The effect of lipopolysaccharide (LPS) on inflammatory markers in blood and brain and on behavior in individually-housed pigs

Janicke Nordgreen[a,f,*], Camilla Munsterhjelm[b], Frida Aae[a], Anastasija Popova[a], Preben Boysen[f], Birgit Ranheim[d], Mari Heinonen[b], Joanna Raszplewicz[g], Petteri Piepponen[e], Andreas Lervik[c], Anna Valros[b], Andrew M. Janczak[a]

[a] Animal Welfare Research Group, Department of Production Animal Clinical Science, Faculty of Veterinary Medicine, Norwegian University of Life Sciences (NMBU), Oslo, Norway
[b] Research Centre for Animal Welfare, Department of Production Animal Medicine, University of Helsinki, Finland
[c] Department of Companion Animal Clinical Science, Faculty of Veterinary Medicine, Norwegian University of Life Sciences (NMBU), Oslo, Norway
[d] Department of Production Animal Clinical Science, Faculty of Veterinary Medicine, Norwegian University of Life Sciences (NMBU), Oslo, Norway
[e] Division of Pharmacology and Pharmacotherapy, Faculty of Pharmacy, P.O. Box 56, 00014, University of Helsinki, Finland
[f] Department of Food Safety and Infection Biology, Faculty of Veterinary Medicine, Norwegian University of Life Sciences, Oslo, Norway
[g] Small Animal Teaching Hospital, University of Liverpool, Chester High Road, Neston CH64 7TE, UK

ARTICLE INFO

ABSTRACT

Most of us have experienced deterioration of mood while ill. In humans, immune activation is associated with lethargy and social withdrawal, irritability and aggression; changes in social motivation could, in theory, lead to less functional interactions. This might also be the case for animals housed in close confinement. Tail biting in pigs is an example of damaging social behavior, and sickness is thought to be a risk factor for tail biting outbreaks. One possible mechanism whereby sickness may influence behavior is through cytokines. To identify possible mediators between immune activation and behavioral change, we injected 16 gilts with lipopolysaccharide (LPS; O111:B4; $1.5\,\mu g\,kg^{-1}$ IV through a permanent catheter). In LPS-treated pigs, a significant increase in cortisol, TNF-α, IL-1 receptor antagonist, IL-6, and IL-8 was observed alongside decreased activity within the first 6 h after the injection. CRP was elevated at 12 and 24 h after injection, and food intake was reduced for the first 24 h after injection. Three days post-injection, LPS pigs had lower levels of noradrenaline in their hypothalamus, hippocampus and frontal cortex compared to saline-injected pigs. Pigs injected with LPS also had higher levels of the pro-inflammatory cytokine IFN-γ in their frontal cortex compared to saline-injected pigs. Thus, a low dose of LPS can induce changes in brain cytokine levels and neurotransmitter levels that persist after inflammatory and stress markers in the periphery have returned to baseline levels.

1. Introduction

Several studies suggest associations between health and tail biting behavior in pigs [1–6]. A possible mechanism by which health could influence behavior is through the effect of cytokines on hormone levels and neurotransmitter systems. Cytokines are small proteins produced by immune cells. Their effects can be both pro- and anti-inflammatory, and they are part of the mechanisms that help the organism cope with infectious and non-infectious challenges. Knowledge about the effects of cytokines on behavior primarily comes from two areas: descriptions of the behavioral consequences of naturally occurring illness—so-called 'sickness behavior' in mammals [7, 8]—and observations of the side

effects experienced by human patients subject to immune therapy, e.g. for hepatitis or metastatic cancer [9, 10]. Sickness behavior is mainly elicited by the pro-inflammatory cytokines interleukin 1β (IL-1β), tumour necrosis factor α (TNF-α) and interleukin 6 (IL-6) and manifests as anorexia, lethargy and decreased social motivation [7]. However, lethargy and social withdrawal are not the only behavioral changes brought about by cytokine increase. Depression, irritability and short temper, anger/hostility, extreme emotional lability, tearfulness and cognitive impairment have been reported in clinical studies on the effects of treatment with pro-inflammatory cytokines such as IL-2 and interferon alpha [9–12]. There also are indications that inflammatory proteins may play a role in aggression, as elevated levels of IL-6 and C-
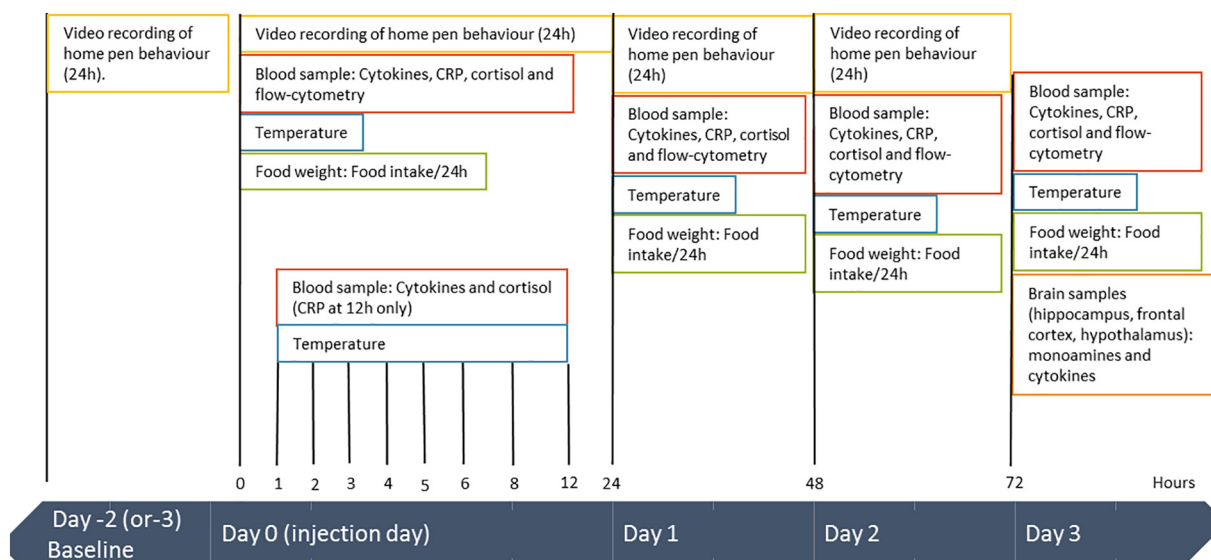
Fig. 1. An overview of the experimental design. The timing of samples, tests and other registrations are shown on the timeline from three days before LPS injection to three days after LPS injection.

reactive protein (CRP) have been found in psychiatric patients with a diagnosis of intermittent explosive disorder [13]. However, in many of these reports, which came first—the cytokine response or the behavior—cannot be ascertained.

If cytokines do play a role in the aetiology of tail biting, it must be by increasing the likelihood that a pig will become either a victim or a biter. Our hypothesis is that if a pig is ill to such an extent that it shows social withdrawal, lethargy or signs of depression, it could be singled out as a victim since it would differ from the rest of the group. Sick animals may be preferred as competitors, as victory is more certain if one competes with a sick conspecific than with a healthy one [14]. On the other hand, irritability, emotional lability and short temper in a pig housed in close confinement could lead to biting behavior.

Findings in rodents and humans indicate that two possible mechanisms by which cytokines may exert their effects on behavior are by altering monoaminergic signalling [15, 16] or by stimulating glutamatergic signalling [17]. Interestingly, monoaminergic signalling seems to differ between biters, victims and neutral pigs. Both biters and victims have been found to have increased serotonin turnover compared to neutral pigs [18]. Ursinus et al. [19] showed a lower level of serotonin storage in blood platelets in biters and victims, which could indicate an increased use of tryptophan for the formation of central serotonin. Dopamine turnover was increased in the victims [18].

The study of causal relationships between health and behavior in pigs held under commercial conditions is difficult because of a lack of both control and standardisation. Experimental models of immune stimulation are easier to work with, as the strength, type and timing of immune stimulation can be controlled. Bringing animals into an experimental facility also provides the opportunity to perform detailed observations that may be difficult to perform in the field. The results from model experiments can therefore be more easily used to identify possible mechanisms underlying the phenomenon of interest, and to guide later field studies. The injection of lipopolysaccharide (LPS), a component of the cell wall of gram-negative bacteria, is one relatively well-characterised model treatment that leads to immune activation. LPS binds CD14 and Toll-like receptor 4 and leads to the activation of transcription factors such as NF-κB. The activation of these transcription factors increases the production of pro-inflammatory cytokines, stimulates the production of acute phase proteins in the liver, and activates the HPA-axis [20–26]. Pigs injected with LPS at moderate doses show decreased activity, exploration and eating behavior [27], and an

increased latency to approach a human in the home pen [28]. With higher doses, lethargy may be more pronounced and longer-lasting [29]. However, pigs are rarely studied for longer than 12 h, precluding the possibility of detecting long-lasting effects on behavior and physiology. Most experiments on pigs focus on LPS-induced changes in TNF-α, IL-6 and sometimes IL-1β [30–33]. However, other cytokines may also be influenced by LPS injection and could contribute to changes in physiology and behavior. To identify candidate mechanisms linking immune activation with behavior, detailed information about the time-course of changes in the levels of more than the three 'classical' pro-inflammatory cytokines (TNF-α, IL-6 and IL-1β), as well as CRP, cortisol, monoaminergic neurotransmitters and behavior is necessary.

We therefore injected pigs with a low dose of LPS and measured changes in time budgets and food intake over three days post-injection. We measured the time-course of changes in 13 different cytokines, and characterised effects on leukocytes, cortisol, CRP and skin temperature. At euthanasia, 72 h post-injection, brain samples were collected for monoamine and cytokine analysis.

2. Materials and methods

2.1. Experimental design and ethical permit

This experiment was approved by the national animal research authority (FOTS id 7002). An overview of the experimental design is provided in Fig. 1. Pigs were kept in the experimental unit for four weeks before the surgical fitting of a permanent central venous catheter into one jugular vein. During this time, they were habituated to the environment and handlers. LPS was injected five to six days after surgery. Relative to the time of injection, blood samples and temperature measurements were taken 30 min before LPS injection (referred to as 0, or baseline) and at 1, 2, 3, 4, 6, 8, 12, 24 and 72 h post-injection. Video-recordings of behavior in the home pen ran continuously throughout the study. Food was weighed in the morning every 24 h. Hay was provided in the afternoon of every day. At euthanasia, brains were removed and samples dissected and snap-frozen in isopentane on dry ice within 10 min. The hippocampus, frontal cortex and hypothalamus were analysed for monoamines (dopamine, serotonin and noradrenaline and their metabolites) and 13 different cytokines.

2.2. Animals and husbandry

Sixteen female pigs (Landrace Yorkshire x Duroc Duroc) were used for this study. As gender may influence the immune response [34], we decided against including both gilts and barrows as it would have necessitated a larger sample size. Female pigs were chosen as they are not castrated. Castration is a surgical procedure and thus has the potential to induce an immune response, potentially influencing the response to later LPS treatment [35].

Eight pigs were allocated to the LPS treatment (LPS), and eight pigs were allocated to the control treatment (saline injection: SAL). All pigs were transported from their farm to the experimental facility on the day of weaning, i.e. at approximately five weeks of age. According to the farmers, the pigs had no previous history of illness and had not been treated with antibiotics before arrival at the experimental unit. The pigs for the two replicates came from two different commercial farms within one hour's driving distance from the experimental facility. The eight pigs per replicate consisted of four sibling pairs. In each sibling pair, one pig was allocated to the LPS group and the other to the control (SAL) group. The allocation was done in a balanced way within the room so that there was an equal number of LPS and control pigs close to and farther away from the entrance. Each sister pair could see and hear one another through the pen division, and they could also have limited tactile contact through the fence dividing the pens. The pigs were fed ad libitum with a piglet diet ('Ideal Junior', Norgesfôr, Oslo, Norway), and had free access to drinking water. The pens (115 cm × 163 cm) had solid concrete flooring with a rubber mat covering part of the area, and wood shavings and hay were added after the pens were cleaned every day. The lights were on from 8:00 am to 4:00 pm every day, and the room was also partly lit by daylight from windows. In addition, lights were turned on after 4:00 pm, during sampling. The temperature varied from 18 to 22 °C. The catheters (see below for a description of the surgical protocol) were flushed with heparinized saline after sampling, and in addition four times per day: 09:00, 12:00, 15:30 and 21:00.

2.3. Surgical procedure and anaesthetic protocol

A complete overview of all substances and doses is provided in Table 1. All pigs were premedicated in their home pen with a mixture containing ketamine, midazolam, and medetomidine injected intramuscularly with a standard hypodermic needle attached to a syringe with extension tubing. Next, a catheter was placed in the auricular vein. Propofol was administered to effect in order to allow orotracheal intubation, and anaesthesia was maintained with isoflurane mixed with 100% oxygen. All pigs were mechanically ventilated to maintain normocapnia. Ampicillin was administered to prevent infection of the

surgical wound. Buprenorphine and flunixin were administered intravenously to provide postoperative analgesia, and Ringer-acetate was administered at 5 ml/kg/h throughout the procedure. A heating mattress was used to prevent hypothermia. Pigs were monitored by a trained veterinary anaesthetist (AL and JR) until they had fully recovered.

An experienced surgeon was responsible for central venous catheterisation in all 16 pigs. After aseptic preparation of the incision site, the pig was placed in dorsal recumbency. An incision was made ventrally in the midline of the neck, from the rostral end of the sternum and cranially towards an imaginary line running between the angles of the mandible. A combination of sharp and blunt dissection was used to reach the internal jugular vein. The vein was ligated by placing a suture. Caudal to that suture, a rubber tube was used to stabilise the vein for cannulation. Before cannulation, a custom-made steel cannula was used to make a subcutaneous tunnel from the incision side up to the dorsal aspect of the neck, where the sharp end was used to perforate the skin. The catheter (Ernæringssonde 31,010,181, length 1000 mm, 2.7 mm outer diameter, OneMed, Oslo, Norway), was pulled through the steel cannula, and the cannula was removed so that the catheter remained in the tunnel. With the catheter ready to be inserted into the vein, the vein was elevated by pulling on the suture and the rubber tube. A pair of scissors was used to make a small incision in the vein, with one blade inserted into the incision to keep the gap open as the catheter was inserted. The catheter was eased approximately 5 cm into the vein in the caudal direction and secured by a suture encompassing the vein and catheter. The incision was closed with two subcutaneous sutures and one skin suture. Bandages and a custom-made backpack protected the catheter and ensured easy access.

For the first two days following the surgery, the pigs received flunixin for pain management and ampicillin to prevent infection (see Table 2 for a complete overview of all substances used in this experiment).

2.4. LPS injection

The study was run in two blocks of eight pigs each. Within each block, pigs were injected with LPS five or six days after surgery. Two sister pairs were injected on the same day, including two saline- and two LPS-treated pigs. The remaining two sister pairs within each block were injected on the following day. The reason for injecting only half of the pigs on each day was to allow sampling within a short period, thus minimising disturbance to the animals. All pigs were injected between 09:20 and 10:10 in the morning. The average weight on the day before injection was 25.9 ± 3.5 kg.

Before the injection day, the lyophilised LPS (from Escherichia coli

Table 1
Overview of substances used, with dose and route of administration indicated.

Active substance	Generic name and concentration of active substance	Dose per kg bw and route of administration	Procedure
Ketamine	Ketalar (100 mg ml^{-1})	6 mg kg^{-1} IM	Premedication
Tiletamin	Zoletil forte vet	2.845 mg kg^{-1} IV	Anaesthesia prior to euthanasia
Zolazepam		2.845 mg kg^{-1} IV	Anaesthesia prior to euthanasia
Midazolam	Midazolam (5 mg ml^{-1})	1 mg kg^{-1} IM	Premedication
Medetomidine	Domitor vet (1 mg ml^{-1})	0.04 mg kg^{-1} IM	Premedication
		0.057 mg kg^{-1} IV	Anaesthesia prior to euthanasia
Buprenorphine	Vetergesic vet (0.3 mg ml^{-1})	0.02 mg kg^{-1} IM	Premedication
Butorphanol	Butomidor (10 mg ml^{-1})	0.181 mg kg^{-1} IV	Anaesthesia prior to euthanasia
Ampicilline	Pentrexyl (powder dissolved in 0.9% NaCl)	40 mg kg^{-1} IV	Administered just before surgery as prophylaxis; continued the first two days post-surgery
Propofol	Propovet (10 mg ml^{-1})	to effect	As needed for endotracheal intubation
Isoflurane		to effect	Gas-anaesthesia during surgery
Ringer-acetate		5 ml kg^{-1} h^{-1}	Fluid administration during surgery
Flunixin meglumine	Finadyne vet (50 mg ml^{-1})	2.2 mg kg^{-1} IV	Post-operative pain management (how many days)
LPS	Serotype 0111:B4 of *Escherichia coli* (Sigma) dissolved in 0.9% sterile saline to a concentration of 20 µg ml^{-1}	1.5 µg kg^{-1} IV	Activation of the immune system

Table 2
Ethogram for scoring of time budgets.

Behavior category	Behavior	Definition
Alert but inactive (ALE-INA)	Lying alert	Lying down with head up
	Sitting alert	Dog-sitting with head up
	Standing alert	Standing with head up
Performing active behavior (ACTIVE)	Moving	Walking, running or jumping with head up
	Exploration	Snout touching bedding, enrichment material or pen fixtures except for the inside of the feeder or drinker
	Social behavior	Attempt to touch another pig with the snout through the fence, with both pigs touching the fence
	Feeding	Snout in feeder
	Drinking	Snout in waterer
	Elimination	Defecating or standing in crouched position
	Comfort behavior	Rubbing body against pen fixtures or rolling on the ground
Lying inactive (SLEEP)	Lying inactive	Head resting against the ground and not moving, body (parts) may make sharp, sudden, short-lasting movements

0111:B4 (Sigma-Aldrich, Darmstadt, Germany)) was dissolved in sterile 0.9% saline to a concentration of $2\,mg\,ml^{-1}$ and frozen in glass vials. On the injection day, a vial was thawed, and the LPS solution was further diluted in sterile 0.9% saline to a final concentration of $20\,\mu g\,ml^{-1}$. Each pig was weighed before injection. The LPS solution was injected into the catheter using a Hamilton glass syringe. The LPS dosage was $1.5\,\mu l\,kg^{-1}$. Immediately after injection, the catheter was flushed with 10 ml of sterile saline to ensure that all of the LPS reached the circulation.

2.5. Recording and scoring of home pen behavior

Twenty-four-hour video recordings were performed to test how the LPS injection would influence the home pen behavior of the pigs. An infrared camera was positioned above the centre of the pen and recordings were made using the Media Recorder system from Noldus (Wageningen, the Netherlands). Daytime behavior was scored by scan-sampling with 10-min intervals for 2 5-h segments of time per day according to the ethogram in Table 2. If a person was in the room at the time of a scan, the video was rewound 2 min; if the person was already in the pen at that time, the scan was excluded from analysis. Two hours were omitted between the segments due to husbandry and sampling. The first scan was sampled 10 min after the injection of LPS. The time was equal within sister pairs and kept the same for all days of observation.

Synchronisation of activity within sister pairs was defined as a scan where both sisters were either active (i.e. behavior category ACTIVE in Table 3) or inactive (i.e. behavior category SLEEP or ALE-INA in Table 3). Synchronisation was assessed for each scan throughout the observation of time budgets.

Behavior was scored over four days altogether, including one day before LPS injection, the day of injection, and two days post-injection.

2.6. Blood sampling

Blood was sampled by syringe through the catheter with minimal stress to the pigs (Fig. 1). After sampling, the catheter was always flushed with 5- to 10-ml of sterile 0.9% saline. The blood was transferred to EDTA tubes for cytokine analysis and flow cytometry, and to additive-free tubes for CRP analysis. The EDTA tubes for cytokine analysis were centrifuged for 10 min at $1000 \times g$ and plasma was transported on ice to a $-80\,°C$ freezer. The blood for CRP analysis and flow cytometry was brought directly to the lab for analysis upon sampling. Sample volume was kept to a minimum: 1–2 ml for cytokine analysis, 1 ml for haematology, 3 ml for flow cytometry and 1 ml for CRP measurement.

2.7. Sampling of brain tissue

On the day of euthanasia, the pigs were injected intravenously

Table 3
Results from the mixed model analysis (or non-parametric between-group comparisons) for all 12 cytokines. The result for the treatment by time interaction is shown, as are the post hoc between-group comparisons. The Bonferroni-corrected critical p-value is 0.004. Details concerning transformations and alternatives to the mixed model are shown in the rightmost column. All analyses were run on fluorescence intensity data, as detailed in materials and methods section.

Cytokine	Time by treatment interaction	Post hoc testing for between-group comparisons (12 comparisons, critical p-value = .004)	Statistical method or transformation
IFN-γ	NS		
IL-1α	Significant, but no relevant post hoc results for between-group comparisons		
IL-1β	Significant, but no relevant post hoc results for between-group comparisons		
IL-1ra	$F_{10, 110} = 62.26; p < .0001$	Significantly higher levels in the LPS group at 2, 3, 4, 6, 8, 12 and 24 h ($p < .0001$ for all comparisons); Tendency to a difference at 1 ($p = .0048$) and 48 ($p = .0067$) h	Mixed model, Box-cox transformation
IL-2	NS		
IL-4	NS		
IL-6	Significant, but could not be transformed to give satisfying homogeneity of variance	Significantly higher levels in the LPS group at 1, 2 and 3 h ($p = .0034$ for all)	Wilcoxon test comparing LPS and SAL at each time point
IL-8	$F_{10, 110} = 27.44; p < .0001$	Significantly higher levels in the LPS group at 2 and 3 after injection ($p < .0001$ for both)	Mixed model, Box-cox transformation
IL-10	Significant, but could not be transformed to give satisfying homogeneity of variance	No differences	Wilcoxon test comparing LPS and SAL at each time point
IL-12	Significant, but no relevant post hoc results for between-group comparisons		
IL-18	Significant, but could not be transformed to give satisfying homogeneity of variance	No differences	Wilcoxon test comparing LPS and SAL at each time point
TNF-α	$F_{10, 110} = 50.89; p < .0001$	Significantly higher levels in the LPS group at 1, 2 ($p < .0001$) and 3 ($p < .0007$) h after injection	Mixed model, Box-cox transformation

through their catheters with a mixture of tiletamine ($2.845\,\mathrm{mg\,kg^{-1}}$), zolazepam ($2.845\,\mathrm{mg\,kg^{-1}}$), butorphanol ($0.181\,\mathrm{mg\,kg^{-1}}$) and medetomidine ($0.057\,\mathrm{mg\,kg^{-1}}$). This injection was administered in the home pen, and the pigs lost consciousness within seconds after the injection. After transport to the dissection room, they were euthanised by an injection of pentobarbital into the catheter. The skull was opened using a bone saw and chisel, and the brain was removed. Samples from the following parts were immediately dissected and frozen in isopentane on dry ice: the hippocampus (left and right), the hypothalamus (left and right) and the frontal cortex (left and right). Following freezing, the samples were stored at $-80\,^{\circ}\mathrm{C}$ until analysis. The frontal cortex was sampled by placing a transverse section approximately 2 cm caudal to the apex of the frontal lobe. The hippocampus was obtained by blunt dissection after having cut through the corpus callosum to separate the left and the right hemisphere down to the level of the thalamus. The hypothalamus was collected by using the optic chiasm and the corpus mammilare (included in the sample) as reference points. Underlying tissue was included by placing two section lines at 45° to the imaginary line between the optic chiasm and the corpus mammilare so that in essence, the tissue block resembled a triangle. The frontal cortex was sampled due to the importance of this area for the control of behavior, the regulation of mood, and the perception of external stimuli [36–40]. The hippocampus was included based on its role in cognition and memory [41–43] and the hypothalamus was collected due to its importance in the regulation of the stress response, appetite and fever [44–46].

2.8. Multiplex cytokine analysis in blood and brain tissue

Cytokines were measured in plasma and brain tissue by a multiplex assay including the cytokines GM-CSF, IFN-γ, IL-1α, IL-1ra, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-18 and TNF-α (PCYTMAG 23 K, (Merck, Darmstadt, Germany)). The detection limits can be found in the supplementary materials (Table S1). The treatment of brain tissue before analysis is described below. Plasma samples or brain homogenate were thawed and centrifuged at $4\,^{\circ}\mathrm{C}$ at $1000 \times g$ for 10 min, and the supernatant was transferred to new Eppendorf tubes. A mixture of antibody-coupled microspheres was incubated with standards, samples, quality controls or blanks (wells receiving buffer only) in a total of 75 µl. Incubation on a plate shaker at 2–$6\,^{\circ}\mathrm{C}$ overnight was followed by a wash step and 2 h of incubation with biotinylated detection antibodies at room temperature. The microspheres were then washed again and incubated with 0.1 µg PE-labeled streptavidin for 30 min. Following an additional wash and resuspension in sheath fluid, the microspheres were analysed on the Luminex100 (Bio-Rad, Hercules, CA) using the BioPlex Manager 6.0 software (Bio-Rad, Hercules, CA). All assays were incubated in darkness.

Tissue blocks were cut from the frontal cortex, hippocampus and hypothalamus samples. Each block was weighed (frontal cortex: average weight ± sd: 87.7 ± 39.0 mg; hippocampus: 74.4 ± 26.1 mg; hypothalamus: 38.3 ± 21.4 mg). Two blocks per sample (except for the hypothalamus, which could only be cut into two blocks in total) were analysed for monoamines (see paragraph below), and one block per sample was analysed for cytokines (as described here). The tissue was homogenised using a modified published procedure [47]. Tissue blocks were weighed and placed in 2-ml round bottomed Eppendorf tubes, each containing 0.5 ml of cell lysis buffer (from The Cell Lysis Kit (#171–304,012), Bio-Rad; see Table 1). The solution contained a protease inhibitor cocktail (#171–304,012, Bio-Rad) and one µl of a stock solution containing 500 mM phenylmethylsulfonyl fluoride (#P-7626) in dimethyl sulphoxide (#D2650, both from Sigma, St. Louis, MO). Each tube also contained a 5-mm tungsten bead. Samples were mechanically homogenised at room temperature for 4 min using a TissueLyser II (Cat.No 85300, Qiagen) set at 20 Hz. The homogenate was centrifuged at 4400 x g for 15 min at $4\,^{\circ}\mathrm{C}$ (Heraeus Multifuge 3SR+ Centrifuge, Thermo Fisher Scientific, MA, USA). We then collected 200 µL supernatant from each tube and stored it at $-80\,^{\circ}\mathrm{C}$ until the bead array analysis of cytokine content described above.

2.9. Cortisol analysis

Cortisol was measured in plasma using an enzyme immunoassay kit (DetectX®, Catalog number K003-HW5, Arbor Assays, MI, USA). The kit reagents were prepared according to the kit protocol.

Plasma samples were thawed and centrifuged at $4\,^{\circ}\mathrm{C}$ at $1000 \times g$ for 10 min. After the dissociation reagent (DR) had been allowed to warm to room temperature, five µl of DR were transferred to Eppendorf tubes—one tube per sample—and 5 µl of plasma supernatant were added to the DR in each tube. The mixture was diluted by adding 490 µl of assay buffer (1:1000 dilution), then vortexed and incubated at room temperature for at least 5 min. All samples were used within 2 h of preparation.

All standards, quality controls and samples were run in duplicates. Fifty microliters of samples, quality control high/low or standards, were pipetted in appropriate wells. Each well then received 25 µl of DetectX® cortisol conjugate, followed by 25 µl of DetectX® cortisol antibody using a repeater pipet. After incubation on a shaker at room temperature for 1 h, the plate was aspirated, and each well was washed four times with 300 µl of wash buffer. Then, 100 µl of TMB substrate was added to each well, and the plate was incubated for 30 min at room temperature. Fifty microliters of stop solution were added before the optical density generated from each well was read.

2.10. C-reactive protein analysis

Sentrallaboratoriet at NMBU's Faculty of Veterinary Medicine (www.sentrallaboratoriet.no) uses a polyethylene glycol (PEG) enhanced immunoturbidimetric assay to measure CRP in serum on an Advia®1800 Chemistry System (Siemens AG, Erlangen, Germany). The sample is reacted with specific antiserum to form a precipitate that is measured turbidimetrically at 340 nm [48].

2.11. Haematology and flow-cytometry

Blood samples were collected in EDTA-containing tubes. Haematological differential counts were retrieved in an Advia® 2120 Haematology System (Siemens AG, Erlangen, Germany). Peripheral blood mononuclear cells (PBMCs) were isolated at each time point by density gradient centrifugation ($2210 \times g$, 30 min) on Lymphoprep™ (Axis-Shield, Dundee, Scotland) and immediately cryopreserved using Recovery™ cell culture freezing medium (Gibco, Thermo Fisher Scientific) for further storage in liquid nitrogen. On the day of flow cytometric analysis, cells from all comparable time points were thawed. The cells were first stained using a LIVE/DEAD® Fixable Yellow dead cell stain kit (Life Technologies/Invitrogen, Oslo, Norway), following the manufacturer's instructions (Table S6 in the supplementary materials provides an overview of the antibodies and secondary reagents used for flow cytometric immunophenotyping in this study). Next, the cells were suspended in a buffer containing 10% porcine plasma, and incubated with monoclonal antibodies and subsequent secondary reagents. For intracellular antigens, the cells were permeabilised and fixed using an Intracellular Fixation & Permeabilization Buffer Set (Affymetrix/eBioscience, Thermo Fisher Scientific). Flow cytometry was performed with a 3-laser Gallios flow cytometer (Beckman Coulter, CA, USA), and gating was based on staining with secondary antibodies only or isotype controls. Data were analysed using Kaluza software (Beckman Coulter, CA, USA). Absolute lymphocyte counts were calculated from haematological analyses as follows: The absolute count of peripheral blood mononuclear cells (PBMC), calculated as lymphocyte + monocyte counts, was multiplied with the relative percentage of each cell subset obtained from flow cytometry and divided by 100, resulting in absolute cell subset counts $*10^{9}/l$.

2.12. Analysis for monoamines in brain tissue

The brain samples were treated in accordance with [18], with some modifications. They were cut from frozen blocks of tissue, weighed (frontal cortex: average weight ± sd: 49.7 ± 20.7 mg; hippocampus: 53.6 ± 18.5 mg; hypothalamus: 32.2 ± 9.7 mg) and homogenised in 0.5 ml of homogenisation solution consisting of six-parts 0.2MHCLO4 and one-part antioxidant solution containing oxalic acid in combination with acetic acid and L-cysteine. The homogenates were centrifuged at 20,800 ×g for 35 min at 48 °C. The supernatant was removed to 0.5 ml Vivaspin filter concentrators (10,000MWCO PES, Sartorius, Stone-house, UK) and centrifuged at 8600g at 4 °C for 35 min. Filtrates containing monoamines were analysed using high-pressure liquid chromatography with electrochemical detection. The analytes were separated on a Phenomenex Kinetex 2.6 μm, 4.6 × 100 mm C-18 column (Phenomenex, Torrance, CA). The column was maintained at 45 °C with a column heater (Croco-Cil, Bordeaux, France). The mobile phase consisted of 0.1 M NaH2 PO4 buffer, 120 mg l^{-1} of octane sulfonic acid, methanol (5%), and 450 mg l^{-1} of EDTA; the pH of the mobile phase was set to 3 using H3PO4. The pump (ESA Model 582 Solvent Delivery Module; ESA, Chelmsford, MA) was equipped with two pulse dampers (SSI LP-21, Scientific Systems, State College, PA) and provided a flow rate of 1 ml min^{-1}. One hundred microliters of the filtrate were injected into the chromatographic system with a Shimadzu SIL-20 AC autoinjector (Shimadzu, Kyoto, Japan). Monoamines and their metabolites were detected using an ESA CoulArray Electrode Array Detector with 12 channels. The chromatograms were processed and concentrations of monoamines calculated using ESA's CoulArray for Windows® software. Analyses of dopamine (DA) and its main metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), noradrenaline (NA) and its main metabolite 3-methoxy-4-hydroxyphenylglycol (MOPEG), and serotonin (5-HT) and its main metabolite 5-hydroxyindoleacetic acid (5-HIAA) were performed.

2.13. Surface temperature and food intake

To avoid stressing the pigs, we measured temperature on the skin surface using an infrared thermometer (Fluke 574 cf., SR Automation AS, Asker, Norway). The same person always measured temperatures, taking care to keep a constant distance between the thermometer and the skin surface (approximately 40 cm). The temperature was always measured on a shaved area of the back, just behind the dressing that secured the catheter. Food uptake was measured by weighing the feeder with food each morning, and calculating the change in weight over the preceding 24 h.

2.14. Data processing and statistical analysis

2.14.1. General description of data processing and statistical analysis

Due to occlusion problems with the catheters, the final group size consisted of 7 pigs in the LPS group and 6 in the SAL group. Analyses of continuous variables except for behavior were run in JMP Pro12 (SAS, NC, USA). All variables were measured several times for each individual and were analysed with mixed models, as detailed below. For variables with a time repeat (all behavioral variables and all substances measured in blood), pig nested in treatment is included as the random effect, and time, treatment and their interaction as fixed effects. For variables with a spatial repeat (brain monoamines and cytokines, as there was one measurement per hemisphere and pig), pig nested in treatment is included as a random effect, and hemisphere, treatment and their interaction are fixed effects. Analyses were run for each brain area separately, as a difference between brain areas was not of interest. Also, neurotransmitter values were in general 10 times higher in the hypothalamus than the frontal cortex and hippocampus, so a joint analysis of all brain areas in one model was not suitable.

For variables with repeats over time, the primary question was

whether there was a significant time by treatment interaction, as neither a main effect of time nor treatment was interesting in itself. Thus, only the interaction is reported in detail in the Results section, and only if the post hoc test yielded relevant results, i.e. differences between treatment groups at the same time points. For variables with a spatial repeat (brain monoamines and cytokines), the main effects of treatment and hemisphere and their interaction were of interest, and results for these are reported. All post hoc tests were done using the t-test with Bonferroni correction. The critical p-value for post hoc tests, including LPS–SAL comparisons, for all 12 time points (this is relevant for blood cytokines and cortisol) is 0.004 (i.e. 0.05 divided by 12). The critical p-value for post hoc tests including five comparisons (CRP, food intake) is 0.01. For variables not described separately below, their analysis followed the description in this paragraph without exception. Results are presented as mean ± sd or median (range).

2.14.2. Statistical analysis of behavioral data

The SPSS statistical package (version 22.0) was used for analysis of behavioral variables. Behavioral variables were considered on the hourly level, as activity was expected to have a certain rhythm over the day and the effects of LPS were expected to last only a few hours.

Time budgets were analysed only on the level of the behavioral categories given in Table 2, as the number of observations was low for most behaviors. Behavioral category variables were expressed as the sum of scans within each category for each hour. Synchronisation of activity was expressed as the percentage of synchronised scans for each hour.

Time budgets and synchronisation of activity were analysed for the effect of day within treatment and time budgets, and also for the effect of treatment within day. For analysis of day effects, the data were split into LPS and CTR for separate analysis in both treatment groups. Behavioral variables for the injection days and the two days following were compared pair-wise to pre-injection values using the Wilcoxon Signed-Rank Test (hereafter Wilcoxon), considering day within individual as the repeated effect. For analysis of treatment effects, CTR was compared to LPS using the Wilcoxon test, considering sister pair within day as the repeated effect.

The non parametric Spearman correlation between the pro-inflammatory cytokines IL-1β, IL-6, IL-8 and TNF-α and number of scans in which the pigs were lying inactive were tested separately for each treatment at baseline and 1, 2, 3, 4 and 6 h after injection.

2.14.3. Statistical analysis of cytokine data

For all thirteen cytokines, a proportion of the analysed samples were below the lower limit of detection (LOD), and the calculated cytokine levels were consequently censored by the analysis software. Dealing with censored data is a common problem in automated biological measurements; a lack of consensus on how to deal with this has resulted in many different analytical approaches, as discussed by Antweiler (2015) and Breen et al. (2016) [49, 50]. Antweiler [49] concludes that a comparison of instrument-generated values that include values below LOD performs well—provided that no > 40% of the measurements have been censored on the basis of LOD—and recommends non-parametric statistical analysis. Breen et al. [50] discuss Luminex analysis specifically, and conclude by recommending a similar approach. In the present study, we excluded analytes from the statistical analysis where > 40% of measurements were censored. A table showing the percentage of censored values for each cytokine can be found in the supplementary material (Table S2). We then used all measured fluorescence intensity (FI) values without subtracting blank and ran a mixed model (as described in the previous paragraph) if the data fulfilled the requirements or could be transformed to do so. Average cytokine values and standard deviations in ng ml^{-1} were obtained from the concentration values provided by the Bioplex manager software, to facilitate comparison with existing studies. It is important to note that these are slightly overestimated compared to the FI values, as the LOD values could not be included.

2.14.4. Biological vs statistical significance

The cytokines and flow cytometry results are presented here with statistical tests and *p*-values. However, the results indicate that some of the variables that were not statistically different between groups nevertheless had an important biological effect in the LPS pigs only. We think this is the case for IL-12 and NK cells, and maybe also for IL-18. The rationale for and implications of this claim will be presented in the discussion, and tables providing an overview of the median cytokine concentration in plasma (min-max) can be found in the supplementary material (Tables S3 and S4).

3. Results

3.1. Measures of immune activation: C-reactive protein and cytokines in blood

C-reactive protein was elevated after LPS injection both at 12 (LPS mean (sd): $34.1\,mg\,l^{-1}$ (7.9) SAL: $15.3\,mg\,l^{-1}$ (4.7), $p < .0001$) and 24 h (LPS: $27.1\,mg\,l^{-1}$ (2.5), SAL: $16.3\,mg\,l^{-1}$ (6.1), $p < .0007$) (F $_{4, 43.02}$ = 31.85; p < .0001), but not at 48 or 72 h. The two groups did not differ at baseline (LPS: $24.2\,mg\,l^{-1}$ (3.2), SAL: ($19.2\,mg\,l^{-1}$ (6.6)).

For several cytokines, there was marked baseline variation, as well as considerable variation over time for both SAL and LPS pigs. Three cytokines differed from this pattern: IL-1ra, IL-8 and TNF-α had very low baselines, little spread and a distinct peak for the LPS group, and hardly any deviations from baseline within the SAL group. IL-1ra peaked either at 3 or 4 h after injection. IL-8 peaked at 2 h post-injection, and TNF-α peaked at 1 h. Also, though it had considerably more unexplained variation, IL-6 showed significant between-group differences at 1, 2 and 3 h after injection, peaking at 2 h. No other cytokines differed significantly between groups. The results for IL1-β, IL-6, IL-8 and TNF-α in blood and IFN-γ in brain tissue are shown in Fig. 2, and median values (range) for all cytokines can be found in Tables S2 and S3 in the supplementary material. The results from the statistical analysis of the effect of treatment and time on cytokine levels can be found in Table 3.

3.2. Brain cytokines

An overview of percent censored values in brain tissue, and the number of censored samples from each group, can be found in the supplementary material (Table S5). As screening of the raw data revealed differences between brain areas regarding the level of cytokines, censoring was calculated per brain area, and cytokines that were censored < 40% in one brain area were analysed for that area even though the overall level of censoring was above 40%. As a result, GM-CSF, IFN-γ, IL-1α, IL-2, IL-8 and IL-18 were analysed for all brain areas, while IL-1β was analysed for the hypothalamus only, IL-1ra was analysed for the hippocampus only, and IL-4, IL-6, IL-10, IL-12 and TNF-α were not analysed at all.

There were no treatment or hemisphere effects on the levels of IL-1β in the hypothalamus or IL-1ra in the hippocampus. In the frontal cortex, LPS pigs had higher levels of IFN-γ than SAL pigs (F (treatment) $_{1, 11.19}$ = 7.27; $p = .02$). In the hippocampus, there was a tendency towards a higher level of IL-18 in the right hemisphere of LPS pigs than in the right hemisphere of SAL pigs (F(treatment * hemisphere) $_{1,11}$ = 3.83; $p = .076$. $P = .1$ for the post hoc Tukey comparison between the right hemispheres of the two groups). There were no other significant differences between treatment groups or hemispheres.

3.3. Brain monoamines

The concentration of monoamines for each brain area is shown in Table 4.

Noradrenaline (NA) levels were considerably lower in all three brain areas of LPS pigs compared to SAL pigs (frontal cortex: F(treatment) $_{1, 11}$ = 22.98, $p < .0006$; hippocampus: F(treatment) $_{1, 11}$ = 5.31, $p < .042$; hypothalamus F(treatment) $_{1, 11}$ = 4.32, $p < .062$; see Fig. 2). In the frontal cortex, NA turnover (measured as the ratio of MOPEG to NA) was also affected (F(treatment)$_{1,11}$ = 5.6; $p = .037$) and was lower in LPS than in SAL brains. In the hippocampus, the lower NA level in LPS pigs was only found in a comparison between the left hemispheres ($p < .01$), as the right hemispheres did not differ between the two treatments (F(hemisphere * treatment)$_{1,11}$ = 4.05; $p = .07$). Furthermore, NA turnover in the hippocampus was not different between treatments, but there was an asymmetry in the MOPEG/NA ratio between the left (lowest turnover) and right hemisphere in LPS pigs (highest turnover, $p < .008$) that could not be detected in SAL pigs (F(hemisphere * treat) $_{1,11}$ = 7.32; $p = .021$). In the hypothalamus, NA turnover was not affected.

Dopamine did not seem to be affected by the LPS treatment, as no differences between treatments were found for dopamine or dopamine turnover (calculated as (DOPAC + HVA)/DA).

In the hippocampus, there was a tendency towards higher levels of serotonin in the right hemisphere of LPS pigs compared to the right hemisphere of SAL pigs (post hoc *t*-test, $p = .015$). We made four relevant comparisons: between each hemisphere and treatment and between hemispheres within treatment (the Bonferroni-corrected critical *p*-value is 0.0125). The difference between hemispheres for the LPS pigs was significant ($p = .004$) (F(treatment * hemisphere)$_{1, 11}$ = 9.63; p = .01, analysis on box-cox transformed data). The hippocampal 5-HIAA/5-HT ratio showed trends in the same direction as the 5-HT results: $p = .031$ for the comparisons between hemispheres in the LPS groups, and $p = .095$ for the comparison between right hemispheres for SAL and LPS pigs (F(treatment * hemisphere)$_{1, 11}$ = 4.19; $p = .065$). Serotonin and serotonin turnover in the frontal cortex and hypothalamus were not affected by treatment.

3.4. Cortisol

Cortisol increased after injection and was significantly higher in the LPS than in the SAL group at 1, 2, 3 and 4 h ($p < .0001$ for all time points; F(time * treatment)$_{10, 110}$ = 14.05; $p < .0001$; analysis was done on box-cox transformed values). The timing of the peak varied between pigs and was at 2, 3 or 4 h after injection. The cortisol increase was seen in all LPS pigs and no SAL pigs (Fig. 2A).

3.5. Haematology and flow-cytometry

In haematological analysis, absolute numbers of peripheral blood mononuclear cells (PBMCs) increased following LPS injection, significant at both 48 ($p = 0,0011$, Bonferroni-corrected critical *p*-value is 0,0125) and 72 h ($p = 0,0008$, Bonferroni-corrected critical *p*-value as above) post-injection (F(time * treatment)$_{3, 32}$ = 5,09; $p = .0054$) (Fig. 2C). Total monocytes, neutrophils, eosinophils and basophils displayed no significant changes after injection. Cellular subsets were measured in multi-colour flow cytometric immunophenotyping. To detect which leukocyte subset contributed to this fluctuation, we calculated the absolute cellular subset numbers by combining haematology and immunophenotyping data as stated in the materials and methods section. In this manner, we found that B-cells (CD21 + /CD3 −) constituted the most prominently responding lymphocyte subset, significantly elevated at 48 h ($p = 0,0067$, Bonferroni-corrected critical *p*-value as above) (Fig. 2C) (F(time * treatment)$_{3, 31}$ = 3,64; $p = .023$). T-cells (CD3 + /CD21 −) did not account for a significant rise (Fig. 2C). Natural killer (NK) cells (CD3 − /CD8 + /NKp46 − , CD3 − /CD8 + /NKp46 + and CD3 − /CD8 − /NKp46 + [51] tended to be higher in LPS pigs at 72 h ($p = 0,027$, Bonferroni-corrected critical *p*-value as above) (F(time * treatment)$_{3, 31}$ = 14,9; $p < .00001$). The expression of the surface activation markers CD25 and CD44 were measured on each subset of NK cells, but there was no measurable increase detected in either case (not shown). Similarly, intracellular perforin content was

not significantly changed in total NK cells or any of the subsets at the measured time points following LPS injection (not shown). Monocytes were analysed on the level of two previously defined subsets: CD14^{bright}/CD163-and CD14^{dim}/CD163+ (Fairbairn et al. [61]). The former monocyte subset showed an early drop, significant at 24 h ($p = 0.009$, Bonferroni-corrected critical p-value as above), and then stabilised at the initial level (F(time ∗ treatment)$_{3, 31}$ = 7,6; $p = .0006$) (Fig. 2C). In contrast, CD14^{dim}/CD163+ monocytes increased at a later time point, significantly at 72 h post-injection (p = 0,002, Bonferroni-

corrected critical p-value as above; (F(time ∗ treatment)$_{3, 31}$ = 9,7; $p < .0001$)).

3.6. Food intake

The LPS group significantly decreased their food intake compared to the SAL group during the first 24 h after injection (F $_{3,33}$ = 4,03; $p = .01$; post hoc t-test with a p(critical) of 0.0125: $p < .0001$; LPS: average ± sd intake of 0.8 ± 0.4 kg/pig/24 h; Saline: average intake
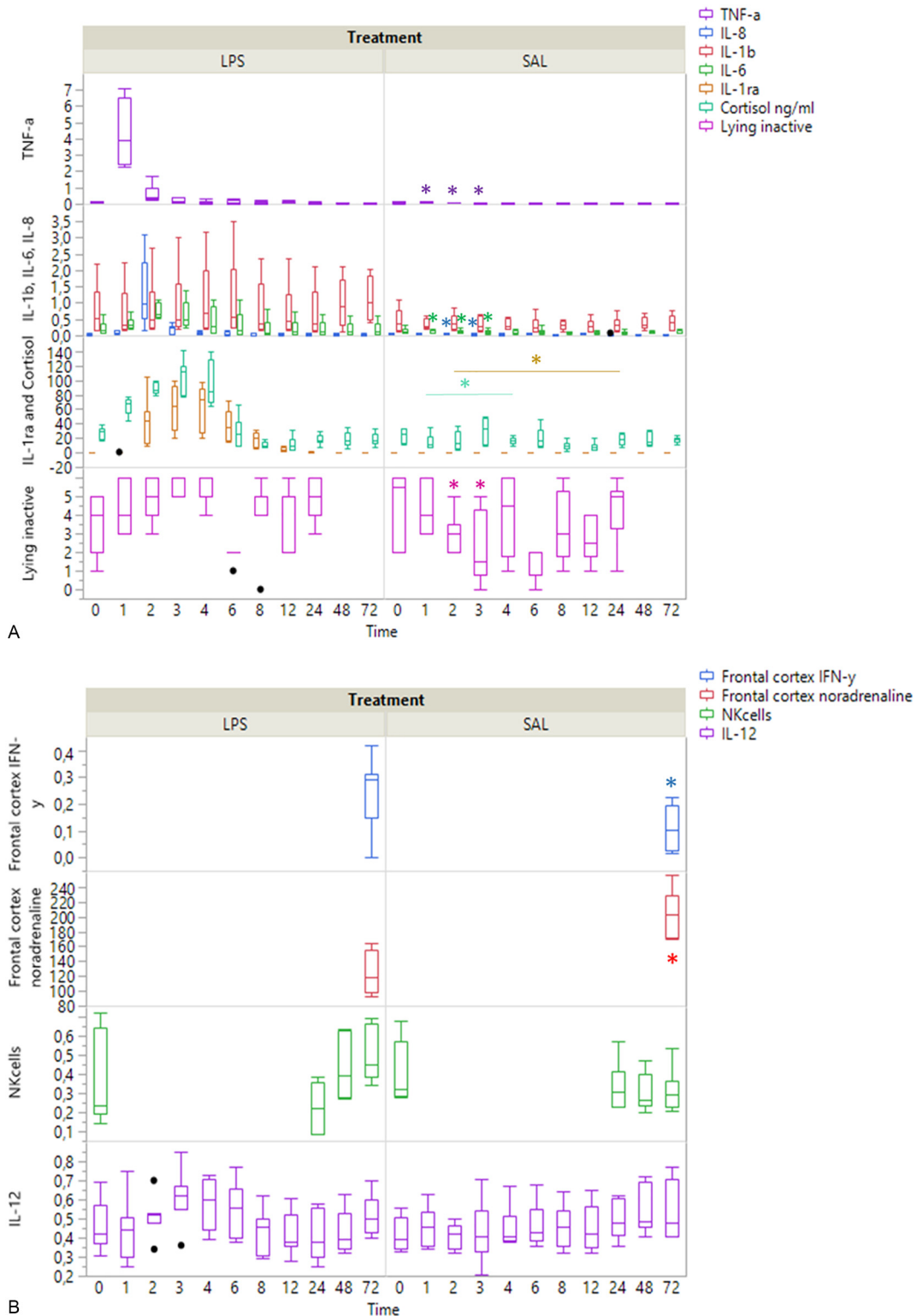
Fig. 2. Outlier box plots of selected behavioral and physiological variables plotted against time (0–72 h relative to injection) for LPS- (left panel) and SAL (right panel) pigs. Significant differences between the LPS and saline group are marked with an asterisk over the saline group. Outliers are marked as black dots. Units of measurements are given in the figure caption. A. Lying inactive, IL-1ra and cortisol levels and IL-1β, IL-6, IL-8 and TNFα levels in LPS (left panel) and SAL (right panel) pigs at baseline (0) and up to 72 h after LPS injection. The concentrations of serum cytokines and cortisol are given as ng ml^{-1}. The behavior is given as number of scans with the indicated behavior. Significant differences between the LPS and saline group are marked with an asterisk over the saline group. In the case of longer-lasting differences between the groups, a line marks all the time points at which the post-hoc comparison between the groups was significant. For *p*-values and test statistics, see the Results section. B. Frontal cortex IFN-γ (ng mg^{-1}) and noradrenaline (ng g^{-1}), NK cells (absolute counts (×10ˆ9/l)) and IL12 (ng ml^{-1}) (in blood or serum respectively) plotted against time for LPS- (left panel) and SAL (right panel) pigs at baseline (0) and up to 72 h after LPS injection. Significant differences between the LPS and saline group are marked with an asterisk over the saline group. In the case of longer-lasting differences between the groups, a line marks all the time points at which the post-hoc comparison between the groups were significant. For *p*-values and test statistics, see the Results section. C. Absolute counts (×10ˆ9/l) of T cells, B cells, classical monocytes, intermediary monocytes, and peripheral blood mononuclear cells plotted against time for LPS- (left panel) and SAL (right panel) pigs. See materials and methods section for the calculation of absolute counts. Significant differences between the LPS and saline group are marked with an asterisk over the saline group. In the case of longer-lasting differences between the groups, a line marks all the time points at which the post-hoc comparison between the groups was significant. For *p*-values and test statistics, see the Results section.

of 1.4 ± 0.4 kg/pig/24 h). However, the food intake of the LPS group during the first 24 h after injection was not significantly lower than baseline (*p* = .05 for the comparison with the baseline for the LPS group: 1.1 ± 0.2 kg/pig/24 h). The SAL pigs tended to increase their food intake in the first 24 h after injection (*p* = .02 for the comparison with the SAL baseline). At 48 and 72 h, the food intake of the two groups did not differ (from 24 to 48 h: LPS: average ± sd intake of 1.3 ± 0.3 kg/pig/24 h; Saline: 1.4 ± 0.1 kg/pig/24 h. From 48 to 72 h: LPS: average ± sd intake of 1.2 ± 0.3 kg/pig/24 h; Saline: average intake of 1.2 ± 0.2 kg/pig/24 h.)

3.7. Temperature

Skin temperature was not influenced by the LPS injection. However, surface recording of temperature, as attempted here, is not very reliable. Therefore, these results should be interpreted with caution.

3.8. Time budgets

The animals spent most of their time lying inactive. Active behavior, mostly in the form of exploration, was primarily seen in the later hours of the first 5-h observation slot.

The injection of LPS caused differences within sister pairs during hours 2 and 3 (between-group comparisons: *p* < .1 at hour 2 and *p* < .05 at hour 3), when LPS pigs were less active and slept more compared to SAL pigs. Changes appeared to diminish during hour 4 (Fig. 3).

Changes in time budgets compared to pre-injection levels were evaluated separately in SAL and LPS pigs. Significant differences were present in SAL pigs at only a few distinct hours, whereas LPS pigs showed decreases in ACTIVE and increases in SLEEP in hours 2–5 post-injection (Fig. 3). For ALE-INA (alert but inactive), a few significant results were seen for both treatments, but no pattern could be recognised (data not shown).

3.9. Synchronisation of activity

The activity of the animals appeared to be fairly synchronised, as judged by the general level of 4.5–5.5 synchronised scans out of 6 scans/h over most of the experimental days (Fig. 4). Synchronisation appeared to be on a slightly higher level in the afternoon hours compared to the hours before noon. The level of synchronisation did not change significantly when compared to the pre-injection level during the experiment, although a clear numerical decrease was evident 2–3 h post-injection.

3.10. Correlations between selected physiological and behavioral variables

Inactivity in the LPS pigs (measured as number of scans in which the pigs were scored as lying inactive) showed a significant and positive correlation with TNF-α at 2 (Spearman's rho 0.92; *p* = .004) and 3 h

(Spearman's rho 0.87; *p* = .01) after LPS injection. Within the saline group, there was no positive correlations, but a negative relationship between IL-8 (Spearman's rho −0.9; *p* = .04) and IL-1β (spearman's rho −1.0; *p* = .0001) and lying inactive at 4 h after injection.

4. Discussion

To our knowledge, this is the first experiment measuring 13 cytokines in blood and cytokine and monoamine levels in brain tissue after LPS injection in pigs. Our findings confirm and expand results from previous research on the effect of LPS on pig behavior and physiology [52]. The classical pro-inflammatory cytokines TNF-α and IL-6 peaked at hours 1 and 2 post-injection, respectively. IL-1ra showed the strongest response to LPS, which lasted for almost 48 *h*. IL-8 (also called chemokine CXCL8) peaked at hour 2. Cytokines IFN-γ, IL-1α and -β, IL-2, IL-4, IL-6, IL-10, IL-12 and IL-18 were not significantly affected but were present in measurable quantities in > 60% of the samples. However, as mentioned in the materials and methods section, though they did not reach statistical significance, some of these cytokines may still have exerted a biologically important effect in the LPS pigs (discussed further below). Cortisol was higher in LPS pigs from 1 to 4 h after injection. CRP was not measured as frequently as cytokines and cortisol but was increased at 12 and 24 h post-injection. Food intake dropped during the first 24 h, and the pigs showed the commonly reported lethargy that disappeared by 6 h post-injection. This relatively short duration of overt sickness is similar to that reported in several studies on the effect of low doses of LPS in healthy human subjects [53–55]. Skin temperature was not influenced by the injection. Seventy-two hours after injection, all behavioral signs of sickness were gone, and blood values were back within the pre-injection range. However, brain noradrenaline levels in LPS pigs were considerably lower in all three brain areas investigated (hippocampus, hypothalamus and frontal cortex). We only had enough measurable values to allow analysis for GM-CSF, IFN-γ, IL-1α, IL-2, IL-8 and IL-18, but not for IL-1ra, IL-4, IL-6, IL-10, IL-12 and TNF-α. IFN-γ, a cytokine that did not seem to be influenced by LPS when measured in blood, was found in higher levels in the frontal cortex of LPS-treated pigs than in saline-treated pigs. It should be noted that the limited sample size in this experiment—chosen due to cost, practical considerations and the severity of the treatment—would not allow us to detect small differences between the treatment groups. For the main pro-inflammatory cytokines, the sample size used in this and many other studies of LPS effects is sufficient because the effects of LPS are so strong, but more subtle changes would go undetected. As the main figures and tables in the supplementary materials show, several of the cytokines had considerable baseline variation. This could be caused by a reaction to the catheter as shown in calves for IL-6 [35]. Data on the same 13 cytokines collected from clinically healthy uncastrated boars indicate lower variation [56]. However, there could also be an effect of age and gender and differences in housing conditions, so the validity of direct comparison with the previous study is therefore questionable.
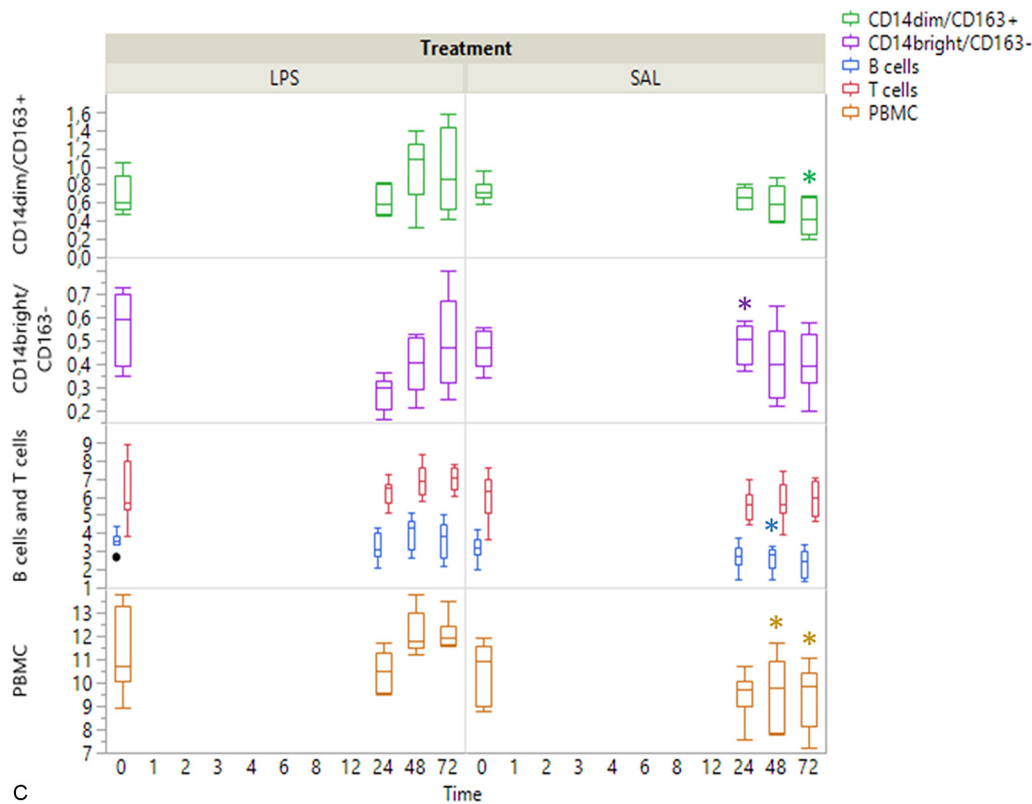
Fig. 2. (*continued*)

Table 4

Monoamine concentrations (ng g^{-1}) in the frontal cortex, hippocampus and hypothalamus of LPS and SAL pigs 72 h after LPS injection. Hemisphere values are shown only for noradrenaline and serotonin in the hippocampus. Between-group differences (LPS vs SAL) at p < critical value are marked in red. Within-group differences (hemispheric differences within treatment) at p < critical value are marked in blue.

Brain area	Treatment					
	LPS			Saline		
	Noradrenaline	Dopamine	Serotonin	Noradrenaline	Dopamine	Serotonin
Frontal cortex	124.1 (30.8)	9.5 (4.3)	82.1 (14.8)	204.2 (52.7)	11.9 (8.9)	69.0 (26.0)
Hippocampus	147.6 (71.3)	6.2 (2.9)	64.7 (45.5)	216.8 (43.3)	6.2 (2.8)	52.3 (30.6)
	Left: 123.6 (55)		Left: 35.9 (7.6)	Left: 221.3 (48)		Left: 57.8 (34.2)
	Right: 171.6 (82)		Right: 93.6 (50)	Right: 212.4 (42)		Right: 46.8 (28.6)
Hypothalamus	1708.0 (816.2)	159.3 (50.9)	284.1 (131.9)	2714.2 (497.4)	193.6 (49.1)	297.1(132.0)

Our results for the three main pro-inflammatory cytokines interleukin 1β (IL-1β), tumour necrosis factor α (TNF-α) and interleukin 6 (IL-6) largely confirm the findings of earlier experiments [30, 33, 52]. We did not see a significant increase in IL-1β, but that was most likely due to excessive baseline variation. The shape of the curve was quite similar to that found by others, with a slow increase and a lack of a distinct peak. When comparing effects of LPS in pigs between studies, the dose and route of administration must be taken into account, as well as the serotype of *E. coli* that the LPS was extracted from, as LPS extracted from different serotypes of *E. coli* differ in potency. We used LPS from *E. coli* O111:B4. This serotype is commonly used in LPS experiments in pigs, and all papers we compare our results to also used O111:B4 unless otherwise mentioned. Historically, high doses of LPS were used, e.g. 25 µg kg^{-1} [30, 33] and 75 µg kg^{-1} [57] (in these three studies, LPS was administered intravenously), and 150 µg kg^{-1} IP [58]. However, de Groot et al. (2007) [59] reported that 4 µg kg^{-1} induced vomiting, which they deemed unacceptable, and 1 µg kg^{-1} gave a variable response. Two µg kg^{-1} induced a clear cortisol increase and behavioral response [28]. Based on this and on results from a pilot

experiment, we chose our dose of 1.5 µg kg^{-1}. The absolute concentrations of TNF-α and IL-6 are considerably higher in the studies using 25 µg kg^{-1} or more than in our results, whereas IL-1β seems to be less affected by dose [33, 57, 58]. Our results are therefore in line with those of de Groot et al. [59]: A dose below 4 µg kg^{-1} is sufficient to produce a reliable response.

Aside from TNF-α and IL-6, only IL-8 and IL-1ra increased significantly in the LPS pigs compared to the control animals. For both IL-8 and IL-1ra, the peak was detected at 2 h post-injection, and the baseline variation was low, with negligible levels before injection and throughout the experiment for SAL pigs. IL-8, also called chemokine CXCL8, is particularly important in aiding migration of neutrophils to extracellular cites. IL-1ra is an anti-inflammatory cytokine that must be present in considerably higher concentrations than IL-1β to exert its effect. Thus, all pigs seemed to have a well-functioning anti-inflammatory response in the form of IL-1ra and cortisol. Cortisol increased from an average baseline of 27 ng ml^{-1} to a peak of 106 ng ml^{-1} at 3 h post-injection. This peak is lower than the concentrations reported in previous studies in which the doses of LPS were
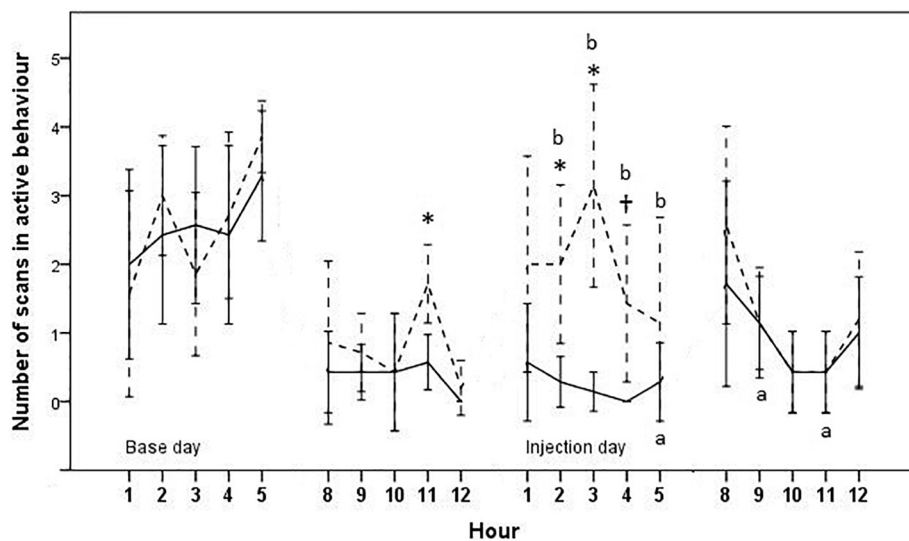
Fig. 3. The average number of scans (± 2 SE) in active behaviors on BASE day (left) and the day of LPS injection (INJ, right). The dashed line represents SAL and the uninterrupted line LPS pigs. Hours are given in relation to the time of LPS injection. Treatment effects (LPS vs SAL) are denoted with * ($p < .05$) and † ($p < .1$) and day effects (BASE as compared to INJ within treatment) with [a] ($p < .05$) for LPS and [b] ($p < .05$) for SAL.
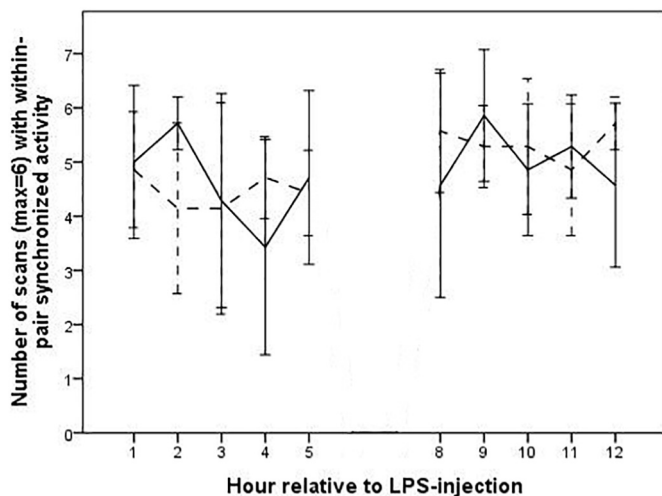

Fig. 4. Within-pair synchronisation of activity on the baseline (dashed line) and LPS injection day (uninterrupted line). Error bars represent ± SD.

higher [30, 31, 33, 57]. The baseline value is considerably lower than baselines reported from sampling by manual restraint and jugular puncture, and similar to the baseline reported when using automated sampling from in-dwelling catheters [60], highlighting how much sampling technique can influence physiological measurements in pigs.

Monocytes in pigs can be divided into subsets grossly similar to humans, whereby CD14^{bright}/CD163− monocytes resemble 'classical' human monocytes and CD14^{dim}/CD163+ resemble 'intermediate', possibly also incorporating 'non-classical' monocytes [61]. The drop in CD14^{bright}/CD163- monocytes observed here 24 h post-injection is in keeping with a human endotoxemia model. In this model, CD14+ monocytes dropped at 24 h [62], possibly due to rapid mobilisation of such cells into lymphoid tissue, inflamed tissue, or both, as observed in cows [63]. The later increase in CD14^{dim}/CD163+ monocytes could be due to replenishment from bone marrow, as well as maturation from classical monocytes [64]. In humans, corresponding cells were found to be an important LPS-responding source of several of the cytokines up-regulated in our experiment, including TNFα, IL-8 and IL-6 [65]. Due to their relatively long lifespan of 4–7 days in the human circulation [64], these monocytes are likely mediators of cytokine production several days beyond the initial acute phase. However, in the present study, the cellular cytokine sources were not specifically addressed. Finally, monocytes should be considered as direct mediators of

neuroimmunology; these cells can cross the blood-brain barrier in diseased individuals, where they may develop into microglia and exert a range of neurological effects [66]. Taken together, circulating monocytes respond phenotypically to an LPS challenge, highlighting them as candidate cells for further investigation.

We observed an increase in IFN-γ in the frontal cortex 72 h post-injection. An early source of IFN-γ is NK cells, which were numerically—though not significantly—increased in the circulation at 48 and 72 h compared to control pigs. During an endotoxin response, myeloid cells produce cytokines such as IL-12 and IL-18, which activate NK cells to produce large amounts of IFN-γ [67]. In the present study, LPS did not increase plasma IL-12, IL-18 or IFN-γ significantly. However, the endotoxin dosage given was low, and though circulating levels may be highly diluted, local effects of monocytes and NK cells in the brain could lie behind the IFN-γ increase in the frontal cortex. In mice injected with LPS, microglia were found to be a likely source of locally produced pro-inflammatory cytokines for several months after injection [68]. IFN-γ is, in fact, the only cytokine that has been measured in plasma after LPS injection in pigs, in addition to the three classical pro-inflammatory cytokines IL-1β, IL-6 and TNF-α [58]. LPS is a potent activator of the innate immune response, and in experiments that are terminated within 12 h, only effects on the innate immune system can be studied. It is nevertheless possible that LPS can induce adaptive immunity either through the antigenic activity of the O-antigen or possibly through induction of autoimmunity [67, 69–71]. Together with the significant elevation in B-cells (CD21+/CD3−) at 48 h, the numerical increase in NK cells and T cells may indicate the beginning of an adaptive immune response in the LPS-injected pigs.

Changes in behavior coincided with the most pronounced physiological effects of the injection, and at 2 and 3 h after the injection, inactivity correlated significantly with TNF-α concentration in plasma in the LPS but not the saline group. Daytime behavioral analyses indicated that LPS induced marked passiveness in the injected pigs from hours 2–5 post-injection, in accordance with previous experiments in pigs [27, 71–73]. The SAL sister appeared to respond to the changed behavior in her LPS-treated sibling by an initial increase in activity in hours 2–3 post-injection, compared to baseline. This was followed by a distinct decrease in activity in hours 4–5, corresponding to levels of activity recorded in the LPS-treated sister. SAL pigs appeared to have synchronised their activity with the LPS-treated pigs after a period of increased activity, which may have been restlessness in response to unexpected behavior by the control-treated sister. These observations were evident in the analysis of synchronisation of activity also, with a non-significant decrease in hours 2–3 followed by a return to baseline levels from hour 4 post-injection. However, it cannot be ruled out that

the passivity of SAL pigs simply shows that they were tired after a period of high activity. Observations were not conducted in hours 6–7 post-injection, but from this time point, the behavior of both groups appeared to be close to normal levels. It is possible that there was a rebound in the form of slightly increased activity, compared to baseline in both groups. No behavioral effects of LPS were evident in the second or third day post-injection. Importantly, the pigs were housed singly so as to avoid problems with the catheters. Thus, the social behaviors they could display were limited and tail biting could not occur. The effect of immune stimulation on social behaviour with more direct relations to tail biting is the focus of a follow-up paper (Munsterhjelm et al., in preparation). The aim of this paper was to detect immune-related candidate mechanisms that could lead to behavioral changes associated with tail biting. The increase in IFN-γ and the decrease in noradrenalin may both influence social behavior, and will be the focus of future experiments. They are discussed in more detail below.

Three days after LPS injection, when behavior and blood values were back within the baseline range, noradrenaline levels were lower in LPS pigs in all brain areas in which noradrenaline was measured, while the level of IFN-γ was higher in the frontal cortex of LPS-treated pigs than in the frontal cortex of saline-treated pigs. IFN-γ induces indoleamine 2,3-deoxygenase (IDO), which metabolises tryptophan to kynurenine [74–76]. The increase in metabolism of tryptophan to kynurenine rather than serotonin can have at least two consequences: there might not be enough tryptophan to produce adequate amounts of serotonin, there may be an increase in metabolites that can potentiate glutamatergic signalling through the NMDA receptor, or both may occur. Serotonin levels tended to be higher in the right hippocampus in LPS-treated pigs compared to SAL pigs, and turnover was not affected by the treatment, indicating that serotonergic signalling was not decreased due to immune stimulation. A similar finding was reported in mice, in which whole brain serotonin turnover was increased 28 h after LPS injection [75]. LPS injected pigs have been found to have an increase in plasma kynurenine to tryptophan ratio [77], but the study was terminated after less than 7 h after injection, so longer term effects could not be measured. As we measured neither kynurenine levels nor IDO activity, we cannot draw conclusions as to the importance of the observed increase in frontal cortex IFN-γ.

The pigs injected with LPS had markedly lower noradrenaline levels in their hypothalamus, left hippocampus and frontal cortex. Dopamine, which is synthesised along the same pathway as noradrenaline, was not affected. This selective effect of immune activation on noradrenaline levels while leaving dopamine levels unchanged corresponds well with earlier findings in rodents [78, 79]. Rats injected with an antigen or with cytokine-containing extracts showed a marked reduction in their hypothalamic noradrenaline content, and no change in dopamine levels [78]. LPS is known to stimulate noradrenergic neurons in locus coerulus (LC) within the first 30 min after injection, with a corresponding increase in noradrenaline levels [80, 81]. Intracerebroventricular administration of IL-1β and IL-6 also stimulate noradrenergic neurons [82]. This initial activation of noradrenergic pathways leads to a noradrenaline increase in the hypothalamus, which is important for the fever response normally observed after an LPS injection [83, 84]. It is possible that a negative feedback mechanism after this initial noradrenergic activation explains the reduced levels three days after immune stimulation. The reduced levels of noradrenaline we observed in the pigs may be important for their cognitive function, their emotional function, or both, as noradrenaline has a role in the regulation of mood and cortical function. Reduced levels of noradrenaline have been postulated as one of the processes underlying depression, as many antidepressants increase noradrenaline availability. However, the comparison between clinical observations in depressed human patients and animal models is complicated by the fact that while antidepressants may have an immediate effect on noradrenaline levels and on behavior in some animal models, they take considerably longer to have a therapeutic effect in human [85, 86]. This makes the inference that low

noradrenaline levels cause depression questionable. However, when improvement is measured as a gradual return of some (but not all) functions, the therapeutic lag is shorter and improved mood and reduced motor retardation may be seen within two weeks after initiating treatment with a selective noradrenaline reuptake inhibitor [87]. This supports the hypothesis that noradrenaline is important in the regulation of mood. Noradrenaline also aids in focusing on salient, meaningful stimuli [88], and is important for effortful attention [39]. How the effect of noradrenaline depletion in controlled tests of attention and working memory translates to the effects on social behaviour in pigs needs to be tested separately.

5. Conclusion

At 72 h post-injection, after the well-documented immediate changes in physiology brought about by LPS injection have subsided, our data suggest that brain noradrenaline levels are markedly reduced in the hippocampus, the hypothalamus and in the frontal cortex. Interferon-γ, a cytokine that induces activity in enzymes involved in tryptophan metabolism, was also increased in the frontal cortex at that time point. Noradrenaline may be important for effortful attention and for focusing on salient cues. Whether IFN-γ induced changes in neurotransmitter balance or noradrenaline depletion may influence the way in which pigs respond to social cues will be studied in group-housed pigs.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.physbeh.2018.07.013.

References

[1] P.K. Almond, G. Bilkei, Effects of oral vaccination against Lawsonia intracellullaris on growing-finishing pig's performance in a pig production unit with endemic porcine proliferative enteropathy (PPE), Deut. Tierärztl. Woch. 113 (2006) 232–235.

[2] M. Barnikol, Observations of tail biting (cannibalism) amongst fattening hogs and breeding pigs in connection with protein and mineral nourishment, Tierärztl. Umschau. 33 (1978) 540–546.

[3] R.B. D'Eath, G. Arnott, S.P. Turner, T. Jensen, H.P. Lahrmann, M.E. Busch, J.K. Niemi, A.B. Lawrence, P. Sandoe, Injurious tail biting in pigs: how can it be controlled in existing systems without tail docking? Animal 8 (2014) 1479–1497.

[4] C. Moinard, M. Mendl, C.J. Nicol, L.E. Green, A case control study of on-farm risk factors for tail biting in pigs, Appl. Anim. Behav. Sci. 81 (2003) 333–355.

[5] J.K. Niemi, A. Sinisalo, A.H.M. Valros, The Timing and Treatment of Tail Biting in Fattening Pigs, Nordic Association of Agricultural Scientists. SLU, Uppsala, Sweden, 2011 (p. 6).

[6] N.R. Taylor, R.M.A. Parker, M. Mendl, S.A. Edwards, D.C.J. Main, Prevalence of risk factors for tail biting on commercial farms and intervention strategies, Vet. J. 194 (2012) 77–83.

[7] R. Dantzer, J.C. O'Connor, G.G. Freund, R.W. Johnson, K.W. Kelley, From inflammation to sickness and depression: when the immune system subjugates the brain, Nat. Rev. Neurosci. 9 (2008) 46–57.

[8] B.L. Hart, Biological basis of the behavior of sick animals, Neurosci. Biobehav. Rev. 12 (1988) 123–137.

[9] L. Capuron, A. Ravaud, R. Dantzer, Early depressive symptoms in cancer patients receiving interleukin 2 and/or interferon alfa-2b therapy, J. Clin. Oncol. 18 (2000) 2143–2151.

[10] A. Constant, L. Castera, R. Dantzer, P. Couzigou, V. de Ledinghen, J. Demotes-Mainard, C. Henry, Mood alterations during interferon-alfa therapy in patients with chronic hepatitis C: evidence for an overlap between manic/hypomanic and depressive symptoms, J. Clin. Psychiat. 66 (2005) 1050–1057.

[11] K.D. Denicoff, D.R. Rubinow, M.Z. Papa, C. Simpson, C.A. Seipp, M.T. Lotze, A.E. Chang, D. Rosenstein, S.A. Rosenberg, The neuropsychiatric effects of treatment with interleukin-2 and lymphokine-activated killer-cells, Ann. Intern. Med. 107 (1987) 293–300.

[12] P.F. Renault, J.H. Hoofnagle, Y. Park, K.D. Mullen, M. Peters, B. Jones, V. Rustgi, A. Jones, Psychiatric complications of long-term interferon alfa therapy, Arch. Intern. Med. 147 (1987) 1577–1580.

[13] E.F. Coccaro, R. Lee, J.R. Fanning, D. Fuchs, M. Goiny, S. Erhardt, K. Christensen, L. Brundin, M. Coussons-Read, Tryptophan, kynurenine, and kynurenine metabolites: relationship to lifetime aggression and inflammatory markers in human subjects, Psychoneuroendocrinology 71 (2016) 189–196.

[14] K.M. Bouwman, D.M. Hawley, Sickness behaviour acting as an evolutionary trap? Male house finches preferentially feed near diseased conspecifics, Biol. Lett. 6 (2010) 462–465.

[15] L. Capuron, J.F. Gumnick, D.L. Musselman, D.H. Lawson, A. Reemsnyder, C.B. Nemeroff, A.H. Miller, Neurobehavioral effects of interferon-alpha in cancer patients: phenomenology and paroxetine responsiveness of symptom dimensions, Neuropsychopharmacology 26 (2002) 643–652.

[16] R.P.A. Gaykema, L.E. Goehler, Ascending caudal medullary catecholamine pathways drive sickness-induced deficits in exploratory behavior: brain substrates for fatigue? Brain Behav. Immun. 25 (2011) 443–460.

[17] A.K. Walker, D.P. Budac, S. Bisulco, A.W. Lee, R.A. Smith, B. Beenders, K.W. Kelley, R. Dantzer, NMDA receptor blockade by ketamine abrogates lipopolysaccharide-induced depressive-like behavior in C57BL/6J mice, Neuropsychopharmacology 38 (2013) 1609–1616.

[18] A. Valros, P. Palander, M. Heinonen, C. Munsterhjelm, E. Brunberg, L. Keeling, P. Piepponen, Evidence for a link between tail biting and central monoamine metabolism in pigs (Sus scrofa domestica), Physiol. Behav. 143 (2015) 151–157.

[19] W. Ursinus, C.G. Van Reenen, I. Reimert, J.E. Bolhuis, Tail biting in pigs: blood serotonin and fearfulness as pieces of the puzzle? PLoS One 9 (2014).

[20] A. Poltorak, X.L. He, I. Smirnova, M.Y. Liu, C. Van Huffel, X. Du, D. Birdwell, E. Alejos, M. Silva, C. Galanos, M. Freudenberg, P. Ricciardi-Castagnoli, B. Layton, B. Beutler, Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene, Science 282 (1998) 2085–2088.

[21] S.T. Qureshi, L. Lariviere, G. Leveque, S. Clermont, K.J. Moore, P. Gros, D. Malo, Endotoxin-tolerant mice have mutations in toll-like receptor 4 (Tlr4), J. Exp. Med. 189 (1999) 615–625.

[22] J. Schletter, H. Heine, A.J. Ulmer, E.T. Rietschel, Molecular mechanisms of endotoxin activity, Arch. Microbiol. 164 (1995) 383–389.

[23] D.M. Steel, A.S. Whitehead, The major acute-phase reactants- C-reactive protein, serum amyloid-P component and serum amyloid-a protein, Immunol. Today 15 (1994) 81–88.

[24] R.J. Ulevitch, P.S. Tobias, Receptor-dependent mechanisms of cell stimulation by bacterial endotoxin, Annu. Rev. Immunol. 13 (1995) 437–457.

[25] S.D. Wright, Toll, a new piece in the puzzle of innate immunity, J. Exp. Med. 189 (1999) 605–609.

[26] S.D. Wright, R.A. Ramos, P.S. Tobias, R.J. Ulevitch, J.C. Mathison, CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding-protein, Science 249 (1990) 1431–1433.

[27] S.L. Moya, L.A. Boyle, P.B. Lynch, S. Arkins, Surgical castration of pigs affects the behavioral response to a low-dose lipopolysaccharide (LPS) challenge after weaning, Appl. Anim. Behav. Sci. 112 (2008) 40–57.

[28] D.C. Lay, H.G. Kattesh, J.E. Cunnick, M.J. Daniels, G. Kranendonk, K.A. McMunn, M.J. Toscano, M.P. Roberts, Effect of prenatal stress on subsequent response to mixing stress and a lipopolysaccharide challenge in pigs, J. Anim. Sci. 89 (2011) 1787–1794.

[29] H. Wyns, S. Croubels, M. Vandekerckhove, K. Demeyere, P. De Backer, B.M. Goddeeris, E. Meyer, Multiplex analysis of pro-inflammatory cytokines in serum of *Actinobacillus pleuropneumoniae*-infected pigs, Res. Vet. Sci. 102 (2015) 45–48.

[30] J.A. Carroll, D.B. Carter, S.W. Korte, R.S. Prather, Evaluation of the acute phase response in cloned pigs following a lipopolysaccharide challenge, Domest. Anim. Endocrinol. 29 (2005) 564–572.

[31] J.A. Carroll, K.J. Touchette, R.L. Matteri, C.J. Dyer, G.L. Allee, Effect of spray-dried plasma and lipopolysaccharide exposure on weaned rigs: II. Effects on the hypothalamic-pituitary-adrenal axis of weaned pigs, J. Anim. Sci. 80 (2002) 502–509.

[32] R.F. Parrott, S.V. Vellucci, J.A. Goode, D.M. Lloyd, M.L. Forsling, Interrelated adrenocortical and neurohypophysial responses associated with fever in endotoxin-treated pigs, Am. J. Physiol.-Regulatory Integ. Comp. Physiol. 273 (1997) R1046–R1052.

[33] P.N. Williams, C.T. Collier, J.A. Carroll, T.H. Welsh, J.C. Laurenz, Temporal pattern and effect of sex on lipopolysaccharide-induced stress hormone and cytokine response in pigs, Domest. Anim. Endocrinol. 37 (2009) 139–147.

[34] L.T. van Eijk, M.J. Dorresteijn, P. Smits, J.G. van der Hoeven, M.G. Netea,

[35] P. Pickkers, Gender differences in the innate immune response and vascular reactivity following the administration of endotoxin to human volunteers, Crit. Care Med. 35 (2007) 1464–1469.

[35] A. Prohl, C.H. Ostermann, C.D. Rummell, J. Rothe, P. Reinholdl, Circulating and broncho-alveolar interleukin-6 in relation to body temperature in an experimental model of bovine *Chlamydia psittaci* infection, PLoS One 12 (2017) 14.

[36] P. Bard, V.B. Mountcastle, Suppression and facilitation of angry behavior by certain forebrain mechanisms, Science 107 (1948) 457.

[37] A.R. Damasio, On some functions of the human prefrontal cortex, in: J. Grafman, K.J. Holyoak, F. Boller (Eds.), Structure and Functions of the Human Prefrontal Cortex, New York Acad Sciences, New York, 1995, pp. 241–251.

[38] A.F.T. Arnsten, Stress signalling pathways that impair prefrontal cortex structure and function, Nat. Rev. Neurosci. 10 (2009) 410–422.

[39] J.A. Milstein, O. Lehmann, D.E.H. Theobald, J.W. Dalley, T.W. Robbins, Selective depletion of cortical noradrenaline by anti-dopamine beta-hydroxylase-saporin impairs attentional function and enhances the effects of guanfacine in the rat, Psychopharmacologia 190 (2007) 51–63.

[40] T.A. Niendam, A.R. Laird, K.L. Ray, Y.M. Dean, D.C. Glahn, C.S. Carter, Meta-analytic evidence for a superordinate cognitive control network subserving diverse executive functions, Cogn. Affect. Behav. Neurosci. 12 (2012) 241–268.

[41] D.A. Drachman, J. Arbit, Memory and hippocampal complex. 2. Is memory a multiple process, Arch. Neurol. 15 (1966) 52.

[42] J.L. McClelland, B.L. McNaughton, R.C. Oreilly, Why there are complementary learning-systems in the hippocampus and neocortex-insights from the successes and failures of connectionist models of lerning and memory, Psychol. Rev. 102 (1995) 419–457.

[43] B.L. McNaughton, F.P. Battaglia, O. Jensen, E.I. Moser, M.B. Moser, Path integration and the neural basis of the 'cognitive map', Nat. Rev. Neurosci. 7 (2006) 663–678.

[44] B.K. Anand, J.R. Brobeck, Hypothalamic control of food intake in rats and cats, Yale J. Biol. Med. 24 (1951) 123–140.

[45] T. Hori, T. Nakashima, H. Koga, T. Kiyohara, T. Inoue, Convergence of thermal, osmotic and cardiovascular signals on preoptic and anterior hypothalamic neurons in the rat, Brain Res. Bull. 20 (1988) 879–885.

[46] C.M. Hueston, T. Deak, The inflamed axis: the interaction between stress, hormones, and the expression of inflammatory-related genes within key structures comprising the hypothalamic-pituitary-adrenal axis, Physiol. Behav. 124 (2014) 77–91.

[47] R.E. Hulse, P.E. Kunkler, J.P. Fedynyshyn, R.P. Kraig, Optimization of multiplexed bead-based cytokine immunoassays for rat serum and brain tissue, J. Neurosci. Methods 136 (2004) 87–98.

[48] M. Kjelgaard-Hansen, A.L. Jensen, A.T. Kristensen, Evaluation of a commercially available human C-reactive protein (CRP) turbidometric immunoassay for determination of canine serum CRP concentration, Vet. Clin. Pathol. 32 (2003) 81–87.

[49] R.C. Antweiler, Evaluation of statistical treatments of left-censored environmental data using coincident uncensored data sets. II. Group comparisons, Environ. Sci. Technol. 49 (2015) 13439–13446.

[50] E.J. Breen, W. Tan, A. Khan, The statistical value of raw fluorescence signal in luminex xMAP based multiplex immunoassays, Sci. Rep. 6 (2016) 13.

[51] K.H. Mair, S.E. Essler, M. Patzl, A.K. Storset, A. Saalmuller, W. Gerner, NKp46 expression discriminates porcine NK cells with different functional properties, Eur. J. Immunol. 42 (2012) 1261–1271.

[52] D.M. Webel, B.N. Finck, D.H. Baker, R.W. Johnson, Time course of increased plasma cytokines, cortisol, and urea nitrogen in pigs following intraperitoneal injection of lipopolysaccharide, J. Anim. Sci. 75 (1997) 1514–1520.

[53] H. Engler, S. Benson, A. Wegner, I. Spreitzer, M. Schedlowski, S. Elsenbruch, Men and women differ in inflammatory and neuroendocrine responses to endotoxin but not in the severity of sickness symptoms, Brain Behav. Immun. 52 (2016) 18–26.

[54] J. Lasselin, S. Elsenbruch, M. Lekander, J. Axelsson, B. Karshikoff, J.S. Grigoleit, H. Engler, M. Schedlowski, S. Benson, Mood disturbance during experimental endotoxemia: predictors of state anxiety as a psychological component of sickness behavior, Brain Behav. Immun. 57 (2016) 30–37.

[55] A. Marraffa, M. Lekander, P. Solsjo, M.J. Olsson, J. Lasselin, J. Axelsson, Yawning, a thermoregulatory mechanism during fever? A study of yawning frequency and its predictors during experimentally induced sickness, Physiol. Behav. 182 (2017) 27–33.

[56] C. Munsterhjelm, J. Nordgreen, F. Aae, M. Heinonen, K. Olstad, T. Aasmundstad, A.M. Janczak, A. Valros, To be blamed or pitied? The effect of illness on social behavior, cytokine levels and feed intake in undocked boars, Physiol. Behav. 179 (2017) 298–307.

[57] J.W. Frank, J.A. Carroll, G.L. Allee, M.E. Zannelli, The effects of thermal environment and spray-dried plasma on the acute-phase response of pigs challenged with lipopolysaccharide, J. Anim. Sci. 81 (2003) 1166–1176.

[58] K.J. Touchette, J.A. Carroll, G.L. Allee, R.L. Matteri, C.J. Dyer, L.A. Beausang, M.E. Zannelli, Effect of spray-dried plasma and lipopolysaccharide exposure on weaned pigs: I. Effects on the immune axis of weaned pigs, J. Anim. Sci. 80 (2002) 494–501.

[59] J. de Groot, G. Kranendonk, M. Fillerup, H. Hopster, W. Boersma, D. Hodgson, K. van Reenen, M. Taverne, Response to LPS in female offspring from sows treated with cortisol during pregnancy, Physiol. Behav. 90 (2007) 612–618.

[60] J.N. Marchant-Forde, D.L. Matthews, R. Poletto, R.R. McCain, D.D. Mann, R.T. Degraw, J.M. Hampsch, S. Peters, G.T. Knipp, C.B. Kissinger, Plasma cortisol and noradrenalin concentrations in pigs: automated sampling of freely moving pigs housed in the PigTurn® versus manually sampled and restrained pigs, Anim. Welf. 21 (2012) 197–205.

[61] L. Fairbairn, R. Kapetanovic, D. Beraldi, D.P. Sester, C.K. Tuggle, A.L. Archibald, D.A. Hume, Comparative analysis of monocyte subsets in the pig, J. Immunol. 190

(2013) 6389–6396.

[62] T. Tak, R. van Groenendael, P. Pickkers, L. Keonderman, Monocyte subsets are differentially lost from the circulation during acute inflammation induced by human experimental endotoxemia, J. Innate Immun. 9 (2017) 464–474.

[63] H. Lund, P. Boysen, C.P. Åkesson, A.M. Lewandowska-Sabat, A.K. Storset, Transient migration of large numbers of cD14++ cD16+ monocytes to the draining lymph node after onset of inflammation, Front. Immunol. 7 (2016), https://doi.org/10.3389/fimmu.2016.00322.

[64] A.A. Patel, Y. Zhang, J.N. Fullerton, L. Boelen, A. Rongvaux, A.A. Maini, V. Bigley, R.A. Flavell, D.W. Gilroy, B. Asquith, D. Macallan, S. Yona, The fate and lifespan of human monocyte subsets in steady state and systemic inflammation, J. Exp. Med. 214 (2017) 1913–1923.

[65] B. Thaler, P.J. Hohensinner, K.A. Krychtiuk, P. Matzneller, L. Koller, M. Brekalo, G. Maurer, K. Huber, M. Zeitlinger, B. Jilma, J. Wojta, W.S. Speidl, Differential in vivo activation of monocyte subsets during lowgrade inflammation through experimental endotoxemia in humans, Sci. Rep. 6 (2016) 30162.

[66] M. Prinz, J. Priller, S.S. Sisodia, R.M. Ransohoff, Heterogeneity of CNS myeloid cells and their roles in neurodegeneration, Nat. Neurosci. 14 (2011) 1227–1235.

[67] Y. Guo, N.K. Patil, L. Luan, J.K. Bohannon, E.R. Sherwood, The biology of natural killer cells during sepsis, Immunology (2017), https://doi.org/10.1111/imm.12854.

[68] L.Y. Qin, X.F. Wu, M.L. Block, Y.X. Liu, G.R. Breese, J.S. Hong, D.J. Knapp, F.T. Crews, Systemic LPS causes chronic neuroinflammation and progressive neurodegeneration, Glia 55 (2007) 453–462.

[69] D. Damotte, C. Goulvestre, J. Charreire, C. Carnaud, LPS and Freund's adjuvant initiate different inflammatory circuits in experimental autoimmune thyroiditis, Eur. Cyt. Network 14 (2003) 52–59.

[70] Y. Liu, H. Yin, M. Zhao, Q. Lu, TLR2 and TLR4 in autoimmune diseases: a comprehensive review, Clin. Rev. Allergy Immunol. 47 (2014) 136–147.

[71] M. Robinet, S. Maillard, M.A. Cron, S. Berrih-Aknin, R. Le Panse, Review on toll-like receptor activation in myasthenia gravis: application to the development of new experimental models, Clin. Rev. Allergy Immunol. 52 (2017) 133–147.

[72] R.W. Johnson, E. von Borell, Lipopolysaccharide-induced sickness behavior in pigs is inhibited by pretreatment with indomethacin, J. Anim. Sci. 72 (1994) 309–314.

[73] E.J. Warren, B.N. Finck, S. Arkins, K.W. Kelley, R.W. Scamurra, M.P. Murtaugh, R.W. Johnson, Coincidental changes in behavior and plasma cortisol in unrestrained pigs after intracerebroventricular injection of tumor necrosis factor-alpha, Endocrinology 138 (1997) 2365–2371.

[74] S. Fujigaki, K. Saito, K. Sekikawa, S. Tone, O. Takikawa, H. Fujii, H. Wada, A. Noma, M. Seishima, Lipopolysaccharide induction of indoleamine 2,3-dioxygenase is mediated dominantly by an IFN-gamma-independent mechanism, Eur. J. Immunol. 31 (2001) 2313–2318.

[75] J.C. O'Connor, M.A. Lawson, C. Andre, M. Moreau, J. Lestage, N. Castanon, K.W. Kelley, R. Dantzer, Lipopolysaccharide-induced depressive-like behavior is mediated by indoleamine 2,3-dioxygenase activation in mice, Mol. Psychiatry 14 (2009) 511–522.

[76] O. Takikawa, Y. Tagawa, Y. Iwakura, R. Yoshida, R.J.W. Truscott, Interferon-gamma-dependent/independent expression of indoleamine 2,3-dioxygenase. Studies with interferon-gamma-knockout mice, in: G. Huether, W. Kochen, T.J. Simat, H. Steinhart (Eds.), Tryptophan, Serotonin and Melatonin: Basic Aspects and Applications, vol. 467, Kluwer Academic/Plenum Publishing, New York, 1999, pp. 553–557.

[77] T. Tesch, E. Bannert, J. Kluess, J. Frahm, L. Huether, S. Kersten, G. Breves, L. Renner, S. Kahlert, H.J. Roethkoetter, S. Daenicke, Relationships between body temperatures and inflammation indicators under physiological and pathophysiological conditions in pigs exposed to systemic lipopolysaccharide and dietary deoxynivalenol, J. Anim. Physiol. Anim. Nutr. 102 (2018) 241–251.

[78] H. Besedovsky, A. Delrey, E. Sorkin, M. Daprada, R. Burri, C. Honegger, The immune-response evokes changes in brain noradrenergic neurons, Science 221 (1983) 564–566.

[79] M.I. Masana, M.P. Heyes, I.N. Mefford, Indomethacin prevents increased catecholamine turnover in rat brain following systemic endotoxin challenge, Prog. Neuro-Psychopharm. Biol. Psychiat. 14 (1990) 609–621.

[80] A.C.E. Linthorst, C. Flachskamm, F. Holsboer, J. Reul, Activation of serotonergic and noradrenergic neurotransmission in the rat hippocampus after peripheral administration of bacterial endotoxin: involvement of the cyclo-oxygenase pathway, Neuroscience 72 (1996) 989–997.

[81] M. Sekio, K. Seki, Lipopolysaccharide-induced depressive-like behavior is associated with alpha(1)-adrenoceptor dependent downregulation of the membrane GluR1 subunit in the mouse medial prefrontal cortex and ventral tegmental area, Int. J. Neuropsychopharmacol. 18 (2015) 12.

[82] N. Kurosawa, K. Shimizu, K. Seki, The development of depression-like behavior is consolidated by IL-6-induced activation of locus coeruleus neurons and IL-1 beta-induced elevated leptin levels in mice, Psychopharmacologia 233 (2016) 1725–1737.

[83] C. Feleder, V. Perlik, C.M. Blatteis, Preoptic norepinephrine mediates the febrile response of Guinea pigs to lipopolysaccharide, Am. J. Physiol. Reg. Integr. Comp. Physiol. 293 (2007) R1135–R1143.

[84] J. Roth, C.M. Blatteis, Mechanisms of fever production and lysis: lessons from experimental LPS fever, Compr. Physiol. 4 (2014) 1563–1604.

[85] A. Frazer, D.A. Morilak, What should animal models of depression model? Neurosci. Biobehav. Rev. 29 (2005) 515–523.

[86] D.A. Morilak, A. Frazer, Antidepressants and brain monoaminergic systems: a dimensional approach to understanding their behavioral effects in depression and anxiety disorders, Int. J. Neuropsychopharm. 7 (2004) 193–218.

[87] M.M. Katz, J.L. Tekell, C.L. Bowden, S. Brannan, J.P. Houston, N. Berman, A. Frazer, Onset and early behavioral effects of pharmacologically different antidepressants and placebo in depression, Neuropsychopharmacology 29 (2004) 566–579.

[88] G. Aston-Jones, J. Rajkowski, J. Cohen, Role of locus coeruleus in attention and behavioral flexibility, Biol. Psychiatry 46 (1999) 1309–1320.