Differential mRNA expression of genes in the porcine adrenal gland associated with psychosocial stress

Eduard Muráni¹, Siriluck Ponsuksili², Richard B D'Eath³, Simon P Turner³, Gary Evans⁴, Ludger Thölking⁵, Esra Kurt⁶, Ronald Klontz⁷, Aline Foury⁸, Pierre Mormède⁸ and Klaus Wimmers¹

¹Research Unit 'Molecular Biology' and ²Research Group 'Functional Genomics', Leibniz Institute for Farm Animal Biology (FBN), Wilhelm-Stahl-Allee 2, D-18196 Dummerstorf, Germany

³Sustainable Livestock Systems, SAC, West Mains Road, Edinburgh, EH9 3JG, UK

⁴PIC UK, 2 Kingston Business Park, Kingston Bagpuize, Oxfordshire, OX13 5FE, UK

⁵PIC Germany, PIC Deutschland GmbH, Ratsteich 31, 24837 Schleswig, Germany

⁶Optimeter, Oyak sitesi 1.kisim 11b blok da:4, Sefakoy, Istanbul

⁷Vion Food Group, Boseind 10, 5281 RM Bostel Postbus1, 5280 AA Bostel, Netherlands

⁸Université Victor Segalen Bordeaux 2, PsyNuGen, UMR1286 INRA, 33076 Bordeaux, France

(Correspondence should be addressed to K Wimmers; Email: wimmers@fbn-dummerstorf.de)

Abstract

To gain insight into the adrenal stress response, we analysed differential mRNA expression of genes associated with psychosocial stress in the pig (*Sus scrofa domestica*). Various levels of psychosocial stress were induced by mixing groups of unfamiliar pigs with different aggressiveness. We selected two experimental groups for comparison, each comprising eight animals, which differed significantly in aggressive behaviour and plasma cortisol levels. To identify differentially expressed genes, we compared the adrenal transcriptome of these two groups of pigs, using the Affymetrix GeneChip porcine Genome Array. Bioinformatic analysis revealed that psychosocial stress induced upregulation of transcripts enriched for functions associated with cholesterol accumulation and downregulation of transcripts enriched for functions associated with cell growth and death. These responses are similar to those induced by ACTH stimulation. Nevertheless, the majority of the differentially expressed genes were so far not described as ACTH responsive. Some, such as *GAL* and *GALP*, may have responded to sympathoadrenal stimulation. Several of the differentially expressed transcripts, such as *AGT*, are associated with processes modulating steroidogenic response of adrenocortical cells to ACTH. One of the most significant findings was upregulation of *LOC100039095*, comprising a precursor of the microRNA miR-202, pointing to a previously unrecognised layer of regulation of adrenal steroidogenesis by microRNA. Our study, performed under entirely physiological conditions, complements previous studies focusing either on a single adrenal tissue and/or on a single stimulus, and contributes to understanding of the fine-tuning of adrenal stress response.

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Introduction

The ability to adapt to challenges is essential for survival. The stress response is one of the most important mechanisms facilitating adaptation. Its malfunction has negative effects on well-being, health status, and in livestock animals on performance (reviewed by Moberg (2000) and Chrousos (2009)). Knowledge of the molecular genetic background of the stress response is fundamental for the identification of the sources of individual variation in adaptation potential and will provide a foundation for the prevention of maladaptive responses.

The adrenal gland plays a pivotal role in the stress response, because it secretes the main stress hormones, glucocorticoids (cortisol in the pig) from the cortex

and catecholamines (adrenaline and to a lesser extent noradrenaline) from the medulla respectively. Secretion of glucocorticoids and catecholamines from the adrenal gland is principally governed by the hypothalamus–pituitary axis via ACTH and by the sympathetic nervous system via the splanchnic nerves respectively. However, the adrenal response to these stimuli is modulated by complex intrinsic regulatory circuits including interactions between cortical and medullary chromaffin cells, paracrine action of neuropeptides, innervation and blood flow (reviewed in Vinson *et al.* (1994), Ehrhart-Bornstein *et al.* (1998), Delarue *et al.* (2001) and Kvetnansky *et al.* (2009)). The action of the modulatory factors may vary depending on the character of the stressor and thus may serve the synchronisation of the response of the two main stress

systems within the adrenal gland (Ehrhart-Bornstein *et al.* 1998).

The current knowledge about the intra-adrenal regulatory circuits comes mainly from morphological and functional studies. Only recently, application of microarray technology has begun to unravel the underlying complex gene networks. So far, the majority of the transcriptome studies have focused on the adrenal response to ACTH treatment *in vitro* (Schimmer *et al.* 2006, Xing *et al.* 2010), but also *in vivo* (Hazard *et al.* 2008, Bureau *et al.* 2009).

In this study, we have used microarray analysis to investigate the adrenal response to stress under physiological settings in the pig (*Sus scrofa domestica*). As a model stressor, we used mixing of unfamiliar pigs with different aggressiveness. Mixing of unfamiliar individuals, which commonly occurs in pig husbandry, disturbs social dominance order and consequently induces aggressive behaviour (Meese & Ewbank 1973). Aggression is a powerful psychosocial stressor, which strongly activates both the sympathoadrenomedullary system and catecholamine release, and the hypothalamic–pituitary–adrenocortical (HPA) axis and cortisol secretion (Fernandez *et al.* 1994, Otten *et al.* 1999, D'Eath *et al.* 2010). We compared the adrenal transcriptome of two groups of animals differing significantly in their aggressiveness and in the amount of aggressive interactions experienced, as well as in the resulting adrenal stress response. In general, we observed transcriptome changes similar to those induced by ACTH treatment. However, we identified differential expression of several genes that may play a key role in the intra-adrenal regulatory circuits, perhaps responding specifically to psychosocial stress.

Materials and methods

Animal experiment

The animal experiment was described in detail by D'Eath *et al.* (2010). Briefly, aggressive temperament (aggressiveness) was measured by counting skin lesions (lesion scoring) immediately before and 24 h after mixing pigs into new groups at ~10–11 weeks of age. The increase in number of skin lesions (particularly at the front of the body) has been shown by Turner *et al.* (2006) to be positively associated with the duration of involvement in reciprocal fighting behaviour. Skin lesions at mixing are also more numerous in groups containing more aggressive individuals (D'Eath 2002). Pigs in each mixed group were ordered by the change in total skin lesions: half of the pigs in each group (those with the most lesions) were designated as high aggressiveness and the remaining half as low aggressiveness. In four slaughter batches, peripubertal pigs

(~190 days old) were assigned to one of four mixing treatments based on their aggressiveness (number of animals per treatment available for this study: high with high $n=63$, high with low $n=61$, low with low $n=65$ and unmixed $n=64$). Pigs were mixed into their treatment groups, as they were loaded onto a vehicle for transport to the abattoir. Skin lesions were counted before mixing and after slaughter on the carcass, dividing the body into front (head, neck, shoulders and front legs), middle (flanks and back) and rear (rump, hind legs and tail) sections. The difference in lesion number was again taken as the lesion score. The mixing treatment had significant effect on aggressive behaviour as reported by D'Eath *et al.* (2010). The animal experiment received approval from the Scottish Agricultural College Animal Experiments Committee.

Measurement of physiological stress parameters

Pigs were stunned by means of CO₂ gas and at exsanguination; a 50 ml sample of trunk blood was collected from each pig in a plastic tube containing 1 ml of 0.5 M EDTA and was stored on ice until plasma preparation, after which they were stored at -80°C . All blood samples were collected at morning between 0600 and 0800 h, at the peak of the circadian rhythm of cortisol secretion in the pig (Désautés *et al.* 1999).

Cortisol levels were measured with the automated analyser Centaur (Siemens Healthcare Diagnostics S.A.S, St Denis, France) using a kit designed for human serum and that we validated for pig serum. The intra- and inter-assay coefficients of variation (%) were 3.4 and 8.0 respectively.

Creatine kinase activity was measured with a clinical biochemistry automat (COBAS-MIRA Plus, Roche Diagnostics).

Transcriptome profiling using microarrays

Immediately after slaughter, the left adrenal gland was dissected, frozen in liquid nitrogen and stored at -80°C . For the microarray experiment, eight animals from each of the high–high and low–low mixing treatments respectively, which differed significantly in the level of psychosocial stress, were selected ($n=16$, for details see the 'Results' section and Table 1). Each slaughter batch was represented by four animals, one female and one castrated male per mixing treatment. All selected animals were free of the *RYRI* mutation associated with the porcine stress syndrome (Fujii *et al.* 1991; details on the genotyping procedure are given in D'Eath *et al.* 2010).

The selected samples were thoroughly ground using mortar and pestle under liquid nitrogen to ensure equal proportions of cortical and medullary tissues. RNA isolation was performed using TRI reagent

Table 1 Differences in indicators of aggressive behaviour and physiological stress parameters between the two microarray experimental groups

Parameter	High-stress group ^a (n=8)	Low-stress group ^a (n=8)	P value*
Skin lesions ^b			
Front	69.6 ± 13.2	4.5 ± 2.1	<0.001
Mid	65.8 ± 15.3	9.4 ± 2.4	0.003
Rear	13.6 ± 3.7	5.4 ± 1.7	0.062
Total	149.0 ± 27.7	19.3 ± 4.0	<0.001
Creatine kinase (U/l)	7547 ± 1359	2777 ± 345	0.004
Cortisol (ng/ml)	69.6 ± 4.7	50.3 ± 3.9	0.007

*P value of a two-sided unpaired *t*-test.^aMean ± S.E.M.^bSkin lesion in body sections: front (head, neck, shoulders and front legs), middle (flanks and back) and rear (rump, hind legs and tail).

(Sigma). After DNaseI treatment, the RNA was cleaned up using the RNeasy kit (Qiagen). The quantity of RNA was determined using the NanoDrop ND-1000 spectrophotometer (NanoDrop, Peqlab, Germany), and the integrity was checked by running 1 µg RNA on 1% agarose gel.

Preparation of antisense biotinylated RNA targets from 500 ng total RNA, hybridisation and scanning on a GeneChip scanner 3000 were performed according to Affymetrix protocols. The quality of hybridisation was assessed in all samples following the manufacturer's recommendations. Data were analysed with Affymetrix GCOS 1.1.1 software using global scaling to a target signal of 500. Data were then imported into Affymetrix Expression Console software for subsequent analysis. The data were first processed with MAS5.0 to generate cell intensity files and filtered (present or absent). Quantitative expression levels of the present transcripts were then estimated using Probe Logarithmic Intensity Error (PLIER). The microarray data related to all samples were deposited in the Gene Expression Omnibus public repository (GEO accession number: GSE25138).

Real-time quantitative PCR

First strand cDNA was synthesised using SuperScript III MMLV reverse transcriptase (Invitrogen) in a reaction containing 2 µg RNA, 500 ng oligo (dT)₁₃VN primer and 500 ng random hexamer primers (Promega), according to the manufacturer's protocol. Real-time quantitative PCR (qPCR) was performed on a LightCycler 480 System using the LightCycler 480 SYBR Green I Master (all Roche Applied Science). Primer and amplicon information is given in [Supplementary Table 1](#), see section on [supplementary data](#) given at the end of this article. The temperature profiles consisted of an initial denaturation step at 95 °C for 10 min and 45 cycles consisting of denaturation at 95 °C for 10 s,

annealing at 60 °C for 15 s (55 °C for *RPL32*) and extension/fluorescence acquisition at 72 °C for 25 s. Melting curve analysis and agarose gel electrophoresis were performed after completion of the qPCR run to confirm specificity of the amplification and absence of primer dimers. Threshold cycles were converted to copy numbers using a standard curve generated by amplifying serial dilutions of an external PCR-generated standard (10⁷–10² copies). To account for variation in RNA input and efficiency of reverse transcription, the calculated copy numbers were normalised by dividing with a normalisation factor derived from the expression of the reference genes *RPL32* and *B2M* according to the method described by [Vandesompele et al. \(2002\)](#).

Data analysis

Before statistical analysis, expression levels for both microarray and qPCR data were log2-transformed. In addition, microarray data were normalised using the median method implemented in JMP Genomics 4.1 Software Suite (SAS Institute, Cary, NC, USA). The effect of the psychosocial stress on gene expression was analysed using ANOVA implemented in JMP Genomics 4.1 for microarray data and in SAS 9.2 (Proc GLM; SAS Institute) for qPCR data respectively. The model included fixed effects of experimental group ('high stress' versus 'low stress', see the 'Results' section), gender, slaughter batch (*n*=4) and microarray batch (*n*=2). Least-square means for the two stress groups were compared using a *t*-test.

Bioinformatic analysis was performed using ingenuity pathway analysis (IPA, Ingenuity Systems, Redwood City, CA, USA). Enrichment of differentially regulated transcripts in specific functional classes and canonical pathways respectively were tested using Fisher's exact test and adjusted using the Benjamini–Hochberg correction. The Affymetrix GeneChip porcine Genome Array probe sets were annotated according to [Naraballoh et al. \(2010\)](#).

Results

Differences in psychosocial stress and in the associated adrenal response between the experimental groups

The samples used in this study were selected from a larger animal experiment, where different levels of aggressive behaviour and psychosocial stress were induced by mixing unfamiliar pigs with different aggressiveness (all three combinations of high with low) or leaving them in familiar groups ([D'Eath et al. 2010](#)). From each of the high–high and low–low mixing treatments respectively, eight animals were selected to

Table 2 Comparison of microarray and quantitative PCR (qPCR) results for transcripts of interest selected for validation

Probe set ID	Gene ID	Function	Microarray		qPCR	
			FC ^a (H/L)	P value	FC ^a (H/L)	P value
Ssc.914.1.S1_at	<i>LOC100039095</i>	Unknown	2.6	0.001	2.2	0.010
Ssc.4875.1.S1_at	<i>GALP</i>	Neuropeptide	1.7	0.043	2.7	0.092
Ssc.713.1.S1_at	<i>GAL</i>	Neuropeptide	1.6	0.038	1.6	0.051
Ssc.18298.1.A1_a_at	<i>RGS2</i>	Signal transduction	1.6	0.007	1.6	0.104
Ssc.16088.1.S1_at	<i>HMGCR</i>	Cholesterol biosynthesis	1.4	0.035	1.4	0.099
Ssc.15388.2.S1_at	<i>TH</i>	Catecholamine biosynthesis	1.3	0.028	1.5	0.026
Ssc.4804.1.S1_at	<i>AGT</i>	Hormone	−1.5	0.024	−1.5	0.032
Ssc.15741.1.S1_s_at	<i>CYP2E1</i>	Steroid metabolism	−1.4	0.034	−2.0	0.033
Ssc.15952.1.S1_at	<i>NR3C1</i>	Transcription factor	−1.3	0.004	−1.3	0.006
Ssc.26267.1.S1_at	<i>CRY2</i>	Circadian rhythm	−1.3	0.000	−1.5	0.031
Ssc.24696.1.S1_at	<i>IRS2</i>	Signal transduction	−1.3	0.007	−1.5	0.086
Ssc.30607.1.A1_at	<i>CALM1</i>	Signal transduction	−1.2	0.003	−1.2	0.010
Ssc.16976.1.S1_at	<i>SREBF2</i>	Transcription factor	1.1	0.100	1.1	0.638

^aFold change in expression levels between the high- and low-stress groups respectively.

create two microarray experimental groups, designated ‘high stress’ and ‘low stress’. The two experimental groups differed significantly in the amount of aggressive interactions induced by the treatment (i.e. in the level of psychosocial stress) as indicated by their skin lesions, primarily at the front of the body, which are known to be acquired during fighting (Table 1), and by their plasma creatine kinase level (a parameter reflecting damage of muscle fibres due to strenuous physical activity, Table 1), as well as in the resulting adrenal response as indicated by significantly different plasma cortisol levels (Table 1). Thus, the two experimental groups provided suitable material to study the adrenal response to psychosocial stress.

Microarray results and validation using qPCR

Out of the 23 937 probe sets on the Affymetrix GeneChip porcine Genome Array, 13 337 probe sets (~56%) were found to be expressed and were evaluated for differential expression. A total of 816 probe sets showed differential expression between the two experimental groups (469 up- and 347 down-regulated; Supplementary Table 2, see section on supplementary data given at the end of this article) at the nominal significance level $P \leq 0.05$. The magnitude of the differential expression ranged from 2.6-fold (upregulated in the high-stress group) to −1.9-fold (downregulated in the high-stress group). Overall, however, the differences in the expression were slight; only 23 probe sets showed higher than ± 1.5 -fold differential expression.

To validate the microarray experiment, we performed qPCR analysis for 13 transcripts that represent various functional classes and show a wide range of fold differences, including one transcript (*SREBF2*) showing only non-significant differences ($P \leq 0.10$, Table 2).

Comparison of the results revealed a high correspondence between both methods in terms of direction and magnitude of the differential expression (Table 2). For eight of the twelve regulated transcripts, statistically significant differential expression was confirmed ($P \leq 0.05$), and for the remaining four transcripts, the differences approached significance ($P \leq 0.10$). For *SREBF2*, qPCR analysis confirmed the absence of statistically significant difference. Taken together, the qPCR analysis proved the validity of the microarray data.

Results of the bioinformatic analysis

To gain insight into processes and pathways in the adrenal gland affected by psychosocial stress, we performed IPA. A total of 768 annotated probe sets, representing 713 unique transcripts (418 up- and 294 downregulated), were used for IPA. We analysed up- and downregulated transcripts separately, since, similar

Table 3 Functional categorisation of transcripts upregulated in the high-stress group, obtained using ingenuity pathway analysis

Molecular and cellular function	−log10 (P value)*	Number of transcripts
Lipid metabolism	5.08	44
Small molecule biochemistry	5.08	78
Vitamin and mineral metabolism	5.08	17
Energy production	1.82	10
Molecular transport	1.82	27
Nucleic acid metabolism	1.82	24
RNA post-transcriptional modification	1.33	13
Carbohydrate metabolism	1.30	30
Cellular assembly and organisation	1.16	31
Cell morphology	1.14	25

*P value after the Benjamini–Hochberg adjustment, significance threshold = 1.30.

Table 4 The ten most enriched ingenuity pathway analysis canonical pathways among the transcripts upregulated in the high-stress group

Pathway	–log ₁₀ (<i>P</i> value)*	Ratio ^a	Number of transcripts
Biosynthesis of steroids	4.25	6.25	8
Purine metabolism	1.93	4.10	18
N-glycan biosynthesis	1.93	7.53	7
Protein ubiquitination pathway	0.90	5.47	11
NRF2-mediated oxidative stress response	0.80	5.46	10
Role of CHK proteins in cell cycle checkpoint control	0.70	11.40	4
LPS/IL1-mediated inhibition of RXR function	0.55	4.65	10
Aminoacyl-tRNA biosynthesis	0.52	4.82	4
One carbon pool by folate	0.48	7.69	3
Amino sugars metabolism	0.36	4.10	5

**P* value after the Benjamini–Hochberg adjustment, significance threshold = 1.30.

^aNumber of genes in a given pathway that meet cut-off criteria, divided by total number of genes that make up that pathway in percentage.

to the study of Schimmer *et al.* (2006), using this approach, we obtained more clear-cut results.

The transcripts involved in metabolism, e.g. lipid metabolism, dominate among those upregulated in response to psychosocial stress (see Table 3 for the overview of significantly enriched functions and Supplementary Table 3 for a complete list of genes assigned to individual functional classes). The most common transcripts in this functional class are those encoding enzymes involved in cholesterol metabolism, primarily in biosynthesis (13 out of 23 in this pathway, e.g. *HMGCR* encoding the rate-limiting enzyme for cholesterol biosynthesis) but also in esterification/storage (e.g. *SOAT1*). The precursor of cholesterol biosynthesis, acetyl-CoA, is derived from glucose or fatty acid metabolism. Enrichment of transcripts related to carbohydrate metabolism and energy production (e.g. *HK2* that increases the rate of glycolysis) may thus indicate stimulation of production of acetyl-CoA to maintain sufficient substrate delivery. Another abundant functional group represents transcripts related to molecular transport, including *LDLR* and *SCARB1* involved in cholesterol uptake. *LDLR* showed higher upregulation compared with *SCARB1* (1.3 vs 1.1) indicating that, similar to humans, the LDLR-mediated endocytic uptake of cholesterol is more important than the selective pathway mediated by *SCARB1* (Kraemer 2007). A constant supply of cholesterol, either via *de novo* synthesis or uptake, is required within adrenal cells to serve as a precursor for the conversion to steroid hormones (Kraemer 2007). Our results indicate that both supply routes play an important role.

Delivery of free cholesterol to the inner mitochondrial membrane is the first and rate-limiting step in steroid hormone biosynthesis. This is facilitated by a macromolecular complex whose essential component is the steroidogenic acute regulatory protein (STAR; Sewer *et al.* 2007). However, neither the *STAR* gene nor genes encoding enzymes of the steroid hormone synthesis pathway (present on the microarray: *CYP11A1*, *CYP17A1* and *HSD3B2*) showed significant differential expression.

In Table 4, ten canonical pathways, showing the highest enrichment among the upregulated transcripts, are shown (genes assigned to individual canonical pathways are listed in Supplementary Table 4, see section on supplementary data given at the end of this article). For three of these, the enrichment reached significance level after the Benjamini–Hochberg correction. The most pronounced enrichment showed the biosynthesis of steroids pathway, which participates in cholesterol biosynthesis. Upregulation of the purine metabolism pathway, linked with ATP synthesis, is in line with the stimulation of energy production but may also indicate stimulation of cAMP synthesis, important for ACTH signal transduction (Sewer *et al.* 2007). The importance of the third enriched canonical pathway, N-glycan biosynthesis, is not obvious in the given context.

The transcripts downregulated in response to psychosocial stress are enriched in functional categories related to cell death, growth and proliferation (see Table 5 for the overview of significantly enriched functions and Supplementary Table 5 for a complete list of genes assigned to individual functional classes). It appears that the effect on cell death and cell growth is bidirectional, since both positive and negative regulators of cell survival (e.g. *SMAD1* and *DEDD* respectively) and growth (e.g. *IGF1* and *SMARCA2* respectively) were downregulated. Another abundant functional class represents transcripts related to gene expression,

Table 5 Functional categorisation of transcripts downregulated in the high-stress group, obtained using ingenuity pathway analysis

Molecular and cellular function	–log ₁₀ (<i>P</i> value)*	Number of transcripts
Gene expression	2.16	59
Cell death	2.03	71
Cellular development	1.56	34
Cellular growth and proliferation	1.46	71
Cell cycle	1.46	39
Lipid metabolism	1.41	14
Small molecule biochemistry	1.41	23
Cellular assembly and organisation	1.13	22
Carbohydrate metabolism	1.07	15
Cellular compromise	1.07	13

**P* value after the Benjamini–Hochberg adjustment, significance threshold = 1.30.

Table 6 The ten most enriched ingenuity pathway analysis canonical pathways among the transcripts downregulated in the high-stress group

Pathway	−log10 (<i>P</i> value)*	Ratio ^a	Number of transcripts
Hepatic fibrosis/hepatic stellate cell activation	1.07	5.97	8
Cell cycle: G ₂ /M DNA damage checkpoint regulation	0.70	9.30	4
IGF1 signalling	0.22	5.00	5
PXR/RXR activation	0.13	4.40	4
Oestrogen receptor signalling	0.13	4.20	5
Glucocorticoid receptor signalling	0.12	2.86	8
DNA methylation and transcriptional repression signalling	0.12	8.70	2
Fatty acid metabolism	0.12	2.60	5
β-Alanine metabolism	0.12	3.06	3
Pantothenate and CoA biosynthesis	0.12	3.12	2

P value after the Benjamini–Hochberg adjustment, significance threshold = 1.30.
^aNumber of genes in a given pathway that meet cut-off criteria, divided by total number of genes that make up that pathway in percentage.

including those involved in steroid hormone signalling (e.g. *NR3C1* encoding glucocorticoid receptor).

As found for upregulated transcripts, genes linked to lipid metabolism were also found among downregulated transcripts. Downregulation of *ABCA1*, which is involved in cholesterol efflux, provides additional evidence of the coordinated regulation of several processes towards accumulation of cholesterol.

In Table 6, ten canonical pathways are shown with the highest enrichment among the downregulated transcripts (genes assigned to individual canonical pathways are listed in Supplementary Table 6, see section on supplementary data given at the end of this article). Considering the enriched functional categories, several of the identified canonical pathways appear meaningful, e.g. the insulin-like growth factor 1 (IGF1) or glucocorticoid receptor signalling pathways related to cell proliferation and gene expression respectively; however, none reached significance after the Benjamini–Hochberg correction for multiple testing.

We compared our list of genes differentially expressed following psychosocial stress with lists of genes reported as ACTH responsive *in vitro* (Schimmer *et al.* 2006) and *in vivo* (Hazard *et al.* 2008, Bureau *et al.* 2009). Only about 10% genes were found to be overlapping (Supplementary Table 7, see section on supplementary data given at the end of this article). Those are mainly associated with cholesterol accumulation, regulation of gene expression and cell proliferation and cell death.

Discussion

This is the first study to report on global adrenal transcriptome response to psychosocial stress. Expectedly, given the known effect of psychosocial stress on HPA axis activity and the higher plasma cortisol levels in

the high-stress group, the observed transcriptome responses in many aspects resemble those induced by ACTH *in vivo* and *in vitro*. One common observation is that both the psychosocial stress and the ACTH treatment induce expression changes of relatively low magnitude (Schimmer *et al.* 2006, Hazard *et al.* 2008, Bureau *et al.* 2009, Xing *et al.* 2010). These small changes were most likely further obscured by inter-individual variability in stress response, which is usually very high (Mormede *et al.* 2002). Moreover, because we analysed transcriptome of the whole adrenal gland, the overall expression changes were potentially under- or overestimated for genes that show anti-parallel or parallel changes respectively in different adrenal compartments.

Genes associated with regulation of gene expression were almost exclusively downregulated. One gene that stands out among these is *NR3C1* encoding the glucocorticoid receptor, which provides negative feedback regulation of the HPA axis activity mainly at the central (Steckler 2001) but also at the adrenocortical level (Gummow *et al.* 2006). Downregulation of *NR3C1* was also described in response to ACTH stimulation *in vitro* (Schimmer *et al.* 2006).

In contrast, upregulated genes were enriched, i.e. for those associated with RNA post-transcriptional modification. This finding, together with several other lines of evidence discussed below, points to an important role of post-transcriptional mechanisms in adrenal stress response, offering a potential explanation for the relatively weak transcriptional response. For instance, the highest magnitude of differential expression showed *LOC100039095* (~2.6-fold upregulated in the high-stress group), comprising a precursor of the microRNA miR-202. MicroRNAs are involved in post-transcriptional regulation of gene expression, mainly by repression of translation but also by promoting mRNA decay (reviewed by Krol *et al.* (2010)). Predicted targets

of miR-202 include *CREM* (John *et al.* 2004), a transcription factor involved in the regulation of *STAR* expression and in the transduction of ACTH signalling (Sewer *et al.* 2007). The predominant expression of miR-202 in steroidogenic tissues such as ovary and testis (Ahn *et al.* 2010, Luo *et al.* 2010) provides additional evidence that miR-202 might be involved in the regulation of steroidogenesis.

Similar to findings reported for the ACTH response, we observed consistent upregulation of transcripts involved in cholesterol biosynthesis (Schimmer *et al.* 2006, Bureau *et al.* 2009) and uptake (Schimmer *et al.* 2006, Hazard *et al.* 2008). This response was likely, at least partly, mediated by the activation of SREBF2, a transcription factor playing a key role in the regulation of cholesterol accumulation (reviewed in Sato (2010)). The activation mechanism was obviously post-transcriptional, because we found no significant differences in *SREBF2* expression. Indeed, proteolytic processing of SREBF2 in the golgi complex is required for its activation (reviewed in Sato (2010)). SREBFs are synthesised as precursor proteins in the endoplasmic reticulum (ER), where they form a complex with another protein, SCAP. The SCAP molecule contains a sterol sensory domain. In the presence of high cellular sterol concentrations, SCAP binds to another ER membrane protein, INSIG, and confines SREBP to the ER. With low cellular concentrations, SCAP escorts SREBP to activation in the golgi complex (reviewed in Sato (2010)). Hence, increased cortisol secretion in the high-stress group most likely depleted cholesterol stores in the adrenocortical cells, leading to activation of SREBF2.

Whereas upregulation of cholesterol accumulation appears to be a robust response to ACTH, transcriptional regulation of steroid hormone biosynthesis depends upon duration of the ACTH stimulus. Upregulation of the steroid hormone biosynthesis pathway occurs after long-term (Schimmer *et al.* 2006), but not after short-term, ACTH stimulation (Hazard *et al.* 2008, Bureau *et al.* 2009). The absence of differential expression of the steroid hormone biosynthesis pathway in our experiment is consistent with the fact that the stress exposure was transient rather than persistent.

Synchronisation between ACTH secretion and the adrenal clockwork is required to achieve appropriate response in the regulation of circadian rhythm of cortisol secretion (Oster *et al.* 2006). However, little is known about the regulation of the clock genes in response to acute ACTH stimulation. We found downregulation of *CRY2*, encoding cryptochrome 2, in the high-stress group. Cryptochrome 2 is a component of the negative limb of the circadian feedback loop. The pattern of circadian expression of cryptochromes is nearly completely opposite to the circadian rhythm of cortisol secretion (Lemos *et al.* 2006). It could

therefore be speculated that downregulation of *CRY2* in the high-stress group may be an indication of the synchronisation of acute ACTH response with the adrenal clockwork action.

Besides its principal role in the regulation of steroid hormone synthesis and secretion, ACTH is also involved in the control of adrenal growth and functional architecture (Coll *et al.* 2004, Karpac *et al.* 2005). Accordingly, ACTH affects expression of transcripts associated with cell structure, adhesion and proliferation (Schimmer *et al.* 2006, Hazard *et al.* 2008). Adrenocortical cells undergo marked morphological changes in response to ACTH *in vitro*, implying reorganisation of their cytoskeleton (Hall & Almahbobi 1997). Cytoskeleton is involved in cholesterol transport and facilitates steroidogenesis by promoting inter-organelle trafficking of intermediary metabolites (Sewer & Li 2008). The enrichment of the functional category 'cellular assembly and organisation' approached significance both among up- and down-regulated transcripts. This finding indicates that remodelling of the cytoskeleton may occur also in response to ACTH *in vivo*. Similar to transcriptional regulation of steroid hormone biosynthesis, the effect of ACTH on proliferation of adrenocortical cells also seems to be dependent upon the duration of the stimulus. Arola *et al.* (1993) have shown that in primary adrenocortical cell culture, ACTH has a biphasic effect with initial growth inhibition followed by later growth stimulation. Thus, the simultaneous downregulation of growth-stimulating and -inhibiting factors in the high-stress group may reflect the biphasic effect of ACTH on adrenocortical cell growth. The balance was perhaps shifted towards growth inhibition, as indicated by downregulation of angiotensinogen (*AGT*), the precursor of angiotensin II, an important factor in the positive regulation of adrenal growth (Huckle & Earp 1994, Vinson 2003). The systemic renin-angiotensin system is the principal activator of aldosterone production in the *zona glomerulosa* (Sewer *et al.* 2007). However, in addition to its role in aldosterone secretion, the *zona glomerulosa* is considered to be the site of proliferation, recruitment and differentiation of adrenocortical cell under the control of the locally produced angiotensin II (Vinson 2003). In vascular smooth muscle, angiotensin II stimulates cell growth through activation of *IGF1R* transcription. This effect is mediated via calcium-dependent activation of protein tyrosine kinase activity (Du *et al.* 1999). We found downregulation of *IGF1R* and *CALM1*, the latter involved in calcium signalling, which indicates that the IGF1 signalling pathway may be a mediator of the effects of angiotensin II on the proliferation of adrenocortical cells.

Given the potent vasoconstrictory effect of angiotensin II, downregulation of *AGT* could serve an additional

function, namely increasing the adrenal blood flow (Ehrhart-Bornstein *et al.* 1998). The observed upregulation of *RGS2* could further augment this effect, because *RGS2* promotes vascular relaxation by attenuating angiotensin II signalling (Goncalves *et al.* 2008). The rate of blood flow modulates glucocorticoid secretion by influencing presentation of ACTH to the adrenal gland (reviewed in Ehrhart-Bornstein *et al.* (1998)). *AGT* and *RGS2* were most likely regulated in response to ACTH, which is well known to stimulate increases in adrenal blood flow. Furthermore, expression of both *AGT* and *RGS2* was previously reported to be ACTH responsive (Schimmer *et al.* 2006, Hazard *et al.* 2008).

The steroidogenic response of adrenocortical cells to ACTH is additionally modulated in a paracrine way by neurotransmitters and neuropeptides released from medullary chromaffin cells (reviewed in Ehrhart-Bornstein *et al.* (1998) and Delarue *et al.* (2001)). This was demonstrated for example by the finding that deletion of the tyrosine hydroxylase (*TH*) gene, encoding the rate-limiting enzyme of catecholamine synthesis, leads to a marked decrease in plasma glucocorticoid level (Bornstein *et al.* 2000). Chromaffin cells express a wide range of neuropeptides (reviewed in Ehrhart-Bornstein *et al.* (1998)). We found upregulation of two members of the galanin neuropeptide family, galanin (*GAL*) and galanin-like peptide (*GALP*). Porcine adrenal medulla contains high concentration of *GAL*, which upon stimulation of the splanchnic nerve is released in large amounts (Holst *et al.* 1991). Moreover, depolarisation of chromaffin cells upregulates expression of *GAL* (Rokaeus *et al.* 1990). Perfusion of isolated porcine adrenal glands with *GAL* increases cortisol secretion (Holst *et al.* 1991). In rodents, two routes of the paracrine stimulation of glucocorticoid secretion by *GAL* were described: through direct activation of adrenocortical *GAL* receptors and through stimulation of adrenomedullary release of catecholamines, which in turn activate β adrenoreceptors located on adrenocortical cells (Andreis *et al.* 2007). With regards to *GALP*, it is known to affect the function of the HPA axis at the central level (reviewed in Suzuki *et al.* (2010)); however, knowledge about its expression and function at the adrenal level is currently lacking. The magnitude of differential expression of both *GAL* and *GALP* was among the highest in our experiment. Furthermore, we found upregulation of *TH* in the high-stress group. Taken together, our results indicate that the adrenomedullary and adrenocortical responses to psychosocial stress might have been synchronised, whereby the *GALP* family might have played an important role.

The large inter-individual variation in stress responsiveness has to some extent genetic background. Differences in cortisol secretion between pig breeds were demonstrated and utilised for quantitative trait

loci mapping (Désautés *et al.* 1999, 2002). This contributes a list of functional candidate genes. For one differentially expressed gene from our list, *NR3C1*, we already found a significant association with cortisol secretion and adrenal size (Murani *et al.* 2010). Another interesting candidate on our list is *CYP2E1*, which is involved in steroid metabolism (Ball *et al.* 1990). Polymorphisms of porcine *CYP2E1* show an effect on skatole level, a compound causing boar taint (Moe *et al.* 2009). The question of whether *CYP2E1* polymorphisms show pleiotropic effects also on cortisol levels merits further investigation.

In summary, to gain insight into the adrenal stress response, we analysed transcriptome changes associated with psychosocial stress, activating both adrenal cortex and medulla.

Several processes (e.g. cholesterol accumulation and cell proliferation) and key molecules/pathways (e.g. *AGT*) responded in a fashion similar to earlier reports of ACTH stimulation. Nevertheless, the majority of the regulated genes were so far not described as ACTH responsive. Some, such as *GAL* and *GALP*, may have responded to splanchnic nerves, i.e. sympathoadrenal stimulation. Several of the differentially expressed transcripts are associated with processes modulating steroidogenic response of adrenocortical cells to ACTH. One of the most significant findings was upregulation of *LOC100039095*, pointing to a previously unrecognised layer of regulation of adrenal steroidogenesis by microRNA. Our study, performed under entirely physiological conditions, complements previous studies focusing either on single adrenal tissue and/or on single stimulus, and contributes to understanding of the fine-tuning of adrenal stress response.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/JME-10-0147>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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