Effects of chronic stress: A comparison between tethered and loose sows

F. Josef van der Staay a,b,c,e,⁎, Teun Schuurman a, Marcel Hulst c, Mari Smits c, Jos Prickaerts d, Gunter Kenis d, S. Mechiel Korte a,⁎

a Biomedical Research, Wageningen University and Research Centre, P.O. Box 65, 8200 AB Lelystad, The Netherlands
b Program Emotion and Cognition, Department of Farm Animal Health, Faculty of Veterinary Medicine, University Utrecht, Utrecht, The Netherlands
c Animal Breeding and Genomics Centre, Animal Sciences Group, Wageningen University and Research Centre, P.O. Box 65, 8200 AB Lelystad, The Netherlands
d Department of Psychiatry and Neuropsychology, Maastricht University, PO Box 616, 6200 MD Maastricht, The Netherlands
e Radolf Magnus Institute of Neuroscience, Utrecht University, Universiteitsweg 100, 3584 CG, Utrecht, The Netherlands

⁎ Corresponding author. Present address: Program Emotion and Cognition, Department of Farm Animal Health, Faculty of Veterinary Medicine, University Utrecht, Yalelaan 7, 3584 CL Utrecht, The Netherlands. Tel.: +31 30 253 4205; fax: +31 30 252 1887.
E-mail address: F.J.vanderStaay@UU.NL (F.J. van der Staay).

Abstract

The present study aimed to investigate whether long-lasting, recurrent tethering of sows leads to enduring effects on measures that may be indicative of chronic stress. Sows that had experienced tethering for about 1.5 or 4.5 years and age-matched sows kept in a social housing system (loose sows) were compared. Immediately after slaughter, blood samples were taken to measure plasma cortisol levels, and the brain, spleen, and adrenals were dissected and weighed. Gene expression in the frontal cortex and hippocampus was analyzed. Plasma cortisol levels were higher in the tethered sows than in the loose sows. The older, but not the younger, tethered sows had heavier adrenal glands than their loose counterparts. The weight of the spleen was not affected by the housing conditions, but the pituitary gland was lighter in tethered sows than in loose sows. Microarray analyses revealed an increased expression of β-globin mRNA in the hippocampus and to a lesser extent in the frontal cortex of the older tethered sows, compared with the older loose sows. Taken together, the findings indicate that chronically stressed pigs develop depression-like symptoms. However, it can be questioned whether the pig subjected to repeated, long-term stress can be regarded an animal model of major depression.

© 2010 Elsevier Inc. All rights reserved.

1. Introduction

Animal models have an essential role in the investigation of neuropsychiatric diseases [61], such as major depression [34], by increasing our understanding of underlying pathological mechanisms and thereby facilitating the development of new therapeutic approaches. Current animal models of depression are either pharmacologically evoked (e.g., reserpine or clonidine-induced) or stress evoked (e.g., inescapable shock-induced learned helplessness, social defeat, social isolation, separation from mother or peers) [34]. However, very few preclinically active antidepressants are clinically effective [14], which suggests there is a need for additional predictive animal models of depression (recent overviews of the current state of models of depression have been compiled by [14,43]). Most animal experimental studies use rodents as the model species. However, pig models offer a number of potential advantages to rodent models [15,41,48,65]. Many of the core symptoms of depression involve higher brain functions that may be more easily modeled in pigs than in rodents, because the pig brain resembles the human brain more closely than does the rodent brain. In particular, the brain regions involved in the regulation of emotions, such as the prefrontal cortex, and components of the limbic system, such as the hippocampus and amygdala, are highly developed in pigs [41]. Depression represents a family of disorders of different pathophysiology [1,27]. A major obstacle to the development of valid and predictive animal models of depression is our limited knowledge of the etiology and pathophysiology of this family of disorders [53]. The main characteristics of depressive disorders appear to be dysregulation of hypothalamic–pituitary–adrenal (HPA) axis activity, as evidenced by increased secretion of corticotropin-releasing hormone and cortisol [1,7], disturbed mineralocorticoid receptor (MR)/glucocorticoid receptor (GR) balance [10,39,72], and reduced serotonergic activity in multiple brain regions [11,21]. These changes may be considered endophenotypes [14,44,59], i.e. phenotypic components of the disorder under study. According to Mello et al. [44], hyperactivity of the HPA axis can be considered an endophenotype of depression. This hyperactivity is evidenced by, among others, raised levels of cortisol, and pituitary and adrenal gland enlargement. The
identification of endophenotypes may facilitate the generalization of results from one species to the other, including humans. Recently, there has been a shift toward an endophenotype-oriented approach to models of depression [14].

Stress is believed to be a major etiological factor in depressive illness (e.g., [22,49]), and chronic stress appears to elicit major depression [36], although some investigators disagree [30]. Pigs exposed to chronic stress can be considered an animal model of depression, if one assumes that stress has a crucial role in the etiology of this mental condition [68]. However, in a previous study in which female piglets underwent repeated defeat, a form of stress, we found only a transient increase in cortisol levels, and no effects on organ weights, number of GR and MR in the ventral hippocampus, or on serotonin turnover in the dorsal hippocampus [62]. Although one could obviously increase the duration, frequency, and/or intensity of the stressful events (e.g., prolongation of the period and increase of the number of defeat experiences) in an attempt to induce depression-like symptoms, we decided to look for an alternative approach. Previous studies have shown that tethering is a chronic stressor to sows [57,60], and indeed tethering has been rated the worst of all sow management systems from an animal welfare perspective [8,9]. As a consequence, tethering has been banned in all European Union member states, as of January 1, 2006 [25].

Before the ban was enforced, we investigated whether recurrent tethering-induced stress brings about lasting changes symptomatic of depression and which may constitute endophenotypes of this family of diseases. In order to explore the effects of “stress load,” we investigated the effects of tethering on gene expression and neuroendocrine function. In particular, the stress hormone cortisol is a crucial player, because cortisol passes the blood-brain barrier to act on the structures of the emotional brain, such as the dorsal hippocampus and central amygdala [16,33], that are involved in emotions, emotional learning, and the regulation of stress responses. Cortisol binds to nuclear corticosteroid receptors in the emotional brain to produce long-term genomic effects, via altered transcription (altered gene expression), on cellular metabolism and excitability in specific neural circuits. The different patterns of gene expression may lead to particular neuronal phenotypes that result in particular behavioral profiles, such as high responsiveness to stress or high resistance to stress (robust animals, i.e. animals that are better able to cope with their environment) [35]. The complex genetic basis of emotional behavior and the influence of the neuroendocrine system make it necessary to examine simultaneously many thousands of genes in order to elucidate variations in emotional, neuroendocrine, and behavioral responses. We expected that studying the above-mentioned gene-function relationships would enable us to identify read-out parameters as objective indicators for animal welfare.

The aim of the present study was to investigate whether long-lasting, recurrent tethering stress in sows leads to enduring effects on measures that may be indicative of (major) depression, i.e. whether recurrent, long-lasting stress is an etiological factor for depressive diseases. To this end, we purchased pigs that had experienced tethering for years from a commercial hog farm, together with age-matched animals from another commercial hog farm where sows were kept in an indoor group housing system. All sows originated from the same breeder. The groups of sows had been housed tethered or loose during 2 to 4, or during 8 to 13 reproductive cycles. We evaluated the persistent effects of long-lasting stress in these animals by weighing the pituitary gland, spleen, thymus, and adrenal glands, by measuring the expression of MR and GR in the spleen (as measures of HPA axis hyperactivity; [13,52]) and plasma cortisol concentrations (as an indicator of stress, e.g. [4]), and by assessing gene expression in the hippocampus, frontal cortex, and olfactory bulbs, regions that are considered to be involved in stress regulation, emotional memory, and mood disorders [24,38,64]. We expected to detect differences in gene expression between loose and tethered sows, in particular in genes that may be linked to stress.

2. Materials and methods

The study was conducted in accordance with the recommendations of the EU directive 86/609/EEC. All efforts were made to minimize the number of animals used and their suffering.

Animals: The sows were purchased from two different farms. All animals had been bred and delivered by Topigs (Tolkamp BV, Aalten, The Netherlands), i.e. they were from the same genetic line (Great Yorkshire/Large White type X Dutch land race), bred and reared under the same highly standardized conditions until they were delivered to the two farms at the age of about 7 months. The normal housing conditions of the loose and tethered sows are schematically represented in Figs. 1 and 2. At one farm, the sows were group-housed during pregnancies, until approximately 10 days before farrowing, when they were transferred to farrowing pens. Groups consisted of 65 to 130 sows. The group pens were provided with solid barriers that permitted sows to avoid confrontations and to escape from fights, a measure that is expected to reduce stress during mixing and housing [2]. However, deviating from this schedule, after weaning of the last litter and before transportation to the slaughterhouse, the loose sows were group-housed, instead of being transferred from the farrowing pens to the service crates. During group housing, about 2.2 m² floor space was available for each sow.

At the other farm, sows were tethered by a thoracic girth during pregnancy and farrowing and lactation in pens measuring 2100 × 1500 mm (see Fig. 1). The sidewalls separating the pens were low enough to allow visual contact with the other sows in the stall; however, direct tactile social interaction between the sows was not possible. The tethered sows stayed in the farrowing crate in the period between weaning of the last litter and transportation to the slaughterhouse.

Fig. 1. Top view and dimensions of a pen. The left sow is lying and strapped by a thoracic girth, whereas the other sow is standing. The dotted lines indicate the position of the solid horizontal bars, which allowed the sow to lie down but restrict her movements and deter her from turning around. F: Position of the food trough. Rows of three pens each were positioned at both sides of a narrow corridor.
Sows were grouped by parity, and the following four groups were compared (see Table 1): Younger loose sows, approximately 2.5 years old, with 2–4 parities; older, approximately 5-year-old loose sows with 8–13 parities; younger tethered sows, approximately 2.5 years old, with 2–4 parities; and older roughly 5-year-old tethered sows with 8–13 parities. The reproductive cycle of tethered and loose sows is schematically shown in Fig. 2.

Both hog farms were about 20 to 30 min from a regional slaughterhouse. The 10 tethered sows were loaded on the cattle carrier by a local, specialized transport company and left the farm at about 7:30. The cattle carrier then picked up the 10 loose sows, leaving the farm at about 8:30. All sows were transported to a local slaughterhouse, where they arrived at about 9:00. On arrival, the animals were unloaded. Care was taken to minimize stress during loading, transport, unloading, time in lairage, and slaughtering [55]. The animals were given the time to voluntarily disembark from the cattle carrier and to enter the lairage. Here, they were allowed a resting time of approximately 1 h, before the first animal was killed. Each of the subgroups of five animals was kept in a separate segment of the lairage, which was made of fences with iron bars. In each of the five successive series of four sows, one sow was taken from each of the four segments of the lairage, i.e. from the four different groups. This order was randomized (the order of slaughter is listed in Table 1). The last sow was slaughtered after a resting time of about 5½ h. A government veterinarian surgeon examined all animals immediately before slaughter and inspected the carcasses after slaughter.

![Reproductive cycle in tethered sows](image)

![Reproductive cycle in loose sows](image)

**Fig. 2.** Schematic presentation of a reproductive cycle in tethered and loose sows. Note that the loose sows were housed socially during part of the pregnancy. Ten days before farrowing, they were transferred to farrowing crates where they stayed until the litter was weaned. The sows of the two tethered groups were transferred from the service crate to the farrowing crate where they were tethered until weaning of the litter. Then, they were transferred to the service crate.

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of litters</th>
<th>Number of living piglets in last litter</th>
<th>Age at slaughter (days)</th>
<th>Slaughter days after weaning</th>
<th>Weight after slaughter (kg)*</th>
<th>Order of slaughter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Younger, loose</td>
<td>2</td>
<td>5</td>
<td>563</td>
<td>6</td>
<td>141.0</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>14</td>
<td>793</td>
<td>4</td>
<td>145.7</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>13</td>
<td>724</td>
<td>13</td>
<td>179.0</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>3</td>
<td>941</td>
<td>13</td>
<td>139.5</td>
<td>16</td>
</tr>
<tr>
<td>Mean ± SEM</td>
<td>3.2 ± 0.4</td>
<td>9.4 ± 2.2</td>
<td>779 ± 65</td>
<td>15.8 ± 4.7</td>
<td>156.0 ± 8.6</td>
<td></td>
</tr>
<tr>
<td>Older, loose</td>
<td>8</td>
<td>7</td>
<td>1405</td>
<td>20</td>
<td>207.4</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>8</td>
<td>1405</td>
<td>13</td>
<td>224.2</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>8</td>
<td>1428</td>
<td>13</td>
<td>219.3</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>11</td>
<td>1543</td>
<td>13</td>
<td>224.7</td>
<td>5</td>
</tr>
<tr>
<td>Mean ± SEM</td>
<td>8.6 ± 0.4</td>
<td>8.8 ± 0.7</td>
<td>1511 ± 68</td>
<td>15.8 ± 1.7</td>
<td>211.9 ± 7.6</td>
<td></td>
</tr>
<tr>
<td>Younger, tethered</td>
<td>3</td>
<td>15</td>
<td>745</td>
<td>6</td>
<td>157.8</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>13</td>
<td>745</td>
<td>10</td>
<td>150.6</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>15</td>
<td>871</td>
<td>6</td>
<td>133.6</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>11</td>
<td>871</td>
<td>21</td>
<td>152.6</td>
<td>7</td>
</tr>
<tr>
<td>Mean ± SEM</td>
<td>3.6 ± 0.2</td>
<td>13.6 ± 0.7</td>
<td>820 ± 31</td>
<td>12.8 ± 3.4</td>
<td>150.5 ± 4.5</td>
<td></td>
</tr>
<tr>
<td>Older, tethered</td>
<td>8</td>
<td>11</td>
<td>1604</td>
<td>6</td>
<td>150.6</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>4</td>
<td>1489</td>
<td>20</td>
<td>204.8</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>13</td>
<td>1972</td>
<td>20</td>
<td>185.3</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>10</td>
<td>2101</td>
<td>6</td>
<td>181.5</td>
<td>13</td>
</tr>
<tr>
<td>Mean ± SEM</td>
<td>10.4 ± 1.0</td>
<td>10.0 ± 1.6</td>
<td>1880 ± 143</td>
<td>11.6 ± 3.4</td>
<td>190.2 ± 12.3</td>
<td></td>
</tr>
</tbody>
</table>

*Weight after slaughter refers to the carcass weight (plus the weight of the head), after the skin, intestines, and stomach have been removed.
A sow was stunned with an electrical stunner, bled (trunk blood was collected), and decapitated. The brain was rapidly excised, the cerebellum was removed, and the brain was transversely cut into three parts (see Fig. 3A). Dissected parts were rapidly frozen in dry-ice cooled isopentane. All brain samples were then stored on dry ice until they were transferred to a freezer where they were stored at −70 °C. The pituitary gland was dissected free from the base of the skull and weighed on a Sartorius L420 scale (accuracy 0.001 g, max. loading 420 g; Sartorius, Elk Grove IL). In contrast to the normal slaughter routine, the carcasses were not dropped into scalding water cooled isopentane. All brain samples were then stored on dry ice until it was transferred to the freezer (−70 °C).

2.1. Cortisol

**Blood sampling:** Blood was collected into heparinised tubes (1 per sow) at bleeding and allowed to clot at room temperature for at least 2 h. Then it was centrifuged for 15 min at 4000×g. Serum was pipetted into a new tube and stored in a freezer at −20 °C. Serum was treated following the same procedure as for sample dilutions.

**Cortisol determination:** The procedure of Dressendorfer et al. [20] was used with modifications. Water, purified by a Milli-Q/UF reagent grade water system (Millipore, Etten-Leur, no. ZFMQ 240 U4), was used in all buffers and dilutions. To avoid carry-over, in particular of grade water system (Millipore, Etten-Leur, no. ZFMQ 240 U4), was filtered through a 0.45-µm D26 filter (Sartorius, Göttingen, no. 6400204), except NSB wells to which 50 µL ethanol diluted 1:62.5 in double concentrated assay buffer was added. Subsequently, 50 µL of sheep anti-cortisol serum diluted 1:75,000 in double concentrated assay buffer was pipetted in all wells. The microtiter plate was shaken for 5 s (DELFIA platewasher, Wallac Oy, no. 1296-001; Turku, Finland), covered with foil, and incubated overnight at ambient temperature.

Eu-labeled streptavidin solution (Wallac Oy, no. 1244-360; Turku, Finland) was diluted 1:1000 in single concentrated assay buffer and filtered through 0.22-µm Milllex-GV (Millipore, no. SLGC 025 BS). The solution was poured into a reagent container and the pipette tips were flushed for a waiting period of 15 min that was found to improve the reproducibility of the duplicates of the low cortisol standard concentrations. The plate was washed three times, followed by the addition of 200 µL of streptavidin–europium solution. The plates were incubated for 30 min at ambient temperature on a plate shaker. The plate was washed six times and 200 µL of ‘enhancement solution’ (Wallac Oy, no. 1244-105; Turku, Finland) was added. After 15 min of incubation at ambient temperature on a plate shaker, the plate was measured in a 1420 multilabel counter (Wallac Oy; Turku, Finland). Counting data were evaluated using Multicalc software (Wallac Oy; Turku, Finland). Samples with duplicate values showing more than 4% difference in relative binding ([(B/B₀)−100%]) were re-analyzed.

**2.2. Gene-expression analysis**

**Dissection of brain samples:** Approximately 60 to 140 mg tissue was dissected from the frozen right and left dorsal hippocampus (see Fig. 3C), using a scalpel. Additional tissue samples weighing between 110 and 280 mg were dissected from the left ventral hippocampus (the latter samples were used for additional investigations not reported in this paper). Frontal cortex samples (see Fig. 3B) weighing between 126 and 266 mg were obtained. All dissections were from brain areas that were superficially visible in the transverse brain cuts obtained by vertically cutting through the freshly dissected brains at the levels indicated in Fig. 3A.

**Isolation of total RNA:** Approximately 1 g of frozen brain tissue was homogenized directly in 10 mL TRIzol® reagent (Gibco BRL) and insoluble material was removed by centrifugation at 12,000×g for 10 min at 4 ºC. Further extraction of RNA from these homogenates was performed according to the instructions of the manufacturer of the TRIzol® reagent. The crude RNA pellet obtained from this isolation procedure was dissolved in 1 mL RNase-free water, and precipitated with 0.25 mL of isopropanol and 0.25 mL of 0.8 M sodium citrate/1.2 M NaCl to remove proteoglycan and polysaccharide contamination. After centrifugation at 12,000×g for 10 min at room temperature, RNA pellets were washed with 75% (v/v) ethanol and dissolved in RNase-free water. Subsequently, the RNA was treated with DNase, extracted with phenol–chloroform, and precipitated with ethanol. RNA pellets were washed with 75% (v/v) ethanol, dissolved in RNase-free water, and stored at −70 °C until further use. The integrity of the RNA was checked by analyzing 0.5 µg on a 0.8% (w/v) agarose gel. A part of the isolated RNA was used to prepare RNA pools for microarray analysis. For the hippocampus, equal amounts of RNA isolated from all five pigs within each group (older tethered, older loose, young...
tethered, and young loose) were mixed and stored at −70 °C until further use. The same was done for the RNA samples isolated from the frontal cortex and olfactory bulbs.

**Preparation of an Expressed Sequence Tagged (EST) library and microarray slides:** The hippocampus, amygdala, and bed nucleus of the stria terminalis (BNST), areas rich in corticoid receptors, were dissected from a 9-week-old pig. Equal quantities of these three parts were pooled, frozen immediately in liquid nitrogen, and stored at −70 °C until shipment on dry ice to Lark Technologies Inc (Houston, USA), where total RNA was extracted and enriched for poly(A)+ RNA, which was used as template to prepare a self-subtracted cDNA library of 10,000 individual EST clones. EST fragments were inserted in the pGEM-T vector (Promega) and cloned in E. coli strain XL-2 Blue (Stratagene). Gel analysis of the inserts of 55 randomly selected library clones revealed an average insert size of 550 bp (ranging from 200 to 1200 bp). The inserts of a total of 2880 library clones were amplified by PCR, purified, sequenced from one orientation [63], and spotted in triplicate on microarray slides (Corning UltraGAPS) along with 204 annotated EST fragments selected from the MarC1 and MarC 2 EST libraries [26,47].

**Microarray hybridization and data analysis:** Dual-color hybridization of slides was performed using the RNA MICROMAX TSA labeling and detection kit (PerkinElmer), as described recently [47]. Briefly, a labeled cDNA target was prepared from 1 µg of RNA template of the RNA pool prepared from loose pigs (see above) using biotin (BI), and a fluorescent (FL). labeled cDNA target was prepared from 1 µg of RNA template of the RNA pool prepared from the tethered pigs. BI- and FL-labeled cDNA targets were simultaneously hybridized to a microarray slide, and cDNA targets hybridized to the spots were sequentially detected with the fluorescent reporter molecule Cy5 (BI) and Cy3 (FL), respectively. For each comparison, a second hybridization experiment was performed in which the BI and FL labels were reversed (dye swap). The slides were scanned for Cy5 and Cy3 fluorescence in a Packard Bioscience Biochip Technologies apparatus (PerkinElmer). The ScanarrayTM-express software package (PerkinElmer) was used to automatically grid spots and raw spot intensities were corrected for local background signal, using the “adaptive circle quantification method.” Spots with intensity less than the background threshold were automatically discarded from the data analysis (flagged). In addition, a visual inspection was performed to eliminate spots with an irregular size and/or shape. Data reports were presented in an M/A plot were M is the \( \log_2 \) ratio of Cy5 over Cy3 \( M = \log_2(Cy5/Cy3) \) and A is the \( \log_2 \) product of Cy5 and Cy3 intensity \( A = \log_2(\sqrt{Cy5 \times Cy3}) \). Intensity-dependent normalization was performed using the Locally Weighted Scatter Plot Smoothing (LOWESS) function in the statistical software package R [70]. Normalization was done with a fraction of 0.2 on all data points. Normalized data files from each hybridization were imported in the Spotfire Decision Site program (version 7.2, BioASP, The Netherlands) to select differentially expressed spots with M values \( \log_2(\text{scale}) \) of < −1.58 or > 1.58. For each probe 6 spots were hybridized, 3 for one slide and 3 for the dye swap. Probes with more than two missing values were removed from further analysis. In the Results section, the mean ratio of spots for each cDNA probe \((n = 4, 5, 6)\) from the two hybridized slides (dye swaps) was presented. Probes that hybridized differently were sequenced as described by Niewold et al. [47]. The sequence results were assembled and compared with the NCBI nonredundant (nr) and mRNA reference sequence database (refseq_rna) and/or the porcine EST databases (TIGR) using blastn options. Microarray comparisons between RNA pools prepared from tethered pigs and loose pigs were performed for the three brain tissues dissected (hippocampus, frontal cortex, and olfactory bulb), for both young and old pigs (a total of 6 comparisons).

**Real-time PCR:** The level of expression of β-globin mRNA and CREB-binding protein (CREBBP) mRNA in individual RNA samples was determined by real-time PCR. For each sample 200 ng of RNA was reverse transcribed in a standard RT reaction using Superscript II reverse transcriptase (Invitrogen) and pd(N)₅ primer. A 19-mer forward primer (5'-TGGCCAGGGCTGCTGGTG-3') and a 20-mer reverse primer (5'-TGCACATCGGTTGGAAGTC-3'), generating a DNA fragment homologous to nt 114 to 411 of the sequence AY610360 posted in the NCBI database, were used to quantify β-globin cDNA in RT reactions in a standard LightCycler (type 1.2 with software version: 5.32; Roche Diagnostics) reaction using Cybergreen as label. A 20-mer forward primer (5'-TGATGGGCAACAGTACA-3') and a 20-mer reverse primer (5'-CAACGTTTACTACTATTG-3'), generating a fragment homologous to nt 156 to 265 of the tentative consensus sequence TC378358 posted in the TIGR database, was used to quantify CREBBP mRNA (all primers were purchased from Isogen Bioscience BV, The Netherlands). The PCR reactions were performed at an annealing temperature of 63 °C for β-globin and 66 °C for CREBBP in a final volume of 20 µL containing 2 µL 10× concentrated DNA Master SYBR Green 1 mix, 2 µL diluted cDNA (dilutions in water: 1/100 for hippocampus, and 1/50 for the frontal cortex and olfactory bulb), at a final concentration of 5 mM MgCl₂, and 0.5 µM for both primers. The relative amount of β-globin and CREBBP mRNA in all RT reactions was calculated by extrapolation of the cycle crossing point on a standard curve prepared from dilutions of an RNA sample extracted from the hippocampus with a relative high concentration of β-globin mRNA (using LightCycler analysis software version: 3.5.28). The concentration of 18S ribosomal RNA (rRNA) was determined in the same RT reactions by real-time PCR and used to normalize the relative amount of β-globin and CREBBP mRNA in all individual samples. The level of expression of 18S RNA in the hippocampus RNA samples was not different \((n = 20, \text{mean expression} \pm \text{SD.;} 1.97 \pm 0.34 \mu g/\mu L^{-1})\).}

### 2.3. Expression of MR and GR in spleen

A real-time PCR was performed to determine the content of MR and GR cDNA. The content of 18S RNA was measured to control for variation in RNA yield and RT-reaction conditions. RNA was isolated according to the manufacturer's instructions (RNaseasy minikit, Qiagen, Venlo, The Netherlands). DNase treatment was performed, and the optical density of the RNA samples was measured at 260 and 280 nm to determine their purity and concentration. The RT reaction was carried out according to the manufacturer's protocol (SuperScript™, Gibco–Invitrogen Corporation, Breda, The Netherlands) with approximately 200 ng RNA per sample and random hexamer primers (Promega Benelux BV, Leiden, The Netherlands). cDNA was diluted in water; 1/50 for MR and GR, and 1/500 for 18S RNA.

Quantitative real-time PCR was performed with the Light Cycler (Roche Diagnostics, Mannheim, Germany). MR PCR reactions were carried out using a LightCycler FastStart DNA Master SYBR Green I kit (Roche Diagnostics, Mannheim, Germany) with 2 µL of cDNA in a 20 µL reaction mixture (3 mM-MgCl₂, 0.5 µM of each primer, 1× LightCycler DNA Master SYBR Green I). Mixtures underwent the following real-time PCR protocol: A denaturation program (95 °C for 10 min) and a three-segment amplification and quantification program (95 °C for 10 s, 60 °C for 10 s, 72 °C for 15 s with a single fluorescence acquisition point) repeated for 40 cycles.

18S rRNA and GR cDNA reactions were carried out using a LightCycler FastStart DNA Master Hybridization Probes kit (Roche Diagnostics, Mannheim, Germany) with 2 µL of cDNA in a 20 µL reaction mixture (5 mM MgCl₂ for GR and 4 mM MgCl₂ for 18S rRNA, 0.5 µM of each primer, and 0.2 µM of each probe, 1× LightCycler FastStart DNA Master Hybridization Probes). The following primers were used: MR, forward primer (CAGCTCAGCTTTGGAATGAC), reverse primer (GAACTGTGGCAAGTGAC); GR, forward primer (CCAAGGATCTGGAAGGATA), reverse primer (CCCAAGTCAAGGCCTC); S18, forward primer (GGTCAAAGGCGAGCAG), reverse primer (GGCCGCCGATCAGCCA). Mixtures underwent the following real-time PCR protocol: A denaturation program (95 °C for 10 min)
and a three-segment amplification and quantification program (95 °C for 10 s, 54 °C for GR for 10 s and 57 °C for 18S rRNA for 10 s with a single fluorescence acquisition point, 72 °C for 15 s) repeated for 40 cycles.

2.4. IGF-II protein levels

The side of the dorsal hippocampus contralateral to that used for the gene-expression array was dissected out (60–140 mg) and homogenized in 1 mL lysis buffer (137 mM NaCl, 20 mM Tris–HCl (pH 8.0), 1% NP-40, 10% glycerol, and a complete EDTA-free protease inhibitor tablet (Roche, Belgium)), using a Mini-Bead beater (Biospec products, Bartlesville, OK, USA). Samples were homogenized at least three times for 30 s each, with cooling of the samples on ice between runs. The concentration of IGF-II was determined with a radioimmunoassay (RIA) kit (Mediagnost, Reutlingen, Germany) according to the manufacturer’s protocol. To our knowledge, there is no RIA that uses a pig IGF-II antibody. The RIAs described in the literature use different human IGF-II antibodies to measure plasma/serum porcine IGF-II (e.g. [19,40]) and we used a similar kit to measure porcine IGF-II in brain homogenates. The assay was reliable within the range of the standard curve. IGF levels are expressed in ng and corrected for the amount of protein (mg) in the hippocampus.

2.5. Statistical analysis

Effects of tethering stress and age and their interaction were statistically evaluated by analysis of variance (ANOVA) with the factors housing (loose vs. tethered) and age group (younger vs. older sows) (SAS, SAS Institute Inc., Cary, North Carolina, USA). In addition, pairwise Sidak post-hoc comparisons were performed between the four housing by age-group combinations, where appropriate. Differences between groups were considered as statistically reliable if the associated p-value did not exceed 0.05.

3. Results

Because the sows were purchased from two different farms, we assessed whether the groups had similar body weights (measured as the carcass weight), age (in days), number of litters, time between...
weaning of the last litter and slaughter (days after weaning), and number of live piglets in the last litter.

**Body weight at slaughter** (see Table 1): The older sows were heavier than the younger sows (age group: \( F_{1,16} = 30.04, p < 0.0001 \)). Housing conditions did not affect body weight (housing: \( F_{1,16} = 2.44, N.S. \); housing by age-group interaction: \( F_{1,16} = 0.88, N.S. \)).

**Age in days** (see Table 1): The tethered sows were older than the loose sows (housing: \( F_{1,16} = 105.48, p < 0.0001 \); housing by age-group interaction: \( F_{1,16} = 3.52, 0.10 > p > 0.05 \)).

**Number of litters** (see Table 1): The older sows had had more litters than the younger sows (age group: \( F_{1,16} = 104.82, p < 0.0001 \). There was a marginal difference in the number of litters between housing conditions (\( F_{1,16} = 3.51, 0.10 > p > 0.05 \); housing by age-group interaction: \( F_{1,16} = 1.38, N.S. \), probably reflecting the older age (and hence more estrus cycles and litters) of the tethered sows.

**Days after weaning** (see Table 1): Age and housing conditions did not influence the time between weaning and slaughter (age group, \( F_{1,16} = 2.04, N.S. \); housing: \( F_{1,16} = 3.37, 0.10 > p > 0.05 \); housing by age-group interaction: \( F_{1,16} = 0.03, N.S. \)).

**Number of live piglets in last litter** (see Table 1): Age and housing conditions did not affect the number of live piglets in the last litter (age group, \( F_{1,16} = 51.29, p < 0.0001 \)). Housing conditions affected the weight of the left adrenals (housing: \( F_{1,16} = 13.34, p < 0.0002 \); housing by age-group interaction: \( F_{1,16} = 15.86, p < 0.0011 \)), with tethered older sows having heavier left adrenal glands than tethered younger sows or loose older and younger sows, as confirmed by Sidak post-hoc comparisons.

**Weight of right adrenal** (see Fig. 4A): The left adrenals of the older sows were heavier than those of the younger sows (age group: \( F_{1,16} = 51.29, p < 0.0001 \)). Housing conditions affected the weight of the left adrenals (housing: \( F_{1,16} = 13.34, p < 0.0002 \); housing by age-group interaction: \( F_{1,16} = 15.86, p < 0.0011 \)), with tethered older sows having heavier right adrenal glands than tethered younger sows or older and younger sows, as confirmed by Sidak post-hoc comparisons.

**Weight of right adrenal** (see Fig. 4B): The right adrenal glands of the older sows were heavier than those of the younger sows (age group: \( F_{1,16} = 19.19, p < 0.0005 \)). Housing conditions affected the weight of the right adrenals (housing: \( F_{1,16} = 4.75, p < 0.05 \); housing by age-group interaction: \( F_{1,16} = 7.86, p < 0.0013 \)), with tethered older sows having heavier right adrenal glands than the other groups of sows; adrenal weight did not differ in the latter three groups.

**Weight of the pituitary gland** (see Fig. 4C): The pituitary glands of the older sows were heavier than those of the younger sows (\( F_{1,16} = 13.74, p < 0.0019 \)), and the pituitary glands of the tethered sows were lighter than those of the loose sows (housing: \( F_{1,16} = 5.83, p = 0.0281 \); housing by age-group interaction: \( F_{1,16} = 0.02, N.S. \)).

**Weight of the spleen** (see Fig. 4D): Two older loose sows had enlarged spleens with vascular abnormalities. After exclusion of these pigs, the older sows had slightly heavier spleens than the younger sows (age group: \( F_{1,14} = 13.74, p = 0.0019 \)). Housing conditions influenced spleen weight (housing: \( F_{1,14} = 5.83, p < 0.0281 \); housing by age-group interaction: \( F_{1,14} = 0.02, N.S. \), with the tethered sows having slightly lighter spleens than the loose sows.

**Plasma cortisol concentration** (see Fig. 4E): Because the data were not homogeneous, cortisol concentrations were logarithmically transformed before statistical analysis. While the older and younger sows had similar cortisol concentrations (\( F_{1,16} = 1.48, N.S. \), the tethered sows had higher cortisol concentrations than the loose sows (\( F_{1,16} = 9.46, p < 0.0072 \)). There were no interactions between the housing conditions and age (\( F_{1,16} = 2.71, N.S. \)).

**Gene-expression analysis**: RNA levels were measured in the pooled samples of hippocampus, frontal cortex, and olfactory bulb prepared from tethered pigs and loose sows, using our “home-made” porcine brain microarray (see Materials and methods). The probes (library clones) that hybridized differently with a mean ratio of 3-fold or more (mean M value [log₂ of > 1.58 or < −1.58 and a p-value < 0.05) in these comparisons were further characterized and annotated by blast(n) analysis. The tethered to loose hybridization ratios for these probes are presented in Table 2. Comparison of RNA pools prepared from the olfactory bulb of tethered and loose pigs did not reveal differential hybridizing probes with a mean ratio greater than 3-fold (data not shown).

The tethered older sows had a higher expression of mRNA for AVL9, prion protein (PRNP), myelin-associated oligodendrocytic basic protein (MOBP), and the mRNA coding for a hypothetical protein with unknown function (MGCB3791) in the hippocampus than did the other sows. The AVL9 protein is homologous to a Golgi/endosomal-associated sorting and transport protein of S. cerevisiae with the same name. MOBP is a structural constituent of the myelin sheath. Strikingly, the expression of β-globin mRNA was almost 25 times higher in the pooled sample for the tethered older sows than in the pooled sample for the loose older sows. This difference was investigated further by measuring the level of β-globin mRNA expression in all individual RNA samples prepared from the hippocampus and frontal cortex, using real-time PCR.

---

**Table 2**

<table>
<thead>
<tr>
<th>Ratio tethered/loose</th>
<th>Hippocampus</th>
<th>Frontal cortex</th>
<th>bp</th>
<th>Blast(n)/refseq-RNA database</th>
<th>E-value</th>
<th>Acc. number</th>
<th>mRNA</th>
<th>WU-BLAST 2.0/TIGR</th>
</tr>
</thead>
<tbody>
<tr>
<td>13 A04 LARK</td>
<td>24.4</td>
<td>6.3</td>
<td>6.9</td>
<td>5.2</td>
<td>335</td>
<td>6.00E−58</td>
<td>gi:73988389</td>
<td>2.90E−53 pigTC218944 Chain B, Hemoglobin (Aquamaet), (Su scrofa Major prion protein precursor (PPP), PIG</td>
</tr>
<tr>
<td>13 D01 LARK</td>
<td>5.7</td>
<td>#</td>
<td>#</td>
<td>#</td>
<td>252</td>
<td>1.00E−08</td>
<td>gi:122056621</td>
<td>1.70E−26 pigTC202089</td>
</tr>
<tr>
<td>2 F02 marc</td>
<td>9.3</td>
<td>3.6</td>
<td>#</td>
<td>#</td>
<td>285</td>
<td>2.00E−79</td>
<td>gi:148747135</td>
<td>3.10E−61 pigTC227471 None</td>
</tr>
<tr>
<td>17 E09 LARK</td>
<td>3.6</td>
<td>#</td>
<td>#</td>
<td>#</td>
<td>539</td>
<td>2.00E−43</td>
<td>gi:1663699</td>
<td>3.00E−86 pigTC199889 Similar to MGCB3791 protein</td>
</tr>
<tr>
<td>2 F06 LARK</td>
<td>6.5</td>
<td>#</td>
<td>#</td>
<td>#</td>
<td>569</td>
<td>2.00E−43</td>
<td>gi:34096477</td>
<td>1.20E−55 Homologue to UPQ035713 (O35713) Myelin-associated/oligodendrocyte basic protein</td>
</tr>
<tr>
<td>23 A08 LARK</td>
<td>4.1</td>
<td>#</td>
<td>#</td>
<td>#</td>
<td>431</td>
<td>3.00E−51</td>
<td>gi:73989800</td>
<td></td>
</tr>
</tbody>
</table>

* #: no statistically reliable difference; ratio<3.
The level of expression of β-globin mRNA in the hippocampus (and to a lesser extent in the frontal cortex) in three (pig nos. 3, 6 and 13) of the tethered older sows was higher than that of the five loose older sows (hippocampus: Age group: $F_{1,16} = 1.08$, NS; housing: $F_{1,16} = 4.31$, $p < 0.05$; housing by age-group interaction: $F_{1,16} = 1.67$, NS; Fig. 5A; frontal cortex: Age group: $F_{1,16} = 3.03$, NS; housing: $F_{1,16} = 14.08$, $p < 0.01$; housing by age-group interaction: $F_{1,16} = 0.08$, NS; Fig. 5B). The level of expression of CREBBP mRNA, which was not different in the pooled analysis, was different in the individual analysis, indicating that the differences in β-globin mRNA expression were not due to variation in sample composition (e.g. types of cells) and/or RNA extraction and handling procedures. Since the role of β-globin in the brain is not clear, we did not follow it up further.

The level of expression of β-globin mRNA in the hippocampus (and to a lesser extent in the frontal cortex) in three (pig nos. 3, 6 and 13) of the tethered older sows was higher than that of the five loose older sows (hippocampus: Age group: $F_{1,16} = 1.08$, NS; housing: $F_{1,16} = 4.31$, $p < 0.05$; housing by age-group interaction: $F_{1,16} = 1.67$, NS; Fig. 5A; frontal cortex: Age group: $F_{1,16} = 3.03$, NS; housing: $F_{1,16} = 14.08$, $p < 0.01$; housing by age-group interaction: $F_{1,16} = 0.08$, NS; Fig. 5B). The level of expression of CREBBP mRNA, which was not different in the pooled analysis, was different in the individual analysis, indicating that the differences in β-globin mRNA expression were not due to variation in sample composition (e.g. types of cells) and/or RNA extraction and handling procedures. Since the role of β-globin in the brain is not clear, we did not follow it up further.

The expression of IGF-II mRNA in the pooled hippocampus sample was higher in the tethered older sows than in the other sows, but this difference was no longer seen when the IGF-II protein levels in the hippocampi (see Fig. 6) based on the samples derived from the individual sows were analyzed (age group: $F_{1,16} = 1.00$, NS; housing: $F_{1,16} = 1.26$, NS; housing by age-group interaction: $F_{1,16} = 1.61$, NS).

GR and MR expression in spleen (see Fig. 7A,B): Grubb’s test indicated that one loose older sow (MR expression in spleen) and one tethered older sow (GR expression in spleen) had extreme values. After exclusion of the data of these two animals from the analyses, neither age nor housing conditions, nor their interaction, affected GR and MR expression in the spleen (GR: Age group: $F_{1,15} = 0.00$, NS; housing: $F_{1,15} = 1.62$, NS; housing by age-group interaction: $F_{1,15} = 0.15$, NS; MR: Age group: $F_{1,15} = 0.18$, NS; housing: $F_{1,15} = 1.42$, NS; housing by age-group interaction: $F_{1,15} = 0.23$, NS).

Neither age (age group: $F_{1,16} = 1.26$, NS; housing: $F_{1,16} = 1.26$, NS; housing by age-group interaction: $F_{1,16} = 1.61$, NS) affected hippocampal IGF-II protein levels.

4. Discussion

In the present study, we investigated whether sows exposed to chronic stress can be considered an animal model of depression, based on the assumption that chronic stress is an important etiological factor for depressive illness (e.g., [22,49]). To this end, we compared the organ weights, cortisol levels, and gene expression in selected brain samples of sows housed socially with those of sows housed individually and tethered during pregnancy and farrowing, i.e. during about 80% of each reproductive cycle. Two age groups of sows (about 2.5 years and about 5 years) were tested per housing condition. We found that plasma cortisol levels were higher in the tethered sows than in the loose sows, and that the older, but not younger, tethered sows had heavier spleens than the older or younger loose sows. The weight of the spleen and the expression of GR and MR in this organ were unaffected by the housing conditions. The pituitary gland was slightly lighter in the tethered sows. Microarray analyses revealed an increased expression of β-globin mRNA in the hippocampus and to a lesser extent in the frontal cortex of the older tethered sows than in the older loose sows. These findings suggest that recurrent stress over 4.5 years, but not over 1.5 years, has lasting neuroendocrine effects.

In an earlier study of piglets experiencing repeated social defeat [62] we found no neuroendocrine symptoms of depression but behavioral adaptation, i.e. this approach appeared to be ineffective to induce depression-like symptoms in pigs. We assumed that the stress...
experienced by the “losers” had not been severe and/or long enough to induce changes that may be characteristic for symptoms of depression. Therefore, we decided to analyze the consequences of recurrent long-lasting episodes of “severe” stress in pigs. Since tethering has been rated as a system that induces severe stress in sows [57,60], we expected that tethering would cause chronic stress, which could trigger the development of depression-like symptoms. It should be noted that this study was performed before the EU ban on tethering was introduced in 2006. The pigs used in this study had been bred and supplied by the same breeder. While the groups of tethered and loose sows were similar regarding number of reproductive cycles and number of viable piglets per litter, the mean age of the older loose sows was nearly 1 year younger than that of the corresponding older group of tethered sows. We cannot exclude that this age difference affected some of our findings.

The uncompromised reproductive success of tethered sows has been advanced as argument against the notion that tethering impairs welfare. However, it is possible that tethered sows adopt efficient coping strategies to reduce the stress of tethering — pigs are “generalists” that are able to adapt to a wide range of environmental conditions [5]. Such coping strategies may also reduce the physiological and neurological expression of the adverse effects of repeated, long-lasting tethering. Our findings appear to suggest that older sows are less able than younger sows to reduce the adverse physiological and neurological consequences of tethering. An alternative explanation is that long-term (over 4.5 years) rather than short-term (over 1.5 years) tethering produces adverse effects.

Our approach can be classified as a “natural experiment” or a “quasi-experiment.” We did not actively manipulate the independent variable (management system and housing conditions), but studied the existing management systems and housing conditions of two hog farms. Despite the quasi-experimental set-up of the study, i.e. we could not control the assignment of the pigs to the different farms, the common genetic background and similar ages of the sows made it possible for us to directly compare the effects of the two housing conditions on organ weights, cortisol concentrations, and gene expression. Moreover, unsystematic observations during visits to Farm B confirmed that the tethered sows engaged in well-described, characteristic stereotypic behavior (e.g. [57]). The frequency and intensity of this behavior appeared to intensify in anticipation of feeding.

4.1. Organ weights

In our study, the older, but not the younger, tethered sows had heavier adrenals than their socially housed counterparts, whereas the pituitary gland of tethered sows was lighter than that of loose sows. In humans, the adrenal glands and pituitary are heavier in depressive patients than in non-depressed individuals [7]. While the spleen was slightly lighter in the tethered sows, we previously found that stress did not affect the weight of the spleen of piglets [62] and thus tentatively conclude that spleen weight is relatively insensitive to the effects of stress.

4.2. Plasma cortisol level

Plasma cortisol levels were higher in the tethered sows than in the loose sows. Elevated cortisol levels are considered as an index of stress [4,46] and may be taken as an endophenotype of major depression [44]. Chronically elevated cortisol levels may have detrimental effects in the brain and may increase vulnerability to major depressive disorders [29]. Higher basal cortisol levels have been found to reduce the cell number and volume of the left, but not the right, dentate gyrus, suggesting that the left dentate gyrus is more sensitive to stress than the right dentate gyrus [60]. Chronic stress, mediated by raised levels of cortisol, may influence neuroanatomical parameters. However, van der Beek et al. did not detect neuropathological changes after 5 months of tethering [60].

Plasma cortisol in pigs shows a circadian rhythm, with a clear decrease during the second half of the day [6,18,29,32]. We collected the blood samples when basal cortisol concentrations would be low. However, there is some evidence that tethering may disrupt or modulate the circadian rhythmicity of plasma cortisol [6]. Moreover, cortisol may not reliably reflect the stress level of pigs [32], although chronic stress usually produces a prolonged activation of the HPA axis [42]. Sows may adapt to the chronic effects of tethering and the responsiveness of the stress systems may decline to a level that is undistinguishable from that in non-stressed individuals [66].

4.3. Expression of PrP and β-globin in frontal cortex and hippocampus

4.3.1. PrP

The physiological function of prion protein (PrP) is not known. Nonpathogenic cellular PrP may modulate neuronal survival and positively regulate neural precursor proliferation during neurogenesis [12,58]. The increased expression of major prion protein precursor in the older sows is interpreted as a counter-regulatory process to prevent further neuronal damage and neuronal loss.

4.3.2. Expression of β-globin

Hemoglobin-like molecules occur widely, in organisms ranging from bacteria to man. They transport and store oxygen, which is essential for the oxidative (aerobic) generation of energy by mitochondria; other functions include oxygen scavenging, transporting and detoxifying nitric oxide, and enzymatic activities [45]. Since the brain samples we collected inevitably contained blood, we cannot be sure whether the β-globin measured is blood or brain tissue associated, or whether it reflects the expression of this gene in either tissue. The function of β-globin in hemoglobin synthesis is well known [28] but it is not known whether β-globin has a function in brain tissue. Recently, it has been shown that the expression of neuroglobin is higher in those brain regions that are responsive to oxidative stress (i.e., hippocampus, thalamus, and hypothalamus) [69]. Thus, we hypothesize that the increased β-globin expression in the tethered sows compared with the loose sows is part of a counter-regulatory process to protect against stress by scavenging reactive oxygen species (see also [50]).

4.3.3. Expression of IGF in hippocampus

Hippocampal IGF-I receptor mRNA expression was reduced in adult rats subjected to neonatal isolation, but neither IGF-II receptor expression nor IGF-I and IGF-II mRNA levels were affected [23]. Yet, IGF-II mRNA levels in the hippocampus plus choroid plexus, an area with very high expression of IGF-II, were reduced in adult rats subjected to maternal separation, which is considered to be more severe than neonatal isolation [37]. This suggests that IGF-II signaling may be affected in psychopathology. Of note, the physiological role of IGF-II in the brain is not well understood. We found an increased IGF-II gene expression, but not increased IGF-II protein levels, in the dorsal hippocampus in the older tethered sows. This suggests that the increase in IGF-II mRNA expression could be a mechanism to compensate for possible reductions in IGF-II protein levels.

4.3.4. Expression of GR and MR in spleen

There is some experimental evidence that GR levels in the spleen are decreased in individuals with depression-like symptomatology [52]. However, in a previous study [62] of the effects of social defeat in piglets, we did not find differences in the expression of GR and MR in spleen. Our current findings show that repeated long-lasting tethering over 1.5 or 4.5 years did not affect the expression of corticoid receptors in spleen.
4.3.5. Putative stressors in the two systems (loose vs. tethered)

Tethering induces different types of stress, in particular restraint stress and social isolation stress. However, according to Pedersen et al. [54] a positive human–animal relationship may attenuate the cortisol response and improve the immunological responsiveness of sows to tethering. The hog farmers from whom the sows were derived were dedicated stockmen, and the animals from both farms (and hence housing conditions) were in good shape. A positive human–animal relationship may be able to attenuate or counteract the effects of adverse environmental factors [for a review see [67]].

Although the tethering system is considered the more stressful housing condition, social housing may also induce recurrent stress, for example each time that the sows are returned to the group and a dominance hierarchy is re-established [56]. After their piglets were weaned, the sows were returned to the group housing pen and may have experienced stress due to re-grouping, i.e. the difference in the stress effect of the two housing conditions may have been diminished by the stress the loose sows had experienced a few days (younger loose sows 15.8 ± 4.7 days; older loose sows 15.8 ± 1.7 days) before they were transported to the slaughterhouse. Re-mixing induces aggression during establishment of the social hierarchy, and the associated physiological stress appears to be short lasting [2]. Longer lasting effects, however, such as recurrent fighting at feeding times, bullying by dominant animals, high feed intake in dominant animals and low feed intake in subordinate animals, and vulva biting cannot be ruled out [17].

The position of the loose sows in the social hierarchy could affect their plasma cortisol levels: Low- and high-ranking sows have lower cortisol levels than middle-ranking sows [71]. Although we do not know their social rank, the sows most likely had already established a social hierarchy days before the blood and tissue samples were taken. It is suggested that it takes between a few days and 3 weeks to establish a stable hierarchy [2]. We agree with van der Beek et al. [60] that it is difficult to include a suitable “stress-free” group for comparison, because housing of sows in freely moving groups induces marked, hierarchy-related differences in stress levels.

4.3.6. The DNA microarray approach to assess effects of chronic stress

The tethered sows experienced chronic stress, as reflected by the larger adrenal glands and raised cortisol levels. The identification of specific gene-expression profiles that reflect the accumulative “stress load” during life (monitoring), and as a consequence the validation of high-throughput DNA-microarrays for the objective measurement of animal welfare, was partly successful. It is conceivable that the expression of mRNA coding for certain receptors etc in the pig brain was below the detection level.

4.4. Limitations of the current study

There are some limitations to our current study that merit consideration. We cannot rule out that acute stress effects caused by transportation to the slaughterhouse and the time in lairage before slaughter may have been strong enough to induce acute hormonal changes and affect gene expression, overshadowing the eventual long-lasting effects of the two different housing conditions. The tethered pigs were loaded first and thus spent about 1 h longer in the truck than the loose sows did. However, Janssens et al. [31] observed that plasma cortisol levels after 10 to 11 weeks of tethering were similar to those of loose nulliparous control gilts. After acute stress, induced by a nose-sling, the cortisol response was not affected by the housing conditions, and cortisol levels returned to normal within 2 h. These findings suggest that the unavoidable acute stress of loading, transporting, unloading, and time in lairage did not affect cortisol levels at slaughter or may have affected all groups to a similar extent (due to the randomization of the order of slaughter; see Table 1).

The housing conditions of the tethered sows were very different from those of the loose housed sows, in particular regarding the unpredictable frequency, duration, and intensity of social interactions in socially housed pigs. The average age of the tethered sows was higher than that of the loose sows (as indicated by a main effect of housing). Although the means in Table 1 suggest that the age difference was larger in the older sows, this was not confirmed statistically (because there was only marginal interaction effect between age and housing). The older groups had had more litters (i.e. reproductive cycles). We cannot exclude that this affected stress-relevant variables. The effects of these putative confounding factors could not be controlled experimentally, nor could their possible effects on our results be estimated.

4.5. Conclusion

Typically, an animal model of depression is based on exposure of an animal to a stressful condition that is believed to induce depression-like symptoms at the physiological and behavioral level [51]. In view of the literature on the long-lasting effects of a single exposure of adult animals, mainly rodents, to a stressful event (reviewed by [3]), our findings are unexpected because tethering is considered a severe stressor [57,60]. We had assumed that recurrent chronic tethering would produce robust depression-like effects in brain regions associated with emotions. Although tethering stress may exert other effects than the stress caused by, for example, repeated defeat, it produced a few changes at the physiological, anatomical, and gene-expression level that are consistent with a “depression”–like symptomatology. However, considering the weak expression of only a subset of symptoms that are associated with the depressive phenotype, it can be questioned whether the pig subjected to repeated, long-term stress can be regarded an animal model of major depression. Further research including behavioral measures is warranted, to verify whether recurrent chronic severe stress induces symptoms of (‘major’) depression in pigs.

Acknowledgments

The authors would like to thank Rita Hoving-Bolink for the coordination of the collection of samples, Sander Boymans and Ulike Brouwer for technical assistance, Leo Krujit for the analysis of GR and MR in spleen, and Marianne Markerink-van Ittersum for the IGF-II protein measurements.

Drs. Jos Prickaerts and Gunter Kenis are supported by the EU Framework 6 Integrated Project NEWMOOD (LSHM-CT-2004-503474).

References


