</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>bp</td>
<td>base pairs</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine 3',5-mono phosphate</td>
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<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
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<tr>
<td>Cx</td>
<td>connexin</td>
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<td>CRH</td>
<td>corticotropin releasing hormone</td>
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<tr>
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<td>kilodaltons</td>
</tr>
<tr>
<td>MGA</td>
<td>mean gestational age</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
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<tr>
<td>MW</td>
<td>molecular weight</td>
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<td>nitric oxide</td>
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<td>PCR</td>
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<td>prostaglandins</td>
</tr>
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</tr>
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<tr>
<td>PGHS</td>
<td>prostaglandin H synthase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>PKA</td>
<td>protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PLC</td>
<td>phospholipase C</td>
</tr>
<tr>
<td>POMC</td>
<td>pro-opiomelanocortin</td>
</tr>
<tr>
<td>PTL</td>
<td>pre-term labour</td>
</tr>
<tr>
<td>PVN</td>
<td>paraventricular nucleus</td>
</tr>
<tr>
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<td>reverse transcriptase</td>
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<td>RT-PCR</td>
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<td>sq-PCR</td>
<td>semi-quantitative polymerase chain reaction</td>
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<tr>
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CHAPTER 1: INTRODUCTION

Human parturition arises as a result of complex interactions within the maternal-placental-fetal unit. Spontaneous term human labour is defined as “the onset of regular uterine contractions of increasing intensity and frequency associated with progressive effacement and dilatation of the cervix following the completion of 38 weeks of gestation” (Cunningham et al., 1993) and is the initial stage in a series of events leading to birth. An understanding of the mechanisms regulating human parturition is vital for the prevention and treatment of labour complications including dystocia, pre-term labour (PTL) and post-term pregnancy each of which is associated with increased morbidity, mortality, health care costs and emotional distress. PTL is the most frequent complication of pregnancy and occurs in about 7% of all pregnancies; however, despite the advances in perinatal medicine over the past 20 years little change has been made in the incidence of PTL and delivery (Cunningham et al., 1993). Given the potential threats of labour complications on the health and survival of both the mother and the fetus, there exists a crucial need for scientists to study the mechanisms involved in the onset, the progress and the termination of human labour.

1.1 OVERVIEW

There are currently several hypotheses regarding the mechanisms involved in the onset of labour. These include the progesterone withdrawal theory and the myometrium activation-stimulation theory. The progesterone withdrawal theory
proposes that progesterone maintains uterine quiescence throughout pregnancy and that a decrease in maternal plasma concentrations of progesterone close to term triggers uterine contractility. While this theory applies to the sheep model of parturition (Thorburn and Challis, 1979), studies in humans have failed to observe a decrease in circulating concentrations of progesterone prior to the onset of labour (Tulchinsky et al., 1972). The myometrium activation-stimulation theory forwarded by Challis and Lye (1994) proposes that with the approach of labour the myometrium is activated (increased muscle excitability), by the synthesis of “contraction-associated proteins”, that renders the myometrium more responsive to stimulation by uterotonic agents.

Several factors, produced by the placenta and the fetal membranes, such as corticotropin-releasing hormone (CRH) (Riley et al., 1991), have the ability to stimulate the myometrium (Quartero et al., 1989) The human placenta synthesizes and secretes increasing concentrations of placental CRH (hCRH-pl) into the maternal circulation in the third trimester of pregnancy and throughout labour (Goland et al., 1986; Okamoto et al., 1989). The physiological significance of hCRH-pl in human pregnancy still remains to be established. CRH exerts its actions via specific G-protein coupled receptors (Chen et al., 1993; Lovenberg et al., 1995a) of which two subtypes have been identified, CRH-R1 and CRH-R2 (Chen et al., 1993; Lovenberg et al., 1995a). CRH-R1 messenger ribonucleic acid (mRNA) and CRH-R2 mRNA are expressed in the myometrium of both nonpregnant and pregnant women (Rodriguez-Linareis et al., 1998). CRH receptors have been linked to secondary pathways associated with both relaxation and contraction in pregnancy (Grammatopoulos, 1994).
suggesting that hCRH-pl may potentially have differential effects on the human myometrium.

As of yet no studies have examined the mRNA expression and protein levels of CRH-R1 and CRH-R2 at the time of labour. Given the increase in circulating levels of hCRH-pl in the maternal circulation at that time and the presence of CRH receptors in human myometrium it has been suggested hCRH-pl may play a role in pregnancy and/or labour. One of the overall objectives of our lab is to understand the role of hCRH-pl in the initiation and/or progression of labour. The specific aims of the present study were a) to determine the expression of CRH-R1 mRNA and CRH-R2 mRNA in the myometrium both in pregnancy and at the time of labour and b) to establish a mammalian model to study the regulation of CRH receptor expression in the myometrium.

1.2 THE PLACENTA AND FETAL MEMBRANES

The human placenta is classified as a hemochorioendothelial placenta. The maternal blood does not directly contact the fetal blood and the exchange of gases, nutrients and wastes occurs via a chorioendothelial membrane. Shortly following fertilization, the cells of the early blastocyst differentiate into the inner cell mass and the trophoblasts. The blastocyst descends into the uterus and the trophoblasts differentiate into the outer syncytiotrophoblast layer and the inner proliferative cytotrophoblast. The syncytiotrophoblast layer results from the fusion of cytotrophoblast cells. The cytotrophoblasts and the syncyial layer form the primary
placental villi. The placental villi are the functional units of the human placenta and are classified as primary, secondary or tertiary. Primary villi become secondary in structure following the invasion of mesenchymal cells from the extraembryonic mesenchyma. Tertiary villi result from angiogenesis within the mesenchyme of the secondary villi and consist of a central core of fetal blood vessels surrounded by the cytotrophoblasts and syncytiotrophoblast layer. With advancing gestation, the ratio of cytotrophoblasts to the syncytiotrophoblast component decreases progressively, until the syncytial layer is the dominant trophoblastic component at term (Petraglia et al., 1996a). The syncytiotrophoblast layer is a major site of protein and steroid hormone synthesis within the human placenta (Petraglia et al., 1996a).

The amnion and the chorion are the fetal membranes and together form the amniotic sac which holds the fetus and the amniotic fluid. The amnion is a thin avascular membrane comprised of a single layer of epithelial cells on a loose connective matrix. The chorionic membrane is composed of an inner connective tissue layer and an outer epithelial layer. It is in direct contact with the decidua except at the site of placenta implantation. The decidua is composed of the decidua basal is; the region directly beneath the site of implantation of the embryo, the decidua capsular is; the region surrounding the embryo and the decidua vera which lines the remainder of the uterus. The three layers of the decidua represent a vascular anatomical interface that allows communication between the fetal membranes and the uterine myometrium (see Figure 1.1) (Petraglia et al., 1996a).
1.3 THE UTERUS

The uterus is a muscular, pear shaped organ located in the pelvic cavity between the bladder and the rectum (Pritchard et al., 1985). The size and the shape of the uterus vary markedly, dependent on age and parity. The uterus is composed of four regions: the fundus, the corpus, the isthmus, and the cervix. The fundus is the uppermost and widest region followed by the corpus, the isthmus and the narrow cervix. The wall of the uterus is composed of three layers: the outer perimetrium, the middle myometrium and the inner endometrium. The myometrium is comprised of an outer layer of longitudinal muscle fibers parallel to the long axis of the uterus and an inner layer of circular muscle fibers perpendicular to the long axis of the uterus (Pritchard, et al., 1985). The longitudinal and the circular muscle fibers of the myometrium originate from the subserosal connective tissue and the paranealplic ducts, respectively (see rev. Challis and Lye., 1994).

The myometrium undergoes sporadic, low pressure minimal intensity contractions known as Braxton-Hicks contractions throughout pregnancy. Labour is characterized by high-frequency, high amplitude, intense contractions of the myometrium. At the time of labour, the human uterus differentiates into an actively contracting upper segment and a relatively quiescent lower segment (see Figure 1.2). Uterine contractions of high intensity and long duration are present in the fundal segment when compared to the activity of the lower segment (see Figure 1.3). The
development of regions of opposing contractile activity within the uterus allows for the effective and forceful expulsion of the fetus from the uterus.

1.4 STIMULATION OF MYOMETRIAL ACTIVITY

Challis and Lye (1994) hypothesized that the activity of the myometrium, in pregnancy, was finely regulated by the synthesis of "contraction-associated proteins" and the balance of stimulatory versus inhibitory factors acting on the myometrium. Lye coined the term "contraction-associated" proteins to refer to the proteins involved in increasing the excitability of the myometrial smooth muscle including the gap junctions and the uterotonic agonist receptors.

Gap junctions are specialized regions of the cell membrane through which direct cell-to-cell contact is maintained. They are formed from the aggregation of hundreds or thousands of hydrophilic protein channels (connexins) on the plasma membranes of adjacent cells. Gap junctions provide the structural basis necessary for coordinated synchronized myometrial contraction (Mackenzie and Garfield, 1985; Kilarski et al., 1994). Petrocelli and Lye, (1993) reported that, one of the gap junction proteins, connexin 43 (Cx 43) mRNA and protein levels were low in rat myometrium in pregnancy and increased markedly prior to labour attaining a peak during delivery.

Oxytocin (OT) is a nonapeptide hormone that stimulates uterine smooth muscle contraction. This hormone is thought to be vital for the initiation of labour in humans, but maternal plasma OT concentrations do not rise prior to labour. In addition, serial studies showed that the maternal plasma concentrations of oxytocin only increased
during the expulsive phase of labour (Leake et al., 1981). Fuchs et al., (1982) reported a significant correlation between the concentration of OT receptors and the sensitivity of the uterus to OT. This led to the proposition that the concentrations of OT receptors could be a possible regulatory mechanism mediating the effects of OT. However, despite a reported increase in oxytocin receptor numbers with the onset of labour (Fuchs et al., 1994), it is not clear whether OT is important for the initiation of labour. It has been suggested that increased OT receptor numbers facilitate the effects of OT on an already "activated" myometrium (see rev. Challis and Lye, 1994).

Prostaglandins (PGs) play a central role in stimulating myometrial contractility in many species (see rev. Challis and Lye, 1994). A role for PGs in labour is suggested by the demonstration that prostaglandin E2 (PGE\(_2\)) and prostaglandin F2 alpha (PGF\(_{2\alpha}\)) stimulate the contraction of myometrium collected from pregnant women (Embrey and Morrison, 1970), that the concentrations of PGF\(_{2\alpha}\) and PGE\(_2\) in amniotic fluid and of their metabolites in maternal plasma and urine increase at labour (see rev. Challis and Lye, 1994) and that the inhibition of PG synthesis suppresses uterine activity and prolongs the length of pregnancy (Anderson et al., 1981).

PGs are formed from arachidonic acid, that is liberated from cellular phospholipids by phospholipase C (PLC) and PLA\(_2\) enzyme activity. Arachidonic acid is metabolized by the cyclooxygenase activity of prostaglandin H synthase to the unstable endoperoxide, PGG\(_2\), that is reduced to PGH\(_2\). PGH\(_2\) is unstable and isomerizes readily to PGE\(_2\), PGF\(_{2\alpha}\) and PGD\(_2\). PGs are metabolized by an oxidized form of nicotinamide adenine dinucleotide type I 15-hydroxyprostaglandin
dehydrogenase to form 15-keto compounds. The 15-keto compounds are reduced by \( \Delta^{13,14} \) reductase to 13-14 dihydro-15 keto derivatives that are oxidized and excreted in urine.

1.5 PROGESTERONE AND ESTROGEN IN LABOUR

The placenta plays an essential role as an endocrine organ of pregnancy and is actively involved in the synthesis and secretion of progesterone and estrogen. The effects of progesterone and estrogen are complimentary, as well as, antagonistic to each other (Roy and Arulkumara, 1991). In addition to other effects, both steroids regulate the formation of gap junction proteins and the synthesis of OT, and therefore have critically important roles in regulating the key physiological events vital to the initiation and progression of labour.

Progesterone inhibits the appearance of gap junctions between rat myometrial cells and reduces cell-to-cell coupling (Garfield et al., 1990). Specifically, Petrocelli and Lye (1993) have shown that progesterone blocks the expected rise in Cx 43 mRNA at the time of labour in rat myometrium. The myometrium under progesterone dominance is characterized by its refractoriness to stimulation by OT and PGF\(_{2\alpha}\) (Neulen and Breckwoldt, 1994). These bioactive effects of progesterone are necessary for the maintenance of human quiescence (see rev. Challis and Lye, 1994).

Maternal plasma progesterone concentrations increase from the sixth week of gestation on through to term (Tulchinsky et al., 1972). Progesterone is formed from circulating low-density lipoproteins (LDL) linked to cholesterol. LDL bind to specific
membrane receptors on the syncytiotrophoblast and are internalized by endocytosis. Within the cell cytoplasm, LDL fuse with lysosomes and are hydrolyzed to amino acids and cholesterol esters. Cholesterol esters are further hydrolyzed to fatty acids and cholesterol and cholesterol is cleaved by the \( P_{450} \) side chain cleavage \( (P_{450Scc}) \) enzyme to pregnenolone \( (C21) \) that is hydroxylated by the mitochondrial placental type I 3β-hydroxysteroid dehydrogenase: \( \Delta5-4 \) isomerase enzyme \( (3\betaHSD) \) to progesterone.

Estrogens accelerate the biosynthetic pathways involved in placental progesterone production (Pepe and Albecht, 1995). They upregulate LDL receptor expression and increase the expression of \( P_{450Scc} \) in the baboon syncytiotrophoblast layer. However, estradiol \( (E2) \) stimulates the development of myometrial gap junctions in the pregnant rat (Mackenzie and Garfield, 1985) and Lye \textit{et al.}, (1993) have shown that the rise in Cx 43 mRNA expression in labour is associated with an increase in the plasma estrogen:progesterone ratio. Estrogens also increase the responsiveness of the myometrium to OT and PGF\(_{2\alpha} \) (Leslie \textit{et al.}, 1994), therefore in general estrogens promote myometrial contractility (see rev. Challis and Lye, 1994).

Maternal plasma concentrations of E2 rise throughout gestation (Tulchinsky \textit{et al.}, 1972). The synthesis of estrogens in human pregnancy requires an obligatory interaction between the placenta and the maternal and fetal adrenal glands. The human placenta lacks the \( P_{450C17} \) enzyme and is therefore unable to convert C21 steroids (progestins) directly to C19 steroids (androgens). Estrogen \( (C18) \) synthesis is dependent on the production of dehydroepiandrosterone sulphate \( (DHEAS) \) in the maternal and fetal adrenals. DHEAS is extracted from the fetal and maternal
circulations by the placenta. It is desulfonated to DHEA that is converted to androstenedione. Androstenedione is readily aromatized to estrone and E2. The majority of DHEAS produced in the fetal adrenal is converted in the liver to 16α-hydroxy-DHEAS before entering the placenta. 16α-OH-DHEAS is cleaved to 16α-OH-DHEA which is converted to 16α-OH-androstenedione and aromatized to estriol.

1.6 CORTICOTROPIN RELEASING HORMONE

In addition to steroid hormone synthesis, the human placenta, the decidua and the fetal membranes produce a large number of hypothalamic-like peptides which include luteinizing hormone releasing hormone and CRH (Khodr and Siler-Khodr, 1978; Riley et al., 1991). These peptides which appear to be structurally and bioactively identical to their hypothalamic counterparts, are thought to modulate autocrine and/or paracrine mechanisms within the uterus. This review will initially discuss the properties of hypothalamic CRH. This will be followed by an in depth explanation of the synthesis and secretion of hCRH-pl and discussion of the putative roles of hCRH-pl in human pregnancy and labour.

CRH is a 41 amino acid peptide, initially characterized in the ovine hypothalamus by Vale et al., (1981). It is synthesized predominantly in the perikarya of neurons within the medial parvocellular region of the paraventricular nucleus (PVN) and released into the hypophyseal portal system in response to stress (Vale et al., 1981; Antoni, 1983). The gene for prepro CRH, the biosynthetic precursor of CRH, was isolated from a human genomic DNA library with an ovine CRH (oCRH)
complementary DNA (cDNA) probe (Shibahara et al., 1983). The deduced amino acid sequence of human CRH exhibits seven amino acid substitutions when compared to the ovine CRH (Vale et al., 1981): glutamic acid for glutamine (at position 2), alanine for threonine (22), arginine for lysine (23), methionine for leucine (38), glutamic acid for aspartic acid (39) and isoleucine for alanine (41). All of these substitutions represent changes in chemically similar amino acids resulting from single nucleotide amino acid substitutions except for the isoleucine/alanine substitution in the carboxyl terminus of CRH (Shibahara et al., 1983; Vale et al., 1981). The rat CRH (rCRH) gene has a similar structural organization to that of the human CRH gene and the deduced amino acid sequence of human and rat CRH (rCRH) appears to be identical (Rivier et al., 1993). In addition, a well-conserved CRH amino acid sequence has also been isolated and characterized in the mouse and the dog hypothalamus (Keegan et al., 1994; Mol et al., 1994).

CRH acts on the corticotrophs of the pituitary to upregulate pro-opiomelanocortin (POMC) mRNA expression and the release of adrenocorticotropic hormone (ACTH). ACTH increases the expression of key enzymes involved in corticosteroid synthesis in the fetal adrenal gland (Fujieda et al., 1981) leading to an increase in plasma glucocorticoid concentrations. Glucocorticoids feedback on the hypothalamus and the pituitary, via specific receptors to decrease ACTH release from the pituitary (Yang et al., 1990; Lu et al., 1991).
1.6.1 CRH-Related Peptides

Sauvagine and urotensin I are 50% homologous to h/r CRH at the amino acid level with high homology particularly in the carboxy terminus (see Figure 1.4) (Montecucchi and Henschen, 1981; Lederis et al., 1982; Ichikawa et al., 1982). Sauvagine is a 40 amino acid peptide, isolated from the skin of the frog Phyllomedusa sauvagei (Montecucchi and Henschen, 1981) and urotensin I is a 41 amino acid peptide isolated from the caudal neurosecretory system of both Catostomus mersoni (sucker fish) and Cyprinus carpio (carp fish) (Lederis et al., 1982; Ichikawa et al., 1982). Both sauvagine and urotensin I stimulate the release of ACTH from anterior pituitary cell cultures (Lederis et al., 1982; Tran et al., 1990).

Recently, another CRH-related peptide has been isolated and characterized in the rat midbrain and from a human genomic DNA library (Vaughan et al., 1995; Donaldson et al., 1996). Urocortin is a 40 amino acid peptide that is highly homologous to urotensin I (63%) and CRH (45%). This peptide also stimulates ACTH release from dispersed rat anterior pituitary cells in culture (Donalson et al., 1996). Urocortin mRNA and peptide have been identified in the human placenta and fetal membranes (Petraglia et al., 1996b), corresponding to a site of CRH synthesis and secretion.

1.7 HUMAN PLACENTAL CRH

Shibasaki et al., (1982) were the first to demonstrate CRH-like bioactivity in human placenta. These investigators showed that CRH extracted from purified
placental extracts, obtained from term spontaneous deliveries, was biologically active in vitro, stimulated the release of ACTH and β-endorphin from cultured rat anterior pituitary cells. Later studies by Frim et al., (1988) revealed that hCRH-pl mRNA was expressed in human placental tissue in human placenta from the seventh week of gestation. Human CRH-pl levels increased more than 20-fold during the last 5 weeks of pregnancy, concomitantly with an increase in hCRH-pl peptide (Frim et al., 1988). These scientists reported that the expression of hCRH-pl mRNA and the levels of peptide were low in the first trimester of pregnancy, increased during mid-gestation and increased exponentially during the 5 weeks prior to labour (Frim et al., 1988). Sasaki et al., (1988) reported the presence of three molecular species with CRH immunoreactivity in extracts of human term placenta. While, the major species eluted with rCRH the other two had apparent molecular weights of 18 kilodaltons (kDa) and 8 kDa, respectively. These two higher molecular weight species did not stimulate ACTH release from cultured rat anterior pituitary cells (Sasaki et al., 1988) and are thought to represent a precursor and a biosynthetic intermediate. The CRH gene is expressed in human placenta and hCRH-pl mRNA is the same size (1.3 kilobases; kb) as hypothalamic CRH (Grino et al., 1987)

Jones et al., (1989b) showed that hCRH-pl was synthesized in human placenta, decidua and fetal membrane cell cultures. These investigators showed that significantly more CRH was produced in placental and fetal membrane tissues collected after spontaneous term labour than tissues obtained at elective cesarean sections. It is now generally accepted that CRH immunoreactivity is present on the syncytiotrophoblast of
term placenta (Riley et al., 1991). Earlier studies by Saijonmaa et al., (1988) and Petraglia et al., (1987) had localized immunoreactive (ir)-hCRH-pl on the cytotrophoblasts in early and term human placenta, respectively. However, Riley et al., (1991) and showed that ir-hCRH-pl is present on the syncytiotrophoblast layer but that it was undetectable in the cytotrophoblasts (Cooper et al., 1994; Warren and Silverman, 1995; Perkins and Linton, 1995). Ir-hCRH-pl staining on the syncytiotrophoblast layer increased from the ninth week of gestation onwards. Positive staining for ir-hCRH-pl was also present on the intermediate trophoblasts cells, the chorionic trophoblasts and the epithelial cells of the amnion in term placenta (Riley et al., 1991; Warren and Silverman, 1995; Perkins and Linton, 1995). Ir-hCRH-pl is also present in the decidua and increases with gestational age (Petraglia et al., 1992).

Human CRH-pl is secreted into the maternal and fetal circulation and released into the amniotic fluid throughout human pregnancy (Sasaki et al., 1987; Goland et al., 1988). Plasma concentrations of ir-hCRH-pl in the maternal and fetal circulation increase in parallel to hCRH-pl levels in the placenta (Frim et al., 1987). In the first trimester of pregnancy plasma concentrations of h-CRH-pl are barely detectable (5.9 ± 0.8 pg/mL) (Sasaki et al., 1987; Goland et al., 1986; Maser-Gluth et al., 1985; Campbell et al., 1987). Plasma ir-hCRH-pl concentrations rise progressively from the second trimester of pregnancy (35.4 ± 5.9 pg/mL) (Sasaki et al., 1988) to term (~ 1000 pg/mL) (Campbell et al., 1987; Sasaki et al., 1987). With a significant increase throughout labour (~ 4000 pg/mL) (Okamoto et al., 1989; Petraglia et al., 1990). Immediately post-partum maternal plasma hCRH-pl concentrations decrease rapidly to
barely detectable levels (Sasaki et al., 1987; Campbell et al., 1987; Schulte and Healy 1987; Goland et al., 1988; Stalla et al., 1989). No information has been reported on the concentrations of the other CRH molecular weight species.

Human CRH-pl is thought to be secreted predominantly into the maternal circulation. At the time of delivery, mean umbilical cord hCRH-pl concentrations are 1000 fold lower than those reported in the maternal circulation (Sasaki et al., 1988). The umbilical venous plasma hCRH-pl concentration (50.6 ± 6.1 pg/mL) was significantly greater than that in simultaneously-obtained umbilical arterial plasma (41.8 ± 4.9 pg/mL) (Schulte and Healy, 1987). The decrease in maternal plasma hCRH-pl concentrations post-partum and the elevated concentrations of hCRH-pl in the umbilical vein when compared to the umbilical artery confirm the placenta as a source of hCRH-pl. Human CRH-pl concentrations, in the amniotic fluid, increase progressively throughout gestation (Maser-Gluth et al., 1987; Campbell et al., 1987; Laatikainen et al., 1988). Contrary to plasma hCRH-pl concentrations, however, amniotic fluid concentrations of hCRH-pl are not altered during labour (Petraglia et al., 1990).

Maternal plasma hCRH-pl concentrations are significantly elevated in PTL patients in comparison to gestational-age matched controls. This rise is apparent prior to the onset of labour (Linton et al. 1987; Warren et al., 1992; McLean et al., 1995).
1.8 CRH-BINDING PROTEIN

CRH binding protein (CRH-BP) is a 37 kDa peptide that binds hCRH-pl and inhibits its ACTH-releasing activity in anterior pituitary cultures (Orth and Mount, 1987; Linton et al., 1988). In addition CRH-BP decreases PG release from cultured maternal decidua and dampens the potentiated contractile activity of CRH and PGF$_{2\alpha}$ on human myometrium in vitro. CRH-BP is predominantly secreted by the maternal liver (Potter et al., 1991) but has also been identified on the syncytiotrophoblast, the intermediate trophoblast cells, the stromal cells of the decidua and the epithelial cells of the amnion (Petraglia et al., 1993; Ramirez et al., 1995).

Maternal plasma CRH-BP concentrations (~ 5 nmol/L) fall significantly (~ 2 nmol/L) in late pregnancy returning to basal concentrations 48 hours postpartum (Linton et al., 1993). In pregnancies complicated with PTL the decrease in plasma CRH-BP concentrations is more pronounced when compared with gestational-age matched controls (Orth, 1992). Mean CRH-BP concentrations, in fetal plasma also decrease prior to term (Linton et al., 1993). The mechanisms controlling the decrease of CRH-BP concentrations, in both pre-term and term pregnancies are still under investigation. Woods et al., (1994) suggested that the association of CRH-BP with CRH in blood may triggers the clearance of CRH-BP from the circulation. A decrease in plasma CRH-BP in both the maternal and the fetal circulations, would result in a net increase in the circulating concentrations of “biologically active” hCRH-pl (Linton et al., 1988), primarily because the majority of hCRH-pl in both the maternal and fetal circulations is bound to CRH-BP (Salminen-Lappalainen and Laatikainen, 1990).
1.9 OTHER REGULATORS OF CRH

It is well established that glucocorticoids inhibit hypothalamic CRH release in humans (Vale and Rivier, 1977; Owens and Nemeroff., 1991; Orth, 1992). However, in human pregnancy hCRH-pl concentrations rise paradoxically with plasma concentrations of cortisol (Fencl et al., 1980; Carr et al., 1981). Robinson et al., (1988) and Jones et al., (1989b) have shown that dexamethasone, a synthetic glucocorticoid, stimulates hCRH-pl synthesis and secretion from primary cultures of term human placentà, decidua, amnion and chorion. In addition, the neurotransmitters, norepinephrine and acetycholine, stimulate hCRH-pl output (Petraglia et al., 1989). In agreement with the mechanisms regulating hypothalamic CRH secretion interleukin-1β, neuropeptide Y, angiotensin II (Ang II), arginine vasopressin and OT increase the release of hCRH-pl in vitro from cultured trophoblast cells (Petraglia et al., 1991). In contrast, progesterone and nitric oxide (NO) inhibit hCRH-pl output from term human placenta, decidual, amnion and chorion (Jones et al., 1989b; Karialis and Mazoub, 1994; Sun et al., 1994).

1.10 MYOMETRIAL CONTRACTILITY

CRH does not have the intrinsic ability to stimulate human myometrial activity. Quartero and Fry (1989) have shown that CRH has both a priming and potentiating effect on the myometrial contractile response to OT. In addition, hCRH-pl stimulates a 3 to 4 fold increase in ir-OT release from placental cells in vitro (Florio et al., 1996). The rise in circulating concentrations of hCRH-pl in pregnancy coincides with an
increase in the sensitivity of the myometrium to OT (Caldeyro-Barcia and Serono, 1961; Fuchs et al., 1994) and may suggest a role for h-CRH in the regulation of OT receptor numbers. McLean et al., (1994) showed a positive association between maternal plasma hCRH-pl concentrations and the frequency of uterine contractions in women who entered labour following OT infusion.

CRH also enhances PGF$_{2\alpha}$-stimulated myometrial activity (Benedetto et al., 1994) and stimulates PGF$_{2\alpha}$ and PGE$_2$ release from primary cell cultures of placenta, chorion and amnion (Jones and Challis, 1989a). A role for hCRH-pl in the initiation of labour is also suggested by the presence of CRH binding sites in the myometrium (Hillhouse et al., 1993) and the recent identification of CRH receptor mRNA (Rodriguez-Linâres et al., 1998). This is central to our study and will be discussed later.

1.11 OTHER ROLES FOR CRH

McLean et al., (1995) proposed that the maternal plasma concentrations of hCRHpl at 16-20 weeks of human pregnancy could be an indicator of the timing of delivery and a determinator of patients at risk of PTL. The role of CRH as a marker of PTL has been controversial, because of wide interpatient variations in plasma CRH concentrations and the stability of hCRH-pl in plasma samples (Warren et al., 1992).

CRH-pl is a potent vasodilator in the human placenta vasculature and mediates its vasoactive effects via NO (Clifton et al., 1994). In addition, it has been hypothesized that hCRH-pl might contribute to the activation of the fetal pituitary
adrenal axis in late pregnancy, thus indirectly contributing to prenatal lung maturation (Petraglia et al., 1991). Finally, it has been suggested that there is a local CRH-POMC-ACTH axis within the placenta responsible for the increased concentrations cortisol in maternal plasma and urine (Carr et al., 1981; Goland et al., 1986; Margioris et al., 1988). This suggestion was made based on the presence of POMC mRNA and POMC-derived peptides in the placenta (Margioris et al., 1988).

1.12 CRH RECEPTORS

CRH binding sites have been identified in several tissues including the pituitary gland (De Souza et al., 1984), the brain (De Souza et al., 1985), the placenta (Petraglia et al., 1990; Clifton et al., 1995) and the myometrium (Hillhouse et al., 1993) by radioligand binding, autoradiographical and in situ hybridization techniques. There are at least two major classes of mammalian CRH receptors, CRH-R1 and CRH-R2.

Chen et al., (1993) recently reported the cloning of a cDNA encoding a CRH-R1 from a human corticotropic tumour library. The CRH receptor is comprised of seven putative membrane-spanning domains and belongs to the calcitonin/vasoactive intestinal peptide/growth hormone releasing hormone subfamily of G-protein coupled receptors. The cloned CRH-R1 cDNA encoded two apparent spliced forms of the receptor, CRH-R1α and CRH-R1β (Chen et al., 1993). CRH-R1α encodes a 415 amino acid peptide and appears to be the predominant form (see rev. Dieterich et al., 1997). CRH-R1β encodes a 444 amino acid peptide which has not been identified in any other species or specific tissue other than the human pituitary. Recently, cDNA's
encoding CRH-R1 homologs have been isolated and characterized in the human cortex, brainstem, hippocampus and testis, mouse pituitary (Vita et al., 1993) and rat brain (Perrin et al., 1993). Two other spliced variants of CRH-R1 have been reported; a receptor with a 40 amino acid deletion in the amino terminal domain (Ross et al., 1994) and a truncated receptor that contains a frameshift that only encodes the first 185 amino acids of CRH-R1α (Chang et al., 1993) (see Table 1.1). All species homologs of the CRH-R1 are of comparable size and are 98% homologous to one another.

CRH-R1 is a glycoprotein (Grigoriadis and DeSouza, 1989) and contains five potential N-linked glycosylation sites in the N-terminal extracellular domain (Chen et al., 1993). While the amino acid sequence of rat brain and rat pituitary CRH-R1 are 97% identical differences have been reported in their molecular weights which appear to be due to differential tissue specific glycosylation of CRH-R1 (Grigoriadis and De Souza, 1989a). In addition, five potential protein kinase C (PKC) phosphorylation sites have also been identified in the first and second intracellular loops and in the C-terminal tail (Chen et al., 1993) Both a casein kinase II and a protein kinase A (PKA) phosphorylation site are present in the third intracellular loop (Chen et al., 1993; Perrin et al., 1993).

CRH-R2 is encoded by a separate and distinct gene This receptor subtype has been cloned from rat brain (Lovenberg et al., 1995a), human (Liaw et al., 1996), mouse heart and skeletal muscle (see Table 1.2) (Kishimoto et al., 1995; Perrin et al., 1995; Stenzel et al., 1995). Two alternatively spliced forms of the receptor, CRH-R2α and CRH-R2β, encoding a 411 amino acid peptide and a 431 amino acid peptide
respectively, have been identified. Their amino acid numbers differ in that the first 34 amino acids in the N-terminal of CRH-R2α are substituted with a unique sequence of 54 amino acids in the CRH-R2β protein (see Figure 1.5). CRH-R2α and CRH-R2β have been identified in distinct anatomical zones. (Lovenberg et al., 1995b). CRH-R2α mRNA is abundantly expressed in rat brain and CRH-R2β mRNA is predominantly expressed in the rat heart and skeletal muscle. In the human CRH-R2α the prevailing CRH-R2 subtype in the heart and skeletal muscle (Valdenaire et al., 1997).

The CRH-R2 variants also have 5 potential N-linked glycosylation sites, identical to those in CRH-R1, as well as potential PKC phosphorylation sites (Lovenberg et al., 1995a). Both rat and human CRH-R2α share 70% sequence identity to CRH-R1α with the difference being the insert of amino acids in the first cytoplasmic loop (Lovenberg et al., 1995a). The receptors show high homology particularly in respect to the complete identity of the third intracellular domain (see rev. Dieterich et al., 1997). It has been reported that a major determinant of the coupling of G-proteins receptors to adenylate cyclase is located in the third intracellular loop (Okamoto et al., 1991). This is consistent with the demonstrated abilities of both CRH-R1 and CRH-R2 to increase intracellular cAMP (Chen et al., 1993; Lovenberg et al., 1995) and suggests conservation of second messenger function between the CRH-R1 and the CRH-R2 receptor genes. Nabhan et al., (1995), however, have reported that CRH-R2 is not well coupled to the G-protein in LLCPK-1 cells and requires elevated levels of CRH to stimulate intracellular cAMP levels. The CRH-R1 gene is present in the 17q12-q22
interval of the long arm of chromosome 17 (Polymeropuoulos et al., 1995) but the CRH-R2 gene has not yet been localized.

CRH binds to its receptor to form a complex that subsequently binds and activates a guanosine-5’ triphosphate stimulatory binding protein (Gs), causing GDP to dissociate from the inactive G protein, and GTP to bind the alpha (α) subunit of the G protein. The G protein α subunit dissociates from the βγ subunits, binds and stimulates the adenylate cyclase enzyme, which catalyses the intracellular cAMP synthesis from ATP. The released cAMP can bind cAMP dependent PKA. It is well documented that CRH binds its receptor with high affinity and stimulates adenylate cyclase activity leading to increased intracellular cyclic adenosine 3’5’ monophosphate (cAMP) in the rat pituitary (Labrie et al., 1982; Aguilera et al., 1983; Bilezikjian and Vale, 1983), the brain (Battaglia et al., 1987; Chen et al., 1986) and the myometrium (Grammatopoulos et al., 1994). The EC₅₀ values in stimulating cAMP production in the above mentioned tissues are similar (E₅₀ = 0.2 - 0.5nM). In addition, the binding of ¹²⁵I-radiolabeled oCRH (¹²⁵I-oCRH) to the receptor is inhibited by guanyl nucleotides, confirming that CRH receptors are coupled to the adenylate cyclase pathway by a guanyl nucleotide regulatory protein (Aguilera et al., 1987).

It has been suggested that optimal CRH receptor expression requires both the PKC and the adenylate cyclase pathway (Lutz-Bucher et al., 1990). The PKC pathway appears to be an important modulator of CRH function. A role for PKC in regulating CRH function is substantiated by the ability of arginine vasopressin and angiotensin II, which use PKC as their secondary messenger, to potentiate CRH-stimulated ACTH
from the pituitary (Abou-Sambara et al., 1986) and the placenta (Petraglia et al., 1991), and the demonstrated ability of direct activators of the PKC pathway to stimulate ACTH secretion and cAMP production in corticotrophs (see rev. Dieterich et al., 1997). Possible cross talk between PKC and adenylate cyclase is of interest as in certain sites, such as the rat Leydig cells, CRH receptors are not directly coupled to a G-protein, but mediate their actions via the direct or indirect actions of PKC (Dufau et al., 1989). Activation of several receptors from the 7 transmembrane receptor family has shown that they are able to couple to PLC and stimulate phosphoinositol hydrolysis when in the presence of high receptor or ligand concentrations (Jelinek et al., 1993; Spengler et al., 1993; Usdin et al., 1993; Nabhan et al., 1995).

The pharmacological properties of CRH binding sites are usually determined by the relative ability of a variety of CRH-related and unrelated peptides to displace specifically bound $^{125}$I oCRH or $^{125}$I rat/human CRH (r/h CRH) (see rev. Dieterich et al., 1997). The binding of $^{125}$I CRH to specific sites in the CNS and the periphery appears to be both saturable and reversible (De Souza et al., 1984; 1985; Hillhouse et al., 1993; Hatzoglou et al., 1996). In addition, CRH and CRH analogs bind these sites with high affinity binding, an apparent dissociation constant ($K_d$) of 0.2 - 0.8 nM. The similar $K_d$ of CRH and CRH fragments suggest that similar structural ligand requirements are shared by CRH receptors in the pituitary, the brain, the placenta and the myometrium. Recent work by Liaw et al., (1997) has found three regions in a chimeric receptor construct of CRH-R1α and CRH-R2α, that were required for the optimal binding of $^{125}$I-r/h CRH and/or receptor activation.
Increased exposure of target tissues to peptide hormones is usually linked to the
downregulation and desensitization of the homologous receptors. Desensitization
reflects either a short term, (affinity change, conformational alteration of the receptor
protein, uncoupling from the corresponding G-protein) or a long-term (downregulation
of the receptor) adaptation process (Dieterich et al., 1997). CRH regulates the
expression of its receptor in a tissue-specific manner, such that exogenous CRH
decreases CRH receptor concentrations in the rat anterior pituitary both in vivo and in
vitro (Wynn et al., 1983; 1988; Hauger and Aguilera., 1993; Lu et al., 1994; Sakai et
al 1996; Pozzoli et al., 1996) whereas intracerebroventricular administration of CRH
stimulates a significant increase in CRH-R1 mRNA in the PVN (Mansi et al.
1996). The expression of CRH receptors are also regulated by other factors such as
stress, adrenalectomy and glucocorticoids. Stress leads to a rise in circulating CRH,
causing a decrease in CRH receptor numbers in the hypothalamus (Makino et al.,
1995). Adrenalectomy results in an increase in circulating hypothalamic CRH, due to
withdrawal of adrenal cortisol negative feedback. This increase in plasma CRH causes
a transient decrease in CRH-R1 mRNA expression in the anterior pituitary and an
increase in CRH-R1 mRNA expression in the PVN (Luo et al., 1995). This effect was
removed with exogenous glucocorticoid administration (Wynn et al., 1983). High
circulating concentrations of glucocorticoids are known to decrease the expression of
CRH receptors in the PVN, the anterior pituitary and the brain (Makino et al., 1995)
and it has been proposed that high concentrations of glucocorticoids act synergistically
with CRH to decrease CRH receptor mRNA in the anterior pituitary (Makino et al.,
Recent studies by Makino et al., (1997) have shown that CRH-R1α mRNA and CRH-R2α mRNA are differentially regulated in the rat hypothalamic PVN after adrenalectomy and glucocorticoid administration: CRH-R1α mRNA was decreased in following these treatments whereas CRH-R2 mRNA levels were unaltered. This suggested that the expression of CRH-R1 mRNA and CRH-R2 mRNA is differentially regulated in the rat brain. This may also be reflected at other sites that express both the receptor subtypes.

CRH binds CRH-R1 with a higher affinity than CRH-R2 (Lovenberg et al., 1995a) whereas urotensin I and sauvagine bind CRH-R2α with a higher affinity than CRH in transfected mouse Ltk- cells and in rat heart and muscle cells (Lovenberg et al., 1995a; Perrin et al., 1995). It has been suggested that CRH is not the preferential ligand of CRH-R2 implying the possible existence of another endogenous ligand for this receptor subtype. A possible candidate is urocortin which has been proven to bind the CRH-R2, with a higher affinity than CRH (Vaughan et al., 1995).

Despite an understanding of some of the pharmacological and second messenger characteristics of CRH receptors, the mechanisms involved in the differential post-translational processing of CRH receptors remain to be elucidated. Differences have been reported in the molecular weight of CRH receptors present in the rat pituitary (58 kDa) (Grigoriadis and De Souza 1988; 1989b), the rat brain and the human placenta (75 kDa; Grigoriadis and DeSouza, 1989a; De Souza et al., 1985; Grigoriadis et al., 1993; Clifton et al., 1995) and the human myometrium (40 and 45 kDa; Castro et al., 1996). Grigoriadis et al., (1989a) deglycosylated the carbohydrate
moiety from the rat brain and pituitary CRH receptor and found that it was predominantly composed of N-acetylglucosamine and/or terminal sialic acid residues, as well as some high mannose chains. The native CRH receptor protein revealed, after deglycosylation, had molecular weight of 40 – 45 kDa. This was consistent with the molecular weight of CRH-R1 (Chen et al., 1993).

Differences can occur in the CRH receptors at the transcriptional, the post-transcriptional, the translational and the post-translational level. To understand the mechanisms functioning at these each of these stages it is important to determine the site of expression of CRH receptor mRNA and the protein levels. The regional and cellular expression of CRH receptor message has been characterized extensively in the rat brain and the rat pituitary (Potter et al., 1994; Chalmers et al., 1995). These studies report that CRH-R1 mRNA expression is abundant in the cerebral cortex, the subcortical limbic structures, the amygdala and in the anterior and intermediate lobe of the pituitary. CRH-R2 mRNA expression was found in distinct subcortical regions of the rat brain including the lateral septal nucleus, the ventromedial hypothalamic nucleus and the choroid plexus (Chalmers et al., 1995). It was also measured at lower levels in the olfactory bulb, amygdaloid nuclei, the paraventricular and supraoptic nuclei of the hypothalamus, as well as in the cerebral arteries throughout the brain. Based on the separate pattern of distribution of CRH-R1 mRNA and CRH-R2 mRNA in the brain it has been proposed that CRH-R1 is primarily a neuroendocrine receptor mediating the bioactive effects of CRH in the pituitary and that CRH-R2 is involved in
the hypothalamic neuroendocrine, autonomic and behavioral actions of central CRH (see rev. Dieterich et al., 1997).

The majority of the work reported on CRH receptor expression has been in the brain. However the pleiotropic effects of CRH in the human (Quartero and Fry, 1989; Clifton et al., 1994) suggests the presence of putative CRH receptors at multiple other sites. Recently CRH receptors have been identified within the structures of the human uterus including the placenta and the myometrium. This would suggest CRH may play a role in the mechanisms leading to parturition.

$^{125}$I-oCRH binding sites, have been localized almost exclusively on the syncytiotrophoblast of human placenta (Hatzoglou et al., 1996) and are thought to represent a single population of CRH receptors (Clifton et al., 1995; Hatzoglou et al., 1996). The binding of $^{125}$I-oCRH and CRH analogs to the placenta is dependent on time, temperature, pH and the presence of divalent ions and reversed on addition of excess oCRH (Clifton et al., 1995; Hatzoglou et al., 1996; Saeed et al., 1997). Interestingly, the number of $^{125}$I-oCRH binding sites in placenta obtained after vaginal delivery was increased compared to placenta delivered by elective cesarean section. This suggests an upregulation in CRH receptor numbers in the placenta at the time of labour in human pregnancy (Petraglia et al., 1990; Clifton et al., 1995). Clifton et al.,(1995) also reported that in vaginally delivered placenta, the CRH binding sites had a much lower affinity than in elective cesarean delivered placenta, they suggested that the high concentrations of hCRH-pl in the maternal circulation at term could regulate the binding properties of the CRH receptor. Despite the apparent presence of CRH
receptors in the human placenta, no studies have, as of yet, examined the expression of
the CRH receptor subtypes in this tissue or determined if these subtypes are
differentially regulated with pregnancy and/or labour.

Specific $^{125}$I-oCRH binding sites are present in human myometrium (Hillhouse
et al., 1993). These binding sites display similar properties to those in the placenta
(Clifton et al., 1995). A majority of the studies investigating the binding and second
messenger properties of the CRH receptor in the human myometrium have been
performed by the group of Hillhouse and Grammatopoulos. These investigators have
used myometrial samples solely from the lower uterine segment, have failed to
differentiate between the existence of CRH-R1 and/or CRH-R2 and have not studied
receptor concentrations or regulation throughout the time of labour.

Recently CRH-R1 mRNA and CRH-R2 mRNA expression have been
characterized in myometrium from nonpregnant and pregnant women (Rodriguez-
Lináres et al., 1998). Hillhouse et al., (1993) had initially identified the presence of a
single population of CRH receptors in the human myometrium. Subsequently they
reported the presence of multiple isoforms of the CRH receptor in the myometrium
collected from nonpregnant and pregnant women (Grammatopoulos et al., 1995). The
CRH receptor is reported to have a higher affinity in the myometrium of pregnant
women, when compared to the myometrium of nonpregnant women. While this
suggests a role for CRH in myometrial function in pregnancy, it fails to specify
whether there is a differential change in the affinity of all the CRH receptor isoforms. It
appears that other binding properties of CRH binding sites are in altered in pregnancy.
In the myometrium of nonpregnant women, the binding of $^{125}$I-oCRH rose with increasing concentrations of myometrial membranes, then stabilized at a plateau, while in the myometrium of pregnant women, the binding of $^{125}$I-oCRH myometrial membranes increased to a peak, and then decreased (Grammatopoulos and Hillhouse, 1994). The authors suggest that the presence of an inhibitor of CRH binding exists in the myometrium in term pregnancy. This is in contrast with the previous report from this group that demonstrated that the CRH receptor increased in affinity for CRH in late pregnancy (Hillhouse et al., 1993). However this is in agreement with recent work from this group where they suggest that in human term pregnancy OT is able to desensitize the CRH receptors in the myometrium by stimulating PKC-mediated phosphorylation of the receptor (Grammatopoulos et al., 1997). A role for CRH in regulating myometrial tone in pregnancy is evidenced by the ability of CRH to potentiate contractions of human myometrium in the presence of OT and PGF$_2$α (Quartero and Fry, 1989; Benedetto et al., 1994). Despite the demonstrated ability for CRH to act on human myometrium and the rise in plasma hCRH-pl concentrations throughout labour (Okamoto et al., 1989) no studies have examined the change in CRH receptors at the time of labour in the human myometrium.

The ability of CRH receptors to mediate the actions of CRH is dependent largely on the secondary messenger to which the receptor is linked. CRH activates a dose-dependent increase of both cAMP and PGE$_2$ from human myometrial membranes obtained in term pregnancies. This indicates that CRH receptors in the myometrium in term pregnancies are coupled to both the adenylate cyclase and the cyclo-oxygenase
secondary messenger pathways (Grammatopoulos et al., 1994). The ability of the CRH receptor to stimulate an increase in intracellular cAMP, via the adenylate cyclase pathway, is reduced at the end of pregnancy concurrent with the ability of CRH to stimulate PG synthesis (Grammatopoulos et al., 1994:1996). This would suggest a shift from the relaxation state of the myometrium, which is associated with increased intracellular cAMP, to the contractile state, associated with cyclooxygenase activity at term. However, no studies have examined the secondary messenger pathway mechanisms associated with the CRH receptors at the time of labour or post-partum.

This review has focused on the roles and mechanisms of CRH and the CRH receptor subtypes in human pregnancy. The human placenta secretes increasing concentrations of hCRH-pl into the maternal circulation in the last trimester of pregnancy and throughout labour, suggesting a role for this hormone in the mechanisms of parturition. The presence of CRH receptors in the human myometrium, suggests a local effect of CRH on the muscular component of the uterus. The demonstrated ability of CRH to stimulate PG synthesis and to enhance the contractile activity of the human myometrium, suggests CRH plays an active role in human parturition. To potentiate the reported roles of myometrial CRH receptors in the regulation of myometrial tone in labour, the mRNA expression and peptide concentrations of the CRH receptor subtypes have to be determined both in the absence and presence of labour. Furthermore, to determine the putative regulatory mechanisms involved in myometrial CRH receptor expression, it is important to establish a suitable mammalian model. Determining the presence and regulation of CRH receptors in the
human myometrium at the time of labour in pre-term and term pregnancies is important
to further our knowledge in the role of CRH in the mechanisms of parturition.
Figure 1.1 Schematic diagram of the intrauterine environment showing the position of the maternal and the fetal tissues in pregnancy. A. The membranes (left to right): the myometrium, the decidua *vera*, the chorion and the amnion. B. The membranes at the site of implantation (right to left): the perimetrium, the myometrium, the decidua *basalis*, the placental villi, the chorion and the amnion (Modified from Pritchard et al., 1995).
Figure 1.2 Schematic diagram showing the change in the size and shape of the uterus in term pregnancy and the development of the upper active and the lower passive segment at the time of labour (Modified from Pritchard et al., 1985).
Uterus in the Nonpregnant Woman

Uterus in Term Pregnancy

Uterus at the Time of Labour
Figure 1.3  Schematic diagram depicting the high intensity and long duration uterine contraction tracings in the upper active segment and the relative absence of contractions in the lower passive segment (Modified from Pritchard et al., 1985)
Figure 1.4 Comparison of the amino acid sequences of human CRH, frog sauvagine, sucker urotensin I and carp urotensin I. The one letter amino acid notation is used. The sets of identical residues are enclosed in solid lines (modified from Shibahara et al., 1983)

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
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<tbody>
<tr>
<td>A</td>
<td>Alanine</td>
</tr>
<tr>
<td>D</td>
<td>Aspartic Acid</td>
</tr>
<tr>
<td>E</td>
<td>Glutamic Acid</td>
</tr>
<tr>
<td>F</td>
<td>Phenylalanine</td>
</tr>
<tr>
<td>G</td>
<td>Glycine</td>
</tr>
<tr>
<td>H</td>
<td>Histidine</td>
</tr>
<tr>
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<td>Isoleucine</td>
</tr>
<tr>
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<td>Lysine</td>
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<tr>
<td>L</td>
<td>Leucine</td>
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<tr>
<td>M</td>
<td>Methionine</td>
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<tr>
<td>N</td>
<td>Asparagine</td>
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<td>Glutamine</td>
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<td>S</td>
<td>Serine</td>
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<td>Theorinine</td>
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<tr>
<td>V</td>
<td>Valine</td>
</tr>
<tr>
<td>Y</td>
<td>Tyrosine</td>
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</tbody>
</table>
Figure 1.5 Schematic diagram depicting the seven transmembrane structure and the amino acid sequence of CRH-R2\(\alpha\) and CRH-R2\(\beta\). The arrows point to the site in the N terminus where the sequence of CRH-R2\(\alpha\) and CRH-R2\(\beta\) diverge (Modified from Chalmers et al., 1996)

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Identification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yellow shaded amino acid</td>
<td>amino acid specific to CRH-R2(\alpha)</td>
</tr>
<tr>
<td>Red shaded amino acid</td>
<td>amino acid specific to CRH-R2(\beta)</td>
</tr>
<tr>
<td>Blue shaded amino acid</td>
<td>amino acid that differs between CRH-R1 and CRH-R2</td>
</tr>
<tr>
<td>(\Psi)</td>
<td>Potential sites for the phosphorylation of PKC</td>
</tr>
<tr>
<td>(\Delta)</td>
<td>Potential N glycosylation sites</td>
</tr>
<tr>
<td>Authors</td>
<td>Source of cDNA</td>
</tr>
<tr>
<td>--------------------</td>
<td>--------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Chen et al., 1993</td>
<td>Human corticotropic tumor library</td>
</tr>
<tr>
<td>Perrin et al., 1993</td>
<td>Rat brain cDNA library</td>
</tr>
<tr>
<td>Vita et al., 1993</td>
<td>Mouse pituitary tumor cell (AtT20) and human brain cDNA library</td>
</tr>
<tr>
<td>Chang et al., 1993</td>
<td>Cloned cDNA based on identified regions amongst all known members of 7-TMD Gs coupled receptors</td>
</tr>
<tr>
<td>Ross et al., 1994</td>
<td>Human hippocampus and fetal brain cDNA library</td>
</tr>
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</table>
### Table 2: Different Species Homologs of CRH-R2

<table>
<thead>
<tr>
<th>Authors</th>
<th>Source of cDNA</th>
<th>Receptor Expression</th>
<th>Receptor Properties</th>
<th>Second Messenger Pathway</th>
<th>Nomenclature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Perrin et al., 1995</td>
<td>Mouse heart cDNA library</td>
<td>Mouse heart, GIT, epididymis and brain</td>
<td>431 aa 16 additional aa in the N-terminal in comparison to CRH-R1α</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lovenberg et al., 1995</td>
<td>Rat hypothalamus cDNA library</td>
<td>Predominantly in rat brain, heart and skeletal muscle</td>
<td>411 aa Splice variant with a different N-terminal domain encoding a 431 aa</td>
<td>Binds sauvagine with a higher affinity than CRH to stimulate cAMP</td>
<td>CRH-R2α, CRH-R2β</td>
</tr>
<tr>
<td>Kishimoto et al., 1995</td>
<td>Mouse heart cDNA library</td>
<td>Mouse heart and skeletal muscle</td>
<td>430 aa 70 % homology with CRH-R1α</td>
<td>Binds sauvagine with a higher affinity than CRH to stimulate cAMP</td>
<td></td>
</tr>
<tr>
<td>Stenzel et al., 1995</td>
<td>Mouse heart cDNA library</td>
<td>Mouse heart, brain and lung</td>
<td>411 aa 94% identical to rat CRH-R2α</td>
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<td></td>
</tr>
<tr>
<td>Liaw et al., 1996</td>
<td>Human front cerebral cortex cDNA library</td>
<td>Human front cerebral cortex</td>
<td>411 aa 94% identical to rat CRH-R2α</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
CHAPTER TWO: RATIONALE AND HYPOTHESIS

2.1 STUDY 1

CRH-R1 mRNA and CRH-R2 mRNA are expressed in the myometrium of nonpregnant and pregnant women (Rodriguez-Lináres et al., 1998). CRH acts synergistically with OT (Quartero and Fry, 1989) and PGF$_{2\alpha}$ (Benedetto et al., 1994) to potentiate myometrial contractility suggesting it may contribute to the complex mechanisms involved in the onset and progression of labour in humans. To substantiate the reported roles of CRH, in the myometrium, the mRNA expression and the protein concentrations of both the CRH-R1 and CRH-R2 have to be determined in the upper fundal region and the lower segment of the uterus both in the absence and presence of labour. Furthermore, the expression of CRH-R1 mRNA and CRH-R2 mRNA in human myometrium have to be compared in pre-term and term pregnancies to assess the regulatory effects of gestational age and PTL on CRH receptor expression.

Based on the presence of elevated levels of bioactive hCRH-pl in the maternal circulation at the time of labour, in both term and PTL pregnancies, the presence of CRH receptors in the myometrium and the ability of CRH to enhance myometrial activity we hypothesized that the expression of CRH-R1 mRNA and/or CRH-R2 mRNA expression and protein concentrations in the human myometrium would be upregulated at the time of labour.
Our specific aims were:

1. To establish the presence of CRH-R1 mRNA and CRH-R2 mRNA and the localization of their proteins in human myometrium from both nonpregnant patients and pregnant patients.

2. To determine whether CRH-R1 mRNA and/or CRH-R2 mRNA expression in human myometrium is upregulated at the time of labour in term and/or in preterm pregnancies.

3. To determine whether CRH-R1 mRNA expression is differentially expressed in the myometrium from the upper fundal and the lower segment in nonpregnant, pregnant, labouring and postpartum.

4. To determine whether CRH-R1 mRNA and/or CRH-R2 are expressed in human decidua, chorion and amnion.

5. To determine if CRH-R1 mRNA and/or CRH-R2 mRNA expression was increased at the time of labour in the decidua, chorion and amnion.

2.2 STUDY 2

CRH receptors are abundantly expressed in rat brain and rat pituitary (Wynn et al., 1983; Aguilera et al., 1987). Extensive studies have been done in the rat brain and pituitary to determine the factors regulating pituitary and brain CRH receptor expression (Makino et al., 1995; Pozzoli et al., 1996; Sakai et al., 1996). Currently, no studies have examined the factors regulating the expression of CRH receptors in the
myometrium. Determining the endogenous factors regulating the transcription, translation and/or posttranslational expression of CRH receptors in the myometrium is a prerequisite to establish the role of CRH in pregnancy. Because of the obvious difficulties of performing these studies in vivo it is crucial to identify a mammalian model that expresses CRH-R1 mRNA and/or CRH-R2 mRNA in the myometrium.

The goal of our study was to establish a mammalian model to study the regulation of CRH receptor expression in the myometrium in pregnancy, in labour and post-partum. Based on the identical structure of the human and the rat CRH receptors and the similar bioactivity of human and rat CRH peptide we hypothesized that CRH-R1 mRNA and CRH-R2 mRNA expression would be increased at the time of labour in rat myometrium.

Our specific aims were:

1. To determine if CRH-R1 mRNA and CRH-R2 mRNA are expressed in the rat myometrium from day 15 of gestation to 1 day postpartum.

2. To determine if CRH-R1 mRNA and CRH-R2 mRNA expression is increased in rat at the time of labour.
CHAPTER THREE: CRH-R1 mRNA IS SIGNIFICANTLY UPREGULATED IN THE HUMAN MYOMETRIUM AT THE TIME OF LABOUR.

3.1. INTRODUCTION

The human uterus differentiates into an actively contracting upper segment and a relatively quiescent lower segment at the time of labour (Pritchard et al., 1985). The factors involved in this anatomic functional difference of the uterus remain unclear. CRH is a 41 amino acid peptide hormone, that has both a priming and potentiating effect on myometrial contractility (Quartero and Fry, 1989; Benedetto et al., 1994). Two distinct subtypes of CRH receptors, CRH-R1 and CRH-R2 have been isolated and characterized (Chen et al., 1993; Vita et al., 1993; Lovenberg et al., 1995; Liaw et al., 1996). The cloned cDNA of CRH-R1 was isolated from human pituitary (Chen et al., 1993) and rat brain (Perrin et al., 1993). Recently, the cDNA of CRH-R2 was isolated from rat (Lovenberg et al., 1995a; 1995b) and human (Liaw et al., 1995; Valdenaire et al., 1997) and encodes two anatomically distinct spliced forms of the receptor (Lovenberg et al., 1995a; 1995b).

The human placenta secretes increasing concentrations of hCRH-pl into the maternal circulation in the third trimester of pregnancy (Goland et al., 1987 reaching a peak at the time of labour (Okamoto et al., 1989; McLean et al., 1995). Recently CRH-R1 mRNA and CRH-R2 mRNA have been identified in the myometrium of pregnant women (Rodriguez-Linares et al., 1998). This strongly suggests that CRH may play an
important role in human parturition. We hypothesized that the expression of CRH-R1 mRNA and/or CRH-R2 mRNA expression and protein concentrations in the myometrium would be upregulated at the time of labour in human pregnancies. We examined the expression of CRH-R1 mRNA and CRH-R2 mRNA and their respective proteins in human myometrium in term and preterm pregnancies prior to at the time of labour. Moreover, we distinguished between myometrium collected from the upper segment and from the lower segment of the uterus. Finally we examined the level of expression of CRH-R1 mRNA and CRH-R2 mRNA in the decidua and fetal membranes in term pregnancies prior to and at the time of labour so to establish if the regulation of CRH receptor expression at the time of labour was specific to the myometrium.

3.2 MATERIALS AND METHODS

3.2.1 Biopsy Samples

Myometrial samples were collected from 2 groups of women: a) nonpregnant and b) pregnant. In the nonpregnant, premenopausal women (age 42 ± 1.5 years; n = 4) the myometrial samples were collected from the lower region of the uterus at hysterectomy, performed due to fibroid invasion. Pregnant patients (age 31 ± 2.4 years), were divided into the following groups: preterm no labour (mean gestational age (MGA) = 32 weeks; n = 5), PTL (MGA = 32 weeks; n = 6), term no labour (MGA = 39 weeks; n = 7) and term in labour (MGA = 39 weeks; n = 7). The myometrial samples were collected from the incision line within the lower segment. Regional myometrial
samples were also collected from the both the upper and the lower segment of the uterus collected in the nonpregnant patients (aged 40 ± 1.3 years; n = 4) at hysterectomy performed as a result of fibroids. Upper segment and lower segment myometrial samples were also collected from subjects (aged 32 ± 2.1 years; n = 4) at elective cesarean followed by a hysterectomy performed for progressive cervical cancer and in active labour (aged 35 years; n = 1) at a classical cesarean section performed due to remove conjoint twins. Finally, a myometrial sample was collected from the upper and the lower uterine segment at a hysterectomy performed post-partum (aged 34 years; n=1) for disseminated intravascular haemorrhage. The pregnant patients were not administered glucorticoids or oxytocin. All tissue samples were immediately snap frozen in liquid nitrogen and stored at -80°C.

Decidua (n=8), chorion (n = 4) and amnion (n = 4) were collected from term patients in the absence (MGA = 39 weeks) and the presence of labour (MGA = 39 weeks). The individual chorion and the decidua were separated by gentle scraping and immediately individually snap frozen in liquid nitrogen. All tissue biopsies were collected at Mount Sinai Hospital in Toronto, Ontario. Informed consent was obtained from all the patients and ethical approval for the study was obtained from the Bioethic Committee of Mount Sinai Hospital and the University of Toronto.

To obtain positive and negative control tissues virgin female Wistar rats (250-280 grams; Charles River Canada, St. Constant, Quebec, Canada) were decapitated and pituitaries and liver, respectively. Sheep pituitaries were also collected from fetuses at term delivery (day 145 gestation) following euthanasia with Euthanyl (5 mL) via
cardiac puncture to use as positive controls. All tissue samples were immediately snap frozen in liquid nitrogen and stored at -80°C.

All animal experimental protocols were approved by the Samuel Lunefeld Institute Animal Care Committee and Animal Care Committee of the University of Toronto according to the Guidelines of the Canadian Council of Animal Care.

3.2.2 Total RNA Extraction

Total RNA was extracted from the samples of myometrium, decidua, chorion, and amnion using the methods described by Chomczynski and Sacchi (1987). Briefly, frozen tissue samples (2-5 mg) were powdered under liquid nitrogen and homogenized in 1 mL of a denaturing solution (4M guanidinium thiocyanate, 25 mM sodium citrate, 0.5% sodium lauroylsarcosine, 0.1M β-mercaptoethanol (v/v)) using an ULTRA-TURAXX homogenizer (Janke & Henkel, IKA-Labortechnik, ON, Canada). Sodium acetate buffer (0.1 mL of 2M ; pH 4) was added to the tissue homogenate, followed by phenol (water-saturated) (1 mL) , and a chloroform-isoamyl alcohol mixture (0.2 mL ; 49:1). Each addition was followed by thorough mixing. The samples were incubated on ice for 15 minutes then centrifuged (Sorvall RC-5B, Du Pont Instruments, MA, USA) at 6,500g for 40 minutes at 4°C. The supernatant was transferred to a fresh polypropylene tube (12 mL; Becton and Dickinson, New Jersey, USA) and mixed with isopropanol (1 mL) and incubated for 1 hour at -20°C to allow RNA precipitation. The samples were centrifuged again at 6,500g for 1 hour 4°C. The resulting RNA pellet was dissolved in the same denaturing solution (see above; 1 mL), transferred to an
ependorf tube (1.5 mL, Diamed, Ont, Canada) and precipitated with an equal volume of isopropanol overnight at -20°C. The samples were then centrifuged for 15 minutes at 4°C. The RNA pellet was resuspended in 70% ethanol, (1 mL) vacuum dried and redissolved in double distilled water (ddH₂O) with 0.1% diethylpyrocarbonate (DEPC) water. The total RNA purity and recovery for each sample was determined with a UV spectrophotometer (Model DU-64, Beckman Instruments, Inc., CA, USA) at 260 and 280 nanometers.

3.2.3 Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Total RNA from myometrium, decidua, chorion, amnion, rat liver and rat pituitary was converted by reverse transcription into cDNA. The reverse transcription reaction mix consisted of 1 μg of total RNA, 1 x PCR buffer (10 mM Tris-HCl, 50 mM KCl, Perkin Elmer, Cetus), 5 mM MgCl₂ (Perkin Elmer, Cetus), 1 mM each of the dNTP (dATP, dCTP, dGTP, dTTP, Pharmacia), 5 ng/μL random (Pharmacia), 1 U/μL RNase inhibitor (Boehringer Mannheim) and 100U Moloney Murine Leukemia Virus Reverse Transcriptase (MMLV-RT; GibcoBRL, Gaithersburg, MD) in 21 μL DEPC water. Negative controls were prepared as above but without RNA, to confirm there was no contamination of the PCR reagents. The reaction mixture was incubated at 25°C for 10 minutes, then at 42°C for 30 minutes and finally at 99°C for 5 minutes. The resultant cDNA (reverse transcriptase; RT) mixture was stored at -20°C until it was used.
PCR was performed using the RT mixture. The PCR mixture consisted of 10 μL of the RT mixture, 0.25 mM dNTP, 50 ng of each specific PCR primer (ACGT Corporation, Toronto), 2.5 U Taq polymerase (Boehringer Mannheim) in 1.25 mM MgCl₂, 50 mM KCl, 10 mM Tris-HCl (pH 8.3) in 24.5 μL DEPC water. Each PCR reaction underwent an amplification regime characterized by a pre-incubation stage (95°C, 5 minutes), a denaturation stage (94°C, 30 seconds), a primer annealing stage (62°C, 30 seconds), an extension stage (72°C, 30 seconds) and a long extension stage (72°C, 8 minutes) in a thermal cycler (MJ Research Inc, Mass, USA). PCR reactions were also performed using RNA that had not been reverse transcribed to establish the extent of genomic DNA contamination in the RNA. PCR products were electrophoresed on a 2% agarose gel, stained with 1.5% ethidium bromide in Tris-acetate/EDTA buffer to allow visualization of the PCR product under a transilluminator (Lighttools Research, Ont, Canada).

Specific primers were used to identify a 333 base pair (bp) product for CRH-R1 in human myometrium, decidua, and fetal membranes (Slominski et al., 1995). A sense primer 5' GCC CTG CCC TGC CTT TTT CTA 3' and an antisense primer 5' GCT CAT GGT TAG CTG GAC CA 3' corresponding to positions 235-255 and 549-568, respectively were used (Accession number L23332) (Chen et al., 1993). Similarly, primers were designed to identify a 781 bp product for CRH-R2 in human myometrium, decidua and fetal membranes. A sense primer 5' GGC ATC AAG TAC AAC ACG AC 3' and an antisense primer 5' CAT CCA GTA CAG GAA GGC AG 3' corresponding to positions 423-442 and 1184-1204, respectively, were used (Accession
number U16253) (Lovenberg et al., 1995a). β-actin gene expression (internal control) was also determined in all samples to assess the integrity of the RNA. Primers were designed to identify a 218 bp product for β-actin in all the samples. A sense primer 5’ AAG AGA GGC ATC CTC ACC CT 3’ and an antisense primer 5’ TAC ATG GCT GGG GTG TTG AA 3’ corresponding to positions 222- 241 and 420- 439, respectively were used (Accession number M10278). The designed primers do not flank intron segments.

3.2.4 Semi-Quantitative PCR

Further analysis using semi-quantitative PCR (sq-PCR) was performed to compare myometrium obtained after different treatments (Kinoshita et al., 1992). To obtain measurements in the linear range of CRH-R1 cDNA we used 28, 30, and 32 cycles and in the decidua and fetal membranes we used 31, 33 and 35 cycles. To obtain measurements in the linear range of CRH-R2 cDNA in human myometrium we used 31, 33, and 35 cycles. The cycles used for β-actin were 16, 18,20. The relative intensity of cDNA signals was quantified from negatives using a computerized image analysis (Imaging Research Inc., St. Catherine, Ont, Canada).

The identity for the PCR product for CRH-R1 was confirmed using 10U of the restriction enzymes Alu I (GibcoBRL) and BSR I (New England Biolabs) in the appropriate buffer (10 µL) Restriction buffer A, (GibcoBRL) and Buffer 3 (New England Biolabs). The PCR product (0.5 – 2 µg cDNA) was incubated at 37°C (Alu I)
or at 65°C (BSR I) for 2-3 hours. The Alu I enzyme digest was inactivated at 65°C for 10 minutes then products were run on a 2% agarose gel to allow visualization.

3.2.5 Immunohistochemistry

Myometrial samples from nonpregnant patients (n = 4) and term pregnant patients in the absence (n = 4) and the presence of labour (n = 4) were removed and fixed in 4% paraformaldehyde and 0.2% glutaldehyde for 24 hours following tissue collection. The samples were then washed twice daily in phosphate-buffered saline (PBS, 0.01 M, pH 7.4) for three days and stored at in 70% ethanol at 4°C. The samples were embedded in paraffin by the Pathology labs at Toronto General Hospital (Toronto, Ontario, Canada). Sections (5 μm) were cut on a microtome (Leica RM 2035, Nussloch, Germany) and placed on glass slides coated with 2% aminopropyltriethoxy-silane (APTEX; Sigma Chemical Co., Missouri, USA) in acetone and dried for 24 hours at 37°C. The slides were deparaffinized with 3 washes of xylene substitute (EM Diagnostic Systems, NJ, USA) for 5 minutes each, then rehydrated in a series of washes 2 minutes each, in 100%, 95%, 70% and 50% ethanol, followed by washing in 0.01 M PBS (pH 7.4). Endogenous peroxidase activity was quenched by incubation in 3% hydrogen peroxidase (in PBS) followed by incubating the sections with 10% normal rabbit serum to inhibit nonspecific staining.

The slides were incubated with the primary antibodies for CRH-R1 and CRH-R2 at 4°C overnight. The primary antibody for CRH-R1 (Santa Cruz Biotechnology Inc., CA, USA). was a polyclonal antibody raised in a goat against a peptide
corresponding to amino acids 425-444 of the human and rat CRH-R1 (Chen et al., 1993). The primary antibody for CRH-R2 (Santa Cruz Biotechnology Inc., CA, USA) is a polyclonal antibody raised in a goat against a peptide corresponding to amino acids 47-66 of the rat CRH-R2 (Lovenberg et al., 1995). The CRH-R1 antibody cross-reacts with CRH-R2. Therefore, in some sections we pre-absorbed the CRH-R1 antibody with an excess of CRH-R2 peptide (1 μM) (Santa Cruz Biotechnology Inc., CA, USA) to determine CRH-R1 staining. The primary antibodies were diluted to 1:175 in antibody dilution buffer (1g bovine serum albumin, 0.02 g sodium azide in 100 mL PBS; pH 7.5). After 16 hours of incubation with the primary antibody, the slides were washed twice in PBS for 5 minutes each and incubated with biotinylated secondary antibody (1:500; Vectastain ABC kit; Vector Laboratories, CA, USA) at room temperature for 2 hours. The sections were washed twice in PBS for 5 minutes and incubated with the avidin-biotin-peroxidase complex (ABC; Vectastain) in PBS for 2 hours at room temperature. To visualize antibody staining the sections were incubated with 3',3-diaminobenzidine tetrahydrochloride dihydrate (DAB; Sigma Chemical Co., Miss, USA) for 10 minutes. The DAB solution was made by dissolving 50 mg of DAB in 200 mL PBS and adding 2 drops of hydrogen peroxide immediately prior to use. The slides were washed in ddH20 water, counterstained with Carazzi's hematoxylin for 45 seconds, rinsed in ddH20, dehydrated in an alcohol series; 50%, 70%, 95%, 100% for 2 minutes each and incubated in xylene for 3 washes of 5 minutes each. The slides were then mounted with Permount (Fisher Scientific, Ontario, Canada) and viewed with a microscope (Leica, DMRB, Nussloch, Germany). Sheep pituitaries were used as
positive controls for CRH-R1. For the negative controls, the primary antibody was preabsorbed with synthetic receptor peptide (1 μM; Santa Cruz Biotechnology Inc., CA, USA).

3.2.6 Protein Extraction

Protein was extracted from tissue with the use of RIPA lysis buffer. Human myometrial tissue (n=8 term pregnancy and n=7 at the time of labour), rat brain and rat heart (positive controls) and rat liver (negative control) tissue (1-2 grams) was ground in a pestle placed on dry ice. The ground tissue was transferred to a fresh polypropylene tube (12 mL; Becton and Dickinson, New Jersey, USA) and pulverized (Janke & Henkel, IKA-Labortechnik, ON, Canada) for approximately 30 seconds in RIPA lysis buffer (50 mM Tris-HCl; pH 7.5, 150 mM NaCl, 1% Triton X-100; v/v, 1% sodium deoxycholate; w/v, 0.1% sodium dodecyl sulphate (SDS); w/v, 100 μM Na orthovanadate, 100 μg/μL leupeptin, 1 mM phenylmethyl-sulfonyl fluoride while maintained on ice. The tissue homogenates were spun in a microcentrifuge (Sorvall RC-5B, Du Pont Instruments, MA, USA) at 8,500g for 15 minutes at 4°C. The supernatant was collected, aliquoted into eppendorf tubes (1.5 mL, Diamed, Ont, Canada) and stored at -80°C until used.

Prior to use, a sample (10μl) of the protein was assayed to determine concentration with a bicinchoninic acid (BCA) protein assay kit (Pierce, Illinois, USA). A fresh set of standards ranging from 200 μg/ mL to 3 μg/ 2 mL were made by diluting 2 mg/mL bovine serum albumin (BSA) stock standard in BCA working reagent (50
parts BCA reagent A with 1 part BCA reagent B). The standard or protein sample (10 μl) where added to the working reagent (same as above; 2 mL), in a borosilicate tube and thoroughly mixed. The tubes where then maintained (45 minutes) in a water bath (37°C). The protein in the tubes was measured at absorbance 562 nm versus a water reference. A standard curve was prepared by plotting the 562 nm reading for each BSA standard versus its concentration in μg/mL and used to determine the protein concentration of the extracted samples.

3.2.7 Western Blots

Western blot gels where made using a miniprotean II cell (Bio-Rad, California, USA). A 10% separating gel mixture was made in an erlmeyer flask with 1. 5 M Tris-HCl (pH 8.8; 5 mL), 10% SDS, acrylamide/bis (37.5: 1; 3.2 mL) and ddH2O (4 mL) was deaerated for 15 minutes. Then, 10% fresh ammonium persulfate (50 μL) and N,N,N',N-tetramethylenediamine (10 μL; Biorad, Clif, USA) were added. the gel was poured and left to polymerise for 1 hour with a ddH2O overlay. A 4% stacking gel was prepared from 0.5 M Tris-HCl (pH 6.8; 2.5 mL), acrylamide/bis (37.5: 1;1.3 mL), and ddH2O (6.1 mL) and was deaerated for 15 minutes. Immediately after 10% fresh ammonium persulfate (50 μL) and N,N,N',N-tetramethylenediamine (10 μL) were added, the gel was poured on top of the stacking gel, a 10 or 15 well comb was inserted. The mixture was left to polymerise for 1 hour.

An aliquot of the protein samples was thawed and diluted with 0.5 M Tris-HCl (pH 6.8) to a final concentration of 10 μg/μL. The protein (100 μg) from each sample
was mixed with 10 μL of loading buffer (Tris-HCl; 0.15 g, glycerol; 1 mL, 10%SDS; 4 mL, 2-β-mercaptoethanol, 20 mg bromophenol blue and ddH2O; 4 mL) in an eppendorf (1.5 mL, Diamed, Ont, Canada). The samples/loading buffer were heated at 95°C, in a waterbath, for 4 minutes and cooled at room temperature. The gel apparatus was placed into the buffer chamber and the electrode buffer (Tris base; 9 g, glycine; 43.2 g, SDS; 3 g and ddH2O; 600 mL) added. The wells were loaded and the gel was run at 100 volts for 2 hours with a prestained protein marker (Broad range, New England Biolabs). The gel was transferred at 15 volts for 30 minutes at room temperature onto nitrocellulose blotting membrane. The blot was washed (3X 10 minutes) in TTBS (150 mM NaCl and 50 mM Tris-HCl) with 0.05% Tween-20 and incubated in 4% BSA in TTBS with 0.05% Tween-20 for 30 minutes. The blots where incubated in the primary antibodies for CRH-R1 (same as above) and CRH-R2 (same as above) at 4°C overnight. The primary antibodies were used at a range of dilutions (1:200 - 1:2000) to establish the optimal antibody dilution.

The blot was washed (3 X 10 minutes) in TTBS with 0.05% Tween-20 and incubated with biotinylated secondary antibody (1:1000 in TTBS; same as above) for 2 hours. The blot was washed again and incubated in the avidin/ biotin complex (1:1000 in TTBS; Vectastain) for 2 hours. Finally the blots were developed in 12 mg DAB (Sigma Chemical Co., Miss, USA) in ddH2O from 30 seconds to a maximum of 5 minutes to ensure maximal staining of the blot.
3.2.8 Data Analysis

To correct for differences in the initial amount of RNA used for RT-PCR we determined β-actin mRNA expression in all the samples. The ratio of the optical densitometry reading measurements for the expression of CRH-R1 mRNA or CRH-R2 mRNA (at 3 progressive amplification cycles) to that of β-actin mRNA expression (at 3 progressive amplification cycles) was determined for each sample. The mean of the ratio was determined for each treatment from all the samples within that group. A Mann-Whitney Rank Sum Test was performed to judge the change in CRH receptor expression at the time of labour in term and then in preterm pregnancies. To determine the difference amongst all the treatment groups (PTL no labour, PTL in labour, term no labour and term in labour) a Kruskal-Wallis One Way Analysis of Variance (ANOVA) on Ranks was performed followed by an All Pairwise Multiple Comparison Procedure (Dunn’s Method) to isolate the group or groups that differ from the others.

3.3 RESULTS

3.3.1 CRH Receptor Expression in the Myometrium of Nonpregnant Patients

Enzyme digestion of CRH-R1 was used to confirm the identity of the receptor (Figure 3.1) and yielded fragments of the expected size. Digestion with BSR I yielded a 244 bp product and digestion with Alu I produced a. Both the expression of CRH-R1 mRNA and CRH-R2 mRNA were present in the myometrium obtained from the non-pregnant hysterectomy patients (Figure 3.2). We identified the expected band of 333 bp representing CRH-R1 (maximally expressed after 32 cycles) and the expected band of
781 bp representing CRH-R2 (maximally expressed after 35 cycles). CRH-R1 mRNA was present at consistently high levels in all the samples studied whereas CRH-R2 mRNA expression was variable. When using primers designed to identify CRH-R2 we observed a second band at 500 bp. We suggest this band represents a spliced form of the receptor. β-actin mRNA (maximally expressed after 20 cycles) expression was present at similar levels in all the patients.

Both the CRH-R1 and CRH-R2 protein were localized in the circular and longitudinal smooth muscle bundles of the human myometrium (Figure 3.3A and Figure 3.4A, respectively) and in the smooth muscle of the myometrial vasculature (Figure 3.5A and Figure 3.6A, respectively). CRH-R1 and CRH-R2 staining was preabsorbed from the uterine smooth muscle (Figure 3.3C and Figure 3.4C) and from the smooth muscle of the vasculature (Figure 3.5C) with an excess of peptide (1 µM). CRH-R1 was present in the sheep pituitary (Figure 3.7A and Figure 3.7B) and the staining was pre-absorbed with CRH-R1 peptide (Figure 3.7C). CRH-R2 was not detected in the pituitary (Figure 3.7D). CRH-R1 and CRH-R2 were not detected in the human myometrium, the rat heart, brain and liver by Western blot.

3.3.2 CRH-R1 mRNA and CRH-R2 mRNA Expression in Myometrium Pregnant Patients

We identified CRH-R1 mRNA expression in myometrium from the lower segment of women in term and preterm pregnancies (Figure 3.8). CRH-R1 mRNA expression was amplified at 28, 30, and 32 cycles in the samples from term and preterm myometrium to obtain linear measurements in the expression of the gene. CRH-R1
mRNA expression was significantly upregulated (p < 0.03; Mann-Whitney Rank Sum Test) at the time of labour in term pregnancies (MGA= 39 weeks; Figure 3.9). CRH-R1 mRNA expression was also significantly upregulated (p < 0.01; Mann-Whitney Rank Sum Test) at the time of labour in preterm pregnancies (MGA= 32 weeks; Figure 3.10). CRH-R1 mRNA expression showed a trend towards rising from 32 weeks to 39 week's gestation and rose significantly at the time of labour (p < 0.05; All Pairwise Multiple Comparison Procedures) (Figure 3.11). CRH-R1 protein was undetectable in human myometrium at term (Figure 3.3B and Figure 3.5B) but was detected in the uterine smooth muscle and the smooth muscle of the vasculature at the time of labour (Figure 3.3D and Figure 3.5D).

We identified CRH-R2 mRNA in the myometrium of some (28%) of the pregnant patients. We did not find a significant change in CRH-R2 mRNA expression at the time of labour (Table 3.1) In the preterm pregnancy, CRH-R2 was present in the myometrium of 3 of the patients not in labour (n = 5) and in 2 of the patients in labour (n = 6 ). In term pregnancy CRH-R2 mRNA was not detectable in the myometrium of the patients not in labour (n = 7) but was present in 2 of the patients in labour (n = 7). CRH-R2 protein was not detectable in the myometrium of term patients (Figure 3.4B and Figure 3.6B) but was present in the myometrium of pregnant in labour patients (Figure 3.4D and 3.6C) at the antibody concentrations used.
3.3.3 Regional Expression of CRH-R1 in Myometrium From Nonpregnant, Pregnant, Pregnant in Labour and Post-Partum Patients

Samples were taken from the upper and the lower segments of the uterus to examine differences in the regional distribution of CRH-R1 in the myometrium. CRH-R1 mRNA expression was present in the upper and lower segment of human myometrium in nonpregnant (n = 4), pregnant (n = 4), pregnant in labour (n = 1) and in post-partum (n = 1) women (Figure 3.12) CRH-R1 mRNA expression was present at similar levels in myometrium from the upper fundal and the lower segment of the uterus nonpregnant and pregnant women as determined CRH-R1 mRNA/β-actin mRNA expression. CRH-R1 mRNA expression was apparently higher in myometrium from the lower segment when compared to the upper segment in the at the time of labour. CRH-R1 mRNA expression was apparently decreased in the lower segment post-partum and returned to levels similar to that in the nonpregnant patients (Figure 3.12). CRH-R1 mRNA expression appears to stay relatively constant in myometrium from the fundal segment in the nonpregnant, the pregnant, the labouring and the post-partum patients.

3.3.4 CRH-R1 mRNA Expression in the Fetal Membranes and Decidua

CRH-R1 mRNA expression was present in the decidua and the chorion but was not significantly altered at the time of labour as determined by analysis of sq-PCR CRH-R1 mRNA/β-actin mRNA expression (Figure 3.12). CRH-R1 mRNA expression was undetectable in the amnion. CRH-R2 mRNA expression was undetectable in the chorion, the amnion and the decidua after 40 amplification cycles.
3.4 DISCUSSION

Using RT-PCR we have shown that CRH-R1 mRNA and CRH-R2 mRNA expression and their proteins are present in the myometrium of both non-pregnant and pregnant women. CRH-R1 mRNA expression is significantly upregulated at the time of labour in both preterm and term pregnancies. The rise in CRH-R1 mRNA expression appeared to be specific to the lower segment of the uterus and disappeared immediately postpartum. CRH-R2 mRNA expression was not significantly changed and at the time of labour in both term and preterm human pregnancies. CRH-R1 protein was prevalent in the myometrium from nonpregnant women, was undetectable in term pregnancy and was increased at the time of labour in the uterine smooth muscle and the uterine vasculature. CRH-R2 protein was present in the smooth muscle and the vasculature of the uterine vessels in the nonpregnant myometrium, was undetectable in term pregnancy and was modestly increased only in the smooth muscle at the time of labour.

The “classical section”, a vertical incision into the body of the uterus from the lower uterine segment to the upper fundal segment of the uterus is rarely used (Pritchard et al., 1995), therefore making it difficult to obtain samples from the active region of the uterus. Most always the incision is made in the lower uterine segment. Moreover, because the size and shape of the uterus varies with age and parity it is impossible to collect myometrial samples at the same site in all the patients. We only used nonpregnant patients undergoing hysterectomy for fibroidal growth so as to eliminate the possible effect of this condition on gene expression. However, the tissue may not be representative of normal tissue. CRH-R1 mRNA expression in the
labouring patients was variable, possibly due to differing stages of labour and/or the duration of labour prior to the surgery however, we no longer have access to this information.

We have used PCR to detect the expression of CRH-R1 mRNA and CRH-R2 mRNA. There are difficulties associated with the use of this technique for quantitative analysis of gene expression. To allow us to quantitate gene expression we have obtained the linear expression of the CRH-R1 and CRH-R2 gene by gradually increasing the number of amplification cycles. We have used the ratio of the optical densitometry readings obtained within the linear range of expression of CRH-R1 mRNA / CRH-R2 mRNA to β-actin mRNA. Whereas this method is only semi-quantitative it is sensitive and effective method for comparing genes expressed at low levels.

The low levels of CRH-R1 mRNA expression and the undetectable levels of CRH-R1 protein in pregnancy suggests there is a decrease in the transcription of the CRH-R1 gene which is associated with a decrease in the translation of CRH-R1 mRNA in the human myometrium in pregnancy. It is important to note that the CRH-R1 mRNA may be present in the human myometrium at term pregnancy but at levels below the detection limits of the antibody concentration used. The rise in CRH-R1 mRNA expression at the time of labour could suggest an increase in the transcription rate of the CRH-R1 gene or an increase in the stability of CRH-mRNA at the time of labour. The significance of the upregulation CRH-R1 gene transcription is not known however it appears to be restricted to the myometrium from the lower uterine segment
conclusions with regards to the levels of CRH-R1 protein in the human myometrium. We suggest that, at the time of labour, CRH-R1 helps to preserve the quiescence of the lower uterine segment. In this way the low decreased amplitude and intensity of myometrial contractions will be maintained. This proposal is consistent with CRH acting through a cAMP second messenger pathway (Labrie et al., 1982; Aguilera et al., 1987).

In contrast, reports by Quartero and Fry, (1989) report that CRH acts synergistically with OT and PGF$_{2\alpha}$ to potentiate myometrial contractility and that this synergistic effect appears to be mediated via a fall in cAMP levels in the lower uterine segment (Quartero et al., 1992). Benedetto et al., (1994) were unable to obtain CRH-OT potentiated myometrial contractility, however they showed that only when myometrial strips where pre-incubated in CRH they demonstrated enhanced myometrial contractility when stimulated with PGF$_{2\alpha}$. No contractility was observed with PGE$_2$. These studies suggest a role for CRH in potentiating myometrial contractility and are supported by Grammatopoulos et al., (1996). These investigators suggested that there was a modification in the coupling between CRH receptors and adenylate cyclase in term pregnancy. This resulted in a reduction of CRH-stimulated cAMP production at a time when the CRH receptor was coupled to the cyclooxygenase pathway (Grammatopoulos et al., 1994). While this is an attractive theory to explain the shift from the relaxed uterus at term to the contracting uterus at the time of labour, the authors only measured PGE$_2$ concentrations in their myometrial membrane media. PGE$_2$ has the paradoxical ability to mediate relaxation or stimulation dependent on the
receptor subtype present or if it is converted to PGF$_{2\alpha}$ (Nelen and Breckwoldt, 1994). The authors fail to explain why CRH receptors in the myometrium of nonpregnant women pathway are coupled to the cyclooxygenase pathway and not the adenylate cyclase pathway. This data suggests the cyclooxygenase pathway may be constitutive in the myometrium and possibly not be a major component in the onset of human labour.

In all the above mentioned studies the authors have used myometrium from the lower uterine segment. Our data suggests that the regulation of CRH-R1 mRNA expression in the lower segment at the time of labour does not appear to reflect the regulation of CRH-R1 mRNA at this time in the fundal region. Differentially regulated CRH-R1 mRNA expression within the human uterus at the time of labour suggests CRH may mediate different roles in these regions. This brings into question the ability to assess the role of CRH at the time of labour from samples collected solely in the lower uterine segment.

Most of the past studies in the uterus myometrium have failed to differentiate between the CRH receptor subtypes, we are the first group to compare CRH-R1 mRNA and CRH-R2 mRNA expression in the human myometrium at the time of labour. The presence of heterogeneous CRH receptors in the myometrium is not a novel concept, Grammatopoulos et al., (1995) had previously identified heterogenous populations of CRH receptors in myometrium. We show that CRH-R1 mRNA and CRH-R2 mRNA expression are differentially regulated at the time of labour suggesting that these receptor subtypes may mediate distinct functions in the human myometrium at the time
of labour. Differential regulation of the CRH receptor subtypes has previously been reported by Mansi et al., (1996) and Makino et al., (1997).

Little is known about CRH receptors in the myometrium of nonpregnant women. We have detected the expression of CRH-R1 mRNA and CRH-R2 mRNA in myometrium obtained from non-pregnant patients at hysterectomy. This is in accordance with Hillhouse et al, (1993) who identified the presence of specific CRH binding sites on human myometrium. We have shown that CRH-R1 mRNA is the predominant receptor subtype in the lower region of the uterus, and that it is present at similar levels in the fundus and the isthmus region. We have localised CRH-R1 and CRH-R2 protein on the uterine smooth muscle and on the smooth muscle layer of the uterine vasculature in the myometrium of nonpregnant patients. We are the first to identify both CRH-R1 and CRH-R2 protein in the smooth muscle of the myometrial vasculature. Based on the vasoactive effects of CRH (Clifton et al., 1994) we suggest CRH may be involved in the regulation of myometrial blood flow, however, the physiological significance of CRH in the myometrium of nonpregnant women remains to be determined.

In conclusion, CRH-R1 mRNA and CRH-R2 mRNA expression was present in the human myometrium in nonpregnant women and in pregnancy. CRH-R1 mRNA was significantly upregulated in myometrium from the lower uterine segment, at the time of labour. Our finding of CRH receptors in myometrium from pregnant women, is in accordance with reported roles for CRH in the myometrium however the function of CRH receptors in the myometrium are still unclear and requires further studies.
Figure 3.1 Enzyme digestion to confirm the identity of CRH-R1 cDNA: A. original 333 bp product, B. digestion with Alu I (244 bp), C. digestion with BSR I (277 bp).
Figure 3.2 Photograph of a PCR gel showing the detection of CRH-R1 mRNA (expected band size 333 bp; after 32 cycles) and CRH-R2 mRNA (expected band size 781 bp; after 35 cycles) in human myometrium (n = 4) obtained at hysterectomy. A 500 bp band was present in association with the 781 bp band in the CRH-R2 PCR reactions. The detection of β-actin mRNA (218 bp), the internal control is shown.
Figure 3.3 Photograph showing immunostaining for CRH-R1 protein in the human myometrium smooth muscle (SM) and blood vessels (BV). Magnified 200x

A. Myometrium in the nonpregnant
B. Myometrium in term pregnancy
C. Myometrium in nonpregnant (negative control- preabsorption with CRH-R1 antibody)
D. Myometrium at the time of labour.
Figure 3.4 Photograph showing immunostaining for CRH-R2 protein in the human myometrium smooth muscle (SM). Magnified 200x

A. Myometrium in the nonpregnant
B. Myometrium in term pregnancy
C. Myometrium in nonpregnant (negative control-preabsorbtion with CRH-R2 antibody)
D. Myometrium at the time of labour.
Figure 3.5 Photograph showing immunostaining for CRH-R1 in the smooth muscle layer of the vasculature (indicated by the arrow). Magnified 200x.

A. Myometrium in the nonpregnant
B. Myometrium in term pregnancy
C. Myometrium in nonpregnant (negative control- preabsorption with CRH-R1 antibody)
D. Myometrium at the time of labour (Magnified 400x).
Figure 3.6 Photograph showing immunostaining for CRH-R2 in the smooth muscle layer of the vasculature (indicated by the arrow). Magnified 200x

A. Myometrium in the nonpregnant
B. Myometrium in term pregnancy
C. Myometrium at the time of labour.
Figure 3.7 Photograph of CRH-R1 immunostaining in the sheep pituitary.

A. CRH-R1 in the region adjacent to the intermediate lobe (IL) (Magnified 200X)
B. CRH-R1 is absent in the IL (Magnified 200X)
C. Preabsorption of CRH-R1 antibody in the region adjacent to the IL (Magnified 200X)
D. CRH-R2 is absent in the intermediate lobe (Magnified 400X)
Figure 3.8 Photograph of a PCR gel showing the detection of CRH-R1 mRNA (333 bp; after 32 cycles) in the human myometrium in pregnancy (NL) and at the time of labour (L). The detection of β-actin mRNA (218 bp), the internal control is shown.

A. CRH-R1 mRNA detection in term pregnancy (n=7) and at the time of labour (n=7).  
B. CRH-R1 mRNA detection in pre-term pregnancy (n = 6) and at the time of labour (n=5).
Figure 3.9 Analysis of sq-PCR for CRH-R1 mRNA (333 bp; 28, 30, and 32 cycles), in term pregnancy, in myometrium, from the lower segment prior to (NL) and after (L) the onset of labour. CRH-R1 mRNA expression is significantly increased (p < 0.03) at the time of labour (p < 0.03), in human myometrium, in term pregnancy.
No labour  Labour

CRH-R1

β-actin

333 bp

218 bp

CRH-R1 mRNA / β-actin mRNA

No Labour  Labour

p < 0.03
Figure 3.10 Analysis of sq-PCR for CRH-R1 mRNA (333 bp; 28, 30, and 32 cycles) in myometrium, from the lower segment, prior to (NL) and after (L) the onset of labour. CRH-R1 mRNA expression is significantly increased (p < 0.01) at the time of labour, in the human myometrium, in pre-term pregnancy.
CRH-R1

β-actin

No labour  Labour

333 bp

218 bp

CRH-R1 mRNA / B-Actin mRNA

p < 0.01
Figure 3.11 CRH-R1 mRNA expression in the human myometrium increases from 32 weeks \((n = 5)\) to 39 weeks \((n = 7)\) of gestation and rises further to similar levels at the time of labour in pre-term \((n = 6)\) and term \((n = 7)\) pregnancies.
Figure 3.12 Photograph of a PCR gel showing the detection of CRH-R1 mRNA (333 bp; after 32 cycles) in human myometrium from the fundal (F) and lower (L) segment of the human uterus. CRH-R1 mRNA expression is shown in the nonpregnant ($n = 4$), the pregnant ($n = 4$), the labouring ($n = 1$) and the postpartum ($n = 1$) patients. Analysis of sq-PCR for CRH-R1 in human myometrium from the F and L segment of the human uterus in the nonpregnant, the pregnant, the labouring and the postpartum patients. The detection of $\beta$-actin mRNA (218 bp; the internal control) is shown.
Figure 3.13 Analysis of sq-PCR for CRH-R1 mRNA (333 bp; 31, 33, and 35 cycles) in the chorion (n = 4) and the decidua (n = 4) in term pregnancy prior to (NL) and after (L) the onset of labour.
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CHAPTER 4: THE RAT- A SUITABLE MODEL TO STUDY THE
REGULATION OF CRH RECEPTOR EXPRESSION IN
THE MYOMETRIUM?

4.1 INTRODUCTION

We and others (Rodriguez-Linares et al., 1998) detected CRH-R1 mRNA and CRH-R2 mRNA expression in the myometrium from pregnant women. The presence of CRH receptors in the myometrium suggests CRH may be of physiological importance in the initiation and/or progression of human parturition. However, the mechanisms involved in the regulation of CRH-R1 mRNA and CRH-R2 mRNA expression in the myometrium remain unknown. In an effort to further our understanding of CRH-mediated responses, extensive studies have been conducted, in the rat to delineate the factors involved in the regulation of CRH receptor expression in the pituitary (Wynn et al., 1984; Luo et al., 1995; Sakai et al., 1996) and the brain (Makino et al., 1995; Imaki et al., 1996).

The specific aim of this study was to establish a mammalian model in which the regulation of CRH-R1 mRNA and CRH-R2 mRNA expression in the myometrium during pregnancy and at the time of labour could be studied, in view of the presence and distribution of remarkably similar CRH receptors in the human and the rat (Perrin et al., 1995, Liaw et al., 1995; Valdenaire et al., 1997) and the common pharmacological and secondary messenger pathways of the human and rat CRH receptors, we suggested that the rat could be a suitable model to study the regulation of
CRH receptor expression in the myometrium. We hypothesized that CRH-R1 mRNA and/or CRH-R2 mRNA would be upregulated at the time of labour. The objectives of the study were to determine whether CRH-R1 mRNA and CRH-R2 mRNA were expressed in the rat myometrium and to examine whether the expression of CRH-R1 mRNA and/or CRH-R2 mRNA was altered at the time of labour. Measurements were made relative to Cx 43, mRNA because the increased expression of this gap junction protein has been well characterized (Lye et al., 1993; Chow and Lye 1994).

4.2 MATERIALS AND METHODS

4.2.1 Animals

Virgin female Wistar rats (250-280 grams; Charles River Canada, St. Constant, Quebec, Canada) were mated, and the day of the appearance of the vaginal plug was set as 1 day post-coitum. The animals were housed individually, under standard conditions (14 hours lightness and 10 hours darkness; at 22°C) and fed Purina Lab Chow (Ralston, Purina, , MO, USA) and water ad libitum. These animals routinely gave birth on the morning of day 23. The rats were decapitated and the myometrium removed on day 15 (n = 4), day 20 (n = 5), day 21 (n = 4), day 22 (n = 4), labour (n = 6) and on 1 day post-partum (n=4 ). Rat pituitary and liver were also collected. All tissue samples were immediately snap frozen in liquid nitrogen and stored at -80°C.

All experiments were approved by the Samuel Lunefeld Institute Animal Care Committee at the University of Toronto according to the Guidelines of the Canadian Council on Animal Care.
4.2.2 Total RNA Extraction

Total RNA was extracted from the samples of rat myometrium, pituitary and liver using the methods described by Chomczynski and Sacchi (1987). Briefly, frozen tissue samples (2-5 mg) were powdered under liquid nitrogen and homogenized in 1 mL of a denaturing solution (4M guanidinium thiocyanate, 25 mM sodium citrate, 0.5% sodium lauroylsarcosine, 0.1M β-mercaptoethanol (v/v)) using an ULTRA-TURAXX homogenizer (Janke & Henkel, IKA-Labortechnik, ON, Canada). Sodium acetate buffer (0.1 mL of 2M; pH 4) was added to the tissue homogenate followed by of phenol (water-saturated) (1 mL), and by a chloroform-isoamyl alcohol mixture (0.2 mL; 49:1). Each addition was followed by thorough mixing. The samples were incubated on ice for 15 minutes then centrifuged (Sorvall RC-5B, Du Pont Instruments, MA, USA) at 6,500g for 40 minutes at 4°C. The supernatant was transferred to a fresh polypropylene tube (12 mL; Becton and Dickinson, New Jersey, USA) and mixed with isopropanol (1 mL) and incubated for 1 hour at -20°C to allow RNA precipitation. The samples were centrifuged again at 6,500g for 1 hour 4°C. The resulting RNA pellet was dissolved in the denaturing solution (1 mL), transferred to an eppendorf tube (1.5 mL, Diamed, Ont, Canada) and precipitated with an equal volume of isopropanol overnight at -20°C. The samples were then centrifuged for 15 minutes at 4°C. The RNA pellet was resuspended in 70% ethanol, (1 mL) vacuum dried and redissolved in ddH2O with 0.1% DEPC water. The total RNA purity and recovery for each sample was determined with a UV spectrophotometer (Model DU-64, Beckman Instruments, Inc., CA, USA) at 260 and 280 nanometers.
4.2.3 RT-PCR

Total RNA from rat myometrium, pituitary and liver was reverse transcribed into cDNA. The reverse transcription reaction mix consisted of 1 µg of total RNA, 1 x PCR buffer (10 mM Tris-HCl, 50 mM KCl, Perkin Elmer, Cetus), 5 mM MgCl₂ (Perkin Elmer, Cetus), 1 mM each of the dNTP (dATP, dCTP, dGTP, dTTP, Pharmacia), 5 ng/µL random hexamers (Pharmacia), 1 U/µL RNase inhibitor (Boehringer Mannheim) and 100 U MMLV-RT (GibcoBRL, Gaithersburg, MD) in 21 µL DEPC water. Negative controls were prepared as above but without RNA. The reaction mixture was incubated at 25°C for 10 minutes, then at 42°C for 30 minutes and finally at 99°C for 5 minutes. The resultant cDNA RT mixture was stored at -20°C until it was used.

PCR was performed using the RT mixture. The PCR mixture consisted of 10 µL of the RT mixture, 0.25 mM dNTP, 50 ng of each specific PCR primer (ACGT Corporation, Toronto), 2.5 U Taq polymerase (Boehringer Mannheim) in 1.25 mM MgCl₂, 50 mM KCl, 10 mM Tris-HCl (pH 8.3) in 24.5 µL DEPC water. Each PCR reaction underwent an amplification regime characterized by a pre-incubation stage (95°C, 5 minutes), a denaturation stage (94°C, 30 seconds), a primer annealing stage (62°C, 30 seconds), an extension stage (72°C, 30 seconds) and a long extension stage (72°C, 8 minutes) in a thermal cycler (MJ Research Inc, Mass, USA). PCR reactions were also performed using RNA that had not been reverse transcribed to establish the extent of genomic DNA contamination in the RNA. PCR products were electrophoresed on a 2 % agarose gel, stained with 1.5 % ethidium bromide in Tris-
acetate/EDTA buffer to allow visualization of the PCR product under a transilluminator (Lighttools Research, Ont, Canada).

Specific primers were used to identify CRH-R1, CRH-R2, Cx 43 and β-actin gene expression in the rat myometrium. Primers were designed to detect a 333 bp product for CRH-R1 in rat myometrium (Slominski et al., 1995) a sense primer 5' GCC CTG CCC TGC CTT TTT CTA 3' and an antisense primer 5' GCT CAT GGT TAG CTG GAC CA 3' corresponding to positions 235-255 and 549-568, respectively were used (Accession number L23332) (Chen et al., 1993). Similarly, primers were designed to identify a 509 bp product for CRH-R2 in rat myometrium. A sense primer 5' TAG TGC TGC GGA GTA TCC GC 3' and an antisense primer 5' CAT CCA GTA CAG GU GGC AG 3' corresponding to positions 631-650 and 1121-1140, respectively, were used (Accession number U16253). Cx 43 expression was recognized by primers were designed to recognize a 433 bp product. A sense primer 5' CCA AGG AGT TCC ACC AAC TT 3' and an antisense primer 5' AGA CTG ACG GGG TCA ACG TG 3' corresponding to positions 137-156 and 551-570, respectively, were used (Accession number M19317). β-actin gene expression (internal control) was also determined in all samples to assess the integrity of the RNA. Primers were designed to identify a 331 bp for β-actin in all the samples. A sense primer 5' CGT GGG CCG CCC TAG GCA CCA 3' and an antisense primer 5' CCC CCC TGA ACC CTA AGG CCA A 3' corresponding to positions 1343-1363 and 1653-1674, respectively were used (Accession number JOO691).
4.2.4 Sq-PCR

Sq-PCR was performed with the CRH-R2, Cx 43 and β-actin cDNA to compare the expression of these genes in rat myometrium from day 15 of gestation through to labour and 1 day post-partum. To obtain a linear range in the expression of CRH-R2 cycles 28, 30, and 32. Cycles 18, 20 and 22 were used for Cx 43 and for the β-actin gene cycles 16, 18, 20 were used. The relative intensity of cDNA signals was quantified using computerized image analysis (Imaging Research Inc., Ont, Canada).

Enzyme digestion was performed to confirm the identity of the CRH-R2 cDNA product using 10 U of the restriction enzyme Taq I (Pharmacia) in One-Phor-All buffer (Pharmacia) The PCR product (0.5-2 μg cDNA) was incubated at 65°C for 2-3 hours then the products were run on a 2% agarose gel (1.5% ethidum bromide) to allow visualization.

4.2.5 Data Analysis

To correct for differences in the initial amount of RNA used we determined β-actin mRNA expression in all the samples. The ratio of the optical densitometry reading measurements for the expression of CRH-R2 mRNA or Cx 43 mRNA (at 3 progressive amplification cycles) to that of β-actin mRNA expression (at 3 progressive amplification cycles) was determined for each sample of rat myometrium. The mean of the ratio was determined for each treatment from all the samples within that group. To determine the difference in gene expression amongst the treatment groups (day 15, day 20, day 21, day 22, labour and IPP) All Pairwise Multiple Comparison Procedures
4.3 RESULTS

4.3.1 CRH-R1 mRNA and CRH-R2 mRNA Expression in Rat Myometrium On Day 15, Day 20, Day 21, Day 22, At Labour And 1 Day Postpartum

Enzyme digestion (Taq I) of CRH-R2 was used to confirm the identity of the product and yielded the product of expected size (409 bp). We have identified CRH-R2 (expected band of 509 bp) in the rat myometrium from day 15-day 22 of gestation, at the time of delivery and 1 day postpartum (Figure 4.1). The amplification of CRH-R2 expression increased linearly in 28, 30 and 32 cycles in all the samples studied. We observed a significant rise (p < 0.05; Student-Newman-Keuls Method) in CRH-R2 mRNA/β-actin mRNA expression in rat myometrium at the time of labour, with a significant decrease rise (p < 0.05; Student-Newman-Keuls Method) to pre-labour levels 1 day post-partum as determined by analysis of sq-PCR CRH-R2 mRNA/β-actin mRNA expression (Fig 4.2). CRH-R1 mRNA expression was undetectable in the rat myometrium from day 15-day 22 of gestation, at the time of labour and 1 day postpartum. The amplification of β-actin mRNA within the linear expression of the gene (cycles 16, 18, 20).
4.3.2 Cx 43 Expression In Rat Myometrium On Day 15, Day 20, Day 21, Day 22, At Labour And 1 Day Postpartum

Cx 43 mRNA expression (expected band of 433 bp) was present in the rat myometrium at day 15- day 22 of gestation, at the time of labour and 1 day postpartum (Figure 4.1). Cx 43 mRNA expression increased linearly at 18, 20 and 22 cycles in all the samples studied. We observed a significant rise ($p < 0.05$; Bonferroni’s method) in Cx 43 mRNA/ β-actin mRNA expression in rat myometrium at the time of labour and a significant decrease ($p < 0.05$; Bonferroni’s method) to pre-labour levels 1 day postpartum as determined by analysis of sq-PCR CRH-R2 mRNA / β-actin mRNA expression (Fig 4.3).

4.4 DISCUSSION

We report the presence of CRH-R2 mRNA expression in the rat myometrium at day 15- day 22 of gestation, at the time of delivery and 1 day postpartum. CRH-R2 mRNA expression increased significantly and concurrently with Cx 43 mRNA expression at the time of delivery. We were unable to detect CRH-R1 mRNA expression in the rat myometrium in pregnancy and post-partum.

Based on the obvious inappropriate and potentially dangerous reasons for manipulating the human myometrium in vivo and the difficulty in obtaining myometrial samples from pregnant women we have used a rat model to study CRH receptor expression in the myometrium. There is no evidence in the literature supporting the presence of distinct functional regions within the rat uterus. Hence, no
distinctions were made between regions of the uterine horn, instead we used all the myometrium from the horn to determine gene expression. We have used a semi-quantitative technique to assess CRH-R2 mRNA expression in the rat myometrium. We have established linear gene expression of the CRH-R2 gene and determined the ratio of CRH-R2 mRNA to the linear gene expression of the Cx 43 gene to evaluate the change in receptor expression. However, we have not as yet determined the localization of CRH receptor protein in the rat myometrium in pregnancy and delivery and examined if the upregulation of CRH-R2 mRNA expression was reflected at the protein level.

We have previously reported that CRH-R1 mRNA expression is significantly increased at the time of labour in myometrium from the lower segment of the human uterus. This rise was observed in both term and pre-term pregnancies, whereas CRH-R2 mRNA expression remains unaltered. Our results show that CRH-R1 mRNA and CRH-R2 mRNA are differentially expressed in rat and human myometrium, in pregnancy. CRH-R2 mRNA is upregulated at the time of delivery in the rat myometrium whereas in the human myometrium CRH-R2 mRNA expression was only slightly increased. In contrast, CRH-R1 mRNA increased significantly at the time of labour in the human myometrium but CRH-R1 mRNA expression was undetectable in the rat myometrium both in pregnancy and at the time of delivery. In both the rat and the human, however the expression of CRH-R2 mRNA and CRH-R1 mRNA, respectively decreased immediately postpartum suggesting they were regulated by labour specific mechanisms.
CRH-R1 mRNA and CRH-R2 mRNA have been reported at similar sites in the rat and the human (Chalmers et al., 1996; Perrin et al., 1995; Valdenaire et al., 1997). However, it is important to keep in mind that the regulatory elements involved in CRH receptor expression may be species-specific. The mechanisms regulating CRH-R2 mRNA expression in the rat myometrium and the time of labour are unknown. It is well documented that CRH receptors in the rat pituitary and brain are regulated by CRH (Wynn et al., 1983; Lu et al., 1994; Makino et al., 1995). Karialis and Mazoub (1994) have shown that the rat placenta does not produce CRH. CRH mRNA expression and irCRH are present in the rat endometrium in pregnancy (Zoumakis et al., 1996; Makrigiannakis et al., 1997) but the presence of CRH in the rat myometrium has not been reported. It has been suggested that endometrial CRH may play a local role in the regulation of myometrial tone, but the mechanisms of this interaction would need to be determined. Interestingly, it has recently been suggested that urocortin, a CRH related peptide, is the preferential ligand for CRH-R2 (Vaughan et al., 1995. In view of the presence of CRH-R2 in the rat myometrium it would be of interest to determine urocortin mRNA expression in the rat myometrium in pregnancy and delivery.

In conclusion we have determined that the rat is a suitable model to study the factors regulating the expression of CRH-R2 mRNA in the uterus. However, the rat is not an appropriate model to study the regulation of CRH-R1 mRNA, which is the major subtype of the receptor in human myometrium. A clear understanding of the
mechanisms involved in regulating CRH receptor expression will help decipher some of the mechanisms regulating CRH-mediated actions.
Figure 4.1 Enzyme digestion to confirm the identity of CRH-R2 cDNA: A. Taq I digestion (409 bp), B. original size product (509 bp).
- - 509 bp

A --- B
Figure 4.2 Photograph of a sq-PCR gel showing the linear expression of CRH-R2 mRNA, Cx 43 mRNA and β-actin mRNA. The picture shows 3 amplification cycles per sample/gene: CRH-R2 mRNA expression (expected band size 509 bp; cycles 28, 30, 32), Cx 43 mRNA expression (expected band size 433 bp; cycles 18, 20, 22) and β-actin mRNA expression (expected band size 331 bp; cycles 16, 18, 20). The expression of the genes is shown in rat myometrium on day 15 (n = 3), day 20 (n = 3), day 21 (n = 3), day 22 (n = 3) of gestation, at the time of labour (day 23; n = 3) and 1 day postpartum (n = 3).
Figure 4.3 Analysis of sq-PCR for CRH-R2 mRNA expression (cycles 28, 30, 32) in rat myometrium on day 15 (n = 4), day 20 (n = 5), day 21 (n = 4), day 22 (n = 4) of gestation, at the time of labour (day 23; n = 6) and 1 day postpartum (n = 4). CRH-R2 mRNA expression is significantly upregulated at the time of labour in rat myometrium (p < 0.05).
Figure 4.4 Analysis of sq-PCR for Cx 43 mRNA expression (cycles 18, 20, 22) in rat myometrium on day 15 (n= 4), day 20 (n=5), day 21 (n= 4), day 22 (n=4) of gestation, at the time of labour (day 23; n= 6) and 1 day postpartum (n= 4). Cx 43 mRNA expression is significantly upregulated at the time of labour in rat myometrium (p < 0.05).
CHAPTER 5: FINAL DISCUSSION

We have shown that CRH-R1 mRNA and CRH-R2 mRNA expression and their proteins are present in the myometrium of both non-pregnant and pregnant women. CRH-R1 mRNA expression and peptide levels in myometrium are downregulated with pregnancy and significantly upregulated at the time of labour. This rise in CRH-R1 mRNA expression appears to be specific to myometrium from the lower segment of the uterus. Moreover, CRH-R1 mRNA expression was increased in both in both pre-term and term pregnancies. CRH-R1 mRNA expression appears to return to pre-labour levels immediately postpartum. CRH-R2 mRNA expression was not significantly changed at the time of labour in either term or pre-term human pregnancies. We did not observe a significant rise in CRH-R1 mRNA expression in the chorion and the decidua. CRH-R1 mRNA expression was undetectable in the amnion whereas CRH-R2 mRNA expression was absent from the decidua, the amnion and the chorion.

CRH-R1 and CRH-R2 protein was localized to the uterine smooth muscle and to the smooth muscle layer of the uterine vessels in the myometrium from nonpregnant women. CRH-R1 protein staining was undetectable in the myometrium in term pregnancy but was apparent once again, but to a lesser degree at the time of labour. CRH-R2 staining was also undetectable in the myometrium in term pregnancy. At the time of labour, we observed a modest increase of positive CRH-R2 protein staining in the uterine smooth muscle, however, CRH-R2 protein was undetectable in the uterine vasculature.
We also report the presence of CRH-R2 mRNA expression in the rat myometrium from day 15 to day 22 of gestation, at the time of delivery and 1 day postpartum. CRH-R2 mRNA expression increased significantly and concomitantly with Cx 43 mRNA at the time of labour in the rat myometrium. Whereas the rise in CRH-R2 mRNA expression in the myometrium was sudden, the rise in Cx 43 mRNA expression was gradual and attained a peak at the time of labour. Interestingly, the expression of both genes decreased immediately post-partum. We were unable to detect CRH-R1 mRNA expression in the rat myometrium in pregnancy, at the time of delivery and post-partum.

The observed rise in CRH receptor expression at the time of labour is in agreement with a physiological role for CRH in human and rat parturition. The apparent exclusiveness of this significant rise of CRH-R1 mRNA to the lower segment of the human uterus may suggest functional differences for CRH in the fundal and the lower segment of the uterus. As the lower segment of the uterus is relatively quiescent, at the time of labour we have suggested that CRH mediates relaxation of this segment probably via a cAMP-mediated pathway. A cAMP secondary messenger pathway for CRH is in agreement with studies done by Labrie et al., (1982) and Aguilera et al., (1987) who have shown CRH receptors are linked to this pathway in the brain and the pituitary. The increase in CRH-R1 mRNA expression, at the time of labour in both preterm and term pregnancies suggests that the regulation of CRH-R1 mRNA expression is involved in and/or mediated by labour related mechanisms. In addition, the absence of a rise in CRH-R1 mRNA expression in the decidua and fetal membranes
suggests that the upregulation of CRH-R1 mRNA expression is specific to the human myometrium.

We observed a rise in CRH-R1 protein in the myometrium at the time of labour. However this rise did not appear to reflect the significant increase in CRH-R1 mRNA message that we observed. We are a little cautious in our interpretation of this data because of the antibody used. In the literature only two CRH-R1 antibodies have been used. The group of E.A. Linton synthesized a CRH-R1 antibody which they have used in a wide array of immunofluorescence and western blot studies (Castro et al., 1996). Interestingly, they report different molecular weight CRH receptors within and amongst tissues. The identified a 40 and a 45 kDa CRH-R1 in the myometrium (Castro et al., 1996). In very recent studies they showed a heterogeneous spread of CRH-R1 protein staining in the myometrium of nonpregnant and pregnant women (Rodriguez-Linares et al., 1998). We are the first group to our knowledge to use the CRH-R1 antibody in this study. We were unable to detect CRH-R1 protein in the myometrium or in positive control tissues with Western blots. Hence we are unable to determine the size of the product the antibody is detecting. Even though the antibody is pre-absorbed with the CRH-R1 peptide much remains to be done to characterize the antibody. Namely the affinity and specificity of the antibody for the peptide has to be clearly established. In addition, we need to determine if the antibody recognizes different post-translational spliced forms of the receptor or furthermore if the antibody cross-reacts with the CRH-BP.
CRH-R2 mRNA expression was not significantly increased at the time of labour in human pregnancy. However, CRH-R2 mRNA was present in the myometrium of more of the preterm patients. We suggest that while CRH-R2 mRNA is not significantly upregulated at the time of labour, its expression may be associated to gestational age and/or premature activation of the uterus. The absence of CRH-R2 mRNA from the decidua and the fetal membranes suggests that the reported actions of CRH in these tissues (Jones et al., 1989) may be mediated by CRH-R1 or a possible unknown subtype of the CRH receptor.

We have shown that CRH-R2 mRNA expression is upregulated at the time of delivery in rat myometrium. The rise in CRH-R2 mRNA was shown relative to the previously reported increase in Cx 43 mRNA (Petrocelli and Lye, 1994) at the time of delivery in the rat myometrium. The presence of CRH-R2 mRNA in the rat myometrium suggests CRH may play a role in delivery. However, CRH is not secreted by the rat placenta (Robinson et al., 1989). As CRH has not been identified in the rat myometrium, we suggest that endometrial CRH (Makrigiannakis et al., 1995) may act via paracrine mechanisms on the myometrial CRH receptor. We also suggest that the CRH-R2 mRNA may not be associated with relaxation-mediated pathways. This is because the evidence in the literature suggests the rat uterus is one contracting entity at the time of labour. In addition, CRH is not thought to be the preferential ligand of CRH-R2 based on the demonstration that urocortin binds CRH-R2 with higher affinity than CRH (Lovenberg et al., 1995a). However, urocortin mRNA expression has not been determined in the rat myometrium. The absence of CRH-R1 mRNA expression in
the rat myometrium is consistent with studies by Chalmers et al., (1995) Lovenberg et al., (1995b) that have shown CRH-R1 mRNA is restricted to the brain regions and that CRH-R2 mRNA is expressed in the brain and muscle.

The differential rise of CRH-R1 mRNA and CRH-R2 mRNA expression at the time of labour in the human and the rat myometrium, respectively, suggests that perhaps the receptors are involved in mediating different actions within this tissue. An attractive proposal could be that CRH-R1 mediates relaxation whereas CRH-R2 is involved in myometrial contractility. The rise in CRH-R1 mRNA levels rise in the myometrium from the lower segment of the human uterus is in agreement with for the effective expulsion of the fetus from the uterus. In the literature, only the genes of uterotonin receptor mRNA expression have been shown to be turned on at the time of labour in the rat myometrium (Fukai et al., 1984). We suggest that CRH is a uterotonic agent in the rat myometrium and that it mediates its effects via the CRH-R2 receptors. Future interesting studies could be to observe the effects of progesterone on CRH-R2 mRNA expression. Progesterone is well-known in its ability to induce refractoriness of the myometrium to OT and PGF2α hence leading to a prolongation of pregnancy. We could examine CRH-R2 mRNA expression in the rat myometrium, following a) progesterone infusion, b) progesterone infusion stopped after a period to allow the rats to deliver and c) pregnant ovariectomised (progesterone withdrawal) rats.

In this study we predominantly used PCR techniques to assess the level of gene expression. We are aware of the difficulties in quantitatively assessing the data. We have shown a linear correlation between the expression of the gene of interest and β-
actin mRNA (an internal control gene). We have used the ratio of optical densitometry within the linear expression of the gene of interest to the linear expression of β-actin mRNA to assess gene expression. The validity of this technique is well established (Kinoshita et al., 1992).

Based on the presence of CRH-R2 mRNA expression on the rat myometrium and the upregulation of the CRH-R2 gene expression at delivery, we have determined that rat is a suitable mammalian model to study the regulation of CRH-R2 mRNA at the time of labour. However another mammalian model has to be established to study the regulation of the expression of CRH-R1 mRNA in the myometrium. CRH-R1 mRNA is the predominant CRH receptor subtype in the human myometrium and elucidating the factors regulating CRH-R1 mRNA expression may help decipher the role of this receptor in human pregnancy. We suggest that the expression of CRH receptors could also be studied in myometrial cells via culturing or transfection techniques. However, while these in vitro experiments may further our understanding of the factors regulating CRH receptor expression in the myometrium the inherent limitations of in vitro studies prevail.

In conclusion, we have shown that CRH-R1 mRNA expression is significantly upregulated in the human myometrium at the time of labour, whereas, in the rat CRH-R2 mRNA expression is upregulated at the time of delivery. The upregulation of both CRH-R1 mRNA and CRH-R2 mRNA expression in the human and the rat, respectively, are similarly mediated by labour-related mechanisms, as suggested by the decrease in the expression of the receptor subtypes immediately postpartum.
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