Changes in endocrine and neurochemical profiles in neonatal pigs prenatally exposed to increased maternal cortisol

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Abstract

Early life environmental factors are able to influence prenatal development and may cause structural and functional effects on hypothalamic–pituitary–adrenal (HPA) axis and neurotransmitter systems in the offspring. These effects seem to be species specific and may depend on the period of gestation when the factors are effective. Elevated maternal cortisol levels are assumed to play a crucial role as a programming factor during prenatal development. Thus, the present study was performed in order to examine the effects of increased maternal cortisol levels during mid- and late gestation on central and peripheral alterations of the HPA axis and brain neurotransmitter profiles in piglets. Endogenous cortisol release was induced by i.m. administration of ACTH to sows every second day either during mid- (day 49 until 75) or late gestation (day 85 until 107). Controls received injections of saline. ACTH treatment of sows during mid- and late gestation had no effects on the gestation length, the number of total born and the frequency of stillborn piglets. However, ACTH treatment during late gestation caused an increase of birth weight ($P < 0.04$) and affected the organ:body weight ratios (brain and adrenal) in the offspring. There was an impact of increased maternal cortisol on the HPA axis and on central neurotransmitter systems in the offspring. ACTH

treatment during mid gestation caused a significant decrease of plasma corticosteroid-binding globulin (CBG; $P < 0.03$) and an increase of the noradrenergic activity in the locus coeruleus (LC) region ($P < 0.02$). Elevated maternal cortisol during late gestation also produced a significant decrease of plasma CBG ($P < 0.05$), but significantly increased the plasma noradrenaline (NA) concentration ($P < 0.02$) and decreased the serotonergic activity in the LC at both postnatal day 1 ($P < 0.016$) and day 28 ($P < 0.003$). Furthermore, there were sex-specific effects of ACTH treatment on plasma CBG, NA and brain monoamine turnover, with more pronounced changes in male offspring. In conclusion, elevated maternal cortisol levels during mid- and late gestation in pigs affect growth, HPA axis and brain neurotransmitter systems in the offspring in a sex-specific manner. The observed alterations in endocrine and neurotransmitter systems are dependent on the gestational period. Late gestation appears to be a more sensitive phase for cortisol-induced programming in pigs. Moreover, the present data show that there are marked developmental differences between laboratory animals and domestic pigs, and highlight the importance of species-specific studies on prenatal influences.

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Introduction

Numerous studies show that stressors acting upon the organism during pregnancy can have distinct and long-lasting effects on the offspring. A variety of functions and systems can be influenced including behaviour, reproduction, and neuroendocrine, and autonomic and immune system (Ward 1972, Herrenkohl 1979, Weinstock *et al.* 1992, Maccari *et al.* 1995, Kay *et al.* 1998). Environmental factors acting early in life to organize or alter permanently these physiological systems result in ‘prenatal programming’ (Barker *et al.* 1993, Edwards *et al.* 1993). The brain is very sensitive to prenatal programming and glucocorticoids, in particular, have strong brain-programming properties. It was shown that maternal glucocorticoids may underlie the association between low birth weight and adult stress-related cardiovascular, metabolic

and neuroendocrine disorders such as hypertension, type 2 diabetes, ischaemic heart disease and affective disorders (Seckl 1998). Substantial evidence suggests that prenatal stress programmes the hypothalamic–pituitary–adrenal (HPA) axis, and that plasticity of developing brain monoamine systems underlie, in part, these changes (Slotkin *et al.* 1992, Muneoka *et al.* 1997, Weinstock *et al.* 1998). Since an important characteristic of the stress response is the secretion of high levels of glucocorticoids, this steroid has become a primary candidate for the role of a programming factor in the prenatal stress paradigm (Phillips *et al.* 1998, Levitt *et al.* 2000, Maccari *et al.* 2003).

Most of the studies on prenatal stress have been performed on rodents (for a review see Braastad 1998, Matthews 2002), while very few have been performed on farm species. In cattle, transport stress during gestation resulted in offspring

with increased pituitary weights and higher post-restraint cortisol concentrations (Lay *et al.* 1997a,b). Repeated transport stress in the last third of gestation of goats had no effect on basal cortisol concentrations in goat kids, but influenced the sympatho-adrenomedullary system (Roussel *et al.* 2005). Isolation stress during gestation in sheep caused higher basal cortisol concentrations in the offspring (Roussel *et al.* 2004) and it was also demonstrated in sheep that prenatal exposure to synthetic glucocorticoids elevated the basal cortisol levels and increased the cortisol response to corticotrophin-releasing hormone plus arginine vasopressin challenge later in life (Moss *et al.* 2001, Sloboda *et al.* 2002). In pigs, stimulation of maternal cortisol release by weekly maternal adrenocorticotrophic hormone (ACTH) injections and restraint in pregnant sows for 6 weeks resulted in offspring with higher adrenocortical expression of ACTH receptor mRNA and a higher adrenal cortex:medulla area ratio on day 1 of age indicating greater adrenal sensitivity. Under stressful conditions, the pigs in the maternal ACTH-treated group also showed significantly higher plasma cortisol responses (Hausmann *et al.* 2000). Recent studies in pigs have also shown alterations in the offspring's HPA axis activity after hydrocortisone treatment or social stress during gestation dependent on the timing of exposure (Jarvis *et al.* 2006, Kranendonk *et al.* 2006). These alterations may be related to programming of brain corticosteroid receptors. In a previous study of our group, we could show that piglets, whose mothers were subjected to daily restraint stress for the last 5 weeks of gestation, exhibit reduced hypothalamic glucocorticosteroid receptor (GR)-binding sites, but increased hippocampal GR sites with no effect on the hippocampal mineralocorticoid receptor (MR), possibly indicating decreased negative feedback at the paraventricular nucleus (PVN) and enhanced facilitation of the HPA response (Kanitz *et al.* 2003).

The stressors used in most prenatal stress studies may also activate other stress systems, e.g. neurotransmitters and opioids, so that it is unlikely that the observed changes in the offspring are caused by the action of maternal glucocorticoids alone. In order to determine whether elevated maternal glucocorticoids are responsible for the observed effects in postnatal pigs, we developed a model of repeated administration of exogenous ACTH to pregnant sows that stimulates the endogenous maternal cortisol response. It has already been shown that repeated intramuscular administration of ACTH to pregnant sows results in a prolonged elevation of their plasma cortisol levels and that this maternal adrenocortical stimulation increases the fetal cortisol levels, which may affect the development *in utero* (Otten *et al.* 2004, Schwerin *et al.* 2005).

Little is known about the sensitivity of the growing fetus to programming effects of maternal glucocorticoids during specific periods of gestation. The timing of maturation of the HPA axis relative to birth is highly species specific. In animals that give birth to mature young (e.g. guinea pigs, sheep and primates), maximal brain growth and a large proportion of neuroendocrine maturation (including corticosteroid receptor development) take place *in utero*

(Matthews 1998, Challis *et al.* 2000). Conversely, in species that give birth to immature young (e.g. rats, rabbits and mice), much of the brain and neuroendocrine development occurs in the postnatal period (Dent *et al.* 2000). Therefore, manipulations of the maternal environment and/or hormonal milieu in gestation will influence different stages of brain and HPA development depending on the species. The marked developmental differences between rodents and farm animals highlight the value of comparative studies on this topic. Moreover, pigs show a different placental structure compared with rats with a placentation characterized as epitheliochorial, whereas that of the rodent is haemoendothelial. Therefore, the pig placenta may offer a more significant barrier to maternal hormonal influence.

The aim of this study was to examine the effects of increased maternal glucocorticoid levels on central and peripheral alterations of the HPA axis and brain neurotransmitter profiles in neonatal pigs. The possible different impact of increased maternal glucocorticoid levels at specific prenatal ontogenetic periods was studied by incorporating mid-gestation as a period of ongoing HPA axis maturation and fetal-growth acceleration (Klemmke & Christenson 1997, McPherson *et al.* 2004), and late gestation as the period of major brain growth spurt in pigs (Pond *et al.* 2000).

Materials and Methods

Animals and housing

Forty-four pregnant primiparous Landrace sows and their litters were used in two experiments (EXP 1, $n=24$; EXP 2, $n=20$). Sows were individually housed in pens (1.2 × 2.0 m), and fed twice a day with a commercial pig diet for pregnant sows. The ration increased from 2.2 kg/day in week 8 of gestation to 3.2 kg/day in the last week of gestation and was totally consumed by the animals. Water was available *ad libitum*. Approximately, 1 week before farrowing, at day 105 of gestation, sows were weighed (EXP 1, 202.2 ± 2.2 kg; EXP 2, 223.3 ± 3.1 kg) and moved to individual farrowing pens (2.0 × 3.0 m). There were no differences in the body weight of sows between the treatment groups within each experiment. Sows stayed in the farrowing pens with their offspring until weaning on day 28 and during this period, piglets were weighed at day 1, 14 and 28 of age. In both the experiments, no abortions occurred.

All the procedures involving animal handling and treatment were approved by the Committee for Animal Use and Care of the Ministerial Agricultural Department of Mecklenburg-Vorpommern, Germany.

Experimental procedure

In EXP 1 (mid-gestation, MG), 12 sows received i.m. administrations of synthetic ACTH (Synacthen Depot, Novartis Pharma, Brussels, Belgium) at a dose of 100 IU per animal every 2 days (MG-ACTH), beginning on day 49

until day 75 of gestation. Administrations of ACTH were given to the unrestrained sows in the neck at 0800 h and injection sites within the neck region were changed every treatment day. The other 12 sows served as a control (MG-C) and received saline repeatedly. We have already shown in a previous study that this ACTH administration protocol during mid-gestation results in a consistent cortisol response throughout the treatment period with increased cortisol levels until 8 h post-injection (Otten *et al.* 2004).

In EXP 2 (late gestation, LG), ten sows were repeatedly injected with 100 IU synthetic ACTH (LG-ACTH) as described earlier (every 2 days), starting on day 85 until day 107 of gestation, whereas the other ten sows served as a control treated with saline (LG-C). During late gestation, ACTH application also caused a consistent cortisol enhancement throughout the treatment period (W Otten, unpublished observations).

In both EXP 1 and EXP 2, litters were standardized to ten animals after birth, usually five males and five females. Surplus animals were fostered to other sows in the pig unit. Blood samples were taken on postnatal day (PND) 1 and day 28 from all the piglets by anterior vena cava puncture in a supine position (the whole procedure lasted approximately 30 s). For plasma extraction, blood samples were collected in ice-cooled polypropylene sampling tubes containing EDTA (Kabevette, Kabe Labortechnik GmbH, Nürnberg-Elseroth, Germany), immediately placed on ice and subsequently centrifuged at 2000 *g* for 15 min at 4 °C. Plasma was stored at –80 °C until hormone analysis.

Tissue collection

On PND1 and PND28, two male and two female piglets from each litter were euthanized by an i.v. injection of T61 (embutramide/mebezonium iodide/tetracaine hydrochloride, Intervet, Unterschleissheim, Germany). The brains were quickly removed and placed on ice, and the hippocampus, amygdala, hypothalamus including the PVN, and the region of locus coeruleus (LC) were dissected, frozen in liquid nitrogen, and stored at –80 °C. The stereotaxic atlas of the pig brain (Felix *et al.* 1999) served as reference. Both adrenal glands were also removed, weighed and frozen in liquid nitrogen.

All the experimental procedures on the animals were performed between 0800 and 1100 h.

Catecholamine analyses

Plasma concentrations of adrenaline (A) and noradrenaline (NA) were analysed in duplicate using high-pressure liquid chromatography (HPLC) with electrochemical detection after extraction from plasma samples by absorption on aluminium oxide (Otten *et al.* 1997). Intra- and inter-assay coefficients of variation (CV) were 11.5 and 12.4% for adrenaline, and 2.1 and 1.9% for noradrenaline.

Cortisol analysis

Plasma cortisol concentrations were measured in duplicate using a commercially available ¹²⁵I-RIA kit (DRG International Inc., Mountainside, NJ, USA) according to the manufacturer's guidelines. Cross-reactivities of antibody used to any potentially competing plasma steroids (DRG International Inc.) were lower than 5%. The assay was validated for use with porcine plasma. Sensitivity of the assay was 3 ng/ml, and intra- and inter-assay CV values were 5.3 and 9.8% respectively.

Corticosteroid-binding globulin (CBG)

Plasma samples were examined for CBG using a modified binding assay previously described by Kanitz *et al.* (2002). Briefly, 25 µl blood plasma were incubated with 0.78 nM unlabelled cortisol (hydrocortisone, Merck) and 25 pM [³H]-cortisol (specific radioactivity 68 Ci/mmol, Amersham Pharmacia Biotech). Non-specific binding was determined in parallel using a 100-fold excess of cold cortisol. The separation of bound and free [³H]-cortisol was performed by precipitation with dextran-coated charcoal at 4 °C and subsequent centrifugation at 1000 *g* for 10 min. The intra- and inter-assay CV values were 7.8 and 9.1% respectively.

Glucocorticosteroid receptor (GR)-binding assay

The GR-binding assay was conducted as previously described by Kanitz *et al.* (1998). Briefly, tissues of hippocampus and hypothalamus were homogenized in ice-cold 10 mM Tris-HCl, pH 7.5, containing 12.5 mM EDTA, 10 mM sodium molybdate, 0.25 mM saccharose and 1 mM dithiothreitol using a Teflon-glass homogenizer. The homogenate was centrifuged at 120 000 *g* for 60 min at 0–4 °C to obtain cytosol (i.e. the supernatant fraction).

Hippocampal GR binding was evaluated directly in saturation experiments using the pure glucocorticoid [³H]-dexamethasone (specific activity 43 Ci/mmol; Amersham Pharmacia Biotech) over a concentration range of 0.2–24 nM. For the single-point assay of the GR binding in the hypothalamus, a 100 µl aliquot of cytosol was incubated with 5 µl aliquots of a saturating 10 nM concentration of [³H]-dexamethasone. In both single-point and saturation experiments, non-specific binding was determined with a parallel incubation that contained 500-fold excess of RU 28362 (kindly donated by Roussel Uclaf, Romainville, France), which binds selectively to the glucocorticoid receptor. The separation of bound from free ligand was performed by precipitation with dextran-coated charcoal and the receptor-[³H]-steroid complexes were counted in a spectral liquid scintillation counter (LKB Wallac, Turku, Finland) at an efficiency of 50%.

The theoretical maximal number of binding sites (*B*_{max}) and *K*_d for [³H]-dexamethasone binding in hippocampus were derived from saturation experiments using the method of Scatchard (1949). The results of the single-point assays

were expressed in terms of specific binding per milligram protein. Protein content was determined by the method of Lowry *et al.* (1951) with BSA as standard. Data were expressed as femtomole per milligram of protein.

Quantification of monoamines and metabolites

NA, its metabolite 3-methoxy-4-hydroxyphenylglycol (MHPG), dopamine (DA), its metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), 5-hydroxytryptamine (5-HT) and its metabolite 5-hydroxyindole-3-acetic acid (5-HIAA) in the LC were determined using HPLC and electrochemical detection. Samples were weighed and homogenized on ice for 5 min with a hand homogenizer in 0.5 ml of 0.2 M perchloric acid followed by centrifugation at 45 000 *g* for 10 min at 4 °C. After collection of the supernatants, the procedure was repeated. Pooled supernatants of the repeated extractions were again centrifuged at 45 000 *g* for 10 min at 4 °C. Samples of 20 µl were then injected directly in the HPLC system equipped with a 125×4 mm reversed-phase column packed with ProntoSil C18 AQ (Bischoff Analysentechnik, Leonberg, Germany). As mobile phase, 58 mM sodium hydrogen phosphate buffer containing 1.2 mM octansulphonic acid, 0.3 mM EDTA, 0.2 mM potassium chloride and 6% methanol at pH 3.5 was used at a flow rate of 0.8 ml/min. Electrochemical detection was achieved by an ISAAC *in situ* Ag/AgCl cell with a glassy carbon working electrode set at a potential of 600 mV (Shimadzu, Duisburg, Germany). Dihydroxybenzylamine was used as an internal standard for quantification and the concentrations were expressed as picogram per milligram weight tissue. As an index of NA, DA and 5-HT turnover, the MHPG/NA, DOPAC/DA, HVA/DA and 5-HIAA/5-HT ratios were calculated.

Adrenal glands

For histological analyses, the right adrenal gland was sectioned (7 µm) with a cryostat microtome (Reichert-Jung, Leica, Nussloch, Germany), transverse to the long axis, in the mid-glandular region. Sections were stained using haematoxylin and eosin dye for visualization of both the adrenal cortex and the medulla (Fiedler *et al.* 1996). Using a video camera (CF15/2RGB, KAPPA, Gleichen, Germany) and a computer system, images of three sections were recorded. The adrenal capsule, the line of demarcation between adrenal cortex and medulla as well as any open space where a blood vessel resided, were traced. The CV for the area of the three sections within the same gland was 1.6% for the adrenal cortex and 1.9% for the adrenal medulla. The tracings were then used to quantify the area of the entire gland, the cortex and the medulla (excluding open space area) using an image analysis system (SIS, Münster, Germany). For each selected section, the number of labelled cells was counted and the data were expressed as densities (number of cells/mm²).

Statistical analysis

Data analyses were performed using the MIXED model procedure of SAS (Statistical Analysis System Institute 1999). Within each experiment, the following analyses were carried out: (1) body weights, relative tissue weights, glucocorticoid receptor binding, and biogenic amine contents in brain tissues were analysed with a model including the fixed factors treatment and gender, the corresponding interaction and the random factor sow nested within treatment; (2) plasma cortisol, CBG and catecholamines, area and number of cells in adrenal tissues were analysed using the same model with the body weight as an additional covariate. Tukey *post hoc* tests were used, where appropriate, to explore the source of significant effects. Significance was considered at $P < 0.05$ and tendencies at $P < 0.10$. All data are presented as least square mean $\pm$ S.E.M.

Results

Gestation outcome, body and organ weights of the offspring

ACTH treatment of sows during mid- and late gestation had no effect on the gestation length (MG-ACTH: 114.8 $\pm$ 0.3 days, MG-C: 115.2 $\pm$ 0.3 days; LG-ACTH: 115.1 $\pm$ 0.4 days, LG-C: 115.4 $\pm$ 0.4 days) nor on the number of total born piglets (MG-ACTH: 12.3 $\pm$ 1.0, MG-C: 12.2 $\pm$ 1.0; LG-ACTH: 13.6 $\pm$ 0.8, LG-C: 13.2 $\pm$ 0.9). There was also no significant difference in the frequency of stillborn piglets during both the treatment periods (MG-ACTH: 2.9 $\pm$ 1.5%, MG-C: 5.0 $\pm$ 2.1%; LG-ACTH: 5.2 $\pm$ 2.1%, LG-C: 4.4 $\pm$ 2.1%).

Birth and body weights of MG-ACTH piglets did not differ from control piglets and there were no differences in brain and adrenal gland weights expressed as organ:body weight ratio (Table 1). In contrast, ACTH treatment during late gestation significantly increased the birth weight in the offspring ($P < 0.041$) and the body weight on PND14 ($P < 0.019$; Table 1). Furthermore, analysis revealed a significant decrease in brain:body weight ratio on PND1 ($P < 0.049$) and an increase in adrenal:body weight ratio on PND28 (right, $P < 0.05$; left, $P < 0.10$). While the decrease in brain:body weight ratio was caused by an increase in body weight on PND1, the increase in adrenal:body weight ratio on PND28 corresponded to a significant increase in adrenal weights of the LG-ACTH piglets (right (LG-ACTH): 0.488 $\pm$ 0.010 g, LG-C: 0.418 $\pm$ 0.012 g, $P < 0.001$; left (LG-ACTH): 0.493 $\pm$ 0.012 g, LG-C: 0.433 $\pm$ 0.014 g, $P < 0.008$).

Plasma cortisol, CBG and catecholamines

ACTH stimulation of sows during mid- and late gestation did not affect the basal cortisol levels in the offspring either on PND1 or on PND28 ($P > 0.1$; Fig. 1A), but the statistical analyses revealed a significant influence of ACTH treatment on CBG concentrations on PND1. As shown in Fig. 1B,

Table 1 Body weights and organ weights of brain and adrenal glands in piglets from sows treated with adrenocorticotrophic hormone (ACTH) (Synacthen Depot 100 IU i.m.) or saline during mid- and late gestation. Values are least square mean $\pm$ s.e.m. Number of animals per group: BW at birth (MG-ACTH=120, MG-C=120, LG-ACTH=100, LG-C=100), BW PND14 and PND28 (MG-ACTH=72, MG-C=72, LG-ACTH=60, LG-C=60); brain and adrenal gland PND1 and PND28 (MG-ACTH=48, MG-C=48, LG-ACTH=40, LG-C=40).

	Mid gestation		Late gestation	
	ACTH	Control	ACTH	Control
Body weight (BW) (kg)				
At birth	1.18 $\pm$ 0.06	1.22 $\pm$ 0.06	1.34 $\pm$ 0.05*	1.18 $\pm$ 0.05*
PND14	3.70 $\pm$ 0.27	3.97 $\pm$ 0.25	4.82 $\pm$ 0.12*	4.33 $\pm$ 0.13*
PND28	7.09 $\pm$ 0.42	7.49 $\pm$ 0.38	8.60 $\pm$ 0.29	8.06 $\pm$ 0.33
Brain (g/kg BW)				
PND1	19.64 $\pm$ 0.44	19.74 $\pm$ 0.47	18.66 $\pm$ 0.84*	21.39 $\pm$ 0.92*
PND28	5.91 $\pm$ 0.31	5.69 $\pm$ 0.28	5.10 $\pm$ 0.18	5.08 $\pm$ 0.20
Adrenal gland (g/kg BW)				
Left				
PND1	0.131 $\pm$ 0.040	0.128 $\pm$ 0.004	0.110 $\pm$ 0.005	0.113 $\pm$ 0.005
PND28	0.062 $\pm$ 0.003	0.062 $\pm$ 0.003	0.058 $\pm$ 0.003	0.052 $\pm$ 0.003
Right				
PND1	0.120 $\pm$ 0.004	0.117 $\pm$ 0.005	0.102 $\pm$ 0.006	0.102 $\pm$ 0.006
PND28	0.065 $\pm$ 0.004	0.062 $\pm$ 0.004	0.057 $\pm$ 0.002*	0.050 $\pm$ 0.002*

*Within a row denotes significant differences ($P<0.05$) between ACTH and controls.

CBG concentrations were decreased in both MG-ACTH offspring ($P<0.034$) and LG-ACTH offspring ($P<0.049$) on PND1. In addition, a significant sex $\times$ prenatal treatment effect ($P<0.005$) on CBG levels was observed on PND28 in LG-ACTH piglets. *Post hoc* testing revealed that male piglets from ACTH sows had significantly higher CBG concentration ($P<0.009$) compared to male controls.

Plasma NA concentrations after venipuncture were not affected in MG-ACTH piglets ($P>0.05$), but were significantly increased in LG-ACTH piglets on PND1 ($P<0.016$; Fig. 2A). Although there were no main effects, either of sex or of prenatal treatment after ACTH application during mid-gestation, there was a significant sex $\times$ prenatal treatment interaction effect on plasma NA levels of MG-ACTH piglets on PND28 ($P<0.0104$). *Post hoc* testing revealed that male piglets from ACTH sows displayed higher NA values ($P<0.035$) compared with male control piglets at this age.

Plasma adrenaline concentrations of piglets were not affected by ACTH stimulation of their mothers during either of the treatment periods (Fig. 2B).

Brain monoamines and metabolites

A summary of monoamine and metabolite levels in LC region of MG-ACTH piglets at different ages is presented in Table 2. Both sex and prenatal treatment altered monoamine and metabolite levels in the LC of MG-ACTH piglets. There was no significant main effect of prenatal treatment on monoamine and metabolite levels in piglets on PND1. However, a significant sex $\times$ prenatal treatment interaction effect on MHPG level was observed ($P<0.049$), where MHPG concentrations increased in male MG-ACTH piglets and decreased in female MG-ACTH piglets. The 5-HT concentration ($P<0.003$) and the 5-HT metabolite 5-HIAA ($P<0.018$) were elevated in males,

regardless of prenatal treatment, compared with females. As shown in Table 2, on PND28, a significant main effect of prenatal treatment was found on MHPG ($P<0.023$) and in tendency on NA ($P<0.10$) and HVA ($P<0.066$). Additionally, there were significant effects of sex on NA ($P<0.039$) and DA ($P<0.05$), with higher concentrations observed in female MG-ACTH piglets.

The effects of ACTH treatment during late gestation on monoamines and metabolites in the LC of piglets are shown in Table 3. There was also an influence of both sex and prenatal treatment on the neurochemical activity in the offspring. ACTH treatment of the mothers significantly decreased the 5-HT levels in the offspring on both PND1 ($P<0.016$) and PND28 ($P<0.003$) and decreased, in tendency, the 5-HIAA levels on PND1 ($P<0.066$). Additionally, there was a significant effect of sex on 5-HT levels in the LC on PND1 ($P<0.05$), with higher levels observed in males compared with females. A significant sex $\times$ prenatal treatment interaction effect on 5-HIAA/5-HT ratio was observed on PND1, where values decreased in male LG-ACTH piglets and increased in female LG-ACTH piglets ($P<0.019$). Furthermore, the DOPAC/DA ratio was significantly affected by sex on PND1 ($P<0.03$), female LG-ACTH piglets displayed higher values compared with male animals. On PND28, there was a main effect of prenatal treatment on HVA/DA ratio ($P<0.05$), piglets from ACTH-treated sows displayed higher values (Table 3).

Glucocorticoid receptor binding in hypothalamus and hippocampus

There was a tendency for reduced GR binding in the hypothalamus of newborn piglets (PND1) from both treatments compared with controls (MG-ACTH, $P<0.068$; LG-ACTH, $P<0.079$; Fig. 3A). The hippocampal GR

Figure 1 (A) Basal plasma cortisol and (B) CBG concentrations in piglets on PND1 and PND28 from sows treated with ACTH (Synacthen Depot 100 IU i.m.; filled bars) or saline (open bars) during mid- and late gestation. * $P < 0.05$. Number of animals per group: cortisol, PND1 (MG-ACTH=88, MG-C=94, LG-ACTH=100, LG-C=95), PND28 (MG-ACTH=37, MG-C=46, LG-ACTH=60, LG-C=50); CBG, PND1 (MG-ACTH=103, MG-C=103, LG-ACTH=100, LG-C=94), PND28 (MG-ACTH=37, MG-C=46, LG-ACTH=59, LG-C=50).

binding was not affected by treatment either during mid- or during late gestation (Fig. 3B). There were no effects of sex on the GR binding in these brain areas.

Adrenal glands

ACTH-treatment of sows during mid gestation did not induce morphological alterations of the adrenal gland in the offspring. However, ACTH-treatment during late gestation significantly increased the area of adrenal cortex in piglets on

PND28 ($P < 0.037$; Fig. 4A) and increased the density of cells in the adrenal medulla on PND1 ($P < 0.02$; Fig. 5B).

Discussion

The present study indicates that elevated maternal cortisol exposure during gestation in pigs affects growth, HPA axis at different levels and brain neurotransmitter profiles in the

Figure 2 (A) Plasma noradrenaline and (B) adrenaline concentrations in piglets on PND1 and PND28 from sows treated with ACTH (Synacthen Depot 100 IU i.m.; filled bars) or saline (open bars) during mid- and late gestation. * $P < 0.05$. Number of animals per group: noradrenaline, PND1 (MG-ACTH=102, MG-C=101, LG-ACTH=100, LG-C=96), PND28 (MG-ACTH=37, MG-C=40, LG-ACTH=59, LG-C=51); adrenaline, PND1 (MG-ACTH=102, MG-C=101, LG-ACTH=100, LG-C=96), PND28 (MG-ACTH=37, MG-C=49, LG-ACTH=59, LG-C=51).

offspring in a sex-specific manner. The effects on endocrine and neurochemical key parameters depend on the gestational stage, and the period of late gestation appears to be a particularly vulnerable developmental stage during porcine brain differentiation.

Administration of ACTH to pregnant sows during mid- and late gestation had no effect on the gestation length, the number of total born and the frequency of stillborn piglets. This is in accordance with previous studies in pigs using

different stressors such as maternal restraint, ACTH injections or treatments with hydrocortisone acetate (Hausmann *et al.* 2000, Otten *et al.* 2001, Kranendonk *et al.* 2005). However, elevated maternal glucocorticoids during late gestation caused an increase in birth weight of LG-ACTH piglets. Such higher birth weights after prenatal ACTH or stress treatments were described for calves and lambs (Lay *et al.* 1997b, Roussel *et al.* 2004), whereas in rodents and humans, maternal restraint stress or prenatal dexamethasone treatment generally

Table 2 Effects of maternal adrenocorticotrophic hormone (ACTH) (Synacthen Depot 100 IU i.m.) or saline treatment during mid gestation on LC monoamine levels and their turnover in piglets on PND1 and PND28. Values are least square mean $\pm$ s.e.m. Number of animals per group: PND1 (ACTH=20, control=12); PND28 (ACTH=15, control=20).

	PND1		PND28	
	ACTH	Control	ACTH	Control
NA (pg/mg)	1255.5 $\pm$ 103.4	1249.6 $\pm$ 108.7	1364.0 $\pm$ 72.1	1165.8 $\pm$ 74.0
MHPG (pg/mg)	92.7 $\pm$ 12.3	90.9 $\pm$ 12.5	92.2 $\pm$ 4.7*	71.2 $\pm$ 5.1*
MHPG/NA	0.08 $\pm$ 0.01	0.08 $\pm$ 0.02	0.07 $\pm$ 0.01	0.07 $\pm$ 0.01
DA (pg/mg)	172.8 $\pm$ 26.4	168.1 $\pm$ 27.6	151.5 $\pm$ 20.8	122.7 $\pm$ 21.3
DOPAC (pg/mg)	353.9 $\pm$ 28.6	281.5 $\pm$ 37.8	188.4 $\pm$ 23.5	220.4 $\pm$ 21.5
HVA (pg/mg)	688.4 $\pm$ 51.8	602.5 $\pm$ 66.4	209.3 $\pm$ 39.3	331.0 $\pm$ 34.0
DOPAC/DA	1.97 $\pm$ 0.14	1.76 $\pm$ 0.18	1.31 $\pm$ 0.19	1.52 $\pm$ 0.31
HVA/DA	4.03 $\pm$ 0.24	4.16 $\pm$ 0.30	1.71 $\pm$ 0.39	2.65 $\pm$ 0.34
5-HT (pg/mg)	1001.9 $\pm$ 123.6	794.9 $\pm$ 146.3	1052.2 $\pm$ 71.1	1093.7 $\pm$ 67.8
5-HIAA (pg/mg)	1826.8 $\pm$ 290.3	1417.8 $\pm$ 343.9	790.6 $\pm$ 79.8	840.8 $\pm$ 79.3
5-HIAA/5-HT	1.89 $\pm$ 0.09	1.79 $\pm$ 0.12	0.74 $\pm$ 0.04	0.78 $\pm$ 0.04

*Within a row denotes significant differences ($P < 0.05$) between ACTH and controls. NA, noradrenaline; MHPG, 3-methoxy-4-hydroxyphenylglycol; DA, dopamine; DOPAC, 3,4-dihydroxyphenylacetic acid; HVA, homovanillic acid; 5-HT, 5-hydroxytryptamine; 5-HIAA, 5-hydroxyindole-3-acetic acid.

decreases the birth weights (Bloom *et al.* 2001, Lesage *et al.* 2004, Burlet *et al.* 2005). This discrepancy may result from differences in the fetal development as well as species sensitivity to glucocorticoids.

Maternal elevated cortisol during late gestation also affected brain and adrenal weights in the offspring. The effects resulted in a reduction of the brain:body weight ratio on PND1, which was due to the increased body weight of LG-ACTH piglets at birth. On PND28, adrenal:body weight ratio and adrenal weights were increased in LG-ACTH piglets. Similar results were found in guinea pigs after prenatal dexamethasone treatment (Liu *et al.* 2001, Banjanin *et al.* 2004). Our results also revealed an increased area of the adrenal cortex in these animals, indicating that internal morphological changes occurred, which increased the cortical mass. This is in accordance with the results from a previous study in pigs,

where prenatal ACTH treatment and restraint caused an increased adrenal cortex:medulla ratio in the offspring (Hausmann *et al.* 2000). These findings indicate that the adrenal may have a higher capacity for cortisol release under stressful conditions as already shown in prenatally stressed pigs (Hausmann *et al.* 2000, Jarvis *et al.* 2006).

HPA activity is regulated by a negative feedback process in which circulating glucocorticoids act at various target sites in the brain and pituitary. CBG influences the bioavailability as well as metabolic clearance rate of cortisol within the circulation (Bright 1995). The amount of cortisol, which is taken up by target tissue, is approximated by the non-CBG bound (free + albumin) fraction of total circulating steroid (Siiteri *et al.* 1982). Thus, differences in circulating CBG levels determine the magnitude of the glucocorticoid negative feedback signal (Walker *et al.* 1990). In the present

Table 3 Effects of maternal adrenocorticotrophic hormone (ACTH) (Synacthen Depot 100 IU i.m.) or saline treatment during late gestation on locus coeruleus (LC) monoamine levels and their turnover in piglets on PND1 and PND28. Values are least square mean $\pm$ s.e.m. Number of animals per group: PND1 (ACTH=27, control=22); PND28 (ACTH=40, control=32).

	PND1		PND28	
	ACTH	Control	ACTH	Control
NA (pg/mg)	1030.4 $\pm$ 75.3	1203.3 $\pm$ 77.1	1236.0 $\pm$ 98.4	1397.3 $\pm$ 112
MHPG (pg/mg)	120.7 $\pm$ 38.7	91.4 $\pm$ 45.6	68.5 $\pm$ 16.0	102.0 $\pm$ 19.1
MHPG/NA	0.12 $\pm$ 0.03	0.10 $\pm$ 0.03	0.07 $\pm$ 0.02	0.08 $\pm$ 0.02
DA (pg/mg)	163.9 $\pm$ 20.1	164.1 $\pm$ 21.0	137.9 $\pm$ 8.0	151.9 $\pm$ 9.1
DOPAC (pg/mg)	314.7 $\pm$ 38.9	330.7 $\pm$ 41.5	251.8 $\pm$ 32.8	196.3 $\pm$ 32.7
HVA (pg/mg)	420.4 $\pm$ 47.0	428.9 $\pm$ 48.7	378.5 $\pm$ 43.5	293.1 $\pm$ 49.2
DOPAC/DA	1.64 $\pm$ 0.21	1.99 $\pm$ 0.22	1.40 $\pm$ 0.09	1.24 $\pm$ 0.08
HVA/DA	2.74 $\pm$ 0.30	2.85 $\pm$ 0.33	2.57 $\pm$ 0.19*	1.93 $\pm$ 0.22*
5-HT (pg/mg)	828.1 $\pm$ 58.8*	1071.2 $\pm$ 63.7*	959.0 $\pm$ 52.1 [†]	1258.3 $\pm$ 58.9 [†]
5-HIAA (pg/mg)	1276.0 $\pm$ 168.1	1782.1 $\pm$ 185.3	772.8 $\pm$ 62.2	960.5 $\pm$ 70.3
5-HIAA/5-HT	1.59 $\pm$ 0.10	1.68 $\pm$ 0.11	0.84 $\pm$ 0.04	0.77 $\pm$ 0.05

*Within a row denotes significant differences ($P < 0.05$) between ACTH and controls. [†]Within a row denotes significant differences ($P < 0.01$) between ACTH and controls. NA, noradrenaline; MHPG, 3-methoxy-4-hydroxyphenylglycol; DA, dopamine; DOPAC, 3,4-dihydroxyphenylacetic acid; HVA, homovanillic acid; 5-HT, 5-hydroxytryptamine; 5-HIAA, 5-hydroxyindole-3-acetic acid.

Figure 3 Glucocorticoid receptor (GR) binding in (A) hypothalamus and (B) hippocampus of piglets on PND1 and PND28 from sows treated with ACTH (Synacthen Depot 100 IU i.m.; filled bars) or saline (open bars) during mid- and late gestation. There is a tendency for reduced GR binding in the hypothalamus on PND1 during both mid- ($P < 0.07$) and late ($P < 0.08$) gestation. Number of animals per group: hypothalamus, PND1 (MG-ACTH=22, MG-C=21, LG-ACTH=25, LG-C=21), PND28 (MG-ACTH=17, MG-C=20, LG-ACTH=37, LG-C=32); hippocampus, PND1 (MG-ACTH=48, MG-C=37, LG-ACTH=24, LG-C=22), PND28 (MG-ACTH=27, MG-C=14, LG-ACTH=40, LG-C=32).

study, elevated maternal cortisol during both the mid- and late gestation period significantly decreased plasma CBG concentrations in piglets on PND1, whereas total plasma cortisol concentrations were not changed. A stress-associated decline in plasma CBG concentrations was found in rats

(Armario *et al.* 1994, Spencer *et al.* 1996), horses (Alexander & Irvine 1998) and pigs (Heo *et al.* 2003). It has not been demonstrated directly whether stress-related changes in plasma CBG result from changes in production, metabolic degradation and/or transfer to target tissues. However, Heo

Figure 4 Areas of (A) adrenal cortex and (B) adrenal medulla in piglets on PND1 and PND28 from sows treated with ACTH (Synacthen Depot 100 IU i.m.; filled bars) or saline (open bars) during mid- and late gestation. * $P < 0.05$. Number of animals per group: adrenal cortex and medulla, PND1 (MG-ACTH=28, MG-C=25, LG-ACTH=27, LG-C=22), PND28 (MG-ACTH=20, MG-C=24, LG-ACTH=40, LG-C=32).

et al. (2005) found in fetal pigs a positive correlation between plasma CBG concentrations and hepatic CBG mRNA. Therefore, it is assumed that the decrease of CBG levels in newborn piglets may result from direct inhibition of fetal hepatic CBG biosynthesis by elevated maternal cortisol exposure during both mid- and late gestation. This decrease in CBG and the unchanged total cortisol levels indicate a higher amount of biologically available free cortisol, which is also found in animals under conditions of chronic stress (Kim *et al.* 1999, Stefanski 2000, Heo *et al.* 2005).

Glucocorticoids regulate the HPA axis at the level of the pituitary, the hypothalamus, and the supra-hypothalamic limbic structures, particularly the hippocampus and amygdala. As maternal glucocorticoids cross the placenta, albeit with partial degradation by 11β -hydroxysteroid-dehydrogenase, they could affect the maturation of the fetal HPA axis, determining the set-point for feedback. In the present study, elevated maternal cortisol during both mid- and late gestation caused a tendency for reduced hypothalamic GR binding, but did not alter the GR binding in the hippocampus on PND1.

Figure 5 Number of cells per mm² in the (A) adrenal cortex and (B) adrenal medulla of piglets on PND1 and PND28 from sows treated with ACTH (Synacthen Depot 100 IU i.m.; filled bars) or saline (open bars) during mid- and late gestation. * $P < 0.05$. Number of animals per group: adrenal cortex and medulla, PND1 (MG-ACTH=28, MG-C=25, LG-ACTH=27, LG-C=22), PND28 (MG-ACTH=20, MG-C=24, LG-ACTH=40, LG-C=32).

In rats, prenatal glucocorticoid exposure permanently reduced the hippocampal GRs and increased basal corticosterone levels, changes thought to attenuate HPA axis feedback sensitivity in anticipation of stress (Levitt *et al.* 1996, Welberg *et al.* 2001). Unlike rats, injections of dexamethasone during late gestation in guinea pigs increased the GRs in the hippocampus (Dean & Matthews 1999). An increase in hippocampal GRs and a decrease in the number of GRs in the hypothalamus were also found in pigs prenatally stressed by repeated restraint during late gestation (Kanitz *et al.* 2003). The results of the present study demonstrate that elevated

maternal cortisol during both mid- and late gestation periods acts at the level of the hypothalamus by decreasing the GR-binding capacity and may therefore alter the set-point and attenuate the HPA axis feedback. A possible reason for the different results between the brain areas could be their heterochronous development. It has been shown in guinea pigs, a species that, similar to the domestic pig and in contrast to rats, delivers mature young, GRs develop earlier in the fetal hypothalamic PVN than in hippocampus. While hippocampal GRs reach their highest concentrations near term, the level in the PVN is highest at the beginning of the third

trimester, and decreases dramatically in the last 3 weeks (Matthews 1998). A similar reduction in GR mRNA in the PVN has also been noted in the fetal sheep near term (Andrews & Matthews 2000). Although the establishment of porcine fetal HPA axis was shown around gestational day 77 (Schwerin *et al.* 2005), at present, data on GR development and maturation in pigs are not available. However, a related time course of brain development in pigs and guinea pigs is assumed because of similarities in fetal HPA function and brain growth spurt (transient period of growth when the brain is growing most rapidly) during late gestation in these species (Fowden *et al.* 1998, Pond *et al.* 2000). The differences in GR regulation in this study may also be due to different receptor sensitivities between these species.

Some studies in rats and also in guinea pigs indicate that programming of corticosteroid receptors, such as the HPA axis, is possibly sex-specific with the effect of prenatal glucocorticoids being larger in female offspring (Weinstock *et al.* 1992, Dean & Matthews 1999, Szuran *et al.* 2000, Bowman *et al.* 2004, Gerardin *et al.* 2005). In pigs, we found no sex-specific effects on brain GRs, but an interaction of sex and treatment on plasma CBG concentrations in 28-day-old LG-piglets. Although there was no significant effect on CBG concentration at this age, male pigs from mothers treated with ACTH during late gestation expressed higher CBG levels than male control animals. These findings could point to a sex-dependent impact of prenatal elevated cortisol on hepatic CBG production in pigs.

Several reports indicate that prenatal stress in rats may induce an enhanced activation of the sympathetic nervous system and may affect the concentrations and turnover of brain catecholamines (Takahashi *et al.* 1992, Rudeen & Weinberg 1993, Muneoka *et al.* 1997, Weinstock *et al.* 1998). In pigs, maternal restraint stress during late gestation did not cause significant effects on plasma and brain catecholamine levels in the offspring (Otten *et al.* 2001, Kanitz *et al.* 2003). In contrast, the present results revealed an influence of elevated maternal cortisol on plasma NA concentrations and monoamine turnover in the LC, a key brainstem region of the noradrenergic system. While ACTH treatment during mid gestation increased MHPG and NA concentrations in the LC region of 28-day-old piglets and decreased HVA content, elevated cortisol exposure during late gestation increased NA concentrations in plasma samples taken by venipuncture on PND1. These results are partly consistent with a study in rats, where it was found that prenatal stress causes an increased turnover of NA and a reduced DA concentration in the LC (Takahashi *et al.* 1992). Furthermore, it was shown that increases in LC activity were correlated with arousal and a general increase in sympathetic activation (Berridge & Foote 1991). The increased levels of NA and MHPG after prenatal cortisol exposure during mid gestation may therefore reflect a higher noradrenergic activity in the offspring. In contrast, cortisol exposure during late gestation did not affect the noradrenergic activity in the LC region, but persistently decreased 5-HT concentrations on PND1 and PND28. A

study with mice and rats revealed a significant decrease of 5-HT levels in the LC after repeated postnatal restraint stress (Konstandi *et al.* 2000). Serotonergic neurons in the LC are presumed to be involved in stress-induced disorders like depression and it was found that conditioned fear diminishes the 5-HT release in the LC, indicating that anxiety is associated with decreased serotonergic activity in the LC (Kaehler *et al.* 2000). It is suggested that the decrease in 5-HT levels in brain of prenatally stressed offspring may be due to a reduction of tryptophan hydroxylation (Hayashi *et al.* 1998). Our findings demonstrate that prenatal cortisol exposure elicits different effects on the monoamine system in the LC of the offspring with more pronounced effects on the noradrenergic activity after mid-gestational and on the serotonergic activity after late-gestational exposure. This may reflect the different susceptibility of these systems to cortisol during the ongoing prenatal brain development. Differences in these monoamine systems were detectable on PND28 and indicate that there could be long-lasting effects on the behavioural and neurophysiological response to stimuli later in life.

In summary, the present results demonstrate that elevated maternal cortisol levels during mid- and late gestation in pigs cause alterations of the HPA axis and of central neurotransmitter systems in the offspring, and in some cases, in a sex-specific manner. In general, increased cortisol exposure of fetuses during both periods leads to decreased plasma CBG concentrations, which indicate a higher amount of biologically available free cortisol. Elevated maternal cortisol during mid gestation specifically increased the noradrenergic activity in the LC, whereas serotonergic activity in the LC was decreased only after late gestational treatment. Sex-specific effects were generally more pronounced in the male offspring. In the pig, the temporal characteristics of neurotransmitters and HPA axis development are not known and, to our knowledge, we are the first group to report these cortisol-dependent alterations in porcine brain. Furthermore, the main period of sexual brain differentiation in pigs seems to be during late gestation, although sex-specific influences on the noradrenergic system were found after mid-gestational treatment.

The present data show that there are marked developmental differences between laboratory animals and domestic pigs and highlight the importance of species-specific studies on prenatal influences. Increases in maternal cortisol could be one of the mechanisms involved in the effects of prenatal stress in pigs. According to the present findings, further studies are necessary to determine long-term consequences on coping behaviour and the adaptive ability of pigs to husbandry practices.

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