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Research paper

A complete corticotropin releasing factor system localized in human fetal lung

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ABSTRACT

OBJECTIVE: The Corticotropin Releasing Factor (CRF) system (neuropeptides CRF, Ucn I, II, III and binding sites CRFR1, CRFR2, CRF-BP) is responsible for stress regulation and the homeostasis of an organism. Herein we study the CRF system in human normal and pathological fetal lungs. DESIGN: Lung tissues from 46 archival human fetuses were divided into Group A (normal), Group B (chromosomal abnormalities) and Group C (congenital disorders). Presence of elements of the CRF system was evaluated using immunohistochemistry and was correlated to pathology, lung developmental stage and clinicopathological characteristics. RESULTS: Immunoreactivity for all antigens was found in both epithelial and mesenchymal lung cells of the bronchi and alveoli. Ucn I and CRFR1 were more frequently present in Group A. Ucns were more frequently localized at the pseudoglandular stage. There was a positive correlation between the presence of the CRF neuropeptides and between CRFR1 and CRF. Two fetuses with lung malformations showed low or no detectable presence of the CRF system. CONCLUSIONS: We report the presence of a complete CRF system in human fetal lungs correlating its developmental stage and several pathologies. Our results are in agreement with findings in experimental animal models, implicating the CRF system in fetal lung development, its action being more significant in the early stages.

Key words: CRF system, Human fetus, Lung development, Lung pathology

INTRODUCTION

It was Harris[1] who, in 1948, hypothesized the presence of a neurochemical factor regulating the secretion of ACTH (Adrenocorticotropic Hormone) by the pituitary. About three decades later, in 1981, Vale et al isolated from the ovine hypothalamus a 41 amino acid peptide that was named Corticotropin Releasing Factor (CRF).[2] Since then, a series of homologous peptides and binding sites have been described which

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constitute the CRF system. This system is responsible for the regulation of stress response at the neuronal, endocrine and immunological levels. Moreover, it is a fundamental factor for the maintenance of the homeostasis of an organism.[3] Endogenous CRF neuropeptides, i.e. CRF, Urocortin I (Ucn I, 40 amino acids peptide), Ucn II (or stresscopin-related peptide, 38 amino acids) and Ucn III (or stresscopin, 38 amino acids), act via activation of two distinct receptors (CRFR1 and CRFR2), both belonging to the class B/secretin family of G-protein coupled receptors, and also bind to a circulating CRF-Binding Protein (CRF-BP). All neuropeptides bind with CRFR2, while Ucn II and III have moderate or no correlation with CRF-BP and CRFR1.[4-7] Since 40-60% of human brain CRF is bound with CRF-BP, the latter can be considered as a natural storage of endogenous CRF, regulating its bioavailability.[8]

It is well established that the fetal environment plays a critical role in development. Exposure to maternal stress can sometimes have deleterious effects on the fetus, depending on the cause, timing, duration and intensity of the stress.[9] In humans and in other primates, the growth of the "fetal zone" in the fetal adrenal cortex follows the CRF secretion pattern by the placenta and CRF seems to play a key role in triggering cortisol synthesis de novo by the fetal adrenals. This cortisol is fundamental for the maturation of different fetal organs, such as the lungs, which is vital for the survival of the fetus outside the uterus.[10,11] Although it is acknowledged that primates' placenta and fetal membranes synthesize CRF,[12,13] very little is known about the distribution of the entire CRF system of neuropeptides and binding sites during human fetal development. CRF peptide expression has been studied using RIA in fetal tissues of newborn, juvenile and adult baboons and was found in the pituitary, adrenals, kidney, liver and lungs at different concentrations, depending on age and tissue.[14] Moreover, in mice experimental models,[15] CRF mRNA was detected in fetal lungs at different gestation days, but not in term tissues. Another study on fetal baboon lung tissues (125th gestation day) concluded that CRF stimulates surfactant phospholipid synthesis.[16] Other studies have discovered the presence of CRF in the rat and ovine hypothalamus,[17,18] ovine pituitary,[19] hippocampal-amygdala complex, frontal cerebral cortex (FCC) and brainstem,[20] as well as in mouse cerebellum.[21] In the fetal periphery, CRF was detected in rat pancreas and GI tract.[22] Furthermore, Lakshmanan et al described the localization and gestation-dependent pattern of both CRF receptor subtypes in ovine fetal distal colon and hypothesized that down-regulation of CRFR2 with concurrent increases in CRFR1 receptor levels in myenteric-smooth muscle units with advancing gestation sensitizes the colonic motility responses to stressors.[23] In addition, the expression of CRF, Ucn I and CRFR2 was shown in fetal sheep distal colon along with inhibiting actions on colonic contractility.[24] The ontogeny of CRF, CRFR1, CRFR2 and CRF-BP has been studied during murine lung development during late gestation where temporal and spatial modulations in gene expression have been demonstrated, consistent with roles for these genes in lung development.[25] Finally, RT-PCR studies have reported m-RNA CRFR1 presence in human fetal adrenals.[10] CRF expression has also been reported in adult human lung cancer tissues and cell lines,[26] while RT-PCR showed that Ucn II transcripts were abundant in the adult human lung.[5] These results point into a locally expressed CRF system in the fetal lung with a possible function during development.

In the present study, the histological mapping of the localization of CRF neuropeptides and their binding sites was evaluated by immunohistochemistry in the lungs of human fetuses from spontaneous abortions and curettages. Localization patterns were compared between the different developmental stages of the lungs. Associations with diagnosed congenital or chromosomal disorders and other clinicopathological parameters were also studied.

MATERIALS AND METHODS

A. Tissues

Fetal lung tissues were retrieved from 46 archival human fetuses of the Histology-Embryology Laboratory Tissue Bank of Democritus University of Thrace (DUTH), Alexandroupolis, Greece. Standard pathological examination and diagnosis data were available along with relevant medical information on the mothers. Fetuses were derived from spontaneous abortions and curettages due to medical reasons con-

cerning the mother (elective therapeutic termination of pregnancy). Fetuses with no congenital or chromosomal anomalies and no signs of chorioamnionitis were considered as 'normal' (Group A, total no. 17, all male). Fetuses with nuchal cord were excluded from Group A. Pathological fetuses were divided in two groups: Group B (total no. 5, male: 3, female: 2) included fetuses with chromosomal abnormalities (Down and Edward's syndrome) and Group C (total no. 24, male: 15, female: 9) with congenital malformations (of the Central Nervous System, heart/vessels, kidneys, lungs, skeleton, visceral cranium and face). Fetuses were further divided according to their gestational stage, which ranged from 12 to 39 weeks (Table 1): Pseudoglandular stage: 7th-16th gestation week, canalicular stage: 17th-27th gestation week, saccular-alveolar stage: >=28th gestation week. Gestational age was estimated by the mother's last menstrual period (LMP). Tissues were embedded in paraffin and sections were used for immunohistochemistry. The study protocol was approved by the Ethical Committee of the University Hospital of DUTH (Decision no. 45/27th/16-11-2009) and was conducted according to the guidelines for the analysis of fetal cells and tissues.

B. Antisera

The antisera used for CRF, CRFR2, Ucn I, II and III detection were obtained from Phoenix Pharmaceuticals (H-017-06, H-006-24, H-019-14, H-019-30, H-019-28, respectively; Belmont, Calif., U.S.A.). The antiserum used for CRF was raised against the whole human peptide sequence; it is 100% specific for human, rat, mouse, canine and feline CRF and shows no cross-reactivity to other peptides. The specific antiserum used for CRFR2 was raised against aa 385-411 of the human CRFR2 receptor. The specific antiserum used for Ucn I, which was raised against the whole human Ucn I peptide sequence, is 100% specific for human and rat peptide. The specific antiserum used for Ucn II was raised against aa 6-43 of the human Ucn II peptide sequence. The specific antiserum used for Ucn III was raised against aa 3-40 of the human Ucn III peptide sequence. The CRF-BP antiserum, obtained from Santa-Cruz Biotechnology [CRF-BP (C-8): SC-365975], is a mouse monoclonal antibody specific for an epitope mapping between amino acids 299-322 at the C-terminus of CRF-BP of human origin. The anti-CRFR1 antiserum was the IgG-purified fraction of 4467a-CRFR1; it has previously been shown to be specific and selective for CRFR1[27,28] and it was kindly donated by Dr. D. Grigoriadis, Neurocrine Bioscience Inc., San Diego, CA., U.S.A.

C. Immunohistochemistry

Immunohistochemistry was conducted as previously described.[29] Tissue specimens were fixed in

Table 1. Characteristics and grouping of fetuses used in the study (some of the pathological fetuses suffered from more than one pathology). Gestational age was estimated by the mother's last menstrual period (LMP)

	Number of fetuses						
		Gestational stages			Sex		
Group	Total n	Pseudoglandular n	Canalicular n	Saccular/Alveolar n	Male n	Female n	Pathology
A	17	5	10	2	17	0	No pathology
B	5	1	4	0	3	2	Chromosomal abnormalities
C	24	2	19	3	15	9	Congenital disorders
A+B+C	46	8	33	5	35	11	No pathology, chromosomal abnormalities, congenital disorders

n: number of fetuses.
Group A: 'normal' fetuses, with no pathological findings. Group B: pathological fetuses with chromosomal abnormalities, Down syndrome (n=4), Edward's syndrome (n=1), acute non-specific chorioamnionitis (n=1), hydropic degeneration of chorionic villi (n=1). Group C: pathological fetuses with congenital disorders of visceral cranium/face (n=6), skeleton (n=3), kidneys (n=1), heart/central vessels (n=3), lungs (n=2)*, CNS (n=7), with gastroschisis (n=1), hydropic degeneration of chorionic villi (n=2), acute non-specific chorioamnionitis (n=9), acute placentitis (n=2), recessive fetal development (n=2), oligohydramnios (n=1).
*Lung pathologies: lung hypoplasia, atelectasis bilaterally.

formalin and embedded in paraffin, according to standard procedures. Four-micron sections (4μm) of representative blocks were deparaffinized, rehydrated and treated with 0.3% H_2O_2 for 5 min in methanol to prevent endogenous peroxidase activity. After washing, slides were incubated at 4°C overnight with the primary rabbit anti-human polyclonal antiserum (anti-CRF 1:500, anti-Ucn I 1:500, anti-Ucn II 1:1000, anti-Ucn III 1:4000, anti-CRF-BP 1:200, 4467a-CRFR1 1:7000, anti-CRFR2 1:1000, diluted in 10% normal rabbit serum in phosphate buffer saline, PBS). Control slides were incubated for the same period with normal rabbit serum IgG and were used as common negative control for all antibody staining. Immunostaining was detected by the the Dako REAL TM EnVision TM Detection System, Peroxidase/DAB+, Rabbit/Mouse kit (DAKO Denmark A/S, Denmark), using a standard streptavidin/biotin detection method, following the instructions of the manufacturer. Finally, bound antibody complexes were stained for 5 min with 0.05% diaminobenzidine, counterstained with Mayer's haematoxylin, mounted and observed under a Nikon Eclipse 50i microscope.

For each slide, approximately 10 fields of stained sections were evaluated by two independent observers and scored in a blinded fashion. Estimations by the two independent observers had an approximately 10% disagreement in most cases and was therefore considered insignificant. Every stained cell was scored as positive, regardless of its staining intensity. Positivity was graded in a four-scale system as follows: Grade 3 represents >70% positive cells in the total number of cells of the specific cell-type counted per field, Grade 2 between 40-70%, Grade 1 between 10-40% and Grade 0 stands for <10% positively stained cells. The extent of positive cells was assessed in epithelial cells of bronchus, bronchioles and alveolar epithelium and in mesenchymal cells of stroma.

D. Statistical analysis

Logistic regression models in order to explore possible predictors for the presence of each factor were not applicable due to the size of our sample, which did not comply with the criteria set by Green SB.[30] This is why statistical significance was assessed by the chi-square test for categorical variables, using the SPSS 17.0 statistical software (SPSS Inc. Chicago, Illinois, USA). Significance was set at a p value of <0.050. Comparisons were made between positively stained (Grades 1, 2 and 3) and negative (Grade 0) tissues for both epithelial and mesenchymal cells. Fetuses from the saccular and alveolar stages were grouped together due to the low numbers studied.

RESULTS

Immunohistochemistry for all 7 antigens revealed immunoreactivity in both epithelial and mesenchymal cells of the lung parenchyma (bronchi and alveoli), that was localized in the cell cytoplasm in all antigens, except for CRFR1 and CRFR2, which was mainly membranic. In the epithelial compartment, no noticeable differences were observed in staining between the immature canals (proximal/bronchial or distal/saccular/putative alveoli) of the first stages and between bronchi and developing alveoli of the following stages. Blood vessels and arteries were generally positive for all antibodies. Representative pictures from fetuses of different gestational stages, with or without pathology, are shown in Figure 1 and representative fields from twin fetuses (one with normal lung development and a second with lung hypoplasia) are shown in Figure 2. Accumulated results depicting fractions of positive tissues per study group are shown in Figure 3. Gestational age of fetuses was estimated by the mother's last menstrual period (LMP).

A. Immunohistochemical localization of CRF neuropeptides in human fetal lung

Results for CRF, Ucn I, II and III localization with semi-quantitative evaluation are presented in Tables 2, 3, 4 and 5, respectively. CRF, Ucn II and III were present at varying percentages in all groups and stages and statistical analysis revealed no significant differences between groups of 'normal' fetuses (Group A) and fetuses with genetic (Group B) or congenital (Group C) disorders. In contrast, when Ucn I presence was compared between Groups A and B, it was found more frequently in Group A (88.23% vs. 40%, p=0.050). Likewise, comparison between Groups A and C favored Group A (88.23% vs. 62.50%, p=0.022). No difference was revealed between Groups B and C. In fact, at the pseudoglandular stage, Ucn I and Ucn III localization was more frequent than during the canalicular stage (p=0.033 and 0.037, respectively)

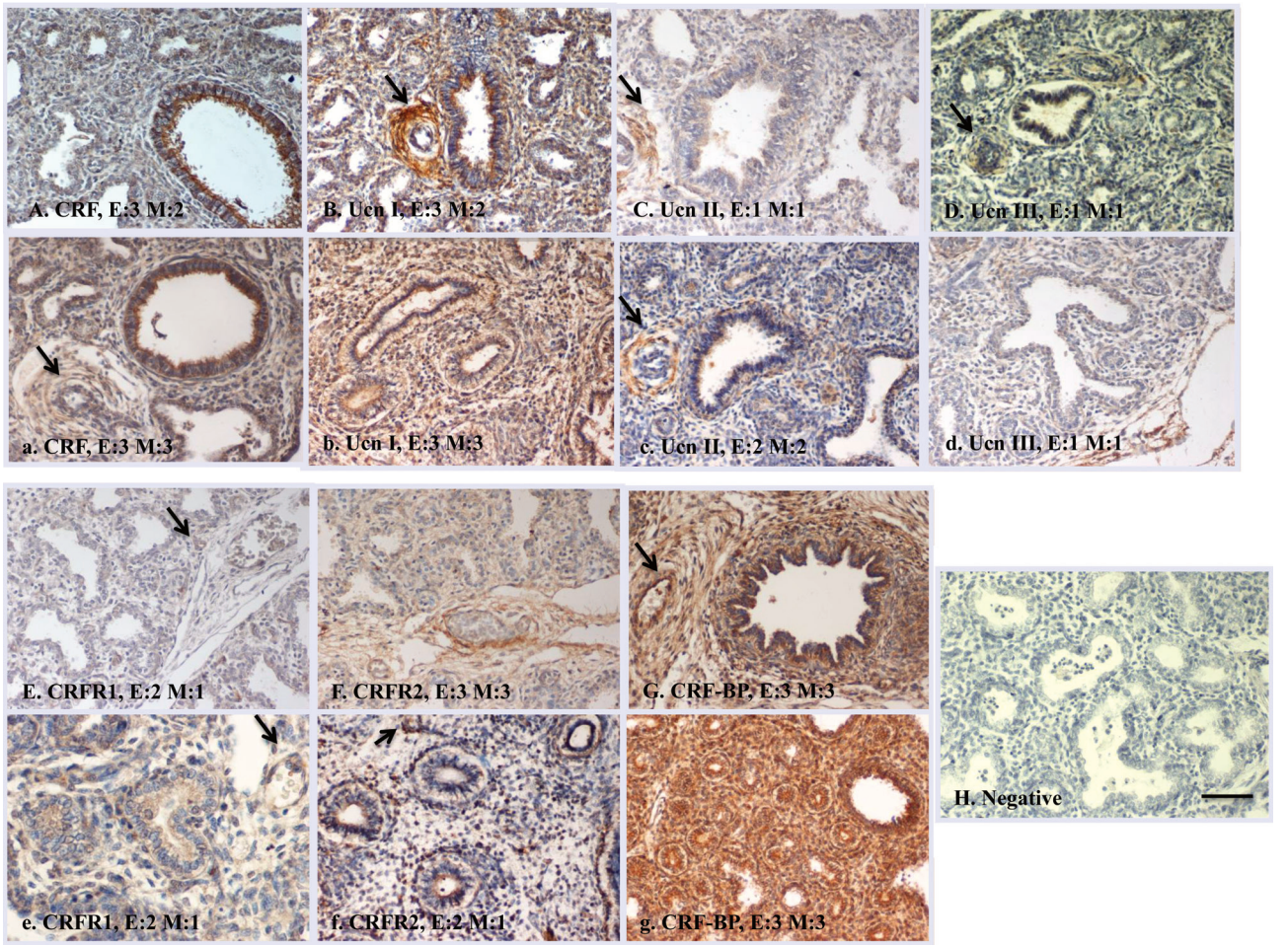

Figure 1. Immunohistochemistry for CRF neuropeptides and binding sites in lung tissues of fetuses of different gestational stages with or without diagnosed pathology. Grading for the epithelial (E) and mesenchymal (M) cellular compartment is shown. Some clearly positive vessels are also shown by arrows. A-G: fetuses with no diagnosed pathology from the canalicular (A, B, D, G, H) or saccular (C, E, F) stage, a-g: pathological fetuses from the pseudoglandular (b, f) or canalicular (a, c, d, e, g) stage, diagnosed with various pathologies other than in the lung. Gestational age was estimated by the mother's last menstrual period (LMP). Original magnification: ×100 (A, B, a, b, C, c, D, d, E, F, G, g), ×200 (f, H), ×400 (e). Scale bar=100μm. H: negative control.

and the saccular/alveolar stage (p=0.034 and 0.031, respectively), whereas the difference between the two first stages for Ucn II was close to statistical significance (p=0.083). In addition, Ucn I was correlated with the fetal sex, being more frequently localized in the male than in the female fetuses (p=0.028) (comparisons made in pooled grouped fetuses of all stages and pathology). No other correlation was found between neuropeptide presence and the presence of chorioamnionitis, maternal age and fetal sex.

Interestingly, there was a positive correlation between the localization of the CRF neuropeptides. Co-presence was found to be statistically significant for CRF/Ucn I (p=0.038), CRF/Ucn II (p=0.001), Ucn I/Ucn II (p=0.013) and Ucn II/Ucn III (p=0.045), whereas for Ucn I/ Ucn III and CRF/Ucn III it was found close to statistical significance (p=0.063 and 0.077, respectively).

B. Immunohistochemical localization of CRF binding sites in human fetal lung

Results for CRFR1, CRFR2 and CRF-BP localization with semi-quantitative evaluation are presented in Tables 6, 7 and 8, respectively. CRFR1 and CRFR2 were present at varying percentages in all groups and stages examined and statistical analysis revealed no significant differences between the four developmen-

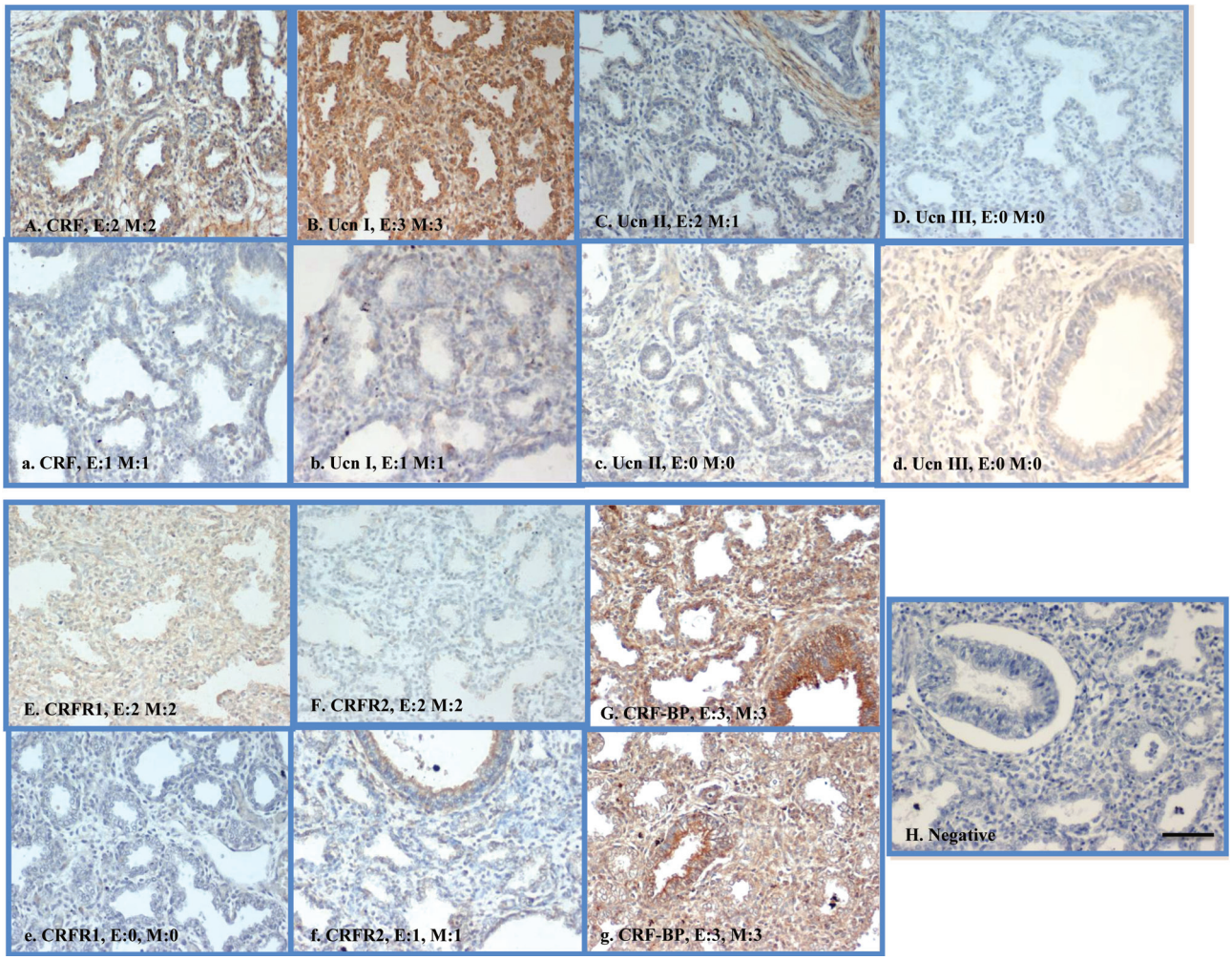

Figure 2. Immunohistochemistry for CRF neuropeptides and binding sites in lung tissues from twin fetuses of the saccular phase, one normal (A, B, C, D, E, F, G) and one with lung hypoplasia (a, b, c, d, e, f, g). Grading for the epithelial (E) and mesenchymal (M) cellular compartment is shown. Gestational age was estimated by the mother's last menstrual period (LMP). Original magnification: X100 (A, B, C, c, D, E, e, F, G, g) X200 (a, b, d, f, H). Scale bar=100μm. H: negative control.

tal stages. CRFR1, however, was more frequently localized in the 'normal' fetuses of Group A than in the fetuses with congenital disorders (Group C) (p=0.045). No other significant correlations were found between receptor localization and the presence of chorioamnionitis, maternal age and fetal sex. However, presence of CRFR1 was significantly correlated to the presence of its ligand CRF (p=0.019) (comparisons made in grouped fetuses from all stages and pathology). Finally, CRF-BP was localized at high levels in all but two fetuses of the canalicular stage, carrying congenital disorders.

C. Immunohistochemical localization of CRF system in two fetuses with lung malformations

Among the fetuses of Group C, there were two fetuses with diagnosed lung pathology, i.e. one fetus with lung hypoplasia/chorioamnionitis (male, 28w, suffering from intra-uterine growth restriction with under-developed lungs) and a second with lung atelectasis bilaterally/bleeding in the tentorium cerebellum and brain stem (female, 39w) that were given special attention. Both fetuses were shown to have noticeably low or no detectable CRF neuropeptides and receptors. Specifically, in the first fetus the localization of CRF, Ucn I and CRFR2 was very low (Grade 1) in

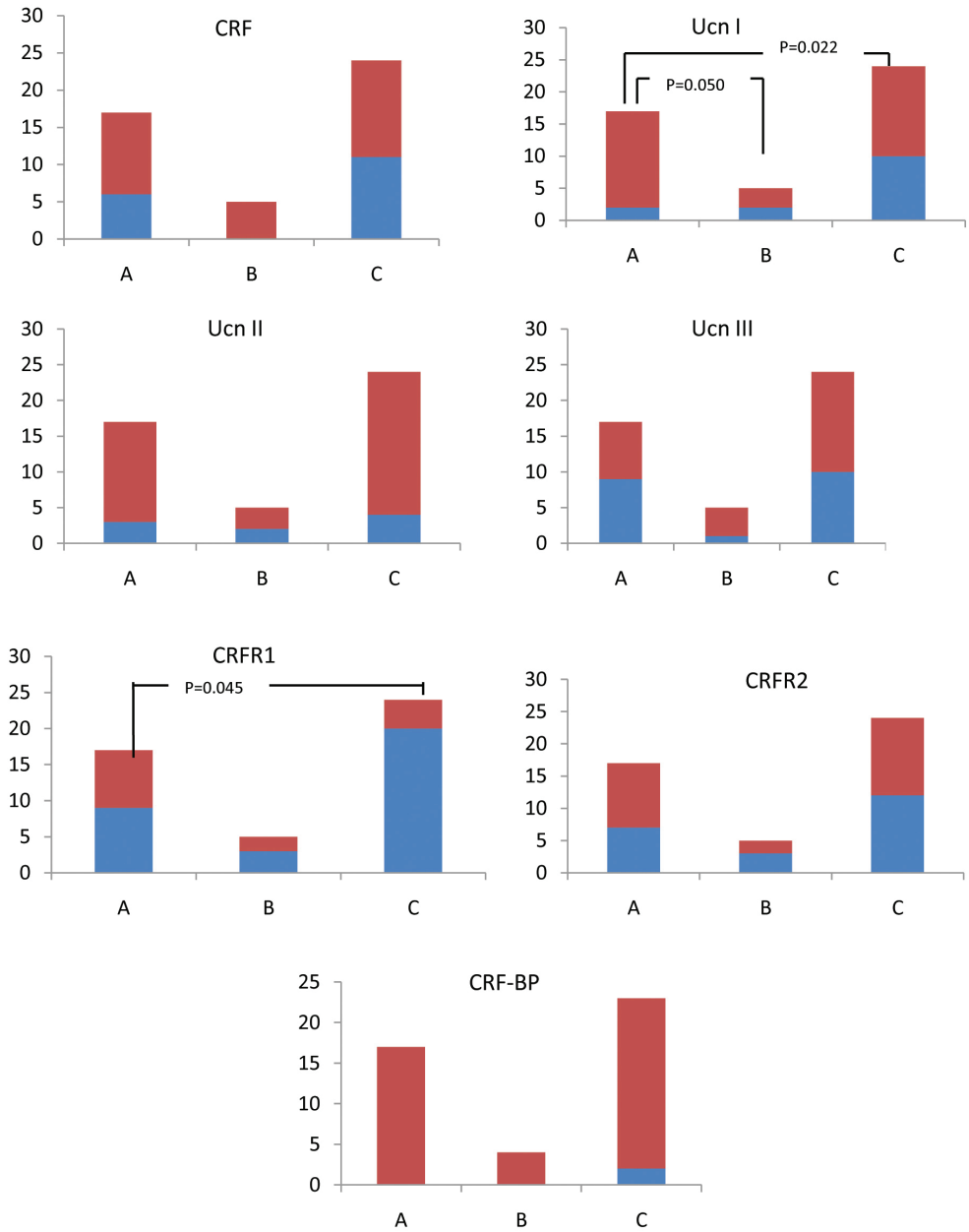

Figure 3. Immunohistochemical detection of CRF neuropeptides and binding sites: accumulated data showing fractions of positively (red) and negatively (blue) stained fetal lung tissues. X axis: A: Group A (fetuses with no pathology), B: Group B (pathological fetuses with chromosomal abnormalities), C: Group C (pathological fetuses with congenital disorders). Y axis: Number of fetuses in every group.

all types of cells (epithelial and mesenchymal), while there was no detectable presence of Ucn II, III and CRFR1. This particular fetus had a twin of the same sex with normal development that showed intense CRF and Ucn I immunoreactivity (Grades 2 and 3, respectively), low presence of Ucn II (Grade 1), moderate presence of CRFR1 and CRFR2 (Grade 2) and no detectable Ucn III (Figure 2). In the second case, there was no detectable presence of CRFR1 and CRFR2, while the presence of Ucn II and III was very weak (Grade 1). CRF and Ucn I were only detected in mesenchymal cells, although poorly again (Grade 1). By contrast, CRF-BP was strongly detected in both types of cells.

DISCUSSION

The CRF system has been proven to play a crucial role not only in stress management, but also in many other physiological processes that support the proper functioning of an organism.[3] Among these there are several lines of evidence that are thought

Table 2. Semi-quantitative estimation for the presence of CRF detected by immunohistochemistry in the epithelial (E) or mesenchymal (M) compartment of fetal lung tissues, shown separately. Numbers represent the number of fetuses in each of the four grades (G), at every gestational stage as estimated by the mother's last menstrual period (LMP) and in the three fetal groups (A: no pathology, B: genetic disorders, C: congenital disorders)

			Gestational stages						Positive n/ Total n per group	
			Pseudoglandular		Canalicular		Saccular/Alveolar			
Peptide	Groups	Grade	E	M	E	M	E	M	E	M
CRF	A (n=17)	G:0	1	1	4	4	1	1	11/17	11/17
		G:1	2	2	3	3				
		G:2	2	2	3	3	1	1		
		G:3								
	B (n=5)	G:0				2			5/5	3/5
		G:1			4	2				
		G:2	1	1						
		G:3								
	C (n=24)	G:0	1	1	8	11	2	1	13/24	11/24
		G:1			8	5	1	2		
		G:2	1		3	3				
		G:3		1						
	Positive n / Total n per stage		6/8	6/8	21/33	16/33	2/5	3/5		

n: number of fetuses.

Table 3. Semi-quantitative estimation for the presence of Ucn I detected by immunohistochemistry in the epithelial (E) or mesenchymal (M) compartment of fetal lung tissues, shown separately. Numbers represent the number of fetuses in each of the four grades (G), at every gestational stage as estimated by the mother's last menstrual period (LMP) and in the three fetal groups (A: no pathology, B: genetic disorders, C: congenital disorders)

			Gestational stages						Positive n/ Total n per group	
			Pseudoglandular		Canalicular		Saccular/Alveolar			
Peptide	Groups	Grade	E	M	E	M	E	M	E	M
UCN I	A (n=17)	G:0			2	2			15/17	15/17
		G:1	1			1				
		G:2	4	5	8	7	1	1		
		G:3					1	1		
	B (n=5)	G:0			2	3			3/5	2/5
		G:1			2	1				
		G:2	1	1						
		G:3								
	C (n=24)	G:0			7	8	2	1	15/24	15/24
		G:1	1	1	5	5	1	2		
		G:2			7	6				
		G:3	1	1						
	Positive n / Total n per stage		8/8	8/8	22/33	20/33	2/5	3/5		

n: number of fetuses.

to suggest a link between the CRF system and fetal lung development. In spite of these findings, limited studies exist on CRF neuropeptides and binding sites expression and role in the human adult lung and none provide information about the human fetal lung. The aim of this work was to investigate the CRF system localization pattern in human fetal lung tissues and discuss the likelihood of its contribution to human lung

Table 4. Semi-quantitative estimation for the presence of Ucn II detected by immunohistochemistry in the epithelial (E) or mesenchymal (M) compartment of fetal lung tissues, shown separately. Numbers represent the number of fetuses in each of the four grades (G), at every gestational stage as estimated by the mother's last menstrual period (LMP) and in the three fetal groups (A: no pathology, B: genetic disorders, C: congenital disorders)

			Gestational stages						Positive n/ Total n per group	
			Pseudoglandular		Canalicular		Saccular/Alveolar			
Peptide	Groups	Grade	E	M	E	M	E	M	E	M
UCN II	A (n=17)	G:0			3	3			14/17	14/17
		G:1	1		4	3		2		
		G:2	4	5	3	4	2			
		G:3								
	B (n=5)	G:0			2	2			3/5	3/5
		G:1	1	1	2	2				
		G:2								
		G:3								
	C (n=24)	G:0			2	4	2	2	20/24	18/24
		G:1	1	1	14	13	1	1		
		G:2	1	1	3	2				
		G:3								
	Positive n / Total n per stage		8/8	8/8	26/33	24/33	3/5	3/5		

n: number of fetuses.

Table 5. Semi-quantitative estimation for the presence of Ucn III detected by immunohistochemistry in the epithelial (E) or mesenchymal (M) compartment of fetal lung tissues, shown separately. Numbers represent the number of fetuses in each of the four grades (G), at every gestational stage as estimated by the mother's last menstrual period (LMP) and in the three fetal groups (A: no pathology, B: genetic disorders, C: congenital disorders)

			Gestational stages						Positive n/ Total n per group	
			Pseudoglandular		Canalicular		Saccular/Alveolar			
Peptide	Groups	Grade	E	M	E	M	E	M	E	M
UCN III	A (n=17)	G:0	1	1	6	6	2	2	8/17	8/17
		G:1	1	1	4	4				
		G:2	3	3						
		G:3								
	B (n=5)	G:0			1	2			4/5	3/5
		G:1	1	1	3	2				
		G:2								
		G:3								
	C (n=24)	G:0			9	9	2	2	13/24	13/24
		G:1	1	1	8	8	1	1		
		G:2	1	1	2	2				
		G:3								
	Positive n / Total n per stage		7/8	7/8	17/33	16/33	1/5	1/5		

n: number of fetuses.

development and pathology by comparing fetuses of different developmental stages and diagnosis.

Immunohistochemistry in 46 archival human fetal lung tissues using specific antisera for all four CRF homologue neuropeptides (CRF, Ucns I, II and III), the two CRF receptors (CRFR1 and CRFR2) and the CRF-BP revealed immunoreactivity for all antigens in the majority of the tissues. In this, the first dem-

Table 6. Semi-quantitative estimation for the presence of CRFR1 detected by immunohistochemistry in the epithelial (E) or mesenchymal (M) compartment of fetal lung tissues, shown separately. Numbers represent the number of fetuses in each of the four grades (G), at every gestational stage as estimated by the mother's last menstrual period (LMP) and in the three fetal groups (A: no pathology, B: genetic disorders, C: congenital disorders)

			Gestational stages						Positive n/ Total n per group	
			Pseudoglandular		Canalicular		Saccular/Alveolar			
Peptide	Groups	Grade	E	M	E	M	E	M	E	M
CRFR1	A (n=17)	G:0	2	1	7	7			8/17	9/17
		G:1	1	2	3	3				
		G:2	2	2			2	2		
		G:3								
	B (n=5)	G:0	1	1	2	2			2/5	2/5
		G:1			2	2				
		G:2								
		G:3								
	C (n=24)	G:0	2	2	15	15	3	3	4/24	4/24
		G:1			3	4				
		G:2			1					
		G:3								
	Positive n / Total n per stage		3/8	4/8	9/33	9/33	2/5	2/5		

n: number of fetuses.

Table 7. Semi-quantitative estimation for the presence of CRFR2 detected by immunohistochemistry in the epithelial (E) or mesenchymal (M) compartment of fetal lung tissues, shown separately. Numbers represent the number of fetuses in each of the four grades (G), at every gestational stage as estimated by the mother's last menstrual period (LMP) and in the three fetal groups (A: no pathology, B: genetic disorders, C: congenital disorders)

			Gestational stages						Positive n/ Total n per group	
			Pseudoglandular		Canalicular		Saccular/Alveolar			
Peptide	Groups	Grade	E	M	E	M	E	M	E	M
CRFR2	A (n=17)	G:0	2	2	5	6			10/17	9/17
		G:1	2	2	3	2				
		G:2	1	1	2	2	2	2		
		G:3								
	B (n=5)	G:0	1	1	2	2			2/5	2/5
		G:1			2	2				
		G:2								
		G:3								
	C (n=24)	G:0	1	1	9	9	2	2	12/24	12/24
		G:1	1	1	6	6	1	1		
		G:2			4	4				
		G:3								
	Positive n / Total n per stage		4/8	4/8	17/33	16/33	3/5	3/5		

n: number of fetuses.

onstration of the localization of the full CRF system in human fetal lung, histological mapping revealed the presence in both epithelial and mesenchymal cells of the lung parenchyma (bronchi and alveoli). Immunoreactivity was found in the cell cytoplasm for all antigens except CRFR1 and CRFR2 whose staining was also membranic. Previous studies, using RIA, have shown expression of immunoreactive CRF

Table 8. Semi-quantitative estimation for the presence of CRF-BP detected by immunohistochemistry in the epithelial (E) or mesenchymal (M) compartment of fetal lung tissues, shown separately. Numbers represent the number of fetuses in each of the four grades (G), at every gestational stage as estimated by the mother's last menstrual period (LMP) and in the three fetal groups (A: no pathology, B: genetic disorders, C: congenital disorders)

			Gestational stages						Positive n/ Total n per group	
			Pseudoglandular		Canalicular		Saccular/Alveolar			
Peptide	Groups	Grade	E	M	E	M	E	M	E	M
CRF-BP	A (n=17)	G:0							17/17	17/17
		G:1	1	1						
		G:2			1	1				
		G:3	4	4	9	9	2	2		
	B (n=5)	G:0							4/4	4/4
		G:1								
		G:2								
		G:3	1	1	3	3				
	C (n=24)	G:0			2	2			21/23	21/23
		G:1		1	1	2				
		G:2	1		1	2		1		
		G:3	1	1	15	13	2	1		
	Positive n / Total n per stage		8/8	8/8	30/32	30/32	4/4	4/4		

n: number of fetuses.

in the lung of the fetus, newborn, juvenile and adult baboon,[14] whereas CRF mRNA has been detected by in situ hybridization in fetal lungs of preterm mice.[15] This, however, seems to be an early-life characteristic, as in the adult human lung CRF has been shown to be expressed only at a very small percentage of small-cell lung cancers,[26] whereas CRF receptors were totally absent.[31]

Our study included fetuses with no pathological characteristics that were considered 'normal', as well as fetuses with diagnosed genetic or congenital malformations. CRF, Ucn II and III were localized at varying percentages in all groups and statistical analysis revealed no significant differences between 'normal' fetuses and those with genetic or congenital disorders (although grouping of fetuses with different pathology was an important limitation of our method). In contrast, Ucn I was more often present in 'normal' fetuses than in those of both pathological groups. Similarly, CRFR1 receptor was more frequently localized in the 'normal' group than in the fetuses with congenital disorders. These results point into a correlation between pathological fetal development and loss of function of the CRF system, although one cannot postulate if this is a causal or a resulting effect. Hormonal and neuroendocrine disturbances accompanying genetic or congenital malformations may affect the local CRF system and its potential interaction with cortisol and the fetal HPA axis. CRF receptors and their endogenous ligands have often been correlated to cell proliferation, apoptosis and migration in adult systems and cancers, which are cellular events that have great significance in ontogeny and fetal tissue development.

The above is further supported by the case of a 28-week male twin pregnancy included in our study that allowed a direct comparison between a pathological and a normal fetus. Specifically, one of these fetuses presenting with lung hypoplasia and chorioamnionitis (intra-uterine growth restriction with under-developed lungs) demonstrated low or no detectable CRF neuropeptides and receptors by comparison with its twin fetus of the same sex that had normal lung development. Another case of lung malformation was that of a fetus presenting bilaterally atelectasis, which also showed no detectable CRF receptors and poor localization of CRF neuropeptides.

A physiological link between the CRF system and lung development is not surprising. Firstly, cortisol, the end product of HPA (Hypothalamic-Pituitary-

Adrenal) axis activation, is an important factor for human fetal development. In humans and in other primates, the fetal adrenal cortex contains a distinct and disproportionately large central zone, called the "fetal zone", which disappears soon after labor.[32-33] The growth of this zone follows the CRF secretion pattern of the placenta. Cortisol produced there has been found to play a crucial part in the maturation of different fetal organs, such as lungs.[11] Moreover, it has been found that the offspring of knockout mice with CRF deficiencies die due to pulmonary hypoplasia, because there is no glucocorticoid production, which is vital for normal lung development. This conclusion was supported even further by the fact that prenatal supplementation of glucocorticoids prevented offspring's death.[34] Exogenous, prenatal glucocorticoids have been shown to decrease the incidence and severity of respiratory distress syndrome in preterm infants[35] as well as to accelerate the maturation of the lung.[36,37] Furthermore, other researchers have connected placental CRF with the maturation of the fetal HPA axis in humans by showing that exposure to elevated CRF levels during the early second trimester but not later in gestation was correlated with delayed maturity in the human fetus at 25 weeks' gestation.[38] However, the contribution of a putative local CRF system needs further investigation.

In our analysis, fetuses were also divided in accordance with the lung developmental stage (pseudoglandular, canalicular, saccular-alveolar). Significant differences in CRF system localization were found between stages, its presence declining with gestational age. In fact, at the pseudoglandular stage, Ucn I and III localization was more frequent than during the canalicular stage and the saccular/alveolar stage, and the same profile for Ucn II was close to statistical significance. For CRF receptors, however, no significant differences were found between developmental stages. It is important to note that fetuses belonging to the saccular and alveolar phase of lung development were under-represented in our study, for obvious reasons. This fact, which was accounted for during statistical analysis, probably impeded an accurate display of statistical significance.

Our results are in agreement with findings in murine experimental models[15] where CRF mRNA has been detected by in situ hybridization in fetal lungs of preterm mice during the period of glucocorticoid-induced lung maturation, but not in term animals. Furthermore, the results of another murine model, in which the expression of genes encoding for CRF, CRFR1, CRFR2b and CRF-BP was studied using RT-PCR and in situ hybridization in fetal lungs at late gestation, supported the presence of autocrine/paracrine actions of CRF and ACTH in developing lungs.[25] Furthermore, in the baboon,[14] in the lungs of 125- and 140-day gestation animals, CRF was higher than in term (185th day of gestation) fetus, juvenile and adult animals. The authors of this study concluded that CRF probably plays a paracrine and/or autocrine role in lung development from the time of intrauterine life until adulthood, this hypothesis further supported by data from baboon fetal lung explants (125d) in which CRF strongly induced surfactant phospholipid synthesis, most likely via CRF receptor.[16] In addition, in mice it has also been found that the expression of CRF and its binding sites was mainly localized in the distal epithelium, which is quite interesting given the fact that the increase of surfactant production in mice occurs on gestational day 17 in several cells of lung epithelium.[25] Here, we have demonstrated the presence of a complete CRF system in this tissue. Indeed, there was a positive correlation between the presence of the CRF neuropeptides and between the presence of CRFR1 and its ligand CRF. In another study in human term fetuses, CRF and Ucn I were shown to stimulate CRFR1 gene expression on the adrenals.[10] In conclusion, the CRF system seems to be more significant at the early stages of lung fetal development, possibly as an inducer of its differentiation and maturation.

The presence of Ucn I was correlated with fetal sex, being more frequently localized in male than in female fetuses, the latter established when comparisons were made in pooled grouped fetuses of all stages and pathology. This finding though is limited by the fact that representation of the two sexes is not balanced within developmental stages in our sample. No other correlation was found between neuropeptide and receptor presence with fetal sex, maternal age and the presence of chorioamnionitis. Chorioamnionitis, however, which in experimental models has been shown to be capable of both injuring and maturing the fetal lung,[39,40] was correlated to

higher CRF, Ucn II and CRFR1 and lower Ucn I and CRFR2 placental mRNA levels in preterm deliveries.[41] Finally, CRF-BP was strongly present in all but two fetuses of the canalicular stage, carrying congenital disorders. CRF-BP, being an endogenous regulator of local CRF/Ucn I bioavailability, may contribute to the function of the local CRF system.

Description of the CRF system in the developing human would be of great interest. It could provide significant information on the regulation of CRF signaling and/or its downstream targets (i.e. glucocorticoids) in the developing lung. The interest lies at both bench research and the clinical level, the latter because it could potentially be used as a pharmacological target in the context of premature birth. In addition, comparing the presence of the CRF system in fetuses with normal lung development to those with lung abnormalities would shed light on its potential role in lung fetal development. On the other hand, it needs to be said that comparisons between fetuses without pathology to those with different genetic or congenital disorders cannot be easily interpreted as their pathology lies in different organs and these embryos might have normal lung development as well. Individual analyses of each fetus with a different pathology would go beyond the scope of this study. Grouping of fetuses with similar pathologies, although inadequate, offered us the ability to carry out a preliminary comparison of normal and pathological fetuses. Studies with a larger number of fetuses with the same pathology are needed in order to reach definitive conclusions. A recent immunohistochemical study of the human neonate, addressing the development of Ucn I-positive neurons and the possible effect of neonatal hypoxic/ischemic encephalopathy on Ucn I expression, showed that in the centrally projecting neurons, Ucn I-immunoreactivity was already evident from 34 weeks of gestation onwards at very low levels, and a positive correlation was found between Ucn I expression and the age of the neonates, but not with hypoxia neuropathological grade.[42]

In this work we present experimental evidence suggesting, for the first time, that all components of the CRF system (neuropeptides and binding sites) are detectable in the lung of normal and pathological human fetuses throughout their development. Our results indicate a correlation to pathological lung development, as both fetuses with lung malformation were shown to have noticeably low or no detectable CRF neuropeptides and receptors. Furthermore, Ucn I and CRFR1 were more frequently detectable in fetuses with no lung pathology. The participation of the CRF system in structural and functional lung development, especially during the early stages, needs further investigation, using also other methods of gene expression detection, such as in situ hybridization or RT-PCR, in order to clarify the exact role of CRF neuropeptides and binding sites in lung maturation and pathology.

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REFERENCES

1. Harris GW, 1948 Neural control of the pituitary gland. Physiol Rev 28: 139-179.
2. Vale W, Spiess J, Rivier C, Rivier J, 1981 Characterization of a 41-residue ovine hypothalamic peptide that stimulates secretion of corticotropin and beta-endorphin. Science 213: 1394-1397.
3. Lovejoy DA, Balment RJ, 1999 Evolution and physiology of the corticotropin-releasing factor (CRF) family of neuropeptides in vertebrates. Gen Comp Endocrinol 115: 1-22.
4. Fekete EM, Zorrilla EP, 2007 Physiology, pharmacology, and therapeutic relevance of urocortins in mammals: ancient CRF paralogs. Front Neuroendocrinol 28: 1-27.
5. Hsu SY, Hsueh AJW, 2001 Human stresscopin and stresscopin-related peptide are selective ligands for the type 2 corticotropin-releasing hormone receptor. Nat Med 7: 605-611.
6. Lewis K, Li C, Perrin MH, et al, 2001 Identification of urocortin III, an additional member of the corticotropin-releasing factor (CRF) family with high affinity for the

CRF2 receptor. Proc Natl Acad Sci USA 98: 7570-7575.
7. Reyes TM, Lewis K, Perrin MH, et al, 2001 Urocortin II: A member of the corticotropin-releasing factor (CRF) neuropeptide family that is selectively bound by type 2 CRF receptors. Proc Natl Acad Sci USA 98: 2843-2848.
8. Behan DP, Heinrichs SC, Troncoso JC, et al, 1995 Displacement of corticotropin releasing factor from its binding protein as a possible treatment for Alzheimer's disease. Nature (London) 378: 284-287.
9. Buss C, Entringer S, Swanson JM, Wadhwa PD, 2012 The Role of Stress in Brain Development: The Gestational Environment's Long-Term Effects on the Brain. Cerebrum 2012: 4.
10. Sirianni R, Mayhew BA, Carr BR, Parker CR, Rainey WE, 2005 Corticotropin-Releasing Hormone (CRH) and Urocortin Act through Type 1 CRH Receptors to Stimulate Dehydroepiandrosterone Sulfate Production in Human Fetal Adrenal Cells. J Clin Endocrinol Metab 90: 5393-5400.
11. Mesiano S, Jaffe RB, 1997 Developmental and Functional Biology of the Primate Fetal Adrenal Cortex. Endocr Rev 18: 378-403.
12. Robinson BG, Arbiser JL, Emanuel RL, Majzoub JA, 1989 Species specific placental corticotrophin releasing hormone messenger RNA and peptide expression. Mol Cell Endocrinol 62: 337-341.
13. Sasaki A, Tempst P, Liotta AS, et al, 1988 Isolation and characterization of a corticotropin-releasing hormone-like peptide from human placenta. J Clin Endocrinol Metab 67: 768-773.
14. Dotzler SA, Digeronimo RJ, Yoder BA, Siler-Khodr TM, 2004 Distribution of Corticotropin Releasing Hormone in the Fetus, Newborn, Juvenile, Adult Baboon. Pediatr Res 55: 120-125.
15. Keegan CE, Herman JP, Karolyi J, O'Shea KS, Camper SA, Seasholtz AF, 1994 Differential expression of corticotrophin-releasing hormone in developing mouse embryos and adult brain. Endocrinology 134: 2547-2555.
16. Emanuel RL, Torday JS, Asokananthan N, Sunday ME, 2000 Direct effects of corticotrophin-releasing hormone and thyrotropin-releasing hormone on fetal lung explants. Peptides 21: 1819 -1829.
17. Chatelain A, Boudouresque F, Chautard T, Dupouy JP, Oliver C, 1988 Corticotrophin-releasing factor immunoreactivity in the hypothalamus of the rat during the perinatal period. J Endocrinol 119: 59-64.
18. Keller-Wood M, Powers MJ, Gersting JA, Ali N, Wood CE, 2006 Genomic analysis of neuroendocrine development of fetal brain-pituitary-adrenal axis in late gestation. Physiol Genomics 24: 218-224.
19. Green JL, Figueroa JP, Massman GA, Schwartz J, Rose JC, 2000 Corticotropin-releasing hormone type I receptor messenger ribonucleic acid and protein levels in the ovine fetal pituitary: ontogeny and effect of chronic cortisol administration. Endocrinology 141: 2870-2876.
20. Keiger CJ, O'Steen WK, Brewer G, Sorci-Thomas M, Zehnder TJ, Rose JC, 1995 Cortisol up-regulates corticotropin releasing factor gene expression in the fetal ovine brainstem at 0.70 gestation. Brain Res Mol Brain Res 32: 75-81.
21. King JS, Bishop GA, 2003 Localization of the type 1 corticotropin releasing factor receptor (CRF-R1) in the embryonic mouse cerebellum. J Neurocytol 32: 305-316.
22. Ohtsuka K, Koshimizu T, Ohyama Y, Yokota Y, 1986 The development of immunoreactive corticotropin-releasing factor (IR-CRF) in the hypothalamus, pancreas and gastrointestinal tract of immature rats. Nihon Naibunpi Gakkai Zasshi 62: 34-44.
23. Lakshmanan J, Magee TR, Richard JD, et al, 2008 Localization and gestation-dependent pattern of corticotrophin-releasing factor receptor subtypes in ovine fetal distal colon. Neurogastroenterol Motil 20: 1328-1339.
24. Lakshmanan J, Oyachi N, Ahanya SA, Liu G, Mazdak M, Ross MG, 2007 Corticotropin-releasing factor inhibition of sheep fetal colonic contractility: mechanisms to prevent meconium passage in utero. Am J Obstet Gynecol 196: 357.e1-7.
25. Simard M, Cote M, Provost P, Tremblay Y, 2010 Expression of genes related to the hypothalamic-pituitary-adrenal axis in murine fetal lungs in late gestation. Reprod Biol Endocrinol 8: 134.
26. Kaprara A, Pazaitou-Panayiotou K, Kortsaris A, Chatzaki E, 2010 The corticotropin releasing factor system in cancer: expression and pathophysiological implications. Cell Mol Life Sci 67: 1293-1306.
27. Chatzaki E, Crowe PD, Wang L, Million M, Taché Y, Grigoriadis DE, 2004a CRF receptor type 1 and 2 expression and anatomical distribution in the rat colon. J Neurochem 90: 309-316.
28. Chatzaki E, Murphy BJ, Wang L, et al, 2004b Differential profile of CRF receptor distribution in the rat stomach and duodenum assessed by newly developed CRF receptor antibodies. J Neurochem 88: 1-11.
29. Lambropoulou M, Papadopoulos N, Tripsianis G, et al, 2010 Co-expression of survivin, c-erbB2, and cyclooxygenase-2 (COX-2): prognostic value and survival of endometrial cancer patients. J Cancer Res Clin Oncol 136: 427-435.
30. Green SB, 1991 How many subjects does it take to do a regression analysis? Multivariate Behavioral Research 26: 499-510.
31. Reubi JC, Waser B, Vale W, Rivier J, 2003 Expression of CRF1 and CRF2 receptors in human cancers. J Clin Endocrinol Metab 88: 3312-3320.
32. Jaffe RB, Seron-Ferre M, Parer JT, Lawrence CC, 1978 The primate fetal pituitary-adrenal axis in the perinatal period. Am J Obstet Gynecol 131: 164-170.
33. Pepe GJ, Albrecht ED, 1995 Actions of placental and fetal adrenal steroid hormones in primate pregnancy. Endocr Rev 16: 608- 648.
34. Muglia L, Jacobson L, Dikkes P, Majzoub JA, 1995

Corticotropin-releasing hormone deficiency reveals major fetal but not adult glucocorticoid need. Nature 373: 427-432.
35. Crowley PA, 1995 Antenatal corticosteroid therapy: a meta-analysis of the randomization trials, 1972 to 1994. Am J Obstet Gynecol 173: 322-335.
36. Ballard PL, 1989 Hormonal regulation of pulmonary surfactant. Endocr Rev 10: 165-181.
37. Surbek D, Drack G, Irion O, Nelle M, Huang D, Hoesli I, 2012 Antenatal corticosteroids for fetal lung maturation in threatened preterm delivery: indications and administration. Arch Gynecol Obstet 286: 277-281.
38. Class QA, Buss C, Davis EP, et al, 2008 Low levels of corticotropin-releasing hormone during early pregnancy are associated with precocious maturation of the human fetus. Dev Neurosci 30: 419-426.
39. Jobe AH, 2012 Effects of chorioamnionitis on the fetal lung. Clin Perinatol 39: 441-457.
40. Westover AJ, Moss TJ, 2012 Effects of intrauterine infection or inflammation on fetal lung development. Clin Exp Pharmacol Physiol 39: 824-830.
41. Torricelli M, Novembri R, Bloise E, De Bonis M, Challis JR, Petraglia F, 2011 Changes in placental CRH, urocortins, and CRH-receptor mRNA expression associated with preterm delivery and chorioamnionitis. J Clin Endocrinol Metab 96: 534-540.
42. Pagida MA, Konstantinidou AE, Tsekoura E, Patsouris E, Panayotacopoulou MT, 2013 Immunohistochemical demonstration of urocortin 1 in Edinger-Westphal nucleus of the human neonate: Colocalization with tyrosine hydroxylase under acute perinatal hypoxia. Neurosci Lett 554: 47-52.