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Exposure to a Complex Cocktail of Environmental Endocrine Disrupting Compounds Disturbs the KiSS-1/GPR54 System in Ovine Hypothalamus and Pituitary Gland

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Running Title: Environmental EDC Exposure affects KiSS-1/GPR54 System

Key Words: environmental chemicals, GPR54, hypothalamus, kisspeptin, pituitary, prenatal exposure, sheep

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Abbreviations:

ARC	arcuate nucleus
EDCs	endocrine disrupting compounds
ERα	estrogen receptor alpha
FSH	follicle stimulating hormone
GnRH	gonadotropin releasing hormone
GPR54	G-protein coupled receptor 54
LHβ	luteinizing hormone beta
POA	preoptic area
PVN	paraventricular nucleus
qPCR	quantitative polymerase chain reaction

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Abstract

Background: Ubiquitous environmental chemicals, including endocrine disrupting chemicals (EDCs), are associated with declining human reproductive health, as well as an increasing incidence of cancers of the reproductive system. Verifying such links requires animal models exposed to 'real life', environmentally relevant concentrations/mixtures of EDC, particularly in utero, when sensitivity to EDC exposure is maximal.

Objectives: To evaluate effects of maternal exposure to a pollutant cocktail (sewage sludge) on ovine fetal reproductive neuroendocrine axes, particularly the kisspeptin/GPR54 system.

Methods: *KiSS-1, GPR54* and *ERα* mRNA expression was quantified in control (C) and treated (T) maternal and fetal (110d) hypothalami and pituitary glands using quantitative PCR and colocalization of kisspeptin with LHβ, and ERα in C and T fetal pituitary glands quantified using dual labeling immunohistochemistry.

Results: Fetuses exposed, in-utero, to the EDC mixture showed reduced *KiSS-1* mRNA expression across three hypothalamic regions examined (rostral, mid and caudal) and had fewer kisspeptin immunopositive cells colocalized with both LHβ and ERα in the pituitary gland. In contrast, treatment had no effect on parameters measured in the adult ewe hypothalamus or pituitary.

Conclusions: This study demonstrates that the developing fetus is sensitive to real-world mixtures of environmental chemicals, which cause significant neuroendocrine alterations. The important role of kisspeptin/GPR54 in regulating puberty and adult reproduction means that in-utero disruption of this system is likely to have long term consequences in adulthood and represents a novel, additional pathway through which environmental chemicals perturb human reproduction.

Introduction

Exposure to ubiquitous environmental chemicals can have detrimental effects on human and animal health, including immune (Rier et al. 1995; Vine et al. 2000) thyroid (Langer et al. 1998), cognitive and motor (Colborn et al. 1993) functions. Exposure to such chemicals has also been associated with increased incidences of reproductive disorders (Dickerson and Gore 2007; Fernandez et al. 2007; Toppari et al. 1996), such as precocious puberty, early menopause and breast cancer in females (Buck Louis et al. 2008; Kortenkamp 2006) and an increased incidence of testicular dysgenesis syndrome (TDS) in males (Olesen et al. 2007; Swan et al. 2000). The chemicals present within the environment include both inorganic (eg. heavy metals such as cadmium and lead) and organic (eg. alkylphenols, phthalates, polychlorinated biphenyls and organochlorine pesticides) compounds and many are termed endocrine disrupting compounds (EDCs) due to their effects on physiological systems. These EDCs are normally present in the environment as complex mixtures and exert their physiological effects via a variety of mechanisms (Safe 1994; Safe et al. 2002; Wilson et al. 2008) thus the effects of each compound can be additive, synergistic or antagonistic (Rajapakse et al. 2002) with the others present within the mixture. Investigation of the complex mechanistic interactions that might occur following exposure to such mixtures is a major emerging issue for research and risk assessment (Kortenkamp 2008). The prenatal and early life stages of development, where fetuses/young are exposed to pollutants through maternal blood and/or milk, are times when the body is particularly vulnerable to the effects of EDCs (Rhind 2005) as during this time EDCs may permanently reprogramme physiological processes influencing later health and/or reproductive function (Rhind et al. 2003).

Sewage sludge, a by-product of treatment of waste water from domestic, agricultural and industrial sources, contains numerous organic and inorganic pollutants (Giger et al. 1984; Smith 1995; Stevens et al. 2003; Webber and Lesage 1989, Lind et al. 2009) which broadly reflect the EDC mixture to which humans are typically exposed. Sewage sludge is widely used for land remediation and as an agricultural fertilizer, including on land used for grazing by farm animals. When applied to pastures, it results in modest increases in EDC concentrations in soil and herbage (Rhind et al. 2002) and provides an ideal model to investigate the effects of 'real-life' exposure to complex mixtures of environmental concentrations of chemicals/EDCs (Rhind 2002; Smith 1995), (Welshons et al. 2003), (Rajapakse et al. 2002). Although the observed concentrations of the individual chemicals in this model would not be predicted to pose a health risk, sheep exposed to this cocktail of pollutants exhibit altered fetal testis (Paul et al. 2005) and ovary (Fowler et al. 2008) development, bone density and morphology (Lind et al. 2009) and altered adult behavior (Erhard et al. 2004).

Normal reproductive function depends on hypothalamic regulation of gonadal function via secretion of gonadotropin-releasing hormone (GnRH) and the pituitary gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), which then act on the ovaries and testes to stimulate gonadal maturation, gametogenesis and steroidogenesis. The hypothalamo-pituitary gonadal (HPG) axis is regulated by a plethora of excitatory and inhibitory hypothalamic inputs and is highly sensitive to the organizational and activational effects of endogenous steroids during fetal and perinatal life (MacLusky and Naftolin 1981; Robinson et al. 2002). Therefore, alterations in reproductive function which manifest later in life, such as the earlier timing of puberty (Euling et al. 2008; Massart et al. 2006) or reduced fertility (Andersen et al. 2006; Carlsen et al. 1992; Skakkebaek et al. 2006) could be the result

of fetal exposure to sex steroid mimetics, such as EDCs, during development of this regulatory axis.

Recent work has shown that the neuropeptide kisspeptin and its receptor G-protein coupled receptor 54 (GPR54) are vital for the central regulation of GnRH neurosecretory activity and timing of puberty (Messager 2005; Roa et al. 2008). Kisspeptins are natural ligands for GPR54 (Kotani et al. 2001; Muir et al. 2001; Ohtaki et al. 2001). Thus, it is possible that the KiSS-1/GPR54 neurosecretory system is a target through which EDCs could perturb normal reproductive function (Navarro and Tena-Sempere 2008). This possibility is highlighted by findings showing that expression of *KiSS-1* mRNA is regulated by estradiol (Navarro et al. 2004; Smith et al. 2005a; Smith et al. 2005b) and that kisspeptin can act as a trans-synaptic modulator for conveying estradiol feedback to GnRH neurons (Navarro and Tena-Sempere 2008).

The objective of the present study was to determine whether exposure of pregnant sheep to environmental concentrations of a mixture of EDCs, via sewage sludge, when used as a fertilizer, disrupts the KiSS-1/GPR54 system, as determined by examination of *KiSS-1*, *GPR54* and *ER*α mRNA expression in the adult and fetal hypothalamus and pituitary and the proportions of kisspeptin positive LH secretory gonadotropes and estradiol receptive gonadotropes within the fetal pituitary gland.

Materials & Methods

Animals

Control (C) and treated (T) pregnant ewes were maintained on pasture treated with conventional inorganic fertilizers, or sewage sludge, respectively. Sewage treatment has been applied to pastures from 1997, as described in detail elsewhere (Paul et al. 2005). Animals were treated humanely and with regard for alleviation of suffering. At 110 days of gestation (GD110) maternal and fetal animals were euthanized (Schedule 1, U.K. Animals (Scientific Procedures) Act, 1986). Hypothalami and pituitary glands were collected from mothers (7 control and 8 treated) and their fetuses (12 control and 17 treated), immediately after euthanasia, and halved. For qPCR studies, tissues were frozen on dry ice and stored at -80°C before later mRNA extraction and analysis. For immunohistochemical studies, hemi-pituitaries from fetuses (7 control and 8 treated) were fixed in 4% paraformaldehyde (PFA) for 24 h, then stored in 30% sucrose, at 4°C, until processed.

Tissue Preparation

While still frozen, maternal and fetal hypothalamic blocks were cut into three coronal slices (4mm thick in adult, 2mm thick in fetuses) using external landmarks on the base of the brain as previously described (Whitelaw et al. 2009). The most rostral slice (Ros) encompassed the preoptic area (POA), the mid slice (Mid) encompassed the paraventricular nucleus (PVN), and the caudal slice (Cau) contained the arcuate nucleus (ARC). From each slice approximately 100-200mg of tissue was harvested for RNA extraction, for each animal. Approximately 100-200mg of tissue was also harvested from the mid sagittal face of maternal and fetal pituitary glands for RNA extraction. Total RNA was extracted from each tissue using TRIzol® (Invitrogen, UK)

as recommended by the manufacturers and mRNA (200-300ng) was then reverse transcribed using Moloney-Murine Leukemia virus (M-MLV) reverse transcriptase (Invitrogen, UK), random hexamers (Promega UK) and Rnasin (Promega, UK) as described previously (O'Shaughnessy and Murphy 1993). Purity and quantity of mRNA and cDNA were assessed using an ND-1000 spectrophotometer (NanoDrop® Technologies).

Quantitative PCR (qPCR)

Oligonucleotide primers and Taqman probes for the *KiSS-1*, *ER*α and β-*actin* genes were designed using published ovine NCBI database (GenBank) sequences, DQ059506, AY033393 and U39357, respectively and Primer Express Software (Applied Biosystems). The *GPR54* primer sequences were obtained from previously published work conducted in the rat (Seminara et al. 2003). The *GPR54* primer sequences used were 100% homologous to the NCBI ovine *GPR54* (KiSS-1 receptor) sequence (EU 272411). The Taqman probes for *KiSS-1*, *ER*α, *GPR54* and β-*actin* were synthesized by Eurogentec and contained FAM as the 5’-reporter and Blackhole Quencher 1 as the 3’-quencher (Sequences shown in Table 1). The qPCR reactions were performed in duplicate and a reagent blank included within each plate to detect contamination by genomic DNA. To validate the primers for *KiSS-1* and *GPR54*, prior to their use in qPCR, they were initially used in an rtPCR reaction, amplified fragments then separated by 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining. PCR products of the predicted size for both genes were obtained and sequenced for verification.

The qPCRs for ovine *KiSS-1*, *GPR54* and the endogenous reference gene β-*actin* were conducted using a Mx3000P™ Real-Time PCR System (Stratagene) according to the manufacturer's instructions. Each qPCR reaction (25µl) contained PCR Buffer II (Applied Biosystems, 50 mM KCl, 10 mM Tris-HCl (pH8.3), 1.5 mM $MgCl_2$, 0.001% (v/v) gelatin), 0.2 mM of each deoxynucleotide triphosphate, 0.625 U AmpliTaq Gold DNA polymerase (Applied Biosystems), template cDNA (2µl), and optimized quantities of the respective probe and primers for the genes of interest and molecular biology grade H_2O (BDH, UK). Thermal cycling conditions were 10 min at 95°C (initial denaturation step) and 45 cycles of 30 sec at 95°C (denaturation step) and 1 min at 60°C (primer annealing and elongation).

KiSS-1, *ER*α and *GPR54* mRNA expression was quantified using the comparative CT (cycle threshold) method (Schmittgen and Livak 2008) and gene expression calculated relative to the reference gene (β-*actin*). Validation studies were conducted to ensure that the amplification efficiencies of the reference gene, *KiSS-1*, *ER*α and *GPR54* were equivalent. This involved generation of relative standard curves for each gene using serial dilutions of cDNA. The resultant ΔCT (y) between the housekeeping gene and *KiSS-1*, *ER*α and *GPR54* were plotted versus log (dilution) (x) and the slope of the line calculated (by linear regression analysis). Slopes for *KiSS-1*, *ER*α and *GPR54* were all <0.1 indicating equivalent amplification efficiencies (data not shown).

Immunohistochemistry

Frozen sections (5µm) of fetal pituitaries were cut on a cryostat and thaw mounted onto Polysine™ coated slides (VWR International). Sections from each animal (n=8 control, n=8 treated) were washed briefly in PBS and underwent microwave antigen

retrieval to unmask epitopic sites (Leong and Milios 1986; Shi et al. 1991). Briefly, slides were immersed in 0.1M sodium citrate buffer and heated in a conventional 750W microwave oven for 2 X 5 minutes as previously described for pituitary tissue (Franceschini et al. 2006). Slides were then left to cool to room temperature for 20 min before being washed in PBS. Sections were blocked with PBS containing 10% normal goat serum (NGS) for 1 h at room temperature then rinsed in PBS before addition of primary antibody. For kisspeptin and LHβ double immunolabeling, sections were incubated in a mixture of polyclonal kisspeptin antibody which was raised against bioactive residues 43–52 (kp10) of mouse metastin (1:1000, IFR 145; INRA, Nouzilly, France, (Richard et al. 2008) and validated in ovine tissue (Franceschini et al. 2006) and a monoclonal anti-LHβ antibody (1:10000, generous gift from Alan McNeilly) in PBS containing 2% NGS, for 72 h at 4°C. For Kisspeptin and ERα double immunolabeling, slides were incubated for 72 h at 4°C with a mixture of polyclonal anti-kisspeptin (as above, 1:1000) and monoclonal anti-human ERα antibody (Dako, 1:200), in PBS containing 2% NGS. Following incubation with the appropriate mixture of primary antibodies, sections were then washed (3 X 5 min) in PBS and incubated for 1 h at room temperature in a mixture of Alexafluor© 488-conjugated goat anti-rabbit IgG and Alexafluor© 594-conjugated goat anti-mouse IgG (both 1:1000 in 2% NGS, Molecular Probes). Sections were washed (3 X 5 min) in PBS before being mounted using Fluorsave™ mounting medium (Calbiochem) and coverslipped.

Immunohistochemical analysis of kisspetin concentrated on expression within the pituitary as the study design did not allow collection of tissue in a manner which would allow visualization of kisspeptin immunopositive cell bodies in the hypothalamus as described by Franceschini et al, 2006.

For LHβ and ERα double immunolabeling, sequential immunostaining using NiDAB and DAB was performed. Briefly, sections were washed in PBS and microwave antigen retrieval carried out as detailed above. Sections were then incubated with 3% H_2O_2 in methanol for 10 min to block endogenous peroxidase activity, washed, blocked in 10% NGS in PBS (1 h at room temperature) and incubated overnight at 4°C with monoclonal anti-human ERα antibody (Dako, 1:200 dilution) in PBS containing 2% NGS. Sections were then washed and incubated with an anti-mouse biotinylated secondary antibody (1:1000, DAKO) for 1 h at room temperature, washed again and treated with ABC (Vectastain Elite Kit, Vector Laboratories). Immunolocalisation of ERα was visualized using NiDAB to produce a blue/black color. Slides were then washed and incubated with polyclonal anti-ovine LHβ, AFP 69707P (1:10000, (Crawford et al. 2000) for 1 h at room temperature before processing as detailed above, except that LHβ immunolocalisation was visualized using DAB, to produce a brown product. Slides were then rapidly dehydrated in increasing concentrations of alcohol, mounted using DPX (BDH) and coverslipped. Pituitary sections from all control and treated animals were run for each antibody at the same time and under the same conditions to ensure comparability. Negative controls for all immunohistochemical procedures were included and indicated specific binding of all antibodies used.

The number of LHβ immunoreactive cells (brown cytoplasmic staining) with or without ERα nuclear immunolabeling (blue/black nucleus) and similarly the number of kisspeptin immunoreactive cells (green) that co-expressed LHβ or ERα (red) immunofluoresence were counted using a Leica DM4000B microscope. Images were captured at X 40 magnification using a Leica DC480 digital camera and Leica Qwin software. Slides were evaluated subjectively but systematically by counting the

number of immunodectectable cells or double-labeled cells from the same three areas for each pituitary gland (top, middle and bottom). As there was no difference in cell number between areas, the mean number of cells from the three areas was calculated for each pituitary and cell numbers were compared between control and treatment groups.

Statistical Analyses

All data are presented as mean ± S.E.M. For real-time data analysis, all data were log transformed and analyzed using two-way ANOVA. For co-localization studies, cell numbers in control and treated animals were compared using Student's t-tests. A *P* value of less than 0.05 was considered statistically significant.

Results

Fetal KiSS-1 and GPR54 mRNA expression

There was no effect of gender on the level of mRNA expression for *KiSS-1* or *GPR54* in either C or T fetuses and so data were pooled. Relative levels of *KiSS-1* mRNA expression in the hypothalamus and pituitary gland of the C and T fetuses are depicted in Figure 1. Hypothalamic *KiSS-1* and *GPR54* mRNA expression did not differ significantly between the areas of the hypothalamus studied. However, T fetuses expressed significantly (P<0.05) less hypothalamic *KiSS-1* mRNA than C fetuses, particularly in the rostral and caudal regions (Fig. 1A). *KiSS-1* mRNA expression was also significantly lower (P<0.05) in the pituitary glands of T than C fetuses (Fig. 1B). Despite the significant effects of treatment on *KiSS-1* mRNA expression, no differences were seen in either hypothalamic or pituitary gland *GPR54* mRNA expression, (Ros: C: 0.14±0.03 T: 0.14±0.02; Mid: C: 0.60±0.51 T: 0.23±0.05 and Cau: C: 0.14±0.07 T: 0.13±0.04; Pituitary: C: 0.28±0.086 T: 0.26±0.06).

Maternal KiSS-1 and GPR54 mRNA expression

There were no significant differences in maternal hypothalamic *KiSS-1* mRNA expression with treatment. (Ros: C: 0.77±0.37 T: 1.46±0.79; Mid: C: 1.64±0.45 T: 1.23±0.76 and Cau: C: 0.82±0.39 T: 0.91±0.91). In the maternal pituitary, *KiSS-1* mRNA expression exhibited a non-significant trend (P=0.15) towards lower expression in the C (1.76±0.58) relative to T animals (0.42±0.61). No significant effects of treatment were seen on *GPR54* mRNA expression in either tissue from the adult ewes (Ros: C: 0.33±0.2 T:0.39±0.17; Mid: C: 0.66±0.54 T: 0.27±0.14 and Cau: C: 0.17±0.03 T: 0.38±0.25: Pituitary C: 0.99±0.75 T: 0.70±0.34).

ERα mRNA expression

Relative levels of *ER*α mRNA expression in the hypothalamus and pituitary gland of the C and T dams are depicted in Figure 2. T dams exhibited a trend towards higher *ER*α mRNA expression than C dams in all areas of the hypothalamus and in the pituitary glands (Fig.2A and 2B). In the fetal animals, there was no effect of exposure to treated pastures on *ER*α mRNA expression in either the hypothalamus or the pituitary gland (Ros: C: 0.041±0.025 T: 0.017±0.003; Mid: C: 0.011±0.003 T: 0.039±0.014 and Cau: C: 0.11±0.10 T: 0.009±0.002: Pituitary C: 0.299±0.138 T: 0.246±0.076).

Kisspeptin and LHβ co-localization in the fetal pituitary

Given the decrease in *KiSS-1* mRNA expression in the fetal animals, described above, using dual immunohistochemistry, we examined whether exposure to sewage sludge treated pastures had any effect on the expression of kisspeptin within the fetal pituitary glands and, if so, in which cells. Representative examples of the staining for LHβ and kisspeptin in the pituitary glands of control and treated fetuses and the mean number of immunopositive cells observed are shown in Figure 3. Exposure to sewage sludge was associated with a significant (P<0.05) reduction in the number of LHβ immunopositive cells in the fetal pituitary gland (Fig.3G) and a significant reduction in the number of cells double labeled for both LHβ and kisspeptin (expressed as the number of LHβ positive gonadotropes per field (P<0.01, Fig.3H) or as a percentage of the total number of LHβ positive cells per field (P<0.001, Fig.3I)).

Kisspeptin and ERα co-localization

Kisspeptin expression was also analyzed relative to ERα expression in the fetal pituitary glands. Representative images of kisspeptin, ERα and double labeled immunoflurescence are and the mean number of cells expressing kisspeptin and ERα in the control and treated fetuses shown in Figure 4. T fetuses exhibited significantly less (P<0.01) ERα positive cells within the fetal pituitary glands compared to C fetuses (Fig. 4G). There were also significantly (P<0.01) fewer ERα positive cells present within the pituitary gland that co-expressed kisspeptin in the T relative to the C animals, expressed as both the number of cells (Fig. 4H) and the percentage of co-localized cells (P<0.001, Fig. 4I).

LHβ and ERα co-localization

Figure 5 (A and B) depicts representative images of pituitary tissue from C and T fetuses immunostained for LHβ and ERα and the mean number of cells expressing LHβ, ERα or co-expressing both antigens is shown in Figure 5C. T fetuses had significantly fewer ERα positive cells (P<0.01) and fewer cells double labeled for LHβ and ERα (per field P<0.005, Fig.5C). However, the number of co-localized cells per field, expressed as a proportion of the total number of ERα immunopositive cells, was similar for C and T fetuses (%): C: 92±1.7 T: 88.7±2.8. As in the study of LHβ and kisspeptin co-expression, significantly fewer (P<0.01) LHβ immunopositive cells per field were observed in T fetuses.

Discussion

For the first time we have shown that maternal exposure to a complex mixture of chemicals/EDCs, at environmental concentrations, negatively affects the fetal KiSS-1/GPR54 neuroendocrine system. Disturbingly, this exposure reduced both the expression of *KiSS-1* mRNA in the hypothalamus and pituitary of exposed fetuses and the proportion of kisspeptin immunopositive pituitary cells that expressed LHβ and ERα. Therefore, our results provide evidence of a mechanism through which low level, long term, exposure to environmental EDCs may alter subsequent adult reproductive function (Navarro et al. 2008; Navarro and Tena-Sempere 2008).

This study showed that fetuses exposed to a mixture of EDCs through maternal contact with chemicals present in sewage sludge have reduced hypothalamic *KiSS-1* mRNA expression, this effect being most pronounced in the regions of the hypothalamus that encompass the POA and ARC. In both areas, kisspeptin cells co-express ERα (Smith et al. 2005b) and in the ARC, kisspeptin immunopositive cells has been implicated in estrogen negative feedback onto GnRH neurons (Franceschini et al. 2006). Therefore, EDC exposure may disturb estrogen feedback systems within the hypothalamus which may have consequences in later life for example the timing of puberty. While this has not yet been examined in this experimental model, kisspeptin signaling is fundamental to the initiation of puberty in rodents (Clarkson and Herbison 2006; Han et al. 2005) and reproductive activity in sheep (Clarke et al. 2009; Smith et al. 2008a) and hypothalamic *KiSS-1* gene expression during puberty is significantly reduced if rats are exposed to estrogenic compounds neonatally (Navarro et al. 2008). Interestingly, despite a significant decrease in *KiSS-1* expression in our exposed fetuses, *GPR54* mRNA expression was not affected in either the hypothalamus or pituitary gland. While regulation of *KiSS-1* mRNA expression by

estradiol is widely accepted, hormonal regulation of *GPR54* mRNA expression is not as well documented and appears to be less sensitive to steroid feedback, at least in the pituitary gland (Richard et al. 2008). Therefore, regulation of the KiSS-1/GPR54 system by estrogenic compounds is likely to be mediated primarily via the ligand rather than its receptor. Although it is not possible to determine which specific compounds were responsible for the effects noted in the T fetuses, the findings of Navarro *et al* (2008) suggest that it is likely due to the activity of estrogenic EDCs known to be present in sewage sludge. Given the critical organizational events occurring in-utero, alterations in the kisspeptin/GPR54 system could have long-term detrimental effects on adult reproductive function and further studies will be required to investigate this possibility.

The results of the current study also indicate that maternal EDC exposure reduces fetal pituitary *KiSS-1* mRNA expression as well as lowering the proportion of gonadotropes which co-expressed kisspeptin and LHβ and the number of LHβ positive gonadotropes. While the current experimental design does not allow us to differentiate between direct effects of EDCs at the level of the pituitary and indirect central actions, the results clearly demonstrate that maternal EDC exposure could affect fetal intra-pituitary modulation of LH release. It should be noted that not all kisspeptin positive cells within the pituitary were LHβ positive; therefore, the observed reduction in *KiSS-1* mRNA expression may also affect other pituitary cell types, including somatotropes, lactotropes and gonadotropes that specifically synthesize FSH. Kisspeptin function within the pituitary gland, particularly during development, may not be restricted to LH release as gonadotropes are also a critical

source of mitogenic and/or differentiation (Tilemans et al. 1992) and anti-apoptotic factors (Melamed et al. 1999).

As with the hypothalamic KiSS-1/GPR54 system, the pituitary kisspeptin system is also sensitive to endogenous estradiol (Smith et al. 2008b). The results of this study show, again for the first time, that kisspeptin is expressed in pituitary cells that also express ERα, demonstrating that there may be important interactions between kisspeptin and estrogens in the fetal ovine pituitary. Moreover, the number of ERα positive cells, as well as the proportion of cells coexpressing ERα and kisspeptin, was significantly reduced in T fetuses. Since 92% of ERα positive cells are also LHβ positive gonadotropes, this reflects a significant disturbance of the interactions between kisspeptin, estrogens and LH by chemical/EDC exposure, *in-utero*.

Our results extend previous work, which reported the presence of kisspeptin and GPR54 in the adult pituitary gland of the rat (Richard et al 2008;Gutierrez-Pascual et al. 2007) and demonstrates for the first time that *KiSS-1* and *GRP54* mRNA and kisspeptin are expressed in the fetal ovine pituitary. The exact role played by pituitary kisspeptin and its relevance in the regulation of LH secretion (Smith et al 2008b) is presently subject to discussion. Our results indicate that kisspeptin could have paracrine/autocrine actions within the pituitary gland and these have not yet been characterized. However, based on studies that have shown kisspeptin to be involved in LH release in the presence of GnRH (Gutierrez-Pascual et al. 2007; Navarro et al. 2005), it has been suggested that kisspeptin might act in the pituitary, in synergy with GnRH (and estradiol) as an endocrine/autocrine/paracrine signal, to modulate hormone secretions from the anterior pituitary (Richard et al. 2009).

The late prenatal and early postnatal developmental stages are particularly sensitive periods of brain development with initiation of sexually dimorphic characteristics (Rhees et al. 1990). It is, therefore, perhaps not surprising, that exposure to exogenous chemicals/EDCs in sewage sludge (Cespedes et al. 2008; Petrovic et al. 2002; Reddy et al. 2005, Lind et al. 2009) during this period, has the potential to alter the KiSS-1 GPR54 systems and thus affect adult reproductive function. Under the present study conditions, the tissue levels of individual EDCs are modest (Rhind et al. 2002) therefore the effects seen must be due to combinations of small amount of many different environmental chemicals (Willingham 2004). Interestingly, the adult female sheep, through which the chemical exposure occurred, were resilient to the effects of sewage sludge exposure, which supports the proposal that the effects of EDCs are more pronounced during the critical, early windows of development than in adulthood. Although the effects shown in fetuses in this study are very likely to be due to exposure of their mothers to a mixture of chemicals present in sewage sludge, identifying which particular chemicals or combination of chemicals are responsible, is extremely difficult. Interpretation, and more importantly extrapolation of the findings of this study to human health, should be attempted with caution as it is impossible to know the exact level of human exposure and whether this is comparable to the levels of chemicals to which sheep are exposed following sewage sludge application to pasture. While there are metabolic and physiological differences between humans and sheep human waste is an important contributor to sewage sludge. We therefore propose that humans themselves are exposed to many of the constituent chemicals/EDCs present in sewage sludge and in fact that the levels of some constituents in this model are probably lower than those to which humans are exposed.

It is concluded that low level exposure of fetuses to a mixture of environmental chemicals via maternal ingestion, throughout development, disrupts both the normal pattern of fetal hypothalamic and pituitary *KiSS-1* mRNA expression and also the number and proportion of kisspeptin positive cells that co-express LHβ and ERα in the fetal pituitary. This indicates that the regulation of gonadotropes by endogenous estradiol could be disrupted. Since kisspeptin plays a major role in many aspects of reproductive axis regulation, including puberty onset, alterations to this system during development of the sexually dimorphic neuroendocrine axis is likely to affect long term reproductive function and fertility and will be examined in future studies of adult animals exposed to EDCs in this experimental paradigm in utero. Humans are also exposed to a cocktail of environmental chemicals on a daily basis throughout their lives, including during development, and so the kisspeptin/GPR54 system is a potential target for environmental EDCs to alter puberty timing and reproductive function in our own species.

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Table 1. qPCR probes and primer sequences

Target	**mRNA Primer Sequence 5'-3'**	**Product (bp)**	**Ref**
β-actin	β-actin-F: TCCTTCCTGGGCATGGAATC β-actin-R: GGGCGCGATGATCTTGATCT β-actin-Probe (FAM labelled): CCTTCCTTCCTGGGCATGGAATCC	199	U39357
KiSS-1	KiSS1-F: CTGGTGCAGCGGGAGAAG KiSS1-R: GCGCAGGCCGAAGGA KiSS1-Probe (FAM labelled): ACGTGTCCGCCTACA	57	DQ059506
ERα	ERα-F:CCCGGAAGACGTGAATCAGA ERα-R: GTTTGCAAGGAATGCGATGA ERα-Probe(FAM labelled): CAGCTGGCCACCACTGGCTGC	66	AY033393
GPR54	GPR54-F: TACATCCAGCAGGTCTCGGTG GPR54-R: ACGTACCAGCGGTCCACACT GPR54-Probe (FAM labelled): CACGTGTGCCACTCTGACCGCC	71	EU272411 Seminara *et al*, 2003

Figure Legends

Figure 1

KiSS-1 mRNA expression in the fetus in A) the rostral (R), mid (M) and caudal (C) regions of the hypothalamus, and B) the pituitary following in-utero exposure to sewage sludge chemicals (Treated). *$P<0.05$, **$P<0.02$ in comparison with respective control value.

Figure 2

Maternal *ER*α mRNA expression in A) the rostral (R), mid (M) and caudal (C) regions of the hypothalamus and B) the pituitary of control and sewage sludge exposed sheep (Treated) were not significantly affected by exposure to sewage sludge.

Figure 3

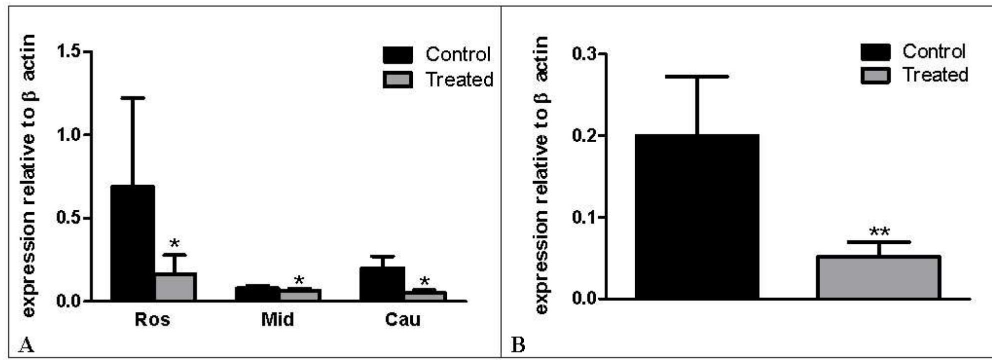
Representative photomicrographs of kisspeptin (green, A&D) and LHβ (red, B&E) and merged image (C&F) in fetal pituitaries from Control (top row) and sewage sludge-exposed (Treated) (bottom row) animals. Cells showing co-localisation are stained yellow (indicated by white arrows, bar=20µm). Quantification per field of view of G) the mean LHβ-immunopositive cell number, H) the mean number of cells immunopositive for both LHβ and kisspeptin, and I) percentage of kisspeptin^{+}/LHβ^{+} cells as a proportion of the total number of LHβ^{+} cells in fetal pituitary tissue sections from control and sewage sludge exposed (Treated) animals. *$P<0.05$, **$P<0.01$, ***$P<0.001$, in comparison with respective control value.

Figure 4

Representative photomicrographs of kisspeptin (green, A&D) and ERα (red, B&E) and merged image (C&F) in fetal pituitaries from Control (top row) and sewage sludge-exposed (Treated) (bottom row) animals. Example of cells showing co-localization are indicated by white arrows (bar=20μm). Quantification per field of view of G) the mean ERα -immunopositive cell number, H) the mean number of cells immunopositive for both ERα and kisspeptin, and I) percentage of kisspeptin^{+}/ ERα^{+} cells as a proportion of the total number of ERα^{+} cells in fetal pituitary tissue sections from control and sewage sludge exposed (Treated) animals, $^{**}P<0.01$, $^{***}P<0.001$, in comparison with respective control value.

Figure 5

Representative photomicrographs of ERα (blue/black) and LHβ (brown) double immunohistochemistry in fetal pituitaries from control (A) and (B) sewage sludge-exposed (Treated) animals. Example co-localized cells are indicated by black arrows (bar=20μm). C) Quantification per field of view of the mean LHβ-, ERα- and LHβ-/ERα immunopositive cell numbers in fetal pituitary tissue sections from control and sewage sludge exposed (Treated) animals. $^{*}P<0.05$, in comparison with respective control value.



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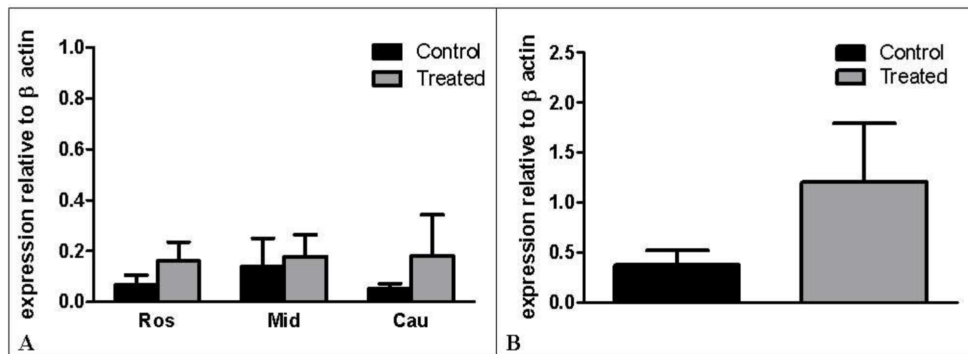

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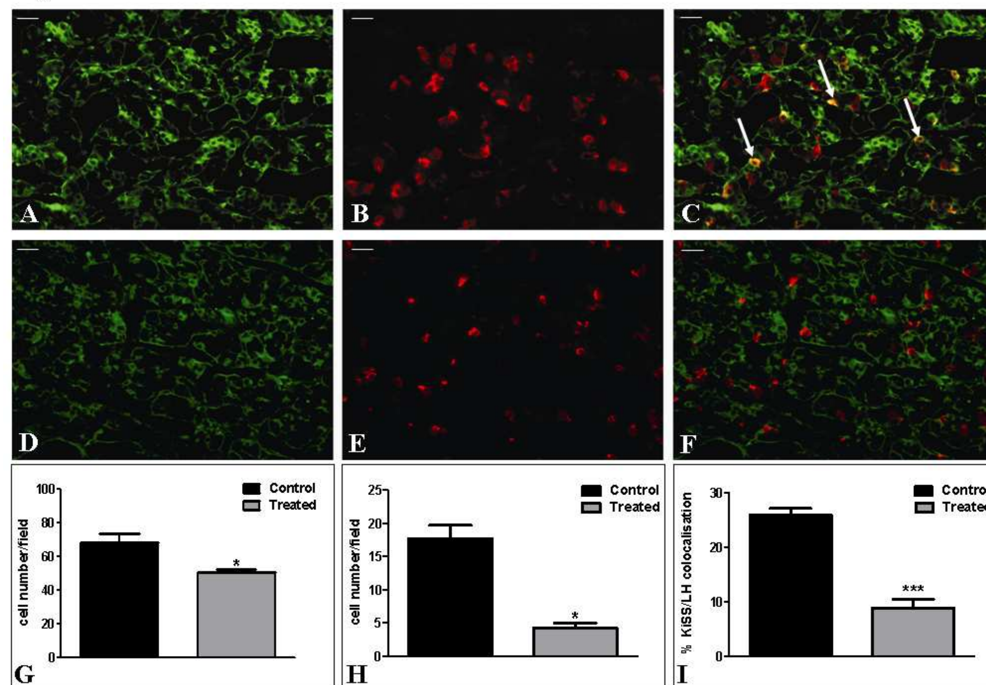

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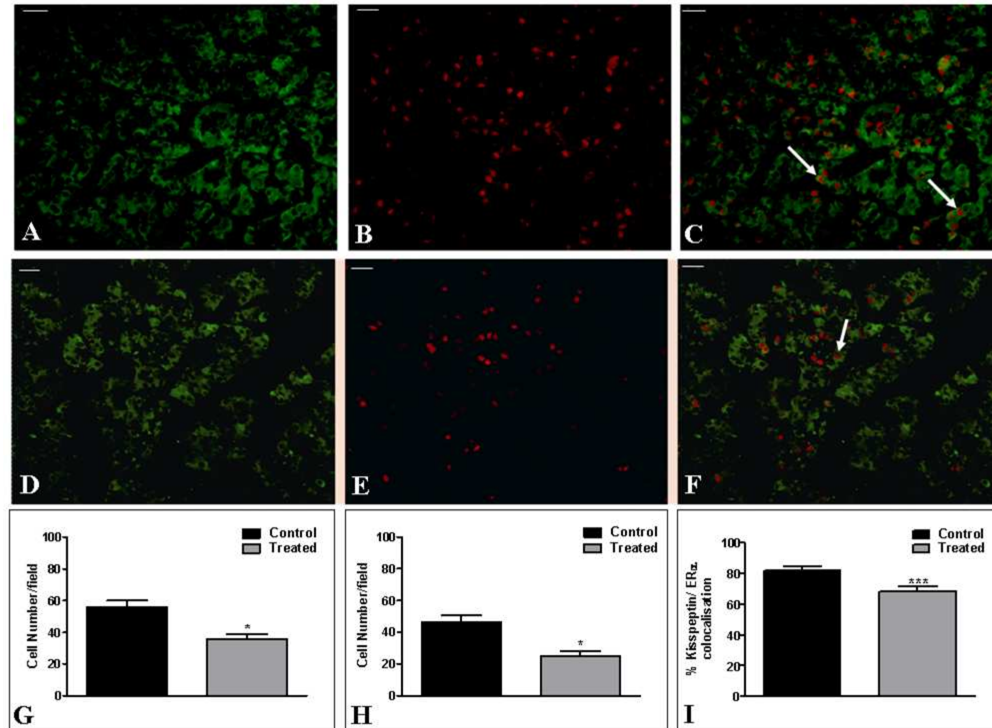
Figure 4



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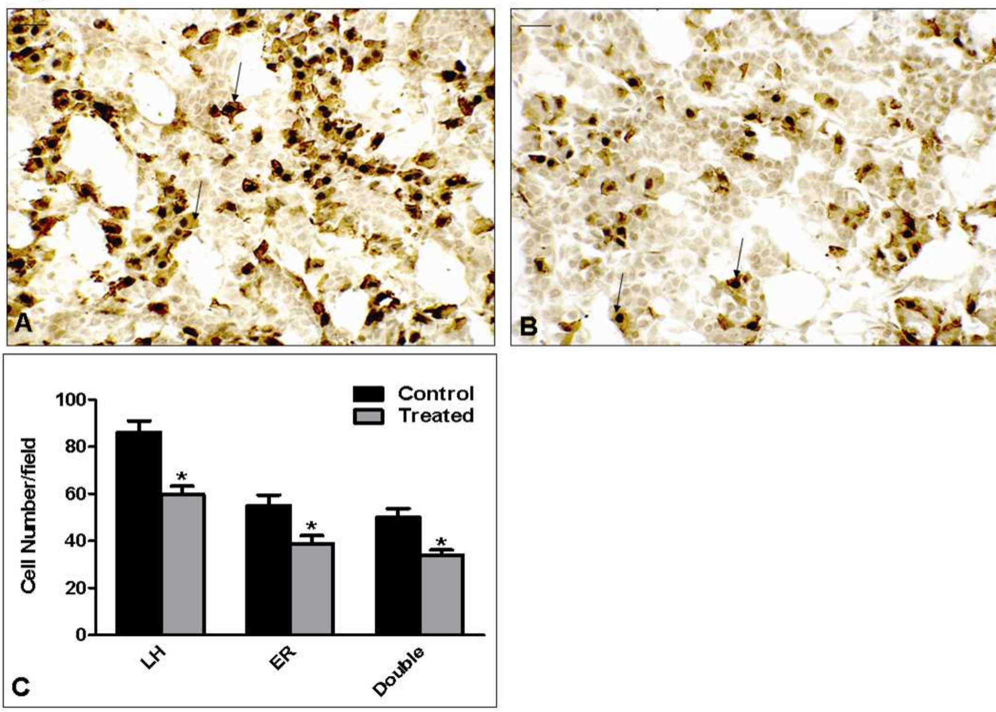

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