Norwegian University of Life Sciences
Faculty of Veterinary Medicine
Department of Paraclinical Sciences

Philosophiae Doctor (PhD)
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Effects of a controlled immune activation on social behaviour, neurophysiology and the acute phase response in pigs

Effekten av en kontrollert immun-aktivering på sosialadferd, nevrofysiologi og akuttfaseresponsen hos gris

Christina Maria Veit

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Table of Contents

Abbreviations

5-HT	5-Hydroxytryptamin/serotonin
ADA	Adenosine deaminase
APP	Acute phase protein
BBB	Blood-brain-barrier
CNS	Central nervous system
COX	Cyclooxygenase
CRP	C-reactive protein
DA	Dopamine
FST	Forced swim test
HP	Haptoglobin
HPA	Hypothalamic-pituitary-adrenal
IDO	Indoleamine 2,3-dioxygenase
IFN	Interferon
IL	Interleukin
KL	Ketoprofen-lipopolysaccharide injected pigs
KS	Ketoprofen-saline injected pigs
KYN	Kynurenine
LPS	Lipopolysaccharide
MADRS	Montgomery–Asberg Depression Rating Scale
MAOI	Monoamine oxidase inhibitors
NA	Noradrenaline
NF-kB	Nuclear factor kappa B
NRI	Selective noradrenaline reuptake inhibitor
NSAID	Nonsteroidal anti-inflammatory drug
OD	Optical density
PAMP	Pathogen-associated molecular pattern
PGE_2	Prostaglandin synthesis

PIG-MAP	Pig-major acute phase protein
SAA	Serum amyloid A
SL	Saline-lipopolysaccharide injected pigs
SNA	Social network analysis
SS	Saline-saline injected pigs
SSRI	Selective serotonin reuptake inhibitor
TCA	Tricyclic antidepressant
TDO	Tryptophan-dioxygenase
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TRY	Tryptophan
TST	Tail suspension test

Summary

Damaging behaviours, such as tail and ear biting, are important animal welfare challenges in commercial pig production. Poor health is a risk factor for damaging behaviours but the mechanisms behind this link remain unknown. We know from studies in humans and rodents that the immune system can influence social motivation, and this could have potentially dire consequences for group-housed production animals, such as pigs. The use of salivary biomarkers, such as proteins of the acute phase response, is a non-invasive method for early detection of diseases on farm. Lipopolysaccharide (LPS) is an important antigenic structure of Gram-negative bacteria and can be used to model aspects of sickness. Recent studies have shown that LPS-injected pigs perform more tail- and ear- directed behaviour compared to saline-injected pigs and suggest pro-inflammatory cytokines may play a role in these behaviours. The overall aim of this thesis was to understand how immune activation influences brain physiology in pigs and how these physiological changes can drive changes in social behaviour. Cytokine activated signalling pathways that may be involved in inducing behavioural alterations were examined by using an LPS-model. In addition, the effect of the nonsteroidal anti-inflammatory drug ketoprofen on the physiological and behavioural effects of LPS was investigated. Fifty-two female pigs (11-12 weeks of age, housed in groups of six with four treatment pigs and two companion pigs per group) were allocated to one of the four treatments, comprising two injections of the following substance combinations: saline-saline, saline-LPS, ketoprofen-saline, and ketoprofen-LPS. The experiment lasted for 72 hours. Activity was scan sampled in the first six hours after injection. Social behaviour with focus on damaging behaviour was observed continuously at specific time intervals one day before and two

days after injection. Saliva samples were taken at baseline and at four timepoints after the injections and analysed for cortisol, haptoglobin and adenosine deaminase. Blood samples were taken for tryptophan and kynurenine analysis at baseline and at 72 hours after the injections. Subsequently, the pigs were humanely killed, and samples of frontal cortex, hippocampus, hypothalamus and brain stem were taken and analysed for cytokines, tryptophan, kynurenine and monoamines. LPS activated the hypothalamic-pituitary-adrenal axis, triggered the acute-phase response and elicited behavioural signs of sickness within six hours after the challenge. Ketoprofen attenuated these effects. Central proinflammatory cytokines (IFN-γ, TNF-α, IL-18) were not affected by LPS at 72 h after the challenge in the brain regions collected. LPS depleted peripheral and central tryptophan. Dopamine concentrations in the hypothalamus of LPS-injected pigs were lower compared to saline-injected pigs. LPS-injected pigs had lower concentrations of serotonin in their hypothalamus and noradrenaline in their hippocampus than pigs that were pre-treated with ketoprofen. Thus, a controlled immune activation altered neurotransmitters and neuromodulators in the brain that are hypothesised to play an important role in the regulation of mood and behaviour. Changes in social interactions in response to a controlled immune activation were detected at both individual and group level. LPS affected the duration and the frequency of ear manipulations in the subsequent days after the challenge but had no effect on other behavioural patterns. LPS-injected pigs manipulated the ears of their pen mates significantly longer compared to saline-injected pigs and received less frequent ear manipulations by their pen mates two days after the challenge. Ketoprofen seemed not to have an impact on social behaviour. The time in relation to injection affected ear-directed behaviour and fighting. The ears of certain individuals in the pen were more frequently manipulated and fights were more evenly spread across all group members in the days after the challenge compared to baseline. There might be long-lasting effects on social behaviour both at individual and group level when even just one individual in a group becomes ill.

Norsk sammendrag

Skadelige adferder, slik som hale- og ørebiting, gir store dyrevelferdsutfordringer i svineproduksjonen. Dårlig helse er en risikofaktor for skadelig adferd, men mekanismene bak sammenhengen er ukjent. Fra klinisk litteratur på humansiden og fra gnagermodeller vet vi at immunsystemet kan påvirke sosial motivasjon, og dette fenomenet kan potensielt ha alvorlige negative konsekvenser for produksjonsdyr som er oppstallet i grupper på begrenset plass, slik som gris. Biomarkører i spytt, for eksempel akutt fase proteiner, kan brukes for å oppdage helseproblemer i svinebesetninger på et tidlig stadium. Lipopolysakkarid (LPS) er en viktig antigen-struktur på gram-negative bakterier og brukes for å modellere deler av immunresponsen ved sykdom. Griser som har vært injisert med LPS retter mer oppmerksomhet mot ørene og halene til sine artsfrender sammenlignet med kontrolldyr, og dette indikerer at pro-inflammatoriske cytokiner spiller en rolle i å utløse disse adferdene. Hovedmålet til denne avhandlingen var å forstå hvordan immunaktivering påvirker hjernefysiologien til gris, og hvordan de fysiologiske endringene kan påvirke sosialadferden til dyrene. Vi brukte LPS som immunstimulator og undersøkte signalveier i hjernen som påvirkes av cytokiner og som man tror kan stå bak adferdsendringer ved sykdom. Femtito unge purker (11-12 uker, oppstallet i grupper på seks med fire forsøksgris og to selskapsgris per gruppe) ble fordelt mellom fire behandlingsgrupper som hver fikk to injeksjoner i en av de følgende fire kombinasjoner: saltvann-saltvann, saltvann-LPS, ketoprofen-saltvann og ketoprofen-LPS. Forsøket varte i 72 timer. Grisenes aktivitet ble registrert de første seks timene etter injeksjonen. Sosialadferd med fokus på skadelig adferd ble observert en dag før og to dager etter injeksjonsdagen. Spyttprøver ble samlet inn før injeksjon og fire ganger etter injeksjon og analysert for

kortisol, haptoglobin og adenosin deaminase. Blodprøver ble tatt før injeksjon og 72 timer etter injeksjon og analysert for tryptofan og kynurenin. Deretter ble grisene humant avlivet, og hjerneprøver fra frontal cortex, hippocampus, hypothalamus og hjernestammen ble analysert for cytokiner, tryptofan, kynurenin og monoaminer. LPS aktiverte hypothalamus-hypofyse-binyrebark aksen, utløste en akutt-fase respons og fremkalte tegn på sykdom ila de første seks timene etter injeksjon. Ketoprofen hemmet disse effektene. LPS reduserte konsentrasjonen av tryptofan i plasma, og reduserte konsentrasjonen både av tryptofan og kynurenin i flere hjerneområder. Dopamin-nivået i hypothalamus var lavere hos griser som hadde fått LPS sammenlignet med gris som fikk saltvann. Griser som fikk LPS hadde lavere nivå av serotonin i hypothalamus og av noradrenalin i hippocampus sammenlignet med griser som også fikk ketoprofen. LPS endret altså nivåene av nevrotransmittorer i hjernen, og disse nevrotransmittorene kan ha en effekt på sinnsstemning og adferd. LPS hadde også en effekt på sosialadferd hos dyrene, både på individ- og gruppe-nivå. LPS påvirket frekvens og varighet av øre-manipulering i dagene etter injeksjon. Griser som hadde fått LPS manipulerte ørene til de andre i gruppen lengre enn de grisene som hadde fått saltvann, og fikk mindre manipulering av egne ører to dager etter LPS injeksjon. Ketoprofen så ikke ut til å påvirke sosialadferden. I dagene etter LPS injeksjon så vi at slåssing ble jevnere fordelt innad i gruppen enn før injeksjonen. Når bare en gris i en gruppe blir syk kan det skje adferdsendringer både hos individet, men også i hvordan gruppen fungerer som helhet.

List of papers

Paper I

The effect of LPS and ketoprofen on cytokines, brain monoamines, and social behaviour in group-housed pigs

Christina Veit, Andrew M. Janczak, Birgit Ranheim, Judit Vas, Anna Valros, Dale A. Sandercock, Petteri Piepponen, Daniela Dulgheriu, Janicke Nordgreen

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Paper II

Dynamics of salivary adenosine deaminase, haptoglobin, and cortisol in lipopolysaccharide-challenged growing pigs

Virpi Sali, Christina Veit, Anna Valros, Sami Junnikkala, Mari Heinonen, Janicke Nordgreen

Manuscript revised and resubmitted

Paper III

The use of social network analysis to describe the effect of immune activation on group dynamics in pigs

Christina Veit, Simone Foister, Anna Valros, Camilla Munsterhjelm, Dale A. Sandercock, Andrew M. Janczak, Birgit Ranheim, Janicke Nordgreen

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1 Introduction

I will start this chapter with introducing a description of pig behaviour pointing towards welfare problems that arise in commercial pig production and address the hypothesised link between poor health and damaging behaviour (1.1.). I will present behavioural alterations in response to inflammation (1.2.) and introduce an experimental model to study the effects of immune activation on behaviour (1.3.). I will elaborate on mechanisms through which cytokines can influence behaviour by presenting the immune-neural communication during inflammation (1.4.). Subsequently, I will describe the acute phase response (1.5.) and continue with a methodological approach on how to examine pigs' social interactions in depth (1.6.). I will end with pointing out knowledge gaps in the research field (1.7.) and stating the aims of my thesis (1.8.).

1.1 Pig behaviour, husbandry and welfare

The pig (Sus scrofa) is a gregarious and highly explorative species that forms hierarchically organized stable groups of several adults and their offspring (Jensen and Wood-Gush, 1984). Despite its domestication and selective breeding for meat production the behavioural repertoire remains similar to that of its ancestor, the wild boar (Stolba and Wood-Gush, 1989). In semi natural environments pigs spend 75 % of their daily activity with foraging-related behaviour (Stolba and Wood-Gush, 1989). Their explorative behaviour has been described as both extrinsic, i.e. to seek resources, and intrinsic, i.e. motivated by curiosity (Wood-Gush and Vestergaard, 1991). The most common way to keep pigs nowadays is in closed barns with slatted flooring. Space-restriction and barren environments limit their possibilities to perform explorative behaviour, which might then be redirected towards their pen mates. In the semi-

wild, pigs develop their strongest social relationships with littermates, mixing with non-littermates occurs gradually (Newberry and Wood-Gush, 1986) and aggression is limited to periods of feeding and mating (Stolba and Wood-Gush, 1989). However, management procedures in pig husbandry often do not adequately consider the social needs of the animals. Regrouping with unfamiliar conspecifics, which is typically performed several times in the lives of domestic pigs, disrupt established social structures and leads to higher levels of aggression (Spoolder et al., 2000).

Pig production went through a tremendous structural change in the past decades moving from many small farms to fewer bigger farms, which are highly specialized in certain production stages (breeding, farrowing, rearing, fattening). In parallel, genetic selection for rapid growth and high reproductive rate led to a constant increase in litter sizes and daily weight gains. After birth, piglets are subjected to mutilations such as castration, tail docking and teeth clipping and, in that way, "fitted" to the housing conditions they are kept in. Welfare problems can arise due to a mismatch between the pig's behavioural needs and its environment (D'Eath and Turner, 2009). Damaging behaviours, such as tail and ear biting, are associated with a wide range of potential risk factors and are major welfare challenges in commercial pig husbandry. They are defined as one pig taking the tail or the ear of another pig into its mouth and biting it, usually causing an avoidance reaction of the victim. The resulting damage can range from mild bite marks to parts of the tail or ear removed (Valros, 2018). In addition to a reduction of animal welfare through pain, suffering and injuries, the consequences of tail and ear biting also include economical losses due to reduced daily gain, extra veterinary, labour and material costs, increased mortality and carcass condemnations (Valros et al., 2004; Kritas and Morrison, 2007; Camerlink et al., 2012; Harley et al., 2014; D'Eath et al., 2016). Tail docking, which means surgically removing a part of the tail, reduces the risk for tail biting damage (Hunter et al., 1999), but does not solve the underlying

shortcomings of the environment of the pigs. In general, damaging behaviour is a multifactorial problem, where the combined impact of a range of extrinsic and intrinsic factors can trigger biting events when the coping abilities of an animal are overtaxed (Dippel and Schrader, 2016). Environment related factors such as lack of rooting material (Beattie et al., 1995; Petersen et al., 1995; Day et al., 2002; Van de Weerd et al., 2006; Zonderland et al., 2008; Telkänranta et al., 2014; Ursinus et al., 2014), poor climatic conditions (Hunter et al., 2001; Taylor et al., 2012; Scollo et al., 2016), high stocking density (Moinard et al., 2003; Munsterhjelm et al., 2015; Scollo et al., 2016; Grümpel et al., 2018), suboptimal nutrition (Van der Meer et al., 2017) and limited access to resources (Hunter et al., 2001; Moinard et al., 2003; Taylor et al., 2012) have been identified. In addition, pig related factors such as genetics (Breuer et al., 2003; Breuer et al., 2005; Sinisalo et al., 2012), sex (Hunter et al., 1999; Kritas and Morrison, 2004; Valros et al., 2004; Zonderland et al., 2010; Keeling et al., 2012) and reduced health status (Moinard et al., 2003; Taylor et al., 2012) were related to damaging behaviours.

Pigs are very social, but they are also kept in high densities, and under conditions, which are likely to stimulate their immune system (Scott et al., 2006; Pastorelli et al., 2012; Reimert et al., 2014; Van der Meer et al., 2016; Bacou et al., 2017). It has been suggested that cytokines, small proteins produced by immune cells to orchestrate the immune response, play a role in the development of damaging behaviours (reviewed by Nordgreen et al., 2020). Pigs diagnosed with respiratory diseases, tended to perform more ear and tail biting than controls in the days before they were diagnosed as sick (Munsterhjelm et al., 2017). In the same study, significant correlations between cytokines and social behaviour were found.

1.2 Behavioural alterations in response to inflammation

From human clinical reports and experiments, we know that activation of the innate immune system or administration of cytokines can lead to sickness behaviour which can trigger depression. Immune activation is also implicated in other forms of psychological pathologies. Patients undergoing cytokine immunotherapy with interferon-α (IFN-α) and/or interleukin-2 (IL-2) for treatment of cancer or hepatitis experienced neuropsychiatric side effects (Denicoff et al., 1987; Renault et al., 1987; Capuron et al., 2000; Constant et al., 2005). Treatment with IFN-α led to symptoms like irritability and short temper, extreme emotional lability, depression, and tearfulness (Renault et al., 1987). Patients treated with IL-2, scored higher on depression scales (Capuron et al., 2000). From rodent studies, it seems that pro-inflammatory cytokines cause changes in central neurotransmitter balance, which are necessary to cause behaviour and/or mood changes. Noradrenergic systems are markedly activated by IL-1 in the hypothalamus and the hippocampus of rats and mice as indicated by increased turnover rates (Dunn, 1988; Kabiersch et al., 1988; Shintani et al., 1993; Zalcman et al., 1994; Fleshner et al., 1995). IFN-α, IL-1, IL-2, IL-6 and tumour-necrosis factor-α (TNF-α) have been shown to activate serotonergic systems and/or dopaminergic systems in the murine hippocampus and the frontal cortex (Mohankumar et al., 1991; Shintani et al., 1993; Zalcman et al., 1994; Clement et al., 1997; De La Garza and Asnis, 2003). These neurotransmitters and their action in specific brain areas are hypothesised to play an important role in the regulation of mood and thereby behavioural expression in immune activated animals and humans.

Immune activation has been suggested as a major factor influencing social interactions in pigs, with outbreaks of damaging behaviours such as tail biting as a possible result (reviewed by Nordgreen et al., 2020). A key aspect of both sickness behaviour and depression is altered social motivation. Typically, sick individuals employ health restoring and rehabilitative strategies such as the avoidance of activity, conservation of

energy, limiting of social interactions, reduction of food intake, and seeking rest (Hart, 1988). Farm animals housed in groups have only limited possibilities for social withdrawal when they experience a bout of illness, and this might influence their social interactions. Sick animals could behave in a way that either increase the risk for being targeted by biters or increase the risk of becoming a biter (Munsterhjelm et al., 2019). The study of causal relationships between health and behaviour 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 (Nordgreen et al., 2018).

1.3 LPS experimental model of sickness

Lipopolysaccharide (LPS), a component of the cell wall of a Gram-negative bacterium, can be used to study the effects of a controlled immune activation on physiology and behaviour. LPS is a model for bacterial infection and sepsis and has been widely used in rodents and pig research (reviewed by Wyns et al., 2015b). In contrast to a bacterial infection model, the endotoxin mimics many of the acute phase responses without actively infecting the host (reviewed by Burrell 1994). Sensitivity to the effects of endotoxin varies between species and pigs are much more sensitive than rodents (Olson et al., 1995; Schmidhammer et al., 2006). The use of different serotypes, doses, routes and duration of administration determine the response strength/clinical symptoms. Within 15 min after intravenous (i.v.) administration of 15 µg/kg LPS, pigs showed marked tachypnoea followed by severe dyspnoea, as well as anorexia, as demonstrated by a complete loss of interest in feed and drinking water (Wyns et al., 2015a). Vomiting was preceded by clear signs of nausea, including salivation, chewing movements and retching and occurred within 30 min after LPS injection. Following this first phase of general sickness and the onset of respiratory symptoms, challenged pigs experienced a depression phase around 2 h after LPS, which was manifested by lateral decubitus with persistent respiratory distress (Wyns

et al., 2015a). The recovery phase, which is recognized by regaining alertness, vitality and appetite, occurred at around 5.5 h after LPS administration. Most LPS-studies in pigs focus on short-term effects (<= 24 h), thus, rarely report behaviour after overt sickness has ended (Johnson and von Borell, 1994; Webel et al., 1997; Carroll et al., 2005; Ebdrup et al., 2008; Wirthgen et al., 2013). Recently, it has been shown that LPS-injected pigs had a shift in social motivation and performed more tail- and ear-directed behaviour than saline injected pigs at 40 h after injection (Munsterhjelm et al., 2019).

1.4 The innate immune system and its communication with the central nervous system

The innate immune system serves as a fast-acting first line of defence against pathogens. Innate immune cells such as macrophages, neutrophils, mast cells and dendritic cells (so called sentinel cells) express pattern recognition receptors (PRRs) to detect pathogen-associated molecular patterns (PAMPs). PAMPs are structurally conserved molecules such as LPS in the cell walls of Gram-negative bacteria. The most important group of PRRs are Toll-like receptors (TLRs), located either on the surface or inside sentinel cells. LPS is recognized by TLR4 (Poltorak et al., 1998; Qureshi et al., 1999) but binds first to an LPS-binding protein and then to a complex of myeloid differentiation-2/ cluster of differentiation-14/TLR4 (MD2/CD14/TLR4) to activate the cell (Hailman et al., 1994). The transcription factor kappa B (NF-kB) pathway is the most significant signal transduction pathway in the immune system. The activation of the NF-kB pathway via the adaptor protein myeloid differentiation 88 (MyD88) activates transcription of the genes for the proinflammatory cytokines IL-1, IL-6 and TNF-α (Medzhitov et al., 1997; Medzhitov et al., 1998).

The immune system communicates with the central nervous system (CNS) via neuronal (vagal nerve) and humoral (cytokines) transmission. The sensory stimulation of peripheral vagal afferents by IL-1β and TNF-α

can trigger signalling to the brain (Fleshner et al., 1995; Steinberg et al., 2016). The afferent vagus nerve communicates via the lower brain stem to higher brain centres such as the hypothalamus and amygdala and induces cytokine release by macrophages (reviewed by McCusker and Kelley, 2013). Bluthé et al. (1996) demonstrated that vagotomy attenuated the decrease in social exploration induced by intraperitoneal (i.p.) injection of IL-1β, but had no effect when IL-1β was injected subcutaneous (s.c.) or intravenous (i.v.). This indicates that, besides neuronal transmission, humoral pathways are also involved in mediating the ability of cytokines to modulate behaviour. Cytokines can either diffuse directly from the bloodstream into the brain or are transported through the blood-brain-barrier (BBB). LPS can disrupt the BBB (Wispelwey et al., 1988) and some cytokines (e.g. TNF-α) affect the structure and integrity of the BBB. Microglia, the resident immune cell of the CNS, are able to respond to PAMPs via TLRs or peripherally derived cytokines with a central induction of proinflammatory cytokine expression (reviewed by McCusker and Kelley, 2013). Cytokines can act on glia cells (astrocytes, microglia) to modify behaviour and neurons are directly responsive to cytokines via specific receptors (e.g. TNF-R1, IL-1-R1), (Katsuura et al., 1988; Srinivasan et al., 2004).

Proinflammatory cytokines (IL-1, IL-6 and TNF-α) induce the enzyme cyclooxygenase 2 (COX-2) in the periphery (e.g. liver, lung) and in the brain (e.g. hypothalamus), (Ivanov et al., 2002; Konsman et al., 2004). This enzyme metabolizes arachidonic acid into prostaglandin E_2 (PGE_2), which triggers a rise in body temperature via specific receptors in the hypothalamus and other brain regions (Sehic et al., 1996; Ushikubi et al., 1998; Ivanov et al., 2002). The febrile animal shows so called "sickness behaviour" which is characterized by sleepiness, depression, anorexia and reduction in grooming (Hart, 1988). Nonsteroidal anti-inflammatory drugs target cyclooxygenases (COX) and reduce pain and decreases fever and inflammation through inhibition of prostaglandin synthesis (reviewed by Vane and Botting, 1998). COX-1 has generally a homeostatic role

(housekeeping), whereas COX-2 is mainly involved in inflammatory processes (reviewed by Pecchi et al., 2009). Administration of the non-selective COX-1/COX-2 inhibitors ketoprofen and flunixin reduced PGE_2 production and fever response after LPS challenge but had no impact on plasma levels of TNF-α, IL-1β, IL-6 in pigs (Mustonen et al., 2012; Peters et al., 2012; Salichs et al., 2012; Wyns et al., 2015a). A selective COX-2 inhibitor (celecoxib) reduced plasma levels of IL-6 in patients with major depressive disorder (Abbasi et al., 2012). Concentrations of IL-1β in the hypothalamus and IL-1β/TNF-α in the frontal cortex were reduced by celecoxib in a rat model of depression (Myint et al., 2007). The mechanism by which some NSAIDs can influence cytokine levels is by blocking the degradation of the inhibitor-kB-α (I-kB-α), a protein that forms a complex with NF-kB, and thereby prevents the release and subsequent translocation of NF-kB into the nucleus and the expression of inflammatory cytokines (Pierce et al., 1996; Stuhlmeier et al., 1999; Matasić et al., 2000).

Proinflammatory cytokines stimulate the hypothalamic-pituitary-adrenal (HPA)-axis, thus, promoting a release of the corticotrophin-releasing factor (CRF) in the hypothalamic paraventricular nucleus (Berkenbosch et al., 1987; Sapolsky et al., 1987; Cambronero et al., 1989). This in turn stimulates the secretion of the adrenocorticotrophic hormone (ACTH) in the anterior pituitary (Besedovsky et al., 1986; Bernton et al., 1987; Katsuura et al., 1990). Consequently, glucocorticoids (cortisol in humans and corticosterone in rodents and birds) are produced by the adrenal cortex and released into the blood stream (Wang and Dunn, 1998). Glucocorticoids regulate the HPA-axis by negative feedback on the hypothalamus and the pituitary gland (Russell et al., 1969; Lamberts et al., 1986). Glucocorticoids act via specific receptors on immune cells (e.g. macrophages) and suppress proinflammatory cytokine expression (Bhattacharyya et al., 2007; Kleiman et al., 2012). An increased tonic activity of the HPA-axis due to a deficit in the negative feedback

regulation has been reported in major depression (reviewed by Fava and Kendler, 2000).

In addition to the direct effects of cytokines in the CNS, another possible pathway for cytokines to influence behaviour is through influencing tryptophan metabolism. In a normal, non-disease state, a total of 90-95 % of the essential amino acid tryptophan is metabolized to kynurenine by the hepatic enzyme tryptophan-dioxygenase (TDO) and the extra-hepatic enzyme indoleamine 2,3-dioxygenase (IDO), (reviewed by Höglund et al., 2019). TDO is stress-responsive and induced by glucocorticoids, whereas IDO is immune-responsive and induced by proinflammatory cytokines, such as IFN-γ and TNF-α (Byrne et al., 1986; Takikawa et al., 1988; Saito et al., 1991; Fujigaki et al., 2001). The rest of dietary tryptophan is metabolized to serotonin by the enzyme tryptophan hydroxylase. Serotonin (5-hydroxytryptamine/5-HT) is located in enterochromaffin cells of the intestine, in neurons of the central nervous system and in blood platelets. Since it is expressed both at the periphery and in the central nervous system, IDO represents a possible link between the immune system and the serotonergic pathway (Lestage et al., 2002). The synthesis of serotonin in the brain is highly dependent on the bio-availability of tryptophan in the plasma (Fernstrom and Wurtman, 1971). Chronic stress and infection can shunt available tryptophan towards the kynurenine pathway and thereby lower serotonin synthesis (reviewed by Höglund et al., 2019). Kynurenine can cross the blood-brain barrier (Fukui et al., 1991) and is further metabolized to the neuroprotective kynurenic acid in astrocytes and neurotoxic metabolites of quinolinic acid in microglia (Saito et al., 1992). In rodents it has been shown that a blockade of IDO activity prevents the development of depressive-like behaviour, whereas administration of kynurenine induces depressive-like behaviour (O'Connor et al., 2009).

LPS has an effect on central neurotransmitters, which is supposed to be mediated by proinflammatory cytokines (reviewed by Dunn et al., 2005). In rodents, a peripheral administration of IL1-β increased noradrenaline

turnover in the hypothalamus and the hippocampus and caused a depletion of noradrenaline (Dunn, 1988; Kabiersch et al., 1988; Zalcman et al., 1994; Fleshner et al., 1995). Pigs injected with LPS, had markedly lower noradrenaline levels in their hypothalamus, hippocampus and frontal cortex (Nordgreen et al., 2018). Bodies of noradrenergic neurons are located among others in the locus coeruleus, a nucleus of the brain stem, and they project diffusely throughout the cortex, hippocampus, hypothalamus, cerebellum and spinal cord (Agarwal et al., 1993; Piekarzewska et al., 1999; Rang et al., 2016). In the periphery, noradrenaline acts as a transmitter of the sympathetic nervous system. Noradrenaline is important for behavioural arousal, stress response and control of mood (Rasmussen et al., 1986; Pacak et al., 1995; Rosario and Abercrombie, 1999; Katz et al., 2004). When rodents are injected (i.p.) with IL-6, IL-2 or IFN-α, dopamine turnover in the hippocampus and the frontal cortex increases (Zalcman et al., 1994; De La Garza and Asnis, 2003). An injection with LPS did not alter dopaminergic systems in frontal cortex, hypothalamus and hippocampus of pigs (Nordgreen et al., 2018). The neurotransmitter dopamine, the precursor of noradrenaline, is mostly abundant in the corpus striatum that derives branches from cell bodies in the substantia nigra (Rosa-Neto et al., 2004). Dopaminergic cell bodies in the ventral tegmental area project to the hippocampus and frontal cortex (Rang et al., 2016). Dopamine has relevance for motor control, is involved in emotion and the reward system and plays a key role in response to stress (Rougé-Pont et al., 1993; Valenti et al., 2011; Chang and Grace, 2013). Peripheral administration of IL-1β, TNF-α and IFN-α increased serotonin turnover in the hippocampus, frontal cortex and brain stem of rats and mice (Zalcman et al., 1994; Clement et al., 1997; De La Garza and Asnis, 2003). In pigs, an injection with LPS did not alter serotonergic systems in frontal cortex, hypothalamus and hippocampus (Nordgreen et al., 2018). Cell bodies of the serotonergic neurons are found in the raphe nuclei of the brain stem (Piekarzewska et al., 1999; Niblock et al., 2004). The projections of these cells are widely distributed throughout the cortex, hippocampus, basal ganglia, limbic system,

hypothalamus, cerebellum and spinal cord (Rang et al., 2016). As a neurotransmitter, serotonin influences among other functions mood, anxiety, stress, and aggression (Saudou et al., 1994; Grahn et al., 1999; Arroyo et al., 2016).

1.5 The acute phase response

Cytokines not only influences the brain but also have profound peripheral effects, geared towards cleaning the body of pathogens, known collectively as the acute phase response. Proinflammatory cytokines (mainly IL-1β, IL-6, TNF-α) promote the production of acute phase proteins (APPs) in the hepatocytes of the liver. The most important APPs in pigs are pig-major acute phase protein (pig-MAP), haptoglobin (HP), serum amyloid A (SAA) and C-reactive protein (CRP), (Eckersall et al., 1996; Heegaard et al., 1998; Sorensen et al., 2006). CRP and SAA are classified as major responsive with a strong raise (10-100-fold) on stimulation, peaking at 24-48 h and then declining rapidly. HP and Pig-MAP count as moderate responsive with a 5-10-fold increase on activation, a peak after 48-72 h and a slower decrease (Parra et al., 2006; Sorensen et al., 2006; Gutiérrez et al., 2009; Pomorska-Mól et al., 2015; Gutiérrez et al., 2017). Recently, the enzyme adenosine deaminase (ADA) was proposed as a potential inflammatory biomarker in pigs (Gutiérrez et al., 2013; Gutiérrez et al., 2017). ADA is involved in purine metabolism and highly expressed in lymphoid organs (reviewed by Bradford et al., 2017). APP levels can be determined in blood and saliva and are used to identify animals with severe infections or inflammations. APPs are sensitive but not specific indicators of infections and can be used to evaluate the general health status of pigs (Heegaard et al., 1998). APPs can directly neutralize inflammatory agents, help to minimize the extent of local tissue damage, as well as participate in tissue repair and regeneration and thereby restore homeostasis (reviewed by Steel and Whitehead, 1994). In human medicine, CRP is used as a marker for low grade inflammation, as it can predict future risk for coronary heart disease (Koenig et al., 1999) and diabetes (Thorand et al., 2003). A

recent metanalysis (Osimo et al., 2019) revealed that about a quarter of patients with depression, showed evidence of low-grade inflammation (CRP > 3mg/L) and over half of patients had mildly elevated CRP levels (CRP > 1mg/L). Having presented the physiological mechanisms that can drive changes in behaviour, I would like to return to the outcome, the behaviour itself, and introduce a method to describe social behaviour in detail.

1.6 Social network analysis

Social interactions differ in their type as well as their frequency and duration. Behaviour is often described on the level of an individual animal and dyadic interactions. A social network describes "who is connected to whom" and how closely. Animals are not equal in the number of connections they have or whether they occupy central or peripheral positions in the social network. A social network is defined as a finite set of individuals and the relationships or interactions that occur between them (Wassermann and Faust, 1994). Depending on the research question one may choose to study the overall structure of a network (group level) or to quantify the position of an individual (Wassermann and Faust, 1994). Social network analysis (SNA) provides quantitative measures to describe social structures on all levels from the individual to the population (Croft et al., 2008). SNA examines individuals in the context of relationships between group members and makes it possible to study how individual behaviour influences the wider population and how in turn the resulting population affects the individual (Couzin and Krause, 2003; Wey et al., 2008). SNA avoids assumptions that dyads interact independently of their wider social group (Turner et al., 2020) and accounts for the fact that the behaviour of one animal in a group affects the behaviour of others (reviewed by Asher et al., 2009). Observations of social behaviour are the basis on which networks are built and different parameters on individual and group level can be calculated to evaluate the social structure of a group. Centrality is one way to quantify an individual's structural importance in a group (Freeman, 1978/79). *Degree*

centrality is determined by how many interactions this individual has with others. An animal with many interactions will have more influence on those around it and possibly on the whole network (reviewed by Wey et al., 2008). *Eigenvector centrality* considers the number of connections an individual has, but also how well-connected group members are (Makagon et al., 2012; Foister, 2019). *Betweenness centrality* describes how central an individual is based on how often it is a part of the shortest path between other individuals in the network (Wassermann and Faust, 1994). This indicates how important an animal is as a point of social connection and information transfer (Wey et al., 2008; Makagon et al., 2012). *Edge density* is measured at group level and indicates how well the members of the group are connected in terms of their interaction with each other (Foister, 2019). A group with higher *edge density* has more interactions per individual than a group with lower density and therefore, is theoretically more cohesive (Wey et al., 2008). Commercially reared animals are restricted in the behaviours that they can show, which has a considerable impact on how networks will form (Foister, 2019). Furthermore, in commercial systems animals are often housed in group sizes far exceeding that which would occur in the wild and the groups have narrowed variation in age, weight, and size, and in some cases sex ratio. Thus, social network analysis of farm animals provides a unique perspective of variation in network position and structure that is shaped due to the variation and combinations of individual personalities (Foister, 2019). The behaviour of a social animal such as the pig lends itself readily to this method and SNA could give a more comprehensive understanding of how immune activation influences the group, from which it is impossible to withdraw when sick.

1.7 Knowledge gaps

The influence of health on welfare and behaviour is acknowledged in humans, and extensively researched in rodent models. However, even though kept under conditions likely to activate the immune system, we know little about the influence of immune activation on mood and

behaviour in production animals. The LPS model has been used in pigs for controlled immune activation, but most studies focus on rather short-term effects of LPS and little is known about the longer-term effects on the social behaviour of pigs after recovery from acute sickness. There is also a paucity of studies that report changes in neurotransmitters- and modulators in the pig brain in response to LPS, although these changes might play a role in the development of damaging behaviours. Previous LPS studies in pigs that investigated social behaviour and brain physiology used group sizes that did not mimic housing conditions on farms and were therefore less representative.

There is a need for non-invasive methods to evaluate heard health and animal welfare. Saliva samples are used to measure stress parameters and to evaluate the acute phase response. CRP and HP are widely used biomarkers in disease monitoring on farm, but not much is known about the putative biomarker for immune activation: the enzyme ADA. The time course of its activity and its relationship to other biomarkers in response to a controlled immune activation has not been described in pigs to date.

Previous studies addressing damaging behaviours focus on pen level data, dyadic interactions, or related indicators, but they do not tell us much about the complexity of social interactions within the group. So far, the use of social network analysis in captive farm animals is limited, and the method has not been applied to observations of pig social behaviour in response to a controlled immune activation.

1.8 Aims

The overall aim of my PhD was to understand how immune activation influences brain physiology in pigs and how these physiological changes can drive changes in social behaviour. In order to achieve this objective, we examined cytokine activated signalling pathways that may be involved in inducing behavioural alterations by using an LPS-model. The working hypothesis is that episodes of acute proinflammatory signalling in pigs

can, subsequent to acute sickness, elicit longer term negative social behaviour such as tail and ear biting (Fig. 1).

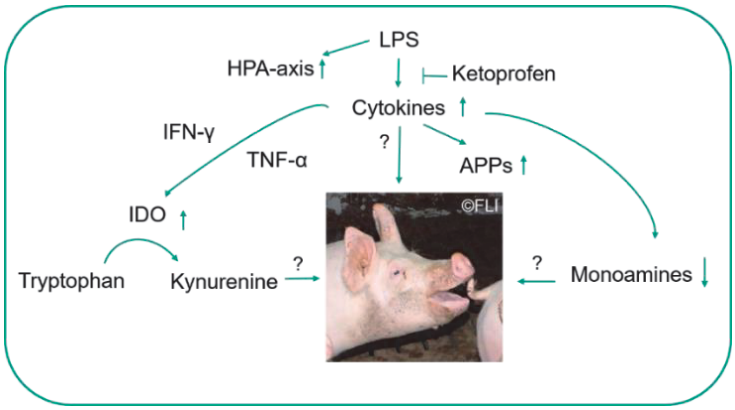


Figure 1: Overview of working hypotheses

Sub aims:

The aim of Paper I was to test the effect of a controlled immune activation with LPS on brain physiology and social behaviour of group-housed pigs. In addition, the aim was to test the effect of a ketoprofen intervention on the physiological and behavioural effects of LPS.

The aim of Paper II was to investigate the dynamics of salivary biomarkers of systemic inflammation in growing pigs exposed to LPS under experimental conditions, and to test whether ketoprofen could attenuate the effect of LPS. In addition, the aim was to describe the correlations between salivary ADA, haptoglobin and cortisol to evaluate their relationship in response to LPS.

The aim of Paper III was to study how pig social behaviour is influenced when one member of a larger group becomes ill and thereby changes its behaviour. To achieve this, social network analysis was used to test the effect of a controlled immune activation and an intervention with ketoprofen on centrality parameters on pig level. In addition, the aim was to test the effect of time relative to injection on general network parameters in order to get a better understanding of changes in social network structures on pen level.

2 Methodological considerations

2.1 Animals and housing

The articles of this thesis are based on the data gathered in one experiment that was conducted in two batches between March 23 and May 15, 2018. Seventy-eight pigs between 11 and 12 weeks of age (52 females and 26 castrated males) were housed in the fattening unit of the Livestock Production Research Center of the Norwegian University of Life Sciences (NMBU), (Fig. 2). The pigs were group-housed by litter with six pigs per pen (four females and two males), resulting in 13 pens in total. Housing details are described in Paper I.

Figure 2: Fattening unit of the experimental facilities at the Livestock Production Research Center of NMBU. The experimental pens were allocated on both sides of the hallway. The pigs in each pen had visual and limited tactile contact with pigs from one adjoining pen

2.2 Experimental design

The four female pigs in each pen were randomly allocated to one of four treatments each, so that all treatments were represented in all pens, resulting in 13 pigs per treatment. The male pigs were companion pigs

(CO) used to increase stocking density and group size. The four experimental treatments consisted of four substance combinations: saline-saline (SS), saline-LPS (SL), ketoprofen-saline (KS) and ketoprofen-LPS (KL). LPS (Serotype 0111:B4) was given at a dose of 1.2 µg/kg and ketoprofen at a dose of 6 mg/kg. Both doses were chosen based on previous experiments (Fosse et al., 2011; Nordgreen et al., 2018). The first substance was administered intramuscularly (i.m.) in the neck and the second substance intravenously (i.v.) through an ear vein catheter on average 1 h after the i.m. injection. The pigs were anesthetized with a mixture of tiletamine and zolazepam (2.84 mg/kg each), butorphanol (0.18 mg/kg) and medetomidine (0.06 mg/kg), and humanely killed with an overdose of pentobarbital (140 mg/kg) at 72 h after the i.v. injection. Each pen was equipped with one video camera placed in the ceiling above the center of the pen. The pigs were individually marked on the back for identification and video recordings of behaviour ran continuously throughout the experiment (Fig. 3).

Figure 3: Bird's eye perspective of one experimental pen; all pigs were individually marked for identification on their back

2.3 Ethical aspects

During the stage of experimental planning, Norwegian legislation required a FOTS-application to the Norwegian Animal Research Authority for permission to conduct the experiment. The approval depended on the fulfilment of the requirements of the Animal Welfare Act (2010) as well as the three R's (2009). The Animal Welfare Act states that "stress/hardship imposed on animals must be as small as possible". The main ethical issue which arises from our experimental design is the LPS injection and induced systemic inflammation in the pigs. The LPS dose used was defined based on prior experiments in our research group where signs of an inflammatory response were evident, but severe symptoms such as vomiting was avoided. The dose used is lower than reported in other publications (Myers et al., 2003; Friton et al., 2006; Peters et al., 2012; Wyns et al., 2015) and the pigs recovered from the challenge 4-6 h after LPS-application. Furthermore, the behaviour of the pigs was observed during 6 h after LPS-application and an end point was defined when intervention would take place. Regarding the reduction of number of animals in the experiment, the sample size was calculated based on prior experiments of the research group and the statistical output. The use of 52 pigs was necessary in order to obtain a sufficient sample size for the different treatments. The replacement of conscious living animals with insentient material was not possible in this instance, because the aim was to study the behaviour expressed by the animals themselves. In addition to the above described optimization of the LPS dosage, it was decided to use mainly saliva sampling instead of blood sampling for cortisol measurements as both parameters are highly correlated (Cook et al., 1996; Schönreiter et al., 1999; Merlot et al., 2011). This meets the refinement criteria of techniques and procedures to reduce pain and distress. The National Animal Research Authority approved the experiment (FOTS ID 15232).

2.4 Sampling procedures

A previous study my thesis is based on (Nordgreen et al., 2018) guided the selection of sampling time points, tissue selection as well as selection of physiological parameters measured. Different samples at different timepoints were taken throughout the experiment (Fig. 4)

Figure 4: The graph shows the time course of the different sampling procedures. Saliva samples are presented as white cotton pads and blood samples are presented as Eppendorf tubes filled with red colour. Injection times are indicated by a syringe. Video recordings (shown by black camera icon) were run throughout the experiment. Details of sampling procedures for saliva, blood and brain tissue are described in Paper I

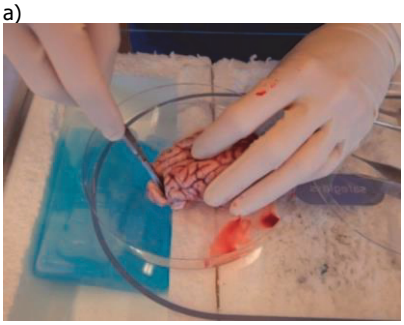
Saliva samples were taken just before i.m. injection (-1 h), as well as 4 h, 24 h, 48 h and 72 h after i.v. injection by letting each pig chew on a cotton pad suspended on a dental cord. This is a less invasive procedure for the pigs than blood sampling (fixation with mouth snare), which in turn might have influenced central parameters through stress responses. Saliva was analysed for cortisol to picture the response of the HPA-axis to LPS and to test the functioning of the LPS-model. Cortisol is a very sensitive parameter and the way of sample collection as well as the timepoint has an impact on the HPA-axis response (Ruis et al., 1997; Merlot et al., 2011). Furthermore, we wanted to monitor the time course of the enzyme ADA and HP in response to LPS to evaluate the acute phase response. Because HP has been stated as a major porcine APP (Parra et al., 2006) and it is widely studied, it was decided to be included

as the most promising APP to be combined with ADA that has previously been reported as a new candidate to monitor heard health (Gutiérrez et al., 2013; Gutiérrez et al., 2017).

Blood samples were taken just before i.v. injections (0 h) as well as 72 h after i.v. injections through a temporarily placed ear vein catheter that was inserted immediately before and removed directly after the procedure. Blood was analysed for tryptophan and kynurenine to investigate the peripheral branch of the IDO pathway. Blood sampling is an invasive method which affects stress responses, that's why the sampling frequency was low.

Brain samples were taken at 72 h after the i.v. injections on average 10 min after cardiac arrest. The head was removed, the skull opened, and the brain collected. The brain regions frontal cortex (Fig. 5a), hippocampus (Fig. 5b), hypothalamus (Fig. 5c) and brain stem (Fig. 5d) with left and right hemisphere respectively were dissected, resulting in eight brain samples per pig. For dissection of the brain regions, a stereotaxic atlas was used (Félix et al., 1999).

a)



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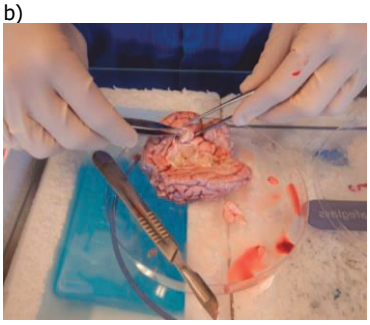

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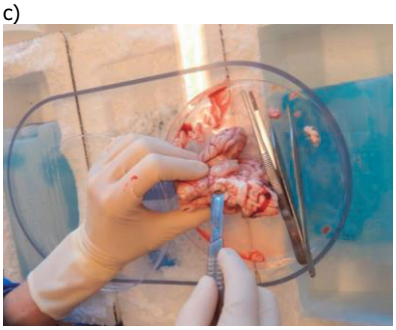

d)

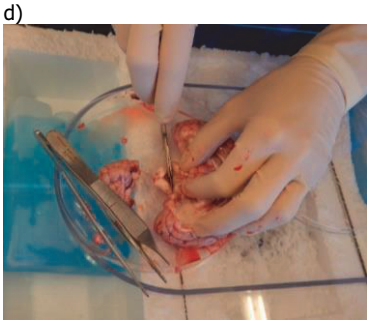

Figure 5: Dissection of the frontal cortex (a), hippocampus (b) hypothalamus (c) and brain stem (d)

The frontal cortex was sampled due to the importance of this area for the control of behaviour, the regulation of mood, and the perception of external stimuli (Milstein et al., 2007; Arnsten, 2009; Niendam et al., 2012). The hippocampus was included based on its role in cognition and memory (Drachman and Arbit, 1966; McClelland et al., 1995; McNaughton et al., 2006). The hypothalamus was collected due to its importance in the regulation of the stress response, appetite and fever (Anand and Brobeck, 1951; Hori et al., 1988; Hueston and Deak, 2014). The brain stem was sampled because it contains the raphe nuclei and locus coeruleus that control serotonergic and noradrenergic signalling (Agarwal et al., 1993; Piekarzewska et al., 1999; Niblock et al., 2004; Rang et al., 2016). Brain samples were used to analyse the monoamines dopamine, noradrenaline and serotonin due to their role in stress responses, behaviour and mood. Furthermore, central cytokines were analysed to test the hypothesis. IFN-γ and TNF-α were included based on previous findings (Nordgreen et al., 2018) and the relevance of these cytokines for kynurenine metabolism through induction of IDO. IL-18 is, in turn, involved in IFN-γ production via NK cell activation and was therefore included. In addition, tryptophan and kynurenine were analysed to investigate the central branch of the IDO pathway.

2.5 Laboratory methods

Full details of sample analysis for saliva, blood and brain tissue are described in Paper I and Paper II. Here, the methods are described in a more general manner.

The stress hormone cortisol was measured in saliva with an enzyme-linked immunosorbent assay (ELISA) kit. A so called "sandwich-ELISA" was used, a method that uses two antibodies, which bind to different sites of the cortisol antigen in the sample. The capture antibody is coated

to the 96-well plate to which the sample is added, followed by addition of the detection antibody to which an enzyme labelled antiglobulin is added. Capture and detection antibodies are from different species and the antiglobulin for the visualization of the detection antibody is species specific. The substrate of the enzyme is added to generate a colorimetric signal that is detected in a spectrophotometric plate reader (as optical density or OD). The intensity of the colour reaction (OD) is proportional to the amount of bound cortisol antigen (Cox et al., 2012; Tizard, 2018).

The acute phase protein HP and the enzyme ADA were measured in saliva by a laboratory in Spain. HP was determined using a time-resolved immunofluorimetry assay (TRIFMA). Streptavidin microtitration strips were coated with a biotinylated capture antibody, the sample was added and incubated with a Europium-labelled detection antibody. The fluorescent signals, which are proportional to the quantity of HP, were measured with a time-resolved fluorometer (Gutiérrez et al., 2009). ADA activity levels were measured using microtitration plates. The method of the assay is based on the measurement of the decrease in absorbance (OD) per minute of a coupled reaction initially catalysed by ADA (Gutiérrez et al., 2017).

The cytokines IFN-γ, TNF-α and IL-18 were measured in homogenized brain tissue with a Milliplex MAP Porcine Cytokine and Chemokine Magnetic Bead Panel Immunology Multiplex Assay. In this method, the capture antibody is coupled to a colour coded bead to which the analyte is added followed by the addition of a biotinylated detection antibody. A streptavidin-conjugated fluorochrome is added and the fluorescent readout is detected by a flow cytometry-based instrument (e.g. Luminex xMAP). The amount of analyte detected is directly proportional to the fluorescent signal. The method allows the simultaneous measurement of several biomarkers in one sample.

The metabolic parameters tryptophan and kynurenine in plasma and homogenized brain tissue were measured with a high-performance liquid chromatography-electrospray ionization tandem mass spectrometry

(HPLC-ESI-MS/MS). The same method was used to analyse the monoamines noradrenaline, dopamine and serotonin and their metabolites in homogenized brain tissue. The method combines the physical separation capabilities of liquid chromatography (LC) with the mass analysis capabilities of mass spectrometry (MS). The interface between both methods is an electrospray ionizer (ESI). The analyte is pumped with a liquid (mobile phase) under high pressure through a column filled with a solid adsorbent material (solid phase). Due to their different degrees of interaction with the solid phase, each component has a different flow rate which leads to a separation of the components (retention time). The liquid containing the analyte is dispersed/nebulized into a fine aerosol (gas phase). A high voltage is applied to charge the components and the ions are transferred to a high vacuum chamber of a mass spectrometer where their mass to charge ratio (m/z) is measured.

2.6 Behavioural analysis

Video recordings were used to analyse pig behaviour one day prior to injections (DAY0), the day of injections itself (DAY1), as well as one (DAY2) and two days after injections (DAY3). Two different behavioural sampling methods were applied, *instantaneous scan sampling* and *continuous observation*. The first method is used to record the behaviour of an individual in a group at predetermined time intervals, thus, frequency of behaviour and not duration is observed (Altmann, 1974). The second method is used to record all activity that occurs while the animals are being watched (e.g. social interactions).

Behavioural signs of sickness were observed by one observer who was blinded to treatment. *Instantaneous scan sampling* was performed every 5 min for 6 h after the injection of the last pig in the pen on DAY1. 13 pens were included in the analysis. The ethogram used is displayed in Table 1.

Table 1: Ethogram for behavioural signs of sickness

Behaviour	**Description**
Lying lateral	Lying on the flank with head resting on the ground and not moving, body (parts) may make rapid, sudden, short-lasting movements
Lying sternal	Lying on the sternum with head resting on the ground, body (parts) may make rapid, sudden, short-lasting movements
Lying alert	Lying (on flank or sternum) with head up
Feeding	Snout in feeder
Active	Any active behaviours in standing position except feeding, including moving, exploration, social behaviour, drinking, elimination, comfort behaviour
Interruption	Person is in the pen, scan not included in data analysis

Social behaviour was observed continuously on DAY0 (baseline) as well as DAY2 and DAY3 by one observer who was blinded to treatment and day. Observations of performers and receivers at certain intervals during the day were performed. The sampling scheme was four 15 min intervals in the morning and six 15 min intervals in the afternoon. Due to inadequate quality of the video material from one pen, only 12 out of 13 pens were included in the analysis. The ethogram used is displayed in Table 2. The data on social behaviour analysis forms the basis for the method applied in Paper III (described below).

Table 2: Ethogram for (negative) social behaviour

Behaviour	**Description**
Tail manipulation	Touching the tail of another pig with the snout, including taking the tail into the mouth
Ear manipulation	Touching the ear of another pig with the snout, including taking the ear into the mouth

Flank nosing	Touching the flank region (=upper part of the lateral side of the body from the beginning of the shoulder until the end of the body, except of tail) of another pig with the snout
Belly nosing	Repetitive up and down movements on the abdomen of another pig that is lying or standing
Manipulation of other body parts	Touching body parts of another pig with the snout except for tail, ear, belly and flank region (e.g. head, legs, back), including taking the body parts into the mouth
Fighting	Biting, hitting, and knocking of another pig with the head. Includes chasing performed immediately after biting, hitting, knocking. Includes parallel pressing after knock, hit or bite. Pig that initiates the fight is the performer, pig that is being attacked is the recipient
Displacement	Pushing away another pig without fighting (as defined above), results in active movement of the recipient and getting access to a resource (e.g. silage, lying space, drinker) for the performer

2.7 Social network analysis

Social behaviour analysis in Paper I focuses on the frequency and the duration of the different behavioural patterns performed and received by the individual pig and disregards the social structure within the pen. In Paper III, social network analysis (SNA) was used as a method to examine social interactions by means of quantitative measures. A social network consists of "nodes" and "edges". Each node represents an individual and each edge represents a social interaction. The data basis for SNA builds a so-called association matrix of the number of interactions between all group members in a pen. The package *igraph* in R 4.0.3 was used to construct networks for each behaviour listed in Table 2 and for each observation day. Tables 3 and 4 list the parameters calculated on pig and pen level. The association matrix and calculated measures can be used to plot network graphs for each pen and day (Fig. 6).

Table 3: Centrality parameters calculated on pig level

Terminology	**Description**
Degree centrality	Number of direct interactions an individual has with other individuals of the group
In-degree	Number of interactions received by an individual
Out-degree	Number of interactions initiated by an individual
Eigenvector centrality	Takes the degree centrality of an individual, as well as the degree centrality of other individuals it is connected with, into account

Networks can be scaled by a theoretical maximum (the maximum possible degree in a network of the same size) or by the highest degree present in the network. In these cases, the node with the highest degree in the network has a degree centrality of 1, and the centrality of every other node will be a fraction of its degree in comparison to the most popular node (Foister 2019).

Table 4: General network parameters calculated on pen level

Terminology	**Description**
Edge density	Amount of actual interactions between individuals divided by the total number of possible interactions in the group
Centralisation	The range or variability of the individuals' centrality values
Degree	Description of whether certain individuals initiate or receive more interactions than the rest of the group
Betweenness	Pens with high values contain individuals who connect other individuals that do not directly interact
Eigenvector	Pens with high values contain a small number of well-connected individuals, with the rest of the group being considerably less well connected

The centralisation of an entire network is calculated by comparing how central the most central node is to all other nodes in the network (Freeman, 1978/79). Centralization provides us with a scale from 0 to 1, with 0 indicating that all individuals in the network have equal centrality and 1 indicating maximum inequality. Freeman's centralization equation can be applied to all the centrality network measures, to provide a group measure that informs us how unequal the individual nodes are in terms of network centrality (Foister, 2019).

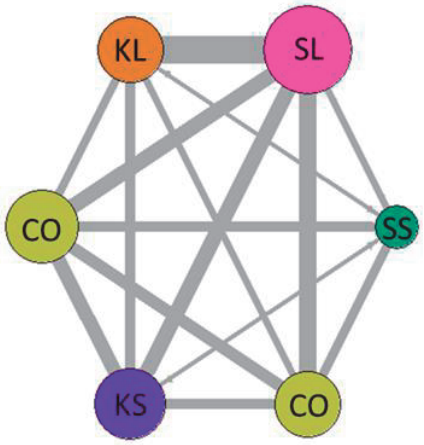

Figure 6: Example of a social network based on all interactions of pigs observed in pen 1 on the second day (DAY3) after injecting the pigs with saline-saline (SS), saline-LPS (SL), ketoprofen-saline (KS) and ketoprofen-LPS (KL). Nodes represent individuals in the pen and size of the nodes represents *degree centrality*; edges represent interactions between individuals, arrows point from the actor to the receiver (directionality) and thickness of the edges represents the frequency (weight)

2.8 Statistical analysis

Behavioural and physiological data were analysed using mixed models in JMP Pro 14.3.0 (SAS, NC, USA) and SPSS (IBM SPSS Statistics 25). Pig nested in treatment was included as a random variable in all models (except for general network parameters). A priori planned contrasts (Student's t-test) were used after running the main models, as there

were predefined predictions, which can be argued to make correction for multiple testing unnecessary (Doncaster and Davey, 2007). SL was compared with SS to elucidate the effect of LPS. In addition, the comparison of SL and KL should answer the question whether ketoprofen attenuates the effects of LPS. Furthermore, it was relevant to compare SS with KS in order to see whether ketoprofen had an effect even in pigs that are not sick. Dependant variables were transformed if the assumptions of normality of residuals and homogeneity of variance were not met (e.g. square root transformation).

2.8.1 Behavioural data (Paper I/III)

For behavioural signs of sickness, the frequency of the respective behavioural pattern was used as dependent variable, and the treatment (SS, SL, KS, KL), the hour after injection (1-6) and the interaction of both were used as independent fixed effects.

For social behaviour, the frequency and duration of the respective behavioural pattern performed and received was used as dependent variable. The treatment, the day in relation to LPS injection (DAY0, DAY2, DAY3) and the interaction of both were used as fixed effects.

For centrality parameters, the calculated values of *degree centrality*, *in-degree centrality*, *out-degree centrality* and *eigenvector centrality* were used as dependent variables. The treatment, the day and the interaction of both were used as independent fixed effects.

Spearman rank coefficient was used to correlate centrality parameters and cortisol concentrations at 4 h after injection, as well as general activity in the first 6 h after injection in SL pigs.

For general network parameters, the calculated values of *edge density*, *degree centralisation*, *in-degree centralisation*, *out-degree centralisation*, *betweenness* and *eigenvector* were used as dependent variables. The day was used as independent fixed effect and the pen was included as a random variable in all models.

2.8.2 Physiological data (Paper I/II)

For salivary and plasma analytes, the concentrations of cortisol, tryptophan, kynurenine and haptoglobin, as well as ADA activity, were used as dependent variables. Treatment, sampling time point in relation to i.v. injection (CORT, HP, ADA: T0, T4, T24, T48, T72 | TRY, KYN: T0, T72) and the interaction of both were used as fixed independent variables.

For brain tissue analytes, the observed concentration of IFN-γ and IL-18, and the fluorescence intensity of TNF-α, as well as the observed concentration of tryptophan, kynurenine, dopamine, noradrenaline, serotonin and respective turnover rates were used as the dependent variables in the models. Treatment and hemisphere were used as independent variables. The time span (TIME) between death of the respective pig until the last brain sample was collected and frozen was included as a covariate in all models.

Spearman rank coefficient was used to correlate the difference between T0 and T4 in measured concentrations of ADA, haptoglobin and cortisol in SL pigs.

3 Summary of papers

My thesis is based on an experiment in which the effect of a controlled immune activation with lipopolysaccharide (LPS), and the effect of the NSAID ketoprofen on cytokines and neurotransmitters in the brain, as well as on social behaviour in group-housed pigs was tested. In this experiment saliva, blood and brain tissues were sampled and cytokine activated signalling pathways that could induce behavioural alterations were analysed (Paper I). Furthermore, video recordings were gathered to evaluate behavioural signs of sickness and social behaviour of the pigs (Paper I). Saliva samples gathered during the experiment were used to analyse different physiological parameters of the acute phase response in order to evaluate inflammatory mediators which could serve as biomarkers at pig herd level (Paper II). Finally, the data from the social behaviour analysis were used to apply "social network analysis" to gain more insights in social interactions on individual and group level (Paper III).

Paper I: The effect of LPS and ketoprofen on cytokines, brain monoamines and social behaviour in group-housed pigs

A controlled immune activation with LPS activated the hypothalamic-pituitary-adrenal-axis and elicited behavioural signs of sickness within six hours after the injection as indicated by an increase in salivary cortisol and decreased activity in LPS-injected pigs. Ketoprofen lowered the effect of LPS on cortisol release and alleviated behavioural signs of sickness. LPS-injected pigs performed longer ear manipulation compared to saline-injected pigs on the second day after injection, but LPS had no effect on any other observed behaviour. A controlled immune activation had no effect on the proinflammatory cytokines IFN-γ, TNF-α, and IL-18

measured at 72 h after the challenge in the frontal cortex, hippocampus, hypothalamus, and brain stem. LPS influenced tryptophan and kynurenine metabolism in plasma and several brain areas. LPS-injected pigs had lower plasma concentrations of tryptophan at 72 h after the challenge compared to baseline. The correlations between tryptophan and kynurenine concentrations in plasma and brain tissue were weak and positive. Both analytes were depleted in frontal cortex and brain stem of LPS-injected pigs compared to saline-injected pigs. A controlled immune activation had an effect on central monoamines. Dopamine concentrations in the hypothalamus of LPS-injected pigs were lower compared to saline-injected pigs. LPS-injected pigs had lower concentrations of serotonin in their hypothalamus and noradrenaline in their hippocampus than pigs that were pre-treated with ketoprofen.

Paper II: Dynamics of salivary adenosine deaminase, haptoglobin, and cortisol in lipopolysaccharide-challenged growing pigs

An injection with LPS stimulated the acute phase response as indicated by an increase in activity of the enzyme ADA and an elevation of salivary haptoglobin concentrations at four hours post-injection. Ketoprofen attenuated this effect. The levels of ADA and haptoglobin were positively correlated, indicative of their parallel dynamics under the influence of bacterial LPS.

Paper III: The use of social network analysis to describe the effect of immune activation on group dynamics in pigs

Changes in social interactions in response to a controlled immune activation were detected at both individual (pig) and group (pen) level using social network analysis. At the pig level, an injection with LPS resulted in a lower *in-degree centrality* in ear manipulation networks two days after the challenge, meaning that the ears of LPS-injected pigs were manipulated to a lesser extent compared to saline-injected pigs. Treatment effects on tail manipulation and fighting networks were not

observed. Ketoprofen seemed not to have an impact on centrality parameters at pig level. For networks of manipulation of other body parts, *in-degree centrality* was positively correlated with the cortisol response at four hours and lying behaviour in the first six hours after the challenge in LPS-injected pigs. This finding indicates that the stronger the pigs reacted to the challenge, the more manipulations were directed towards them (ears and tails excepted) on the following days. At the pen level, the time in relation to injection affected general network parameters for ear manipulation and fighting networks. *In-degree centralisation* was higher in the two subsequent days after injection in ear manipulation networks, thus, certain individuals were more frequently manipulated than the rest of the group compared to baseline. For fighting networks, *betweenness* decreased on the first day after injection compared to baseline, indicating that network connectivity increased following the injection. A decline in *betweenness* suggests that interactions were more evenly spread across all group members and no single individual was responsible for connecting a fragmented network.

4 Discussion

The overall aim of my PhD was to understand how immune activation influences brain physiology in pigs and how these physiological changes can drive changes in social behaviour. We know from human clinical reports and rodent studies that acute sickness can develop into depression or depressive-like behaviours and other psychological problems. To address this, cytokine activated signalling pathways that may be involved in inducing behavioural alterations were examined by using an LPS-model. The working hypothesis was that episodes of acute proinflammatory signalling in pigs can, subsequent to acute sickness, elicit longer term negative social behaviour such as tail and ear biting. The background for this hypothesis is derived partly from studies in humans and rodents where the influence of health on welfare and behaviour is acknowledged and extensively studied (reviewed by Dantzer et al., 2008). The immune system of pigs is very similar to that of humans in terms of anatomy, function, and gene expression (reviewed by Meurens et al., 2012) and the pig brain, which is gyrencephalic, resembles the human brain more in anatomy, growth and development than do the brains of commonly used small laboratory animals (reviewed by Lind et al., 2007). In this chapter, I will discuss and interpret the findings in the light of the current literature, starting with the experimental model used (4.1.) and continuing with the physiological (4.2.) and behavioural (4.3.) alterations in response to a controlled immune activation. I will discuss the effects of the NSAID ketoprofen on the effects of LPS (4.4.) and elaborate how housing conditions can be related to immune activation and damaging behaviours (4.5.). Finally, I will conclude the findings of my thesis (4.6.).

4.1 The LPS-model

In the current experiment, a single low dose of synthetically purified lipopolysaccharide was used to stimulate the immune system and mimic an endotoxemic state. LPS has been widely applied in different species to study Gram-negative bacterial infections and sepsis and the serotype used (O111:B4) is the most frequently used in pig research (reviewed by Wyns et al., 2015b). LPS administration can be performed either as a single or repetitive intravenous (i.v.), intraperitoneal (i.p.) or intramuscular (i.m.) injection or as a continuous infusion. Apart from the route, the chosen dose has an impact on the strength and duration of the clinical symptoms. Johnson and von Borrell (1994) reported a dose dependant reduction in activity within 4 h after LPS administration (0.5 µg/kg, 5 µg/kg, 50 µg/kg i.p.) compared to saline-injected controls.

The ethogram used for the description of behavioural signs of sickness in the current experiment is based on the study by Nordgreen et al. (2018), as a comparable dose and route of administration of LPS (1.2 µg/kg i.v.) was used. Other studies using higher doses of LPS applied more detailed observations of sickness behaviour. Peters et al. (2012) reported skin flushing and laboured breathing within 1-8 h and lethargy from 3-8 h after LPS injection (2 µg/kg i.v.). Friton et al. (2006) and Myers et al. (2016) used clinical scores based on respiratory rate, heart rate, rectal temperature, skin redness, lethargy, coughing, vomiting, diarrhoea and shivering, and reported them to be returned to baseline within 24 h after challenging pigs with LPS (2, 4, 6 µg/kg, i.v.).

The advantage of using LPS instead of live bacteria is that it is more standardized and reproducible (Myers et al., 2003) and can easily be stored in its lyophilized form until use (Fink and Heard, 1990). The disadvantage is that a single LPS-injection might imitate clinical endotoxemia less accurately as the endotoxin remains in the circulation for a longer period during natural infection (Olson et al., 1988; Olson et al., 1995). One possibility to evade this problem is a continuous infusion (Goscinski et al., 2003; Ruud et al., 2007; Ebdrup et al., 2008; Dänicke et

al., 2013; Levenbrown et al., 2013) or a repeated application of LPS (Escribano et al., 2014). Due to the study design, which required group housing and behavioural observations, this strategy was not applicable in the current experiment. A continuous infusion requires a permanent catheter, which would have been removed by companion pigs, and a repeated application would have entailed more frequent handling, which had to be avoided to not interrupt recordings of behaviour.

4.2 Physiological alterations in response to LPS

4.2.1 Cortisol

The cortisol response to LPS was used as a confirmation that the experimental model worked and the sampling time point was chosen based on a previous study, where plasma cortisol concentrations were elevated for 1-4 h in LPS-injected pigs compared to controls (Nordgreen et al., 2018). These findings were confirmed in the current experiment, as LPS-injected pigs showed a significant increase in salivary cortisol concentrations compared to saline-injected pigs at 4 h after injection (Paper I). The magnitude and the time course of the cortisol response depends on the LPS dose and the route of administration. Other studies (Webel et al., 1997; Wright et al., 2000; Terenina et al., 2017) using higher LPS doses reported a peak at 2-4 h and elevated plasma cortisol concentrations until 8-12 h after the injection. Escribano et al. (2014) measured salivary cortisol concentrations after challenging pigs with 30 µg/kg i.v. and reported a significant elevation at 3 h compared to baseline, interestingly, no such peak was found after a repeated administration of LPS.

Glucocorticoid release by the HPA-axis follows a diurnal pattern and basal salivary cortisol concentrations seemed to be influenced by sex and age (Ruis et al., 1997; Sarabdjitsingh et al., 2012). Diurnal variation was accounted for by taking baseline measurements between 08.30 and 10.45 in the morning, and only gilts in the same age group were included in the experiment. Basal salivary cortisol concentrations are

approximately 10 % of those in plasma (Parrott et al., 1989; Cook et al., 1996; Merlot et al., 2011). Compared to other studies measuring salivary cortisol in pigs (Merlot et al., 2011; Escribano et al., 2014), the baseline concentrations in the current experiment were very low (mean = 0.28 ng/ml). It is possible that the good housing and husbandry conditions in Norway in general contribute to producing these low values. Zupan and Zanella (2017) reported similar values for salivary cortisol in a study on Norwegian pig farms (0.42-0.87 ng/ml during circadian rhythm), so it might be that housing environment affects baseline cortisol concentrations. On the other hand, results are obtained in different labs and with potentially different methods or kits, which makes it difficult to compare them directly. Low salivary cortisol concentrations may also reflect cortisol breakdown (e.g. into cortisone, 5β-dihydrocortisol etc). Such breakdown products would not be detected by the assay due to a low cross-reactivity.

4.2.2 Acute phase response

The parameters of the acute phase response were measured to confirm and quantify the effects of the LPS challenge. Haptoglobin, a commonly used biomarker of the acute phase response in pigs, was combined with the rarely investigated enzyme ADA to access their dynamics in response to a controlled immune activation.

Salivary HP concentrations tended to be higher in LPS-injected pigs compared to saline-injected controls at 4 h after injection (Paper II). Salivary and serum HP are highly correlated (Gutiérrez et al., 2012) and the time course and magnitude in response to an LPS-challenge has been described previously. Others found no effect (Frank et al., 2005; Llamas Moya et al., 2006), numerical effects (Wright et al., 2000; Williams et al., 2009) or significant effects of LPS (Escribano et al., 2014) on salivary or plasma HP concentrations measured between 2 and 48 h after injection. Their results suggest a rather moderate and slow reaction of HP in response to infection. HP has also been widely studied as an

inflammatory biomarker both in experimentally infected pigs (Sorensen et al., 2006; Martín de la Fuente et al., 2010; Heegaard et al., 2011; Pomorska-Mól et al., 2013; Pomorska-Mól et al., 2015), as well as in herds undergoing natural infections (Parra et al., 2006; Grau-Roma et al., 2009; Gutiérrez et al., 2009; Gutiérrez et al., 2012). HP follows a circadian pattern (Gutiérrez et al., 2013) and is influenced by age and sex (Frank et al., 2005; Scollo et al., 2013; Reimert et al., 2014; Sánchez et al., 2019). As saliva was collected in the morning and only gilts in the same age group were included in the study, the current findings are only relevant for that time of day, age and sex of the animals used. HP has immunomodulatory effects by inhibition of granulocyte chemotaxis, phagocytosis and bactericidal activity and can thereby dampen the acute inflammatory response (Rossbacher et al., 1999). Moreover, HP plays a role in tissue repair by stimulating angiogenesis (Cid et al., 1993).

In the current experiment, salivary ADA concentrations were significantly higher in LPS-injected pigs compared to saline-injected pigs at 4 h after the challenge and positively correlated to the HP response (Paper II). ADA was shown to be elevated in gastrointestinal and respiratory disorders and local inflammation in pigs (Gutiérrez et al., 2017), but has so far not been measured in LPS studies. Its activity has been reported to be highly correlated with the concentrations of HP and CRP (Gutiérrez et al., 2017; Gutiérrez et al., 2018). ADA was shown to be influenced by sex, age and breed (Gutiérrez et al., 2018; Sánchez et al., 2019). The enzyme plays a role in the differentiation and maturation of the immune system (reviewed by Bradford et al., 2017). Both ADA and HP reliably detected immune activation at a timepoint where the pigs were most severely affected clinically.

4.2.3 Cytokines

The short-term effects of LPS on proinflammatory cytokines in the blood were not monitored as this has been extensively studied in the past. The proinflammatory cytokine TNF-α peaks at 1 h, IL-6 peaks at 2-4 h and IL-

1β at 3-4 h after LPS injection at different doses and routes of administration (Webel et al., 1997; Carroll et al., 2005; Ebdrup et al., 2008; Williams et al., 2009; Peters et al., 2012; Wirthgen et al., 2013; Wyns et al., 2015a; Nordgreen et al., 2018). The focus was beyond the first 24 h after the challenge, as the aim was to test whether the physiological changes last alongside behavioural changes. The duration of effect on physiology is important for the relevance of the LPS model in the study of immune effects on mental health and damaging behaviour in pigs. If the physiological and behavioural changes are short-lived, they may not tell us much about the mechanisms underlying longer-term mental illness and/or damaging behaviour.

There was no effect of LPS on IFN-γ, TNF-α and IL-18 measured in frontal cortex, hippocampus, hypothalamus and brain stem at 72 h after LPS-injection (Paper I). This is in contrast to Nordgreen et al. (2018) who found higher concentrations of IFN-γ in the frontal cortex and a tendency toward an elevation of IL-18 in the right hippocampus of LPS-injected pigs compared to saline-injected pigs at the same time point. This study provided the background for taking measurements at 72 h post-LPS injection in the current study. The difference between both studies is the slightly lower dose of LPS in the current experiment (1.2 µg/kg vs 1.5 µg/kg), the route of administration (ear vein vs. central vein catheter) and the housing system (group-housed vs. single-housed). That no changes in central cytokine concentrations were observed is not to say that LPS didn't induce changes, or indeed, other cytokines in these brain regions closer to the time of LPS injection (i.e. within 5-6 h where some changes in behaviour were observed), but this was not possible to confirm as no samples were taken around that time.

Cytokines released by immune cells, such as macrophages and monocytes, during the course of an immune response are key messengers in immune system-to-brain signalling (Kent et al., 1992). In human medicine, the so called "cytokine-theory of depression" was developed based on clinical reports and the similarity in symptoms

between sickness behaviour and depression. Hepatitis C patients undergoing weekly IFN-α therapy showed an increase in Montgomery–Asberg Depression Rating Scale (MADRS), which was positively correlated with plasma levels of IL-2, IL-6 and TNF-α (Wichers et al., 2007). Cancer patients undergoing cytokine therapy (IL-2/IFN-α) had an increase in MADRSs at five days after therapy started (Capuron et al., 2001). Whereas sickness is an adaptive response to infection by pathogens and fully reversible once the pathogen has been cleared, this is not the case for depression. It is possible that depression represents a maladaptive version of cytokine induced sickness which could occur when the activation of the innate immune response is exacerbated in intensity and/or duration (reviewed by Dantzer et al., 2008).

4.2.4 Tryptophan-kynurenine metabolism

A possible downstream effect of cytokines (IFN-γ, TNF-α) could be the induction of the enzyme IDO. Under inflammatory stages, there is a shunt from serotonin towards kynurenine metabolism of tryptophan through an upregulation of IDO. In the current experiment, a depletion of plasma tryptophan in LPS-injected pigs at 72 h after the challenge was observed, but plasma kynurenine was not affected (Paper I). Jørgensen (2020) reported a decrease in plasma tryptophan for 8 h and an increase in plasma kynurenine for 12 h after injecting pigs with a comparable dose of LPS. Wirthgen et al. (2013) showed that plasma tryptophan was depleted for 24 h (max. observation time), whereas plasma kynurenine was elevated for only 6 h after an i.p. injection with LPS (100 µg/kg). This suggests a rather short-lasting effect of LPS on kynurenine concentrations in the blood and indicates that the increase in IDO activity in response to immune activation is rather short-lived.

Tryptophan is an essential amino acid and the availability depends on dietary uptake. Pigs fed a diet with a high tryptophan content, had higher plasma tryptophan concentrations (Le Floc’h et al., 2010; Poletto et al., 2010) and vice versa (Le Floc'h et al., 2008). In the current experiment,

LPS-injected pigs showed depressed feeding during the first six hours after the challenge, which can be explained by the appetite suppressant effect of cytokines (Plata-Salamán, 1996). This might have affected the dietary intake of tryptophan in LPS-injected pigs on the day of injection but should not have a longer lasting impact on peripheral and central levels of tryptophan measured at 72 h after the challenge.

There were weak positive correlations between plasma and central tryptophan and kynurenine concentrations (Paper I). A relationship between dietary tryptophan and damaging behaviour has been reported previously. Increased levels of orally administered tryptophan reduced aggression (Li et al., 2006; Poletto et al., 2010; Poletto et al., 2014) and tail and ear biting in pigs (Martínez-Trejo et al., 2009), as well as feather pecking in chickens (Van Hierden et al., 2004). Dietary supplementation of tryptophan led to an increase in hypothalamic serotonin in pigs (Shen et al., 2012). In human patients undergoing cytokine therapy, a decrease in serum tryptophan was positively correlated with the development and severity of depressive symptoms (Capuron et al., 2002).

The effect of a controlled immune activation on central tryptophan-kynurenine metabolism has not previously been studied in pigs. Central tryptophan and kynurenine were measured in homogenized brain tissue, thus, the concentrations refer to total (intra- and extracellular) concentrations of the respective analyte. A reduction of tryptophan in the frontal cortex and brain stem of LPS-injected pigs compared to saline-injected pigs at 72 h after the challenge was observed. Kynurenine concentrations were decreased in the frontal cortex, hypothalamus and brain stem at the same time point (Paper I). Metabolites of kynurenine were not measured in the current experiment, but kynurenine concentrations might be lowered due to further metabolism. In mice, a repeated injection of LPS significantly elevated brain kynurenine as well as its metabolites kynurenic acid and quinolinic acid at 24 and 48 h (Larsson et al., 2016). LPS depleted central tryptophan and increased central kynurenine in the hippocampus together with a pronounced IDO

expression at six weeks after chronic administration (Rodrigues et al., 2018). O'Connor et al. (2009) showed that a blockade of IDO activity (measured by means of mRNA expression) prevents the development of depressive-like behaviour in mice at 24 and 28 h after LPS injection, whereas administration of kynurenine (i.p.) induces depressive-like behaviour.

4.2.5 Monoamines

There is a paucity of studies that report central monoamine levels in pigs. So far, different age groups, sexes and phenotypes (stress-susceptible vs stress-tolerant, dominant vs subordinate, tail biter vs victim) have been studied (Adeola et al., 1993; Agarwal et al., 1993; Poletto et al., 2011; Valros et al., 2015) and the effects of novelty tests and acute restraint on monoamine concentrations have been reported (Piekarzewska et al., 2000; Ursinus et al., 2013). The effect of an immune activation with LPS on central neurotransmitters in the pig brain have been tested in only one study (Nordgreen et al., 2018). In the current experiment, the concentrations of noradrenaline in the hippocampus and serotonin in the hypothalamus were numerically lower, whereas dopamine concentrations were significantly lower in the hypothalamus of LPS-injected pigs compared to controls measured at 72 h after the challenge (Paper I). Nordgreen et al. (2018) found lower noradrenaline concentrations in the frontal cortex, hippocampus, and hypothalamus of LPS-injected pigs compared to saline-injected pigs measured at the same time point. Serotonergic and dopaminergic systems were not affected by the immune challenge in the brain regions that were studied.

The mechanism behind these findings might be the effect of proinflammatory cytokines produced by immune cells in response to LPS, which cross the blood-brain-barrier, and act via specific receptors on astrocytes, microglia or neurons and thereby activate monoaminergic signalling (Shintani et al., 1993). Cytokines might also activate vagal afferents, and thereby influence the brain (Fleshner et al., 1995). If one

does not observe changes in monoamine concentrations in homogenised brain tissue, one cannot say that these systems were not affected unless one also looks at the effects of LPS on monoamine receptor function and expression. Quantification of an analyte concentration alone might not be sufficient as there may be too much noise to pull out any potential signal. Single cell isolation and analysis of neuron types might be a way of drilling down on the mechanism of LPS effect in future studies.

In rodents, lower concentrations of noradrenaline and serotonin in the prefrontal cortex (Zhu et al., 2015) and serotonin in the hippocampus (Zhao et al., 2019) after LPS injection were observed. In both studies, mice displayed depressive-like behaviours measured by increased immobility in the forced swim test (FST) and tail suspension test (TST) at 24 h after LPS-challenge. In the forced swim test the animals are placed in a container filled with water from which they cannot escape. The latency to stop active swimming (struggling) and the duration of time spent immobile (floating) are registered. In the tail suspension test the animal is suspended by its tail and the latency to cease struggling and the duration of passive immobility are scored. Immobility in both tests is thought to be indicative of helplessness and despair (Porsolt et al., 1977; Steru et al., 1985).

In humans, the so called "monoamine theory of depression" was developed based on the effectiveness of monoamine oxidase inhibitors (MAOI), selective serotonin and noradrenaline reuptake inhibitors (SSRIs/NRIs) and tricyclic antidepressant (TCA) as treatments of anxiety and depression. That suggested a role for monoamines, their receptors and transporters in the aetiology of mood disorders (Coppen, 1967; Katz et al., 2004). MAOIs inhibit the degradation or metabolism of monoamines in the neuron, whereas SSRIs, NRIs and TCA block the reuptake of either serotonin, noradrenaline or both from the synaptic cleft and thereby increase extracellular serotonin and/or noradrenaline concentrations.

4.3 Behavioural alterations in response to LPS

4.3.1 Sickness behaviour

Sickness behaviour was studied by means of general activity on the day of injection to evaluate the functioning of the LPS-model. A drop in activity and an increase in lying behaviour within 2-5 h after LPS injection compared to saline-injected controls was observed (Paper I). This finding corroborates other studies that showed a marked decrease in activity lasting for a few hours following LPS injection (Johnson and von Borell, 1994; Nordgreen et al., 2018). Behavioural changes can be seen in sick animals but also in their healthy conspecifics. Sickness and social behaviour are closely connected. Acute sickness leads to lethargy and social withdrawal (Hart, 1988). Reduction in time spent investigating an unfamiliar conspecific is a commonly used measure of sickness behaviour (Fishkin and Winslow, 1997; Arakawa et al., 2009). On a population level, the behaviour may be beneficial to prevent spread of infectious diseases by the avoidance of interaction with others (Anders et al., 2013). Immune-challenged animals showed reduced connectivity to their social groups, which happened as a function of their own behaviour, rather than through conspecific avoidance (Lopes et al., 2016). LPS-injected rats were found to exhibit less social behaviour, but spend more time passively huddling with non-injected cage-mates than controls (Yee and Prendergast, 2010). Rodents have been shown to discriminate and avoid conspecifics exhibiting a sickness response (Kavaliers and Colwell, 1995; Avitsur et al., 1997; Lopes et al., 2016). In contrast, signs of sickness failed to provoke avoidance in pigs; LPS-injected pigs and pigs diagnosed with osteochondrosis received increased social attention by pen mates (Munsterhjelm et al., 2017; Munsterhjelm et al., 2019). A sick animal probably attracts the interest of others due to passivity and unresponsiveness. In laying hens, inactive individuals were more likely to become targets of both gentle and severe feather pecks than active individuals (Riber and Forkman, 2007). As a sick animal is weakened, others may prefer to compete with this animal for resources, as success

is more certain than if engaging in a contest with a healthy conspecific. It was shown in finches that males strongly preferred feeding near diseased conspecifics (Bouwman and Hawley, 2010).

If sickness has a role in the aetiology of tail biting it must be by increasing the likelihood of a pig becoming either a victim or a biter (Munsterhjelm et al., 2019). My thesis is concerned with the hypothesis that sickness increases the chance of becoming a biter. Increased attention towards sick animals could also lead to damaging behaviour towards them, but that has not been the main focus. In boars, tail and ear biting tended to increase 0-2 weeks before clinical signs of respiratory infection were visible (Munsterhjelm et al., 2017), thus, behaviour changed already in a preclinical stage of illness. This could also be the case in the phase of recovery when clinical signs abate. Thus, pigs might feel irritable, which might increase the probability of becoming a biter both before and after clinical signs of illness. In humans, neuropsychiatric side effects such as irritability, emotional lability and short temper were reported during cytokine therapy (Denicoff et al., 1987; Renault et al., 1987; Capuron et al., 2000; Constant et al., 2005). These latter symptoms might be more relevant than depression and lethargy for understanding how the immune system can increase the likelihood of becoming a tail or ear biter.

4.3.2 Social behaviour

It is important to disentangle the cytokine-induced sickness behaviour from potential psychological aftereffects on social interactions in immune stimulated animals, when motor activity has returned to normal. Social behaviour was studied on the first and second day after the challenge, as the aim was to describe how social dynamics change after overt sickness has ended. The pigs manipulated mostly the ears and other body parts of their pen mates and were frequently involved in fights. Tail manipulation was shown to a much lesser extent. The proportions of different manipulative behaviour observed were similar to other studies in group-

housed pigs (Bolhuis et al., 2006; Van der Meer et al., 2017). Behaviours that targeted body parts such as tail, ear, head and leg were defined as "manipulative behaviour", as the pigs had the possibility to take these body parts into their mouth. To my understanding "manipulation" is a more comprehensive term than simply "biting" as it includes also more gentle touching. Severe biting which caused an immediate avoidance reaction in the receiver was rarely observed during the experiment and wounds on the targeted body parts were not present. It has been discussed that a "pre-damage" state (Fraser and Broom, 1997), in which pigs perform so called "tail/-ear-in-mouth behaviour" (Schrøder-Petersen et al., 2003; Diana et al., 2019), can develop into a "damage-state". Thus, the gentle tail or ear manipulation observed could be a precursor of more severe biting behaviour. Pigs that perform tail biting also perform a higher frequency of other abnormal behaviours (such as ear biting), which may indicate that these different abnormal behaviours to some extent have the same motivational background (Beattie et al., 2005; Brunberg et al., 2011).

LPS affected the duration of ear manipulation as well as centrality parameters for ear manipulation networks at the individual level. LPS-injected pigs manipulated the ears of their pen mates significantly longer compared to saline-injected pigs (Paper I) and received less frequent ear manipulations (decrease in *in-degree centrality*) by their pen mates two days after the challenge (Paper III). This appears logical as pigs that are involved as performers in an interaction simultaneously reduce the probability of being involved as receivers in an interaction. LPS had no effect on tail manipulation but it cannot be ruled out that this is due to the lower frequency of this behaviour (and maybe less individuals showing it at all). In a previous experiment, it was shown that LPS-injected pigs had a shift in social motivation (seen as more ear- and tail-directed behaviour) 40 h after the signs of acute illness dissipated and this was not accompanied by an increase in activity (Munsterhjelm et al., 2019). When sickness behaviour resolves, mice display depressive-like

behaviours measured by increased immobility in the forced swim test (FST) and tail suspension test (TST) at 24-28 h after LPS-challenge (Frenois et al., 2007; O'Connor et al., 2009; Ge et al., 2015; Zhu et al., 2015; Sulakhiya et al., 2016; Zhao et al., 2019). It is these psychological aftereffects and their potential effect on social interactions that we wanted to investigate with the current experiment in pigs.

The stronger the pigs reacted towards the LPS-challenge (indexed by cortisol response and frequency of lying behaviour in the first 6 h after LPS), the more they were manipulated by pen mates (ears, and tails excepted) the subsequent days (Paper III). When it comes to interpreting manipulation of other body parts Jensen and Wood-Gush (1984) suggested a threatening function of "nose-to-nose" contact and associated "nose-to-body" contact with individual recognition. Camerlink and Turner (2013) found that nosing the tail correlated with tail biting and nosing an ear correlated with ear biting, but nosing other parts of the body was unrelated to such damaging forms of interaction. It is therefore not clear whether manipulation of other body parts can be interpreted as purely affiliative behaviour. However, it is interesting to note that LPS-injected pigs increased ear manipulation of pen mates, which might be a first stage towards damaging behaviour, but received attention of a kind that has not been linked to ear and tail biting. Thus, they did not seem to become victims following their brief bout of sickness.

At the pen level, the ears of certain individuals were more frequently manipulated (increase in *in-degree centralisation*) than the rest of the group in the two subsequent days after injection (Paper III). Network connectivity in fighting networks increased (decrease in *betweenness*) one day after injection suggesting that interactions were more evenly spread across all group members and no single individual was responsible for connecting a fragmented network. The results indicate that the pigs changed the way they directed social activities and that the immune status of individuals affected these changes. Social network analysis is a

method that can potentially provide important insights into the effects of sickness on social behaviour in a gregarious production animal species.

4.4 Effects of the NSAID ketoprofen on the effects of LPS

There are anecdotal reports that NSAIDs are used as a treatment for tail biting outbreaks. Ketoprofen was used to test whether it would prevent or attenuate adverse effects of immune activation on physiology and behaviour, in order to better understand causality. Ketoprofen has analgesic, anti-inflammatory and anti-pyretic effects and is licenced for use in pigs (Fosse et al., 2011). Ketoprofen inhibits the activity of both isoforms (1 and 2) of the enzyme cyclooxygenase (Tobetto et al., 1997), and thereby reduces the production of PGE_2 in endothelial cells which is, as the main pro-inflammatory prostaglandin, responsible for the development of fever. The administration of ketoprofen significantly reduced PGE_2 production compared to LPS-groups (Wyns et al., 2015a). This is one possible pathway through which ketoprofen can influence behaviour. Moreover, some NSAIDs are able to alter the expression of NF-kB and thereby reduce subsequent cytokine expression (Peters et al., 2012), but whether ketoprofen works in this way is not known.

In the current experiment, ketoprofen attenuated the effects of LPS on cortisol, the acute phase response, plasma tryptophan and behavioural signs of sickness (Paper I+II). Central monoamines were lower in LPS-injected pigs compared to those pre-treated with ketoprofen (Paper I). On the other hand, social behaviour, central tryptophan-kynurenine-metabolism, as well as central cytokines were not affected by a pre-treatment with ketoprofen (Paper I+III). Other pig studies using ketoprofen prior or after LPS injection (Mustonen et al., 2012; Salichs et al., 2012; Wyns et al., 2015a) reported an attenuation of behavioural signs of sickness, a reduced PGE_2 production and a decrease or a lack of increase in rectal body temperature. No effect of ketoprofen (Wyns et al., 2015a) or flunixin (Peters et al., 2012) on the increase of plasma concentrations of TNF-α, IL1-β and IL-6 in response to LPS were found;

central cytokine concentrations were not measured in these studies. In mice, the non-selective NSAIDs indomethacin and ibuprofen reversed the effect of LPS on behaviour without changing serum concentrations and hippocampal mRNA expression of TNF-α, IL1-β and IL-6 (Teeling et al., 2010). The effect of NSAIDs on brain monoamines in response to inflammation has not been studied in pigs so far. In rodents, IFN-α increased serotonin turnover in the prefrontal cortex and dopamine turnover in hippocampus, while pre-treatment with the NSAID diclofenac completely prevented these effects (De La Garza and Asnis, 2003). Pre-treatment with indomethacin attenuated LPS or IL-1 induced noradrenaline and serotonin turnover in the hippocampus (Linthorst et al., 1996) and the hypothalamus (Masana et al., 1990; Mefford and Heyes, 1990). A metanalysis in human patients (n= 6262) revealed that the use of NSAIDs and cytokine inhibitors reduced depressive symptoms compared with placebo (Köhler et al., 2014). The ratio of kynurenine to tryptophan (measured in plasma), which represents the activity of IDO, predicted the antidepressant response to NSAID therapy with celecoxib (Krause et al., 2017).

4.5 Effects of housing environment and management on immune system and behaviour

Damaging behaviour is a symptom of the environment not fulfilling the needs of the pig and the effects of risk factors are cumulative (reviewed by Nordgreen et al., 2020). In the current experiment, the pigs were kept at the Livestock Production Research Center of NMBU. The pigs received wood shavings and grass silage twice a day. Many studies showed that provision of enrichment materials reduce or prevent tail biting compared to barren environments (Petersen et al., 1995; Beattie et al., 2000; Day et al., 2008). It has also been reported that environmental enrichment modulates different aspects of the immune system (Manciocco et al., 2011; van Dixhoorn et al., 2016). Enriched housed pigs had lower haptoglobin concentrations than barren housed pigs (Scott et al., 2006; Scollo et al., 2013; Reimert et al., 2014). Brown et al. (2018) showed an

upregulation of genes involved in neuroprotection and synaptic plasticity in the frontal cortex at one hour after stimulating pigs with a straw-filled bag. Microglia activity seemed to be downregulated in enriched compared to barren conditions, where pigs were housed in smaller pens without additional straw (Brown et al., 2018). Microglia are specialised macrophages of the CNS that play a role in neuromodulation, phagocytosis and inflammation (reviewed by Gomez-Nicola and Perry, 2015). The pigs in the current experiment were housed at a rather low stocking density (1.3m^2/pig) compared to commercial conditions and they had a solid lying area, which was half the size of the pen. Slatted flooring, limited feeding space and high stocking density contribute to a higher risk of tail biting (Moinard et al., 2003). Housing environment has also been related to the acute phase response. The pigs in the current experiment were housed with their siblings in the pens they were born in and were not moved to other compartments during the study. Poor sanitary conditions (no cleaning between batches, no preventive treatment with antibiotics) resulted in elevated haptoglobin concentrations (Le Floc'h et al., 2009; Le Floc'h et al., 2010; Pastorelli et al., 2012; Van der Meer et al., 2016), a decrease in plasma tryptophan (Le Floc'h et al., 2009) and an increase in total oral manipulation of pen mates (Van der Meer et al., 2017). Furthermore, the feed of the pigs in the current experiment had an optimal nutrient composition and was available ad libitum. It was shown that pigs fed with a low protein diet showed more ear and tail biting and other oral manipulation directed towards pen mates (Van der Meer et al., 2017). Moreover, management practices that are common in commercial pig production, such as mixing with unfamiliar conspecifics after weaning, were not conducted in the present study. It has been shown that piglets in socialized pens showed a significantly lower *degree centrality*, *eigenvector centrality* and *clustering coefficient* compared to controls (Turner et al., 2020). Regrouping causes stress and activates the immune system as shown by elevated salivary cortisol (de Groot et al., 2001; Merlot et al., 2004; Coutellier et al., 2007) and induced leukocyte mobilization (Bacou et al., 2017). Overall, the housing conditions were

rather optimal regarding the known risk factors for damaging behaviours and they might have contributed to the low levels of tail manipulation (9 % of all social behaviour observed) in the current experiment.

4.6 Conclusion

The overall aim of my PhD was to understand how immune activation influences brain physiology in pigs and how these physiological changes can drive changes in social behaviour. The working hypothesis was that episodes of acute proinflammatory signalling in pigs can, subsequent to acute sickness, elicit longer term negative social behaviour such as tail and ear biting. The results of Paper I showed that a single low dose of LPS had longer-term effects on the social behaviour of pigs after recovery from acute sickness. Social network analysis revealed behavioural changes both at individual and group level in response to immune activation (Paper III). No true damage was observed on the pigs maybe due to the optimal housing conditions (as discussed in 4.5.). It was not possible to confirm that the behavioural changes were elicited by proinflammatory cytokines, but monoamines which are supposed to play an important role in the regulation of mood and behaviour were downregulated in several brain regions in the pigs that received LPS (Paper I). Furthermore, central tryptophan and kynurenine concentrations were altered in response to LPS and their downstream metabolites are hypothesised to have either neuroprotective or neurotoxic effects (dependent on the further metabolic pathway) in the central nervous system (Paper I). The findings of Paper II added to our knowledge the time course of adenosine deaminase activity in response to LPS, suggesting that ADA is a promising inflammatory biomarker in pigs. The NSAID ketoprofen can be used to attenuate the effects of a controlled immune activation on both the HPA-axis and the acute phase response and alleviates behavioural signs of sickness (Paper I+II).

5 Future perspectives

RNA sequencing

To further disentangle the effects of a controlled immune activation on brain physiology and behaviour, it would be relevant to study the gene expression in the sampled brain regions of different factors of interest, such as the enzyme IDO or markers of microglia activation (such as IBA-1). A differential gene expression analysis can detect up- or downregulated genes in response to the LPS treatment, which would tell us more about the pathways involved in behavioural alterations.

Field study

We have pilot data indicating that the immune status of the pig is influenced by the housing environment. It would be interesting to test the effect of housing, (e.g. closed barn vs barn with access to outdoor area vs fully outdoor system) on the innate immune system, brain physiology and behaviour of the pigs. This set up could also be used to validate ADA in a larger sample size under field conditions. It would be further interesting to apply SNA to behavioural data of pens with either healthy pigs or sick pigs in order to compare their social structure.

Behavioural testing

In my PhD, I wanted to investigate the psychological aftereffects following acute sickness and their potential effect on social interactions. As there is no validated test to evaluate depressive-like behaviour in pigs, it would be highly relevant to test if an acute immune activation alters their affective states. This could be evaluated in a cognitive bias test (e.g. spatial judgment task, Düpjan et al., 2017) in which pigs are trained to associate one cue with a positive outcome and a second cue with a

negative outcome (cues are spatial, visual, auditory, olfactory). During the test, the pig is then confronted with a novel and ambiguous cue and the response interpreted as either positively or negatively biased.

Gut-brain-axis

In my PhD, I studied the bidirectional communication between the innate immune system and the central nervous system in relation to behaviour. The gut-brain-axis is another bidirectional communication system that involves the HPA-axis, the innate immune system (cytokines), tryptophan metabolism and neurotransmitters (serotonin). Each individual has a different composition of microbiota in its gastrointestinal tract which is influenced by diet, genetics and other factors. In experiments with germ free mice it has been shown that microbiota is essential for the development of normal social behaviour (Desbonnet et al., 2014). In laying hens, high feather pecking lines have a different gut microbiota compared to low feather pecking lines (Meyer et al., 2013). The hypothesis whether pigs that show tail or ear biting have a different composition of gut microbes than pigs that do not develop damaging behaviours needs to be tested (reviewed by Brunberg et al., 2016). A recent report showed more lactobacilli in the gut microbiota of pigs that neither bit nor were bitten compared to biters and victims (Rabhi et al., 2020).

6 References

Abbasi, S.-H., Hosseini, F., Modabbernia, A., Ashrafi, M., Akhondzadeh, S., 2012. Effect of celecoxib add-on treatment on symptoms and serum IL-6 concentrations in patients with major depressive disorder: Randomized double-blind placebo-controlled study. Journal of Affective Disorders 141, 308-314.

Adeola, O., Ball, R.O., House, J.D., O'Brien, P.J., 1993. Regional brain neurotransmitter concentrations in stress-susceptible pigs. Journal of Animal Science 71, 968-974.

Agarwal, R.K., Chandna, V.K., Engelking, L.R., Lightbown, K., Kumar, M.S.A., 1993. Distribution of catecholamines in the central nervous system of the pig. Brain Research Bulletin 32, 285-291.

Altmann, J., 1974. Observational Study of Behavior: Sampling Methods. Behaviour 49, 227-266.

Anand, B.K., Brobeck, J.R., 1951. Hypothalamic control of food intake in rats and cats. The Yale Journal of Biology and Medicine 24, 123-140.

Anders, S., Tanaka, M., Kinney, D.K., 2013. Depression as an evolutionary strategy for defense against infection. Brain, Behavior, and Immunity 31, 9-22.

Arakawa, H., Blandino, P., Deak, T., 2009. Central infusion of interleukin-1 receptor antagonist blocks the reduction in social behavior produced by prior stressor exposure. Physiology & Behavior 98, 139-146.

Arnsten, A.F.T., 2009. Stress signalling pathways that impair prefrontal cortex structure and function. Nature Reviews Neuroscience 10, 410-422.

Arroyo, L., Carreras, R., Valent, D., Peña, R., Mainau, E., Velarde, A., Sabrià, J., Bassols, A., 2016. Effect of handling on neurotransmitter profile in pig brain according to fear related behaviour. Physiology & Behavior 167, 374-381.

Asher, L., Collins, L.M., Ortiz-Pelaez, A., Drewe, J.A., Nicol, C.J., Pfeiffer, D.U., 2009. Recent advances in the analysis of behavioural organization and interpretation as indicators of animal welfare. Journal of The Royal Society Interface 6, 1103-1119.

Avitsur, R., Cohen, E., Yirmiya, R., 1997. Effects of Interleukin-1 on Sexual Attractivity in a Model of Sickness Behavior. Physiology & Behavior 63, 25-30.

Bacou, E., Haurogné, K., Mignot, G., Allard, M., De Beaurepaire, L., Marchand, J., Terenina, E., Billon, Y., Jacques, J., Bach, J.-M., Mormède, P., Hervé, J., Lieubeau, B., 2017. Acute social stress-induced immunomodulation in pigs high and low responders to ACTH. Physiology & Behavior 169, 1-8.

Beattie, V.E., Breuer, K., O'Connell, N.E., Sneddon, I.A., Mercer, J.T., Rance, K.A., Sutcliffe, M.E.M., Edwards, S.A., 2005. Factors identifying pigs predisposed to tail biting. Animal Science 80, 307-312.

Beattie, V.E., O'Connell, N.E., Moss, B.W., 2000. Influence of environmental enrichment on the behaviour, performance and meat quality of domestic pigs. Livestock Production Science 65, 71-79.

Beattie, V.E., Walker, N., Sneddon, I.A., 1995. Effects of Environmental Enrichment on Behaviour and Productivity of Growing Pigs. Animal Welfare 4, 207-220.

Berkenbosch, F., van Oers, J., del Rey, A., Tilders, F., Besedovsky, H., 1987. Corticotropin-releasing factor-producing neurons in the rat activated by interleukin-1. Science 238, 524.

Bernton, E.W., Beach, J.E., Holaday, J.W., Smallridge, R.C., Fein, H.G., 1987. Release of multiple hormones by a direct action of interleukin-1 on pituitary cells. Science 238, 519.

Besedovsky, H., del Rey, A., Sorkin, E., Dinarello, C.A., 1986. Immunoregulatory feedback between interleukin-1 and glucocorticoid hormones. Science 233, 652.

Bhattacharyya, S., Brown, D.E., Brewer, J.A., Vogt, S.K., Muglia, L.J., 2007. Macrophage glucocorticoid receptors regulate Toll-like receptor 4–mediated inflammatory responses by selective inhibition of p38 MAP kinase. Blood 109, 4313-4319.

Bluthé, R.M., Michaud, B., Kelley, K.W., Dantzer, R., 1996. Vagotomy attenuates behavioural effects of interleukin-1 injected peripherally but not centrally. Neuroreport 7, 1485-1488.

Bolhuis, J.E., Schouten, W.G.P., Schrama, J.W., Wiegant, V.M., 2006. Effects of rearing and housing environment on behaviour and performance of pigs with different coping characteristics. Applied Animal Behaviour Science 101, 68-85.

Bouwman, K.M., Hawley, D.M., 2010. Sickness behaviour acting as an evolutionary trap? Male house finches preferentially feed near diseased conspecifics. Biology Letters 6, 462-465.

Bradford, K.L., Moretti, F.A., Carbonaro-Sarracino, D.A., Gaspar, H.B., Kohn, D.B., 2017. Adenosine Deaminase (ADA)-Deficient Severe Combined Immune Deficiency (SCID): Molecular Pathogenesis and Clinical Manifestations. Journal of Clinical Immunology 37, 626-637.

Breuer, K., Sutcliffe, M.E.M., Mercer, J.T., Rance, K.A., Beattie, V.E., Sneddon, I.A., Edwards, S.A., 2003. The effect of breed on the development of adverse social behaviours in pigs. Applied Animal Behaviour Science 84, 59-74.

Breuer, K., Sutcliffe, M.E.M., Mercer, J.T., Rance, K.A., O'Connell, N.E., Sneddon, I.A., Edwards, S.A., 2005. Heritability of clinical tail-biting and its relation to performance traits. Livestock Production Science 93, 87-94.

Brown, S.M., Bush, S.J., Summers, K.M., Hume, D.A., Lawrence, A.B., 2018. Environmentally enriched pigs have transcriptional profiles consistent with neuroprotective effects and reduced microglial activity. Behavioural Brain Research 350, 6-15.

Brunberg, E., Wallenbeck, A., Keeling, L.J., 2011. Tail biting in fattening pigs: Associations between frequency of tail biting and other abnormal behaviours. Applied Animal Behaviour Science 133, 18-25.

Brunberg, E.I., Rodenburg, T.B., Rydhmer, L., Kjaer, J.B., Jensen, P., Keeling, L.J., 2016. Omnivores Going Astray: A Review and New Synthesis of Abnormal Behavior in Pigs and Laying Hens. Frontiers in Veterinary Science 3, 57.

Burrell, R., 1994. Human responses to bacterial endotoxin. Circulatory Shock 43, 137-153.

Byrne, G.I., Lehmann, L.K., Kirschbaum, J.G., Borden, E.C., Lee, C.M., Brown, R.R., 1986. Induction of Tryptophan Degradation In Vitro and In Vivo: A γ-Interferon-Stimulated Activity. Journal of Interferon Research 6, 389-396.

Cambronero, J.C., Borrell, J., Guaza, C., 1989. Glucocorticoids modulate rat hypothalamic corticotrophin-releasing factor release induced by interleukin-1. Journal of Neuroscience Research 24, 470-476.

Camerlink, I., Bijma, P., Kemp, B., Bolhuis, J.E., 2012. Relationship between growth rate and oral manipulation, social nosing, and aggression in finishing pigs. Applied Animal Behaviour Science 142, 11-17.

Camerlink, I., Turner, S.P., 2013. The pig's nose and its role in dominance relationships and harmful behaviour. Applied Animal Behaviour Science 145, 84-91.

Capuron, L., Ravaud, A., Dantzer, R., 2000. Early Depressive Symptoms in Cancer Patients Receiving Interleukin 2 and/or Interferon Alfa-2b Therapy. Journal of Clinical Oncology 18, 2143-2151.

Capuron, L., Ravaud, A., Gualde, N., Bosmans, E., Dantzer, R., Maes, M., Neveu, P.J., 2001. Association between immune activation and early depressive symptoms in cancer patients treated with interleukin-2-based therapy. Psychoneuroendocrinology 26, 797-808.

Capuron, L., Ravaud, A., Neveu, P.J., Miller, A.H., Maes, M., Dantzer, R., 2002. Association between decreased serum tryptophan concentrations and depressive symptoms in cancer patients undergoing cytokine therapy. Molecular Psychiatry 7, 468-473.

Carroll, J.A., Carter, D.B., Korte, S.W., Prather, R.S., 2005. Evaluation of the acute phase response in cloned pigs following a lipopolysaccharide challenge. Domestic Animal Endocrinology 29, 564-572.

Chang, C.-h., Grace, A.A., 2013. Amygdala β-noradrenergic receptors modulate delayed downregulation of dopamine activity following restraint. The Journal of Neuroscience 33, 1441-1450.

Cid, M.C., Grant, D.S., Hoffman, G.S., Auerbach, R., Fauci, A.S., Kleinman, H.K., 1993. Identification of haptoglobin as an angiogenic

factor in sera from patients with systemic vasculitis. The Journal of Clinical Investigation 91, 977-985.

Clement, H.W., Buschmann, J., Rex, S., Grote, C., Opper, C., Gemsa, D., Wesemann, W., 1997. Effects of interferon-gamma, interleukin-1 beta, and tumor necrosis factor-alpha on the serotonin metabolism in the nucleus raphe dorsalis of the rat. Journal of Neural Transmission 104, 981-991.

Constant, A., Castera, L., Dantzer, R., Couzigou, P., de Ledinghen, V., Demotes-Mainard, J., Henry, C., 2005. Mood alterations during interferon-alfa therapy in patients with chronic hepatitis C: evidence for an overlap between manic/hypomanic and depressive symptoms. The Journal of Clinical Psychiatry 66, 1050-1057.

Cook, N.J., Schaefer, A.L., Lepage, P., Jones, S.M., 1996. Salivary vs. serum cortisol for the assessment of adrenal activity in swine. Canadian Journal of Animal Science 76, 329-335.

Coppen, A., 1967. The Biochemistry of Affective Disorders. British Journal of Psychiatry 113, 1237-1264.

Coutellier, L., Arnould, C., Boissy, A., Orgeur, P., Prunier, A., Veissier, I., Meunier-Salaün, M.-C., 2007. Pig's responses to repeated social regrouping and relocation during the growing-finishing period. Applied Animal Behaviour Science 105, 102-114.

Couzin, I.D., Krause, J., 2003. Self-Organization and Collective Behavior in Vertebrates, Advances in the Study of Behavior, Academic Press, pp. 1-75.

Cox, K., Devanarayan, V., Kriauciunas, A., al., e., 2012. Immunoassay Methods, In: Markossian S, S.G., Grossman A, et al. (Ed.), Assay Guidance Manual Eli Lilly & Company and the National Center for Advancing Translational Sciences, https://www.ncbi.nlm.nih.gov/books/NBK92434/.

Croft, D.P., James, R., Krause, J., 2008. Exploring Animals Social Networks. Princeton University Press, Oxfordshire.

D'Eath, R.B., Niemi, J.K., Vosough Ahmadi, B., Rutherford, K.M.D., Ison, S.H., Turner, S.P., Anker, H.T., Jensen, T., Busch, M.E., Jensen, K.K., Lawrence, A.B., Sandøe, P., 2016. Why are most EU pigs tail docked? Economic and ethical analysis of four pig housing and

management scenarios in the light of EU legislation and animal welfare outcomes. Animal 10, 687-699.

D'Eath, R.B., Turner, S.P., 2009. The Welfare of Pigs, in: Marchant-Forde, J.N. (Ed.), Springer.

Dantzer, R., O'Connor, J.C., Freund, G.G., Johnson, R.W., Kelley, K.W., 2008. From inflammation to sickness and depression: when the immune system subjugates the brain. Nature Reviews Neuroscience 9, 46-56.

Day, J.E.L., Burfoot, A., Docking, C.M., Whittaker, X., Spoolder, H.A.M., Edwards, S.A., 2002. The effects of prior experience of straw and the level of straw provision on the behaviour of growing pigs. Applied Animal Behaviour Science 76, 189-202.

Day, J.E.L., Van de Weerd, H.A., Edwards, S.A., 2008. The effect of varying lengths of straw bedding on the behaviour of growing pigs. Applied Animal Behaviour Science 109, 249-260.

De Groot, J., Ruis, M.A.W., Scholten, J.W., Koolhaas, J.M., Boersma, W.J.A., 2001. Long-term effects of social stress on antiviral immunity in pigs. Physiology & Behavior 73, 145-158.

De La Garza, R., Asnis, G.M., 2003. The non-steroidal anti-inflammatory drug diclofenac sodium attenuates IFN-α induced alterations to monoamine turnover in prefrontal cortex and hippocampus. Brain Research 977, 70-79.

Denicoff, K.D., Rubinow, D.R., Papa, M.Z., Simpson, C., Seipp, C.A., Lotze, M.T., Chang, A.E., Rosenstein, D., Rosenberg, S.A., 1987. The Neuropsychiatric Effects of Treatment with Interleukin-2 and Lymphokine-Activated Killer Cells. Annals of Internal Medicine 107, 293-300.

Desbonnet, L., Clarke, G., Shanahan, F., Dinan, T.G., Cryan, J.F., 2014. Microbiota is essential for social development in the mouse. Molecular Psychiatry 19, 146-148.

Diana, A., Carpentier, L., Piette, D., Boyle, L.A., Berckmans, D., Norton, T., 2019. An ethogram of biter and bitten pigs during an ear biting event: first step in the development of a Precision Livestock Farming tool. Applied Animal Behaviour Science 215, 26-36.

Dippel, S., Schrader, L., 2016. Tail docking in pigs - status quo in Germany. Züchtungskunde 88, 417-428.

Doncaster, C.P., Davey, A.J.H., 2007. Analysis of Variance and Covariance: How to Choose and Construct Models for the Life Sciences. Cambridge University Press, Cambridge.

Drachman, D.A., Arbit, J., 1966. Memory and the Hippocampal Complex: II. Is Memory a Multiple Process? Archives of Neurology 15, 52-61.

Dunn, A.J., 1988. Systematic interleukin-1 administration stimulates hypothalamic norepinephrine metabolism parallelling the increased plasma corticosterone. Life Sciences 43, 429-435.

Dunn, A.J., Swiergiel, A.H., Beaurepaire, R.d., 2005. Cytokines as mediators of depression: What can we learn from animal studies? Neuroscience & Biobehavioral Reviews 29, 891-909.

Düpjan, S., Stracke, J., Tuchscherer, A., Puppe, B., 2017. An improved design for the spatial judgement task in domestic pigs. Applied Animal Behaviour Science 187, 23-30.

Dänicke, S., Brosig, B., Kersten, S., Kluess, J., Kahlert, S., Panther, P., Diesing, A.-K., Rothkötter, H.-J., 2013. The Fusarium toxin deoxynivalenol (DON) modulates the LPS induced acute phase reaction in pigs. Toxicology Letters 220, 172-180.

Ebdrup, L., Krog, J.A.N., Granfeldt, A., Larsen, P.Ø., Vestergaard, C., Hokland, M., Tønnesen, E., 2008. Leukocyte, plasma, and organ-associated cytokine profiles in an animal model of acute inflammation APMIS 116, 352-360.

Eckersall, P.D., Saini, P.K., McComb, C., 1996. The acute phase response of acid soluble glycoprotein, α1-acid glycoprotein, ceruloplasmin, haptoglobin and C-reactive protein, in the pig. Veterinary Immunology and Immunopathology 51, 377-385.

Escribano, D., Campos, P.H.R.F., Gutiérrez, A.M., Le Floc'h, N., Cerón, J.J., Merlot, E., 2014. Effect of repeated administration of lipopolysaccharide on inflammatory and stress markers in saliva of growing pigs. The Veterinary Journal 200, 393-397.

Fava, M., Kendler, K.S., 2000. Major depressive disorder. Neuron 28, 335-341.

Félix, B., Léger, M.-E., Albe-Fessard, D., Marcilloux, J.C., Rampin, O., Laplace, J.P., Duclos, A., Fort, F., Gougis, S., Costa, M., Duclos, N., 1999. Stereotaxic atlas of the pig brain. Brain Research Bulletin 49, 1-137.

Fernstrom, J.D., Wurtman, R.J., 1971. Brain Serotonin Content: Physiological Dependence on Plasma Tryptophan Levels. Science 173, 149.

Fink, M.P., Heard, S.O., 1990. Laboratory models of sepsis and septic shock. Journal of Surgical Research 49, 186-196.

Fishkin, R.J., Winslow, J.T., 1997. Endotoxin-induced reduction of social investigation by mice: interaction with amphetamine and anti-inflammatory drugs. Psychopharmacology 132, 335-341.

Fleshner, M., Goehler, L.E., Hermann, J., Relton, J.K., Maier, S.F., Watkins, L.R., 1995. Interleukin-1β induced corticosterone elevation and hypothalamic NE depletion is vagally mediated. Brain Research Bulletin 37, 605-610.

Foister, S., 2019. The application of social network analysis to understand acute and chronic post-mixing aggression in commercially reared pigs, The University of Edinburgh, Edinburgh.

Fosse, T.K., Toutain, P.L., Spadavecchia, C., Haga, H.A., Horsberg, T.E., Ranheim, B., 2011. Ketoprofen in piglets: enantioselective pharmacokinetics, pharmacodynamics and PK/PD modelling. Journal of Veterinary Pharmacology and Therapeutics 34, 338-349.

Frank, J.W., Mellencamp, M.A., Carroll, J.A., Boyd, R.D., Allee, G.L., 2005. Acute feed intake and acute-phase protein responses following a lipopolysaccharide challenge in pigs from two dam lines. Veterinary Immunology and Immunopathology 107, 179-187.

Fraser, A.F., Broom, D.M., 1997. Farm animal behaviour and welfare. CAB International, Wallingford, OX10 8DE.

Freeman, L.C., 1978/79. Centrality in Social Networks Conceptual Clarification. Social Networks 1, 215-239.

Frenois, F., Moreau, M., O'Connor, J., Lawson, M., Micon, C., Lestage, J., Kelley, K.W., Dantzer, R., Castanon, N., 2007. Lipopolysaccharide induces delayed FosB/DeltaFosB immunostaining within the mouse extended amygdala, hippocampus and hypothalamus, that parallel

the expression of depressive-like behavior. Psychoneuroendocrinology 32, 516-531.

Friton, G.M., Schmidt, H., Schrödl, W., 2006. Clinical and anti-inflammatory effects of treating endotoxin-challenged pigs with meloxicam. Veterinary Record 159, 552.

Fujigaki, S., Saito, K., Sekikawa, K., Tone, S., Takikawa, O., Fujii, H., Wada, H., Noma, A., Seishima, M., 2001. Lipopolysaccharide induction of indoleamine 2,3-dioxygenase is mediated dominantly by an IFN-γ-independent mechanism. European Journal of Immunology 31, 2313-2318.

Fukui, S., Schwarcz, R., Rapoport, S.I., Takada, Y., Smith, Q.R., 1991. Blood–Brain Barrier Transport of Kynurenines: Implications for Brain Synthesis and Metabolism. Journal of Neurochemistry 56, 2007-2017.

Ge, L., Liu, L., Liu, H., Liu, S., Xue, H., Wang, X., Yuan, L., Wang, Z., Liu, D., 2015. Resveratrol abrogates lipopolysaccharide-induced depressive-like behavior, neuroinflammatory response, and CREB/BDNF signaling in mice. European Journal of Pharmacology 768, 49-57.

Gomez-Nicola, D., Perry, V.H., 2015. Microglial dynamics and role in the healthy and diseased brain: a paradigm of functional plasticity. The Neuroscientist 21, 169-184.

Goscinski, G., Lipcsey, M., Eriksson, M., Larsson, A., Tano, E., Sjölin, J., 2003. Endotoxin neutralization and anti-inflammatory effects of tobramycin and ceftazidime in porcine endotoxin shock. Critical Care 8, R35.

Grahn, R.E., Will, M.J., Hammack, S.E., Maswood, S., McQueen, M.B., Watkins, L.R., Maier, S.F., 1999. Activation of serotonin-immunoreactive cells in the dorsal raphe nucleus in rats exposed to an uncontrollable stressor. Brain Research 826, 35-43.

Grau-Roma, L., Heegaard, P.M.H., Hjulsager, C.K., Sibila, M., Kristensen, C.S., Allepuz, A., Piñeiro, M., Larsen, L.E., Segalés, J., Fraile, L., 2009. Pig-major acute phase protein and haptoglobin serum concentrations correlate with PCV2 viremia and the clinical course of postweaning multisystemic wasting syndrome. Veterinary Microbiology 138, 53-61.

Grümpel, A., Krieter, J., Veit, C., Dippel, S., 2018. Factors influencing the risk for tail lesions in weaner pigs (Sus scrofa). Livestock Science 216, 219-226.

Gutiérrez, A.M., Cerón, J.J., Fuentes, P., Montes, A., Martínez-Subiela, S., 2012. Longitudinal analysis of acute-phase proteins in saliva in pig farms with different health status. Animal 6, 321-326.

Gutiérrez, A.M., De La Cruz-Sánchez, E., Montes, A., Sotillo, J., Gutiérrez-Panizo, C., Fuentes, P., Tornel, P.L., Cabezas-Herrera, J., 2017. Easy and non-invasive disease detection in pigs by adenosine deaminase activity determinations in saliva. PLOS ONE 12, e0179299.

Gutiérrez, A.M., Escribano, D., Fuentes, M., Cerón, J.J., 2013a. Circadian pattern of acute phase proteins in the saliva of growing pigs. The Veterinary Journal 196, 167-170.

Gutiérrez, A.M., Martínez-Subiela, S., Cerón, J.J., 2009a. Evaluation of an immunoassay for determination of haptoglobin concentration in various biological specimens from swine. American Journal of Veterinary Research 70, 691-696.

Gutiérrez, A.M., Martínez-Subiela, S., Soler, L., Pallarés, F.J., Cerón, J.J., 2009b. Use of saliva for haptoglobin and C-reactive protein quantifications in porcine respiratory and reproductive syndrome affected pigs in field conditions. Veterinary Immunology and Immunopathology 132, 218-223.

Gutiérrez, A.M., Montes, A., Gutiérrez-Panizo, C., Fuentes, P., De La Cruz-Sánchez, E., 2018. Gender influence on the salivary protein profile of finishing pigs. Journal of Proteomics 178, 107-113.

Gutiérrez, A.M., Nöbauer, K., Soler, L., Razzazi-Fazeli, E., Gemeiner, M., Cerón, J.J., Miller, I., 2013b. Detection of potential markers for systemic disease in saliva of pigs by proteomics: A pilot study. Veterinary Immunology and Immunopathology 151, 73-82.

Hailman, E., Lichenstein, H.S., Wurfel, M.M., Miller, D.S., Johnson, D.A., Kelley, M., Busse, L.A., Zukowski, M.M., Wright, S.D., 1994. Lipopolysaccharide (LPS)-binding protein accelerates the binding of LPS to CD14. Journal of Experimental Medicine 179, 269-277.

Harley, S., Boyle, L., O'Connell, N., More, S., Teixeira, D., Hanlon, A., 2014. Docking the value of pigmeat? Prevalence and financial

implications of welfare lesions in Irish slaughter pigs. Animal Welfare 23, 275-285.

Hart, B.L., 1988. Biological basis of the behavior of sick animals. Neuroscience & Biobehavioral Reviews 12, 123-137.

Heegaard, P.M.H., Klausen, J., Nielsen, J.P., González-Ramón, N., Piñeiro, M., Lampreave, F., Alava, M.A., 1998. The Porcine Acute Phase Response to Infection with Actinobacillus pleuropneumoniae. Haptoglobin, C-Reactive Protein, Major Acute Phase Protein and Serum Amyloid A Protein Are Sensitive Indicators of Infection. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology 119, 365-373.

Heegaard, P.M.H., Stockmarr, A., Piñeiro, M., Carpintero, R., Lampreave, F., Campbell, F.M., Eckersall, P.D., Toussaint, M.J.M., Gruys, E., Sorensen, N.S., 2011. Optimal combinations of acute phase proteins for detecting infectious disease in pigs. Veterinary Research 42, 50.

Hori, T., Nakashima, T., Koga, H., Kiyohara, T., Inoue, T., 1988. Convergence of thermal, osmotic and cardiovascular signals on preoptic and anterior hypothalamic neurons in the rat. Brain Research Bulletin 20, 879-885.

Hueston, C.M., Deak, T., 2014. The inflamed axis: The interaction between stress, hormones, and the expression of inflammatory-related genes within key structures comprising the hypothalamic–pituitary–adrenal axis. Physiology & Behavior 124, 77-91.

Hunter, E.J., Jones, T.A., Guise, H.J., Penny, R.H.C., Hoste, S., 1999. Tail biting in pigs 1: the prevalence at six UK abattoirs and the relationship of tail biting with docking, sex and other carcass damage. Pig Journal (United Kingdom).

Hunter, E.J., Jones, T.A., Guise, H.J., Penny, R.H.C., Hoste, S., 2001. The relationship between tail biting in pigs, docking procedure and other management practices. The Veterinary Journal 161, 72-79.

Höglund, E., Øverli, Ø., Winberg, S., 2019. Tryptophan Metabolic Pathways and Brain Serotonergic Activity: A Comparative Review. Frontiers in Endocrinology 10, 158.

Ivanov, A.I., Pero, R.S., Scheck, A.C., Romanovsky, A.A., 2002. Prostaglandin E(2)-synthesizing enzymes in fever: differential

transcriptional regulation. American Journal of Physiology-Regulatory, Integrative and Comparative Physiol 283, R1104-1117.
Jensen, P., Wood-Gush, D.G.M., 1984. Social interactions in a group of free-ranging sows. Applied Animal Behaviour Science 12, 327-337.
Johnson, R.W., von Borell, E., 1994. Lipopolysaccharide-induced sickness behavior in pigs is inhibited by pretreatment with indomethacin. Journal of Animal Science 72, 309-314.
Jørgensen, C., 2020. Changes in tryptophan and kynurenine concentrations in pig plasma after in vivo immune activation and controlled tissue trauma, Norwegian University of Life Sciences, Oslo.
Kabiersch, A., del Rey, A., Honegger, C.G., Besedovsky, H.O., 1988. Interleukin-1 induces changes in norepinephrine metabolism in the rat brain. Brain, Behavior, and Immunity 2, 267-274.
Katsuura, G., Arimura, A., Koves, K., Gottschall, P.E., 1990. Involvement of organum vasculosum of lamina terminalis and preoptic area in interleukin 1 beta-induced ACTH release. American Journal of Physiology-Endocrinology and Metabolism 258, E163-E171.
Katsuura, G., Gottschall, P.E., Arimura, A., 1988. Identification of a high-affinity receptor for interleukin-1 beta in rat brain. Biochemical and Biophysical Research Communications 156, 61-67.
Katz, M.M., Tekell, J.L., Bowden, C.L., Brannan, S., Houston, J.P., Berman, N., Frazer, A., 2004. Onset and Early Behavioral Effects of Pharmacologically Different Antidepressants and Placebo in Depression. Neuropsychopharmacology 29, 566-579.
Kavaliers, M., Colwell, D.D., 1995. Odours of parasitized males induce aversive responses in female mice. Animal Behaviour 50, 1161-1169.
Keeling, L.J., Wallenbeck, A., Larsen, A., Holmgren, N., 2012. Scoring tail damage in pigs: an evaluation based on recordings at Swedish slaughterhouses. Acta Veterinaria Scandinavica 54, 1-6.
Kent, S., Bluthé, R.-M., Kelley, K.W., Dantzer, R., 1992. Sickness behavior as a new target for drug development. Trends in Pharmacological Sciences 13, 24-28.
Kleiman, A., Hübner, S., Parkitna, J.M.R., Neumann, A., Hofer, S., Weigand, M.A., Bauer, M., Schmid, W., Schütz, G., Libert, C., Reichardt, H.M., Tuckermann, J.P., 2012. Glucocorticoid receptor

dimerization is required for survival in septic shock via suppression of interleukin-1 in macrophages. The FASEB Journal 26, 722-729.

Koenig, W., Sund, M., Fröhlich, M., Fischer, H.G., Löwel, H., Döring, A., Hutchinson, W.L., Pepys, M.B., 1999. C-Reactive protein, a sensitive marker of inflammation, predicts future risk of coronary heart disease in initially healthy middle-aged men: results from the MONICA (Monitoring Trends and Determinants in Cardiovascular Disease) Augsburg Cohort Study, 1984 to 1992. Circulation 99, 237-242.

Konsman, J.P., Vigues, S., Mackerlova, L., Bristow, A., Blomqvist, A., 2004. Rat brain vascular distribution of interleukin-1 type-1 receptor immunoreactivity: Relationship to patterns of inducible cyclooxygenase expression by peripheral inflammatory stimuli. Journal of Comparative Neurology 472, 113-129.

Krause, D., Myint, A.-M., Schuett, C., Musil, R., Dehning, S., Cerovecki, A., Riedel, M., Arolt, V., Schwarz, M.J., Müller, N., 2017. High Kynurenine (a Tryptophan Metabolite) Predicts Remission in Patients with Major Depression to Add-on Treatment with Celecoxib. Frontiers in Psychiatry 8, 16.

Kritas, S.K., Morrison, R.B., 2004. An observational study on tail biting in commercial grower-finisher barns. Journal of Swine Health and Production 12, 17-22.

Kritas, S.K., Morrison, R.B., 2007. Relationships between tail biting in pigs and disease lesions and condemnations at slaughter. Veterinary Record 160, 149-152.

Köhler, O., Benros, M.E., Nordentoft, M., Farkouh, M.E., Iyengar, R.L., Mors, O., Krogh, J., 2014. Effect of anti-inflammatory treatment on depression, depressive symptoms, and adverse effects: a systematic review and meta-analysis of randomized clinical trials. JAMA psychiatry 71, 1381-1391.

Lamberts, S.W.J., Bons, E., Zuiderwijk, J., 1986. High concentrations of catecholamines selectively diminish the sensitivity of CRF-stimulated acth release by cultured rat pituitary cells to the suppressive effects of dexamethasone. Life Sciences 39, 97-102.

Larsson, M.K., Faka, A., Bhat, M., Imbeault, S., Goiny, M., Orhan, F., Oliveros, A., Ståhl, S., Liu, X.C., Choi, D.S., Sandberg, K., Engberg, G., Schwieler, L., Erhardt, S., 2016. Repeated LPS Injection Induces Distinct Changes in the Kynurenine Pathway in Mice. Neurochemical Research 41, 2243-2255.

Le Floc'h, N., LeBellego, L., Matte, J.J., Melchior, D., Sève, B., 2009. The effect of sanitary status degradation and dietary tryptophan content on growth rate and tryptophan metabolism in weaning pigs. Journal of Animal Science 87, 1686-1694.

Le Floc'h, N., Melchior, D., Sève, B., 2008. Dietary tryptophan helps to preserve tryptophan homeostasis in pigs suffering from lung inflammation1. Journal of Animal Science 86, 3473-3479.

Le Floc'h, N., Matte, J.J., Melchior, D., van Milgen, J., Sève, B., 2010. A moderate inflammation caused by the deterioration of housing conditions modifies Trp metabolism but not Trp requirement for growth of post-weaned piglets. Animal 4, 1891-1898.

Lestage, J., Verrier, D., Palin, K., Dantzer, R., 2002. The enzyme indoleamine 2,3-dioxygenase is induced in the mouse brain in response to peripheral administration of lipopolysaccharide and superantigen. Brain, Behavior, and Immunity 16, 596-601.

Levenbrown, Y., Penfil, S., Rodriguez, E., Zhu, Y., Hossain, J., Bhat, A.M., Hesek, A., O'Neil, K.B., Tobin, K., Shaffer, T.H., 2013. Use of insulin to decrease septic shock-induced myocardial depression in a porcine model. Inflammation 36, 1494-1502.

Li, Y.Z., Kerr, B.J., Kidd, M.T., Gonyou, H.W., 2006. Use of supplementary tryptophan to modify the behavior of pigs. Journal of Animal Science 84, 212-220.

Lind, N.M., Moustgaard, A., Jelsing, J., Vajta, G., Cumming, P., Hansen, A.K., 2007. The use of pigs in neuroscience: Modeling brain disorders. Neuroscience & Biobehavioral Reviews 31, 728-751.

Linthorst, A.C.E., Flachskamm, C., Holsboer, F., Reul, J.M.H.M., 1996. Activation of serotonergic and noradrenergic neurotransmission in the rat hippocampus after peripheral administration of bacterial endotoxin: Involvement of the cyclo-oxygenase pathway. Neuroscience 72, 989-997.

Llamas Moya, S., Boyle, L., Lynch, P.B., Arkins, S., 2006. Pro-inflammatory cytokine and acute phase protein responses to low-dose lipopolysaccharide (LPS) challenge in pigs. Animal Science 82, 527-534.

Lopes, P.C., Block, P., König, B., 2016. Infection-induced behavioural changes reduce connectivity and the potential for disease spread in wild mice contact networks. Scientific Reports 6, 31790.

Makagon, M.M., McCowan, B., Mench, J.A., 2012. How can social network analysis contribute to social behavior research in applied ethology? Applied Animal Behaviour Science 138, 152-161.

Manciocco, A., Sensi, M., Moscati, L., Battistacci, L., Laviola, G., Brambilla, G., Vitale, A., Alleva, E., 2011. Longitudinal effects of environmental enrichment on behaviour and physiology of pigs reared on an intensive-stock farm. Italian Journal of Animal Science 10, e52.

Martín de la Fuente, A.J., Carpintero, R., Rodríguez Ferri, E.F., Álava, M.A., Lampreave, F., Gutiérrez Martín, C.B., 2010. Acute-phase protein response in pigs experimentally infected with Haemophilus parasuis. Comparative Immunology, Microbiology and Infectious Diseases 33, 455-465.

Martínez-Trejo, G., Ortega-Cerrilla, M.E., Rodarte-Covarrubias, L.F., Herrera-Haro, J.G., Figueroa-Velasco, J.L., Galindo-Maldonado, F., Sánchez-Martínez, O., Lara-Bueno, A., 2009. Aggressiveness and Productive Performance of Piglets Supplemented with Tryptophan. Journal of Animal and Veterinary Advances 8, 608-611.

Masana, M.I., Heyes, M.P., Mefford, I.N., 1990. Indomethacin prevents increased catecholamine turnover in rat brain following systemic endotoxin challenge. Progress in Neuro-Psychopharmacology and Biological Psychiatry 14, 609-621.

Matasić, R., Dietz, A.B., Vuk-Pavlović, S., 2000. Cyclooxygenase-independent inhibition of dendritic cell maturation by aspirin. Immunology 101, 53-60.

McClelland, J.L., McNaughton, B.L., O'Reilly, R.C., 1995. Why there are complementary learning systems in the hippocampus and neocortex:

insights from the successes and failures of connectionist models of learning and memory. Psychological Review 102, 419-457.

McCusker, R.H., Kelley, K.W., 2013. Immune-neural connections: how the immune system's response to infectious agents influences behavior. Journal of Experimental Biology 216, 84-98.

McNaughton, B.L., Battaglia, F.P., Jensen, O., Moser, E.I., Moser, M.B., 2006. Path integration and the neural basis of the 'cognitive map'. Nature Reviews Neuroscience 7, 663-678.

Medzhitov, R., Preston-Hurlburt, P., Janeway, C.A., Jr., 1997. A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. Nature 388, 394-397.

Medzhitov, R., Preston-Hurlburt, P., Kopp, E., Stadlen, A., Chen, C., Ghosh, S., Janeway, C.A., Jr., 1998. MyD88 is an adaptor protein in the hToll/IL-1 receptor family signaling pathways. Molecular Cell 2, 253-258.

Mefford, I.N., Heyes, M.P., 1990. Increased biogenic amine release in mouse hypothalamus following immunological challenge: antagonism by indomethacin. Journal of Neuroimmunology 27, 55-61.

Merlot, E., Meunier-Salaün, M.-C., Prunier, A., 2004. Behavioural, endocrine and immune consequences of mixing in weaned piglets. Applied Animal Behaviour Science 85, 247-257.

Merlot, E., Mounier, A.M., Prunier, A., 2011. Endocrine response of gilts to various common stressors: A comparison of indicators and methods of analysis. Physiology & Behavior 102, 259-265.

Meurens, F., Summerfield, A., Nauwynck, H., Saif, L., Gerdts, V., 2012. The pig: a model for human infectious diseases. Trends in Microbiology 20, 50-57.

Meyer, B., Zentek, J., Harlander-Matauschek, A., 2013. Differences in intestinal microbial metabolites in laying hens with high and low levels of repetitive feather-pecking behavior. Physiology & Behavior 110-111, 96-101.

Milstein, J.A., Lehmann, O., Theobald, D.E.H., Dalley, J.W., Robbins, T.W., 2007. Selective depletion of cortical noradrenaline by anti-dopamine beta-hydroxylase–saporin impairs attentional function and

enhances the effects of guanfacine in the rat. Psychopharmacology 190, 51-63.

Mohankumar, P.S., Thygarajan, S., Kaleem Quadri, S., 1991. Interleukin-1 stimulates the release of dopamine and dihydroxyphenylacetic acid from the hypothalamus in vivo. Life Sciences 48, 925-930.

Moinard, C., Mendl, M., Nicol, C.J., Green, L.E., 2003. A case control study of on-farm risk factors for tail biting in pigs. Applied Animal Behaviour Science 81, 333-355.

Munsterhjelm, C., Heinonen, M., Valros, A., 2015. Application of the Welfare Quality® animal welfare assessment system in Finnish pig production, part II: Associations between animal-based and environmental measures of welfare. Animal Welfare 24, 161-172.

Munsterhjelm, C., Nordgreen, J., Aae, F., Heinonen, M., Olstad, K., Aasmundstad, T., Janczak, A.M., Valros, A., 2017. To be blamed or pitied? The effect of illness on social behavior, cytokine levels and feed intake in undocked boars. Physiology & Behavior 179, 298-307.

Munsterhjelm, C., Nordgreen, J., Aae, F., Heinonen, M., Valros, A., Janczak, A.M., 2019. Sick and grumpy: Changes in social behaviour after a controlled immune stimulation in group-housed gilts. Physiology & Behavior 198, 76-83.

Mustonen, K., Banting, A., Raekallio, M., Heinonen, M., Peltoniemi, O.A.T., Vainio, O., 2012. Dose-response investigation of oral ketoprofen in pigs challenged with Escherichia coli endotoxin. Veterinary Record 171, 70.

Myers, M., Smith, E., Messenheimer, J., Lewandowski, A., Groesbeck, C., Zhang, W., Swain, T., Chiesa, O.A., Deaver, C.M., 2016. Impact of a nonsteroidal anti-inflammatory drug (NSAID) on serum MCP1 & clinical observations during lipopolysaccharide (LPS) induced inflammation in swine. International Congress of Immunology, John Wiley & Sons, Ltd, Melbourne, Australia, p. 390.

Myers, M.J., Farrell, D.E., Palmer, D.C., Post, L.O., 2003. Inflammatory mediator production in swine following endotoxin challenge with or without co-administration of dexamethasone. International Immunopharmacology 3, 571-579.

Myint, A.M., Steinbusch, H.W.M., Goeghegan, L., Luchtman, D., Kim, Y.K., Leonard, B.E., 2007. Effect of the COX-2 Inhibitor Celecoxib on Behavioural and Immune Changes in an Olfactory Bulbectomised Rat Model of Depression. Neuroimmunomodulation 14, 65-71.

Newberry, R.C., Wood-Gush, D.G.M., 1986. Social relationships of piglets in a semi-natural environment. Animal Behaviour 34, 1311-1318.

Niblock, M.M., Kinney, H.C., Luce, C.J., Belliveau, R.A., Filiano, J.J., 2004. The development of the medullary serotonergic system in the piglet. Autonomic Neuroscience 110, 65-80.

Niendam, T.A., Laird, A.R., Ray, K.L., Dean, Y.M., Glahn, D.C., Carter, C.S., 2012. Meta-analytic evidence for a superordinate cognitive control network subserving diverse executive functions. Cognitive, Affective, & Behavioral Neuroscience 12, 241-268.

Nordgreen, J., Edwards, S.A., Boyle, L.A., Bolhuis, J.E., Veit, C., Sayyari, A., Marin, D.E., Dimitrov, I., Janczak, A.M., Valros, A., 2020. A Proposed Role for Pro-Inflammatory Cytokines in Damaging Behavior in Pigs. Frontiers in Veterinary Science 7, 646-646.

Nordgreen, J., Munsterhjelm, C., Aae, F., Popova, A., Boysen, P., Ranheim, B., Heinonen, M., Raszplewicz, J., Piepponen, P., Lervik, A., Valros, A., Janczak, A.M., 2018. The effect of lipopolysaccharide (LPS) on inflammatory markers in blood and brain and on behavior in individually-housed pigs. Physiology & Behavior 195, 98-111.

O'Connor, J.C., Lawson, M.A., André, C., Moreau, M., Lestage, J., Castanon, N., Kelley, K.W., Dantzer, R., 2009. Lipopolysaccharide-induced depressive-like behavior is mediated by indoleamine 2,3-dioxygenase activation in mice. Molecular Psychiatry 14, 511.

Olson, N.C., Hellyer, P.W., Dodam, J.R., 1995. Mediators and vascular effects in response to endotoxin. British Veterinary Journal 151, 489-522.

Olson, N.C., Salzer, W.L., McCall, C.E., 1988. Biochemical, physiological and clinical aspects of endotoxemia. Molecular Aspects of Medicine 10, 511-629.

Osimo, E.F., Baxter, L.J., Lewis, G., Jones, P.B., Khandaker, G.M., 2019. Prevalence of low-grade inflammation in depression: a systematic

review and meta-analysis of CRP levels. Psychological Medicine 49, 1958-1970.

Pacak, K., McCarty, R., Palkovits, M., Kopin, I.J., Goldstein, D.S., 1995. Effects of immobilization on in vivo release of norepinephrine in the bed nucleus of the stria terminalis in conscious rats. Brain Research 688, 242-246.

Parra, M.D., Fuentes, P., Tecles, F., Martínez-Subiela, S., Martínez, J.S., Muñoz, A., Cerón, J.J., 2006. Porcine Acute Phase Protein Concentrations in Different Diseases in Field Conditions. Journal of Veterinary Medicine, Series B 53, 488-493.

Parrott, R.F., Misson, B.H., Baldwin, B.A., 1989. Salivary cortisol in pigs following adrenocorticotrophic hormone stimulation: Comparison with plasma levels. British Veterinary Journal 145, 362-366.

Pastorelli, H., Le Floc'h, N., Merlot, E., Meunier-Salaün, M.C., van Milgen, J., Montagne, L., 2012. Sanitary housing conditions modify the performance and behavioural response of weaned pigs to feed- and housing-related stressors. Animal 6, 1811-1820.

Pecchi, E., Dallaporta, M., Jean, A., Thirion, S., Troadec, J.-D., 2009. Prostaglandins and sickness behavior: Old story, new insights. Physiology & Behavior 97, 279-292.

Peters, S.M., Yancy, H., Deaver, C., Jones, Y.L., Kenyon, E., Chiesa, O.A., Esparza, J., Screven, R., Lancaster, V., Stubbs, J.T., Yang, M., Wiesenfeld, P.L., Myers, M.J., 2012. In vivo characterization of inflammatory biomarkers in swine and the impact of flunixin meglumine administration. Veterinary Immunology and Immunopathology 148, 236-242.

Petersen, V., Simonsen, H.B., Lawson, L.G., 1995. The effect of environmental stimulation on the development of behaviour in pigs. Applied Animal Behaviour Science 45, 215-224.

Piekarzewska, A., Sadowski, B., Rosochacki, S.J., 1999. Alterations of Brain Monoamine Levels in Pigs Exposed to Acute Immobilization Stress. Journal of Veterinary Medicine Series A 46, 197-207.

Piekarzewska, A.B., Rosochacki, S.J., Sender, G., 2000. The Effect of Acute Restraint Stress on Regional Brain Neurotransmitter Levels in

Stress-Susceptible Pietrain Pigs. Journal of Veterinary Medicine Series A 47, 257-269.

Pierce, J.W., Read, M.A., Ding, H., Luscinskas, F.W., Collins, T., 1996. Salicylates inhibit I kappa B-alpha phosphorylation, endothelial-leukocyte adhesion molecule expression, and neutrophil transmigration. The Journal of Immunology 156, 3961.

Plata-Salamán, C.R., 1996. Anorexia during acute and chronic disease. Nutrition 12, 69-78.

Poletto, R., Cheng, H.-W., Meisel, R.L., Richert, B.T., Marchant-Forde, J.N., 2011. Gene expression of serotonin and dopamine receptors and monoamine oxidase-A in the brain of dominant and subordinate pubertal domestic pigs (Sus scrofa) fed a β-adrenoreceptor agonist. Brain Research 1381, 11-20.

Poletto, R., Kretzer, F.C., Hötzel, M.J., 2014. Minimizing aggression during mixing of gestating sows with supplementation of a tryptophan-enriched diet. Physiology & Behavior 132, 36-43.

Poletto, R., Meisel, R.L., Richert, B.T., Cheng, H.-W., Marchant-Forde, J.N., 2010. Aggression in replacement grower and finisher gilts fed a short-term high-tryptophan diet and the effect of long-term human–animal interaction. Applied Animal Behaviour Science 122, 98-110.

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

Pomorska-Mól, M., Markowska-Daniel, I., Kwit, K., Stępniewska, K., Pejsak, Z., 2013. C-reactive protein, haptoglobin, serum amyloid A and pig major acute phase protein response in pigs simultaneously infected with H1N1 swine influenza virus and Pasteurella multocida. BMC Veterinary Research 9, 14.

Pomorska-Mól, M., Markowska-Daniel, I., Kwit, K., Urbaniak, K., Pejsak, Z., 2015. Correlation Between Serum Acute Phase Proteins, Lung Pathology, and Disease Severity in Pigs Experimentally Co-Infected with H3N2 Swine Influenza Virus and Bordetella Bronchiseptica. Bulletin- Veterinary Institute in Pulawy 59.

Porsolt, R.D., Le Pichon, M., Jalfre, M., 1977. Depression: a new animal model sensitive to antidepressant treatments. Nature 266, 730-732.

Qureshi, S.T., Larivière, L., Leveque, G., Clermont, S., Moore, K.J., Gros, P., Malo, D., 1999. Endotoxin-tolerant mice have mutations in Toll-like receptor 4 (Tlr4). The Journal of Experimental Medicine 189, 615-625.

Rabhi, N., Thibodeau, A., Côté, J.-C., Devillers, N., Laplante, B., Fravalo, P., Larivière-Gauthier, G., Thériault, W.P., Faucitano, L., Beauchamp, G., Quessy, S., 2020. Association Between Tail-Biting and Intestinal Microbiota Composition in Pigs. Frontiers in Veterinary Science 7.

Rang, H.P., Ritter, J.M., Flower, R.J., Henderson, G., 2016. Rang&Dales Pharmacology. 8th ed. Elsevier.

Rasmussen, K., Morilak, D.A., Jacobs, B.L., 1986. Single unit activity of locus coeruleus neurons in the freely moving cat: I. During naturalistic behaviors and in response to simple and complex stimuli. Brain Research 371, 324-334.

Reimert, I., Rodenburg, T.B., Ursinus, W.W., Kemp, B., Bolhuis, J.E., 2014. Selection Based on Indirect Genetic Effects for Growth, Environmental Enrichment and Coping Style Affect the Immune Status of Pigs. PLOS ONE 9, e108700.

Renault, P.F., Hoofnagle, J.H., Park, Y., Mullen, K.D., Peters, M., Jones, D.B., Rustgi, V., Jones, E.A., 1987. Psychiatric Complications of Long-term Interferon Alfa Therapy. Archives of Internal Medicine 147, 1577-1580.

Riber, A.B., Forkman, B., 2007. A note on the behaviour of the chicken that receives feather pecks. Applied Animal Behaviour Science 108, 337-341.

Rodrigues, F.T.S., de Souza, M.R.M., Lima, C.N.d.C., da Silva, F.E.R., Costa, D.V.d.S., dos Santos, C.C., Miyajima, F., de Sousa, F.C.F., Vasconcelos, S.M.M., Barichello, T., Quevedo, J., Maes, M., de Lucena, D.F., Macedo, D., 2018. Major depression model induced by repeated and intermittent lipopolysaccharide administration: Long-lasting behavioral, neuroimmune and neuroprogressive alterations. Journal of Psychiatric Research 107, 57-67.

Rosa-Neto, P., Doudet, D.J., Cumming, P., 2004. Gradients of dopamine D1- and D2/3-binding sites in the basal ganglia of pig and monkey measured by PET. NeuroImage 22, 1076-1083.

Rosario, L.A., Abercrombie, E.D., 1999. Individual differences in behavioral reactivity: correlation with stress-induced norepinephrine efflux in the hippocampus of Sprague-Dawley rats. Brain Research Bulletin 48, 595-602.

Rossbacher, J., Wagner, L., Pasternack, M.S., 1999. Inhibitory effect of haptoglobin on granulocyte chemotaxis, phagocytosis and bactericidal activity. Scandinavian Journal of Immunology 50, 399-404.

Rougé-Pont, F., Piazza, P.V., Kharouby, M., Le Moal, M., Simon, H., 1993. Higher and longer stress-induced increase in dopamine concentrations in the nucleus accumbens of animals predisposed to amphetamine self-administration. A microdialysis study. Brain Research 602, 169-174.

Ruis, M.A.W., Te Brake, J.H.A., Engel, B., Ekkel, E.D., Buist, W.G., Blokhuis, H.J., Koolhaas, J.M., 1997. The Circadian Rhythm of Salivary Cortisol in Growing Pigs: Effects of Age, Gender, and Stress. Physiology & Behavior 62, 623-630.

Russell, S.M., Dhariwal, A.P.S., McCann, S.M., Yates, F.E., 1969. Inhibition by Dexamethasone of the in Vivo Pituitary Response to Corticotropin-Releasing Factor (CRF). Endocrinology 85, 512-521.

Ruud, T.E., Gundersen, Y., Wang, J.E., Foster, S.J., Thiemermann, C., Aasen, A.O., 2007. Activation of Cytokine Synthesis by Systemic Infusions of Lipopolysaccharide and Peptidoglycan in a Porcine Model in Vivo and in Vitro. Surgical Infections 8, 495-504.

Saito, K., Markey, S.P., Heyes, M.P., 1991. Chronic effects of γ-interferon on quinolinic acid and indoleamine-2,3-dioxygenase in brain of C57BL6 mice. Brain Research 546, 151-154.

Saito, K., Markey, S.P., Heyes, M.P., 1992. Effects of immune activation on quinolinic acid and neuroactive kynurenines in the mouse. Neuroscience 51, 25-39.

Salichs, M., Sabaté, D., Ciervo, O., Homedes, J., 2012. Comparison of the antipyretic efficacy of ketoprofen, acetylsalicylic acid, and

paracetamol, orally administered to swine. Journal of Veterinary Pharmacology and Therapeutics 35, 198-201.

Sánchez, J., García, A., Ruiz, J.M., Montes, A.M., Cabezas-Herrera, J., Ros-Lara, S., De la Cruz, E., Gutiérrez, A.M., 2019. Porcine Breed, Sex, and Production Stage Influence the Levels of Health Status Biomarkers in Saliva Samples. Frontiers in Veterinary Science 6, 32.

Sapolsky, R., Rivier, C., Yamamoto, G., Plotsky, P., Vale, W., 1987. Interleukin-1 stimulates the secretion of hypothalamic corticotropin-releasing factor. Science 238, 522.

Sarabdjitsingh, R.A., Joëls, M., de Kloet, E.R., 2012. Glucocorticoid pulsatility and rapid corticosteroid actions in the central stress response. Physiology & Behavior 106, 73-80.

Saudou, F., Amara, D.A., Dierich, A., LeMeur, M., Ramboz, S., Segu, L., Buhot, M.C., Hen, R., 1994. Enhanced aggressive behavior in mice lacking 5-HT1B receptor. Science 265, 1875.

Schmidhammer, R., Wassermann, E., Germann, P., Redl, H., Ullrich, R., 2006. Infusion of increasing doses of endotoxin induces progressive acute lung injury but prevents early pulmonary hypertension in pigs. Shock 25, 389-394.

Schrøder-Petersen, D.L., Simonsen, H.B., Lawson, L.G., 2003. Tail-in-mouth behaviour among weaner pigs in relation to age, gender and group composition regarding gender. Acta Agriculturae Scandinavica Section a-Animal Science 53, 29-34.

Schönreiter, S., Huber, H., Lohmüller, V., Zanella, A.J., Unshelm, J., Henke, J., Erhardt, W., 1999. Salivary cortisol as a stress parameter in piglets. Tierärztliche Praxis. Ausgabe G Grosstiere/Nutztiere 27, 175-179.

Scollo, A., Contiero, B., Gottardo, F., 2016. Frequency of tail lesions and risk factors for tail biting in heavy pig production from weaning to 170 kg live weight. The Veterinary Journal 207, 92-98.

Scollo, A., Di Martino, G., Bonfanti, L., Stefani, A.L., Schiavon, E., Marangon, S., Gottardo, F., 2013. Tail docking and the rearing of heavy pigs: The role played by gender and the presence of straw in the control of tail biting. Blood parameters, behaviour and skin lesions. Research in Veterinary Science 95, 825-830.

Scott, K., Chennells, D.J., Campbell, F.M., Hunt, B., Armstrong, D., Taylor, L., Gill, B.P., Edwards, S.A., 2006a. The welfare of finishing pigs in two contrasting housing systems: Fully-slatted versus straw-bedded accommodation. Livestock Science 103, 104-115.

Scott, K., Taylor, L., Gill, B.P., Edwards, S.A., 2006b. Influence of different types of environmental enrichment on the behaviour of finishing pigs in two different housing systems: 1. Hanging toy versus rootable substrate. Applied Animal Behaviour Science 99, 222-229.

Sehic, E., Székely, M., Ungar, A.L., Oladehin, A., Blatteis, C.M., 1996. Hypothalamic prostaglandin E2 during lipopolysaccharide-induced fever in guinea pigs. Brain Research Bulletin 39, 391-399.

Shen, Y.B., Voilqué, G., Kim, J.D., Odle, J., Kim, S.W., 2012. Effects of increasing tryptophan intake on growth and physiological changes in nursery pigs. Journal of Animal Science 90, 2264-2275.

Shintani, F., Kanba, S., Nakaki, T., Nibuya, M., Kinoshita, N., Suzuki, E., Yagi, G., Kato, R., Asai, M., 1993. Interleukin-1 beta augments release of norepinephrine, dopamine, and serotonin in the rat anterior hypothalamus. The Journal of Neuroscience 13, 3574.

Sinisalo, A., Niemi, J.K., Heinonen, M., Valros, A., 2012. Tail biting and production performance in fattening pigs. Livestock Science 143, 220-225.

Sorensen, N.S., Tegtmeier, C., Andresen, L.O., Piñeiro, M., Toussaint, M.J.M., Campbell, F.M., Lampreave, F., Heegaard, P.M.H., 2006. The porcine acute phase protein response to acute clinical and subclinical experimental infection with Streptococcus suis. Veterinary Immunology and Immunopathology 113, 157-168.

Spoolder, H.A.M., Edwards, S.A., Corning, S., 2000. Aggression among finishing pigs following mixing in kennelled and unkennelled accommodation. Livestock Production Science 63, 121-129.

Srinivasan, D., Yen, J.-H., Joseph, D.J., Friedman, W., 2004. Cell Type-Specific Interleukin-1β Signaling in the CNS. The Journal of Neuroscience 24, 6482-6488.

Steel, D.M., Whitehead, A.S., 1994. The major acute phase reactants: C-reactive protein, serum amyloid P component and serum amyloid A protein. Immunology Today 15, 81-88.

Steinberg, B.E., Silverman, H.A., Robbiati, S., Gunasekaran, M.K., Tsaava, T., Battinelli, E., Stiegler, A., Bouton, C.E., Chavan, S.S., Tracey, K.J., Huerta, P.T., 2016. Cytokine-specific Neurograms in the Sensory Vagus Nerve. Bioelectronic Medicine 3, 7-17.

Steru, L., Chermat, R., Thierry, B., Simon, P., 1985. The tail suspension test: A new method for screening antidepressants in mice. Psychopharmacology 85, 367-370.

Stolba, A., Wood-Gush, D.G.M., 1989. The behaviour of pigs in a semi-natural environment. Animal Science 48, 419-425.

Stuhlmeier, K.M., Li, H., Kao, J.J., 1999. Ibuprofen: new explanation for an old phenomenon. Biochemical Pharmacology 57, 313-320.

Sulakhiya, K., Keshavlal, G.P., Bezbaruah, B.B., Dwivedi, S., Gurjar, S.S., Munde, N., Jangra, A., Lahkar, M., Gogoi, R., 2016. Lipopolysaccharide induced anxiety- and depressive-like behaviour in mice are prevented by chronic pre-treatment of esculetin. Neuroscience Letters 611, 106-111.

Takikawa, O., Kuroiwa, T., Yamazaki, F., Kido, R., 1988. Mechanism of interferon-gamma action. Characterization of indoleamine 2,3-dioxygenase in cultured human cells induced by interferon-gamma and evaluation of the enzyme-mediated tryptophan degradation in its anticellular activity. Journal of Biological Chemistry 263, 2041-2048.

Taylor, N.R., Parker, R.M.A., Mendl, M., Edwards, S.A., Main, D.C.J., 2012. Prevalence of risk factors for tail biting on commercial farms and intervention strategies. The Veterinary Journal 194, 77-83.

Teeling, J.L., Cunningham, C., Newman, T.A., Perry, V.H., 2010. The effect of non-steroidal anti-inflammatory agents on behavioural changes and cytokine production following systemic inflammation: Implications for a role of COX-1. Brain, Behavior, and Immunity 24, 409-419.

Telkänranta, H., Bracke, M.B.M., Valros, A., 2014. Fresh wood reduces tail and ear biting and increases exploratory behaviour in finishing pigs. Applied Animal Behaviour Science 161, 51-59.

Terenina, E., Sautron, V., Ydier, C., Bazovkina, D., Sevin-Pujol, A., Gress, L., Lippi, Y., Naylies, C., Billon, Y., Liaubet, L., Mormede, P., Villa-Vialaneix, N., 2017. Time course study of the response to LPS targeting the pig immune gene networks. BMC Genomics 18, 988.

Thorand, B., Löwel, H., Schneider, A., Kolb, H., Meisinger, C., Fröhlich, M., Koenig, W., 2003. C-reactive protein as a predictor for incident diabetes mellitus among middle-aged men: results from the MONICA Augsburg cohort study, 1984-1998. Archives of Internal Medicine 163, 93-99.

Tizard, I.R., 2018. Veterinary Immunology. 10 ed. Elsevier, St. Louis, Missouri.

Tobetto, K., Yamamoto, Y., Kataoka, M., Ando, T., Sugimoto, K., Himeno, M., 1997. The Effects of a Newly Developed Nonsteroidal Anti-inflammatory Drug (M-5011) on Arachidonic Acid Metabolism in Rheumatoid Synovial Fibroblasts. The Japanese Journal of Pharmacology 75, 371-379.

Turner, S.P., Weller, J.E., Camerlink, I., Arnott, G., Choi, T., Doeschl-Wilson, A., Farish, M., Foister, S., 2020. Play fighting social networks do not predict injuries from later aggression. Scientific Reports 10, 15486.

Ursinus, W.W., Bolhuis, J.E., Zonderland, J.J., Rodenburg, T.B., de Souza, A.S., Koopmanschap, R.E., Kemp, B., Korte-Bouws, G.A.H., Korte, S.M., van Reenen, C.G., 2013. Relations between peripheral and brain serotonin measures and behavioural responses in a novelty test in pigs. Physiology & Behavior 118, 88-96.

Ursinus, W.W., Wijnen, H.J., Bartels, A.C., Dijvesteijn, N., van Reenen, C.G., Bolhuis, J.E., 2014. Damaging biting behaviors in intensively kept rearing gilts: The effect of jute sacks and relations with production characteristics. Journal of Animal Science 92, 5193-5202.

Ushikubi, F., Segi, E., Sugimoto, Y., Murata, T., Matsuoka, T., Kobayashi, T., Hizaki, H., Tuboi, K., Katsuyama, M., Ichikawa, A., Tanaka, T., Yoshida, N., Narumiya, S., 1998. Impaired febrile response in mice lacking the prostaglandin E receptor subtype EP3. Nature 395, 281-284.

Valenti, O., Lodge, D.J., Grace, A.A., 2011. Aversive stimuli alter ventral tegmental area dopamine neuron activity via a common action in the ventral hippocampus. The Journal of Neuroscience 31, 4280.

Valros, A., 2018. Chapter 5 - Tail biting, in: Špinka, M. (Ed.), Advances in Pig Welfare, Woodhead Publishing, pp. 137-166.

Valros, A., Ahlström, S., Rintala, H., Häkkinen, T., Saloniemi, H., 2004. The prevalence of tail damage in slaughter pigs in Finland and associations to carcass condemnations. Acta Agriculturae Scandinavica, Section A — Animal Science 54, 213-219.

Valros, A., Palander, P., Heinonen, M., Munsterhjelm, C., Brunberg, E., Keeling, L., Piepponen, P., 2015. Evidence for a link between tail biting and central monoamine metabolism in pigs (Sus scrofa domestica). Physiology & Behavior 143, 151-157.

Van de Weerd, H.A.V.d., Docking, C.M., Day, J.E.L., Breuer, K., Edwards, S.A., 2006. Effects of species-relevant environmental enrichment on the behaviour and productivity of finishing pigs. Applied Animal Behaviour Science 99, 230-247.

Van der Meer, Y., Gerrits, W.J.J., Jansman, A.J.M., Kemp, B., Bolhuis, J.E., 2017. A link between damaging behaviour in pigs, sanitary conditions, and dietary protein and amino acid supply. PLOS ONE 12, e0174688.

Van der Meer, Y., Lammers, A., Jansman, A.J.M., Rijnen, M.M.J.A., Hendriks, W.H., Gerrits, W.J.J., 2016. Performance of pigs kept under different sanitary conditions affected by protein intake and amino acid supplementation. Journal of Animal Science 94, 4704-4719.

Van Dixhoorn, I.D.E., Reimert, I., Middelkoop, J., Bolhuis, J.E., Wisselink, H.J., Groot Koerkamp, P.W.G., Kemp, B., Stockhofe-Zurwieden, N., 2016. Enriched Housing Reduces Disease Susceptibility to Co-Infection with Porcine Reproductive and Respiratory Virus (PRRSV) and Actinobacillus pleuropneumoniae (A. pleuropneumoniae) in Young Pigs. PLOS ONE 11, e0161832.

Van Hierden, Y.M., Koolhaas, J.M., Korte, S.M., 2004. Chronic increase of dietary l-tryptophan decreases gentle feather pecking behaviour. Applied Animal Behaviour Science 89, 71-84.

Vane, J.R., Botting, R.M., 1998. Mechanism of Action of Nonsteroidal Anti-inflammatory Drugs. The American Journal of Medicine 104, 2S-8S.

Wang, J., Dunn, A.J., 1998. Mouse interleukin-6 stimulates the HPA axis and increases brain tryptophan and serotonin metabolism. Neurochemistry International 33, 143-154.

Wassermann, S., Faust, K., 1994. Social network analysis: Methods and applications. Cambridge university press.

Webel, D.M., Finck, B.N., Baker, D.H., Johnson, R.W., 1997. Time course of increased plasma cytokines, cortisol, and urea nitrogen in pigs following intraperitoneal injection of lipopolysaccharide. Journal of Animal Science 75, 1514-1520.

Wey, T., Blumstein, D.T., Shen, W., Jordán, F., 2008. Social network analysis of animal behaviour: a promising tool for the study of sociality. Animal Behaviour 75, 333-344.

Wichers, M.C., Kenis, G., Koek, G.H., Robaeys, G., Nicolson, N.A., Maes, M., 2007. Interferon-α-induced depressive symptoms are related to changes in the cytokine network but not to cortisol. Journal of Psychosomatic Research 62, 207-214.

Williams, P.N., Collier, C.T., Carroll, J.A., Welsh, T.H., Laurenz, J.C., 2009. Temporal pattern and effect of sex on lipopolysaccharide-induced stress hormone and cytokine response in pigs. Domestic Animal Endocrinology 37, 139-147.

Wirthgen, E., Tuchscherer, M., Otten, W., Domanska, G., Wollenhaupt, K., Tuchscherer, A., Kanitz, E., 2013. Activation of indoleamine 2,3-dioxygenase by LPS in a porcine model. Innate Immunity 20, 30-39.

Wispelwey, B., Lesse, A.J., Hansen, E.J., Scheld, W.M., 1988. Haemophilus influenzae lipopolysaccharide-induced blood brain barrier permeability during experimental meningitis in the rat. The Journal of Clinical Investigation 82, 1339-1346.

Wood-Gush, D.G.M., Vestergaard, K., 1991. The seeking of novelty and its relation to play. Animal Behaviour 42, 599-606.

Wright, K.J., Balaji, R., Hill, C.M., Dritz, S.S., Knoppel, E.L., Minton, J.E., 2000. Integrated adrenal, somatotropic, and immune responses of

growing pigs to treatment with lipopolysaccharide1. Journal of Animal Science 78, 1892-1899.

Wyns, H., Meyer, E., Plessers, E., Watteyn, A., van Bergen, T., Schauvliege, S., De Baere, S., Devreese, M., De Backer, P., Croubels, S., 2015a. Modulation by gamithromycin and ketoprofen of in vitro and in vivo porcine lipopolysaccharide-induced inflammation. Veterinary Immunology and Immunopathology 168, 211-222.

Wyns, H., Plessers, E., De Backer, P., Meyer, E., Croubels, S., 2015b. In vivo porcine lipopolysaccharide inflammation models to study immunomodulation of drugs. Veterinary Immunology and Immunopathology 166, 58-69.

Yee, J.R., Prendergast, B.J., 2010. Sex-specific social regulation of inflammatory responses and sickness behaviors. Brain, Behavior, and Immunity 24, 942-951.

Zalcman, S., Green-Johnson, J.M., Murray, L., Nance, D.M., Dyck, D., Anisman, H., Greenberg, A.H., 1994. Cytokine-specific central monoamine alterations induced by interleukin-1, -2 and -6. Brain Research 643, 40-49.

Zhao, X., Cao, F., Liu, Q., Li, X., Xu, G., Liu, G., Zhang, Y., Yang, X., Yi, S., Xu, F., Fan, K., Ma, J., 2019. Behavioral, inflammatory and neurochemical disturbances in LPS and UCMS-induced mouse models of depression. Behavioural Brain Research 364, 494-502.

Zhu, L., Wei, T., Gao, J., Chang, X., He, H., Miao, M., Yan, T., 2015. Salidroside attenuates lipopolysaccharide (LPS) induced serum cytokines and depressive-like behavior in mice. Neuroscience Letters 606, 1-6.

Zonderland, J.J., Bracke, M.B.M., den Hartog, L.A., Kemp, B., Spoolder, H.A.M., 2010. Gender effects on tail damage development in single- or mixed-sex groups of weaned piglets. Livestock Science 129, 151-158.

Zonderland, J.J., Wolthuis-Fillerup, M., van Reenen, C.G., Bracke, M.B.M., Kemp, B., Hartog, L.A.d., Spoolder, H.A.M., 2008. Prevention and treatment of tail biting in weaned piglets. Applied Animal Behaviour Science 110, 269-281.

Zupan, M., Zanella, A.J., 2017. Peripheral regulation of stress and fear responses in pigs from tail-biting pens. Revista Brasileira de Zootecnia 46, 33-38.

7 Appendix: Papers I-III

Paper I

frontiers in Veterinary Science

ORIGINAL RESEARCH
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The Effect of LPS and Ketoprofen on Cytokines, Brain Monoamines, and Social Behavior in Group-Housed Pigs

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Poor health is a risk factor for damaging behaviors, but the mechanisms behind this link are unknown. Injection of pigs with lipopolysaccharide (LPS) can be used to model aspects of poor health. Recent studies have shown that LPS-injected pigs perform more tail- and ear-directed behavior compared to saline-injected pigs and suggest that pro-inflammatory cytokines may play a role in these behaviors. The aims of this study were to test the effect of LPS on the social behavior of pigs and the neurotransmitters and modulators in their brains and to test the effect of a nonsteroidal anti-inflammatory drug on the effects of LPS. Fifty-two female pigs (11–12 weeks) were allocated to four treatments comprising two injections: saline–saline (SS), saline–LPS (SL), ketoprofen–saline (KS), and ketoprofen–LPS (KL). Activity was scan-sampled every 5 min for 6 h after the last injection in the pen. Social behavior was observed continuously in 10 × 15-min bouts between 8 a.m. and 5 p.m. 1 day before (baseline) and 1 and 2 days after the injection. Saliva was analyzed for cortisol and plasma for tryptophan and kynurenine. The frontal cortex, hippocampus, hypothalamus, and brain stem were sampled 72 h after the injection and analyzed for cytokines and monoamines. LPS activated the HPA axis and decreased the activity within 6 h after the injection. Ketoprofen lowered the effect of LPS on cortisol release and attenuated the behavioral signs of sickness in challenged pigs. SL pigs manipulated the ears of their pen mates significantly longer than SS pigs 2 days after the injection. LPS had no observed effect on IFN-γ, TNF-α, and IL-18. At 72 h after the injection, plasma tryptophan was depleted in SL pigs, and tryptophan and kynurenine concentrations in the frontal cortex and brain stem of SL pigs were significantly lower compared to those in SS pigs. Dopamine concentrations in the hypothalamus of SL pigs were significantly lower compared to those in SS pigs. Serotonin concentrations in the hypothalamus and noradrenaline concentrations in the hippocampus of SL pigs

were significantly lower compared to those in KL pigs. In conclusion, LPS influenced the different neurotransmitters and modulators in the brain that are hypothesized to play an important role in the regulation of mood and behavior.

Keywords: lipopolysaccharide (LPS), ketoprofen, social behavior, sickness behavior, cytokines, kynurenine, tryptophan, monoamines

INTRODUCTION

Sickness in humans and other mammals affects many aspects of behavior, including failure or inability to participate in subsistent activities and social interactions due to malaise, pain, and weakness (1). Typically, sick individuals employ health-restoring and rehabilitative strategies such as the avoidance of activity, conservation of energy, limiting of social interactions, reduction of food intake, and seeking rest. In commercial pig production systems, animals housed in close confinement cannot withdraw from their pen mates when they experience a bout of illness, and this might influence their social interactions. The behavioral components of sickness represent, together with the fever response and the associated neuroendocrine changes, a highly organized strategy of the organism to fight an infection [reviewed by Konsmann et al. (2)]. Infectious microorganisms that invade the body encounter, as a first line of defense, macrophages that express toll-like receptors (TLRs). They bind to extracellular pathogen-associated molecular patterns such as lipopolysaccharide (LPS), a cell wall component of Gram-negative bacteria, and initiate the transcription and release of pro-inflammatory cytokines into the blood stream (3). Of these cytokines, mainly interleukin 1-β (IL1-β), interleukin 6 (IL-6), and tumor necrosis factor alpha (TNF-α) elicit a sickness behavior, which is characterized by social withdrawal, lethargy, and loss of thirst and appetite (4).

An injection with LPS can be used to model aspects of sickness. Recent studies in pigs indicate changes in behavior and brain physiology after an injection of LPS (5, 6). At 2 days post-injection, the LPS-injected pigs exhibited a shift in social motivation and performed more tail- and ear-directed behavior than saline-injected pigs (5). At 3 days post-injection, the LPS-injected pigs had higher levels of IFN-γ in their frontal cortex, a tendency toward an elevation of IL-18 in their right hippocampus, and lower levels of noradrenaline in their hypothalamus, hippocampus, and frontal cortex compared to the saline-injected pigs (6). The downregulation of central monoamines that play an important role in the regulation of mood is one of the possible pathways through which cytokines can influence behavior (7, 8).

Another possible pathway for the cytokines to influence behavior is through the induction of the enzyme indolamine 2,3-deoxygenase (IDO) (9) by IFN-γ and TNF-α. IDO metabolizes tryptophan (TRP) to kynurenine (KYN), which is further metabolized into different neuroactive components. IDO is a critical molecular mediator of inflammation-induced depressive-like behavior. In rodents, it has been shown that a blockade of IDO activity prevents the development of depressive-like behavior, whereas administration of KYN induces a depressive-like behavior (10). A relationship between depressive symptoms and peripheral blood levels of TRP and KYN during IFN-α treatment has been reported in humans (11).

Modulation of the immune response by drugs is of major interest in human and veterinary medicine. Nonsteroidal anti-inflammatory drugs (NSAIDs) are candidate substances for blocking the effect of immune activation on behavior. This drug class targets cyclooxygenases (COX) and reduces pain and decreases fever and inflammation through the inhibition of prostaglandin synthesis (12). Additionally, some NSAIDs are able to alter the expression of NFkappaB (13), a transcription factor that is activated by the binding of LPS to TLR-4 (14), and thereby reduce subsequent cytokine expression. Ketoprofen, which inhibits the COX-1 and COX-2 enzymes (15), is a commonly used NSAID in veterinary medicine and has been repeatedly established as a major anti-inflammatory drug in pigs (16, 17). An effect on NFkappaB has not been shown, but other effects apart from COX inhibition and PGE2 reduction cannot be ruled out (18).

Most studies of LPS effects focus on a relatively short time period (24 h) and rarely describe social behavior in detail (9, 19, 20). In recent LPS studies on social behavior and brain physiology, pigs were either housed in groups of three (5) or singly (6). Thus, the complexity of social interactions that could be studied was limited. The pig is a gregarious species, and investigation of their social behavior in larger groups would increase the understanding of how immune activation can influence social dynamics and the likelihood of damaging behaviors. These behaviors are a major welfare challenge in commercial pig husbandry, and a positive correlation with poor health has been reported (21, 22). Therefore, we tested the effect of a controlled immune activation with LPS on brain physiology and social behavior in pigs housed in groups of six. In addition, we tested the effect of ketoprofen intervention on the physiological and behavioral effects of LPS.

We hypothesized that a controlled immune activation would lead to changes in behavior thought to increase the risk of tail biting or ear biting and that these changes would be associated with changes in neurotransmitter and neurotransmitter precursor levels. We predicted that LPS would first decrease activity and then lead to a more manipulative behavior toward pen mates. Because downregulation of central monoamines and plasma TRP depletion are possible pathways through which cytokines can influence behavior, we predicted that LPS would decrease the levels of noradrenaline, serotonin, and dopamine and increase the cytokine levels in the CNS compared to healthy controls. We also predicted an increase in

peripheral and central kynurenine and a corresponding decrease in tryptophan. NSAID treatment was predicted to attenuate the effects of immune activation on neurotransmitter and cytokine levels and on behavior.

MATERIALS AND METHODS

Animals and Husbandry

The national animal research authority approved the experiment (FOTS ID 15232). The experiment took place between March 23 and May 15, 2018 at the Livestock Production Research Center of the Norwegian University of Life Sciences (NMBU), Ås Campus. Seventy-eight pigs aged between 11 and 12 weeks were used in the study (52 females and 26 castrated males). Only the females were included in the treatment or control groups, and their average weight on the day before treatment was 33.9 ± 9.7 kg. The pigs were group-housed by litter with six pigs per pen (four females and two males), resulting in 13 pens in total, at a stocking density of 1.3 m^2 per pig. The four female pigs in each pen were randomly allocated to one of four treatments each so that all treatments were represented in all pens, resulting in 13 pigs per treatment. The male pigs were companion pigs used to increase the stocking density and group size. The pigs in each pen had visual and limited tactile contact with pigs from an adjoining pen. The lying area (2.4 m × 1.6 m) had a concrete floor; the rest of the pen (2.4 m × 1.6 m) was fully slatted. The pigs were fed pellets *ad libitum*, with an animal-to-feeding-place ratio of 3:1 and a diet composition of 15.5% protein, 9.0 g calcium, and 1.9 g sodium (IDEAL S Die Ekstra, produced by Norgesfôr, Mysen, Norway). Each pen had three nipple drinkers. The staff provided two handfuls of wood shavings per pen, spread on the lying area, and two handfuls of grass silage twice per day (one handful in a rack and one handful on the floor of the lying area, respectively). The slatted area in each pen was equipped with a water sprinkler, which turned on every 10 min for 20 s. Lights were on between 6 a.m. and 10 p.m. During the night, the room was dimly lit by night lights. Average ambient temperature in the unit was set to 20°C.

Experimental Design and Sampling Procedures

The four treatments consisted of four substance combinations: saline–saline (SS), saline–LPS (SL), ketoprofen–saline (KS), and ketoprofen–LPS (KL). The first substance was administered intramuscularly (i.m.) in the neck and the second substance intravenously (i.v.) through an ear vein catheter 60 ± 14 min afterwards on average. The pigs were humanely killed at 72 h after the intravenous injection using a mixture of tiletamine and zolazepam (Zoletil Forte vet.), medetomidine (Domitor vet.), and butorphanol (Dolorex vet.), followed by pentobarbital (Eutasol vet.). Details on drug dosages, suppliers, and routes of administration are given in **Table 1**. Each pen was equipped with one camera placed centrally on the ceiling above the pen. The pigs were individually marked on the back, and video recordings of behavior ran continuously throughout the experiment using the Media Recorder system from Noldus (Wageningen, The Netherlands). Saliva samples were taken at baseline (between 08:30 and 10:45) and at 4, 24, 48, and 72 h after the intravenous injection. Each pig chewed on a dental cotton pad suspended on a dental cord until it was moistened [modified from Munsterhjelm et al. (23)]. Each pad was fixed within the upper part of a 10-ml sampling tube and centrifuged for 5 min at 1,000 × g to extract the saliva. Saliva was pipetted to 2-ml Eppendorf tubes and stored on dry ice until it was moved to a freezer set at −80°C at the end of a sampling day. Blood samples were taken through a 1-ml syringe from a temporarily placed ear vein catheter (20 G, Becton Dickinson Infusion Therapy AB, Sweden) at baseline (between 10:00 and 14:00) and 72 h after the i.v. injection of LPS or saline. The catheter was removed directly after the procedure. The blood was immediately transferred to 2-ml EDTA tubes and centrifuged for 10 min at 1,000 × g. The plasma was pipetted to 2-ml Eppendorf tubes and stored on dry ice until it was moved to a freezer set at −80°C at the end of a sampling day. Brain samples were taken 10 ± 2 min on average after euthanasia (i.e., 72 h after i.v. injection of LPS or saline). The head was removed from the body, and the skull was opened using a wood saw designed to cut when pulled toward the operator and chisel. The brain was removed, and the frontal cortex (left and right), hippocampus (left and right), hypothalamus (left and right), and brain stem (left and right) were collected. The frontal cortex was sampled by placing a transverse section ∼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 hemispheres 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 anatomical 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 the tissue block resembled a triangle. The brain stem was sampled to include both the locus coerulus and the raphe nuclei. Rostrally, the brain stem sample was delimited by a diagonal cut placed from the end of the hypothalamus sample ventrally to just caudal to the inferior colliculi (not included in the sample). Caudally, the brain stem sample was cut approximately at the caudal end of the pons. The samples were snap-frozen in isopentane on dry ice and moved to a freezer set at −80°C at the end of a sampling day.

Video Analysis

All behavioral recordings were analyzed using the Observer XT 14.1 from Noldus (Wageningen, The Netherlands).

Behavioral Signs of Sickness

Behavioral signs of sickness were observed on the day of injection, referred to as DAY1, by one observer (JV) who was blinded to the treatment. Instantaneous scan sampling was performed every 5 min for 6 h after the injection of the last pig in the pen for all treatment and control pigs. All 13 pens were included in the analysis. The ethogram used is displayed in **Table 2**.

Social Behavior

Continuous observation of performers and receivers of social behavior at certain intervals during the day was performed

TABLE 1 | Overview of substances used for each procedure, with dose, and route of administration indicated.

Substance	Generic name, concentration, and supplier	Dose per kilogram body weight and route of administration	Procedure
Butorphanol	Dolorex MSD Animal Health (10 mg ml^{-1}), The Netherlands	0.18 mg kg^{-1} IV	Anesthesia prior to euthanasia
Ketoprofen	Romefen vet (100 mg ml^{-1}) Ceva Santé Animale, France	6 mg kg^{-1} IM	Treatment
Lidocaine	Xylokain 5% ointment Aspen Pharma Trading Ltd., Irland	Topical	Topical application prior to catheterization of the ear vein
Lipopolysaccharide	Serotype 0111: B4 of *Escherichia coli* dissolved in 0.9% sterile saline to a concentration of 100 μg ml^{-1} Sigma, Germany	1.2 μg kg^{-1} IV	Treatment
Medetomidine	Domitor vet. (1 mg ml^{-1}) Orion Pharma International, Finland	0.06 mg kg^{-1} IV	Anesthesia prior to euthanasia
Sodium chloride	9 mg ml^{-1}	IV	Control treatment
Sodium pentobarbital	Euthasol vet. (400 mg ml^{-1}) Le Vet, The Netherlands	140 mg kg^{-1} IV	Euthanasia
Tiletamine	Zoletil Forte vet. (50 mg ml^{-1}) Virbac, Norway	2.84 mg kg^{-1} IV	Anesthesia prior to euthanasia
Zolazepam	Zoletil Forte vet. (50 mg ml^{-1}) Virbac, Norway	2.84 mg kg^{-1} IV	Anesthesia prior to euthanasia

TABLE 2 | Ethogram for behavioral signs of sickness.

Behavior	Description
Lying lateral	Lying on the flank with the head resting on the ground and not moving; body (parts) may make rapid, sudden, short-lasting movements
Lying sternal	Lying on the sternum with the head resting on the ground; body (parts) may make rapid, sudden, short-lasting movements
Lying alert	Lying (on flank or sternum) with the head up
Feeding	Snout in feeder
Active	Any active behaviors in standing position except feeding, including moving, exploration, social behavior, drinking, elimination, and comfort behavior
Interruption	Person is in the pen; scan not included in data analysis

by one observer (CV) who was blinded to the treatment and day of experiment. The sampling scheme for the baseline day (1 day before injection), referred to as DAY0, was four 15-min intervals in the morning (08:00–08:15, 08:30–08:45, 09:00–09:15, and 09:30–09:45) and six 15-min intervals in the afternoon (14:00–14:15, 14:30–14:45, 15:00–15:15, 15:30–15:45, 16:00–16:15, and 16:30–16:45). The same sampling scheme was applied on the day after the injection, referred to as DAY2, and on the second day after the injection, referred to as DAY3. The day of injection itself (DAY1) was not of interest for observation as social behavior was interrupted due to handling for injections and saliva and blood sampling. The sampling scheme resulted in 150 min of continuous observation per pen for each of the days DAY0, DAY2, and DAY3. If a person entered the pen within a 15-min observation interval (e.g., due to saliva sampling, silage feeding, cleaning of pen, marking and weighing of pigs), the observation was postponed until the person had left the pen, and the interval was extended to obtain 15 min of observation. Due to the inadequate quality of the video material from one pen (which was too brightly lit to identify back markings), only 12 out of 13 pens were included in the analysis. The ethogram for social behavior is displayed in **Table 3**.

Lab Procedures and Measurements

Cortisol

Cortisol in saliva was measured using an enzyme immunoassay kit (DetectX®, Catalog Number K0033-H5W, Arbor Assays, MI, USA). The saliva samples were thawed and centrifuged at 4°C at 2,500 × g for 20 min. The kit reagents were prepared according to the manufacturer's protocol. In total, 30 μl of each sample was transferred to Eppendorf tubes, diluted by adding 120 μl of assay buffer (1:4 dilution), and vortexed. All samples were measured within 2 h of preparation. All standards, nonspecific binding wells, blanks, and samples were run in duplicate. Treatments were distributed randomly over the plates. In total, 50 μl of samples, quality controls (high/low), or standards were pipetted into appropriate wells (96-well plate). Each well then received 25 μl of DetectX® cortisol conjugate, followed by 25 μl of DetectX® cortisol antibody (except for low-quality controls), using a repeater pipette. After incubating the plate on a shaker at room temperature for

TABLE 3 | Ethogram for social behavior.

Behavior	Description
Tail manipulation	Touching the tail of another pig with the snout, including taking the tail into the mouth
Ear manipulation	Touching the ear of another pig with the snout, including taking the ear into the mouth
Flank nosing	Touching the flank region (upper part of the lateral side of the body from the beginning of the shoulder until the end of the body, except the tail) of another pig with the snout
Belly nosing	Repetitive up-and-down movements on the abdomen of another pig that is lying or standing
Manipulation of other body parts	Touching body parts of another pig with the snout except for tail, ear, belly, and flank region (e.g., head, legs, back), including taking the body parts into the mouth
Fighting	Biting, hitting, and knocking of another pig with the head; includes chasing performed immediately after biting, hitting, and knocking; includes parallel pressing after a knock, hit, or bite. The pig that initiates the fight is the performer; the pig that is being attacked is the recipient
Displacement	Pushing away another pig without fighting (as defined above); results in the active movement of the recipient and getting access to a resource (e.g., silage, lying space, drinker) for the performer

1 h, the plate was aspired, and each well was washed four times with 300 µl of wash buffer. Then, 100 µl of 3,3′,5,5′-tetramethylbenzidine solution was added to each well, and the plate was incubated for 30 min at room temperature. After this time, 50 µl of stop solution (1 M hydrochloric acid) was added before the optical density of each well was read with the Sunrise Absorbance Reader (Tecan Austria GmbH, Grödig/Salzburg, Austria) at 450 nm using the Magellan 6.4 software. Mean coefficient of variation varied between 4.69 and 7.63%. Sensitivity was determined as 27.6 pg ml^{-1}, and limit of detection was determined as 45.4 pg ml^{-1} according to the manufacturer.

Homogenization of Brain Tissue

The frozen brain tissue blocks were mechanically homogenized using a pestle and a mortar that was placed on dry ice and filled repeatedly with liquid nitrogen to keep the sample frozen. The pulverized brain tissue was transferred into 2-ml round-bottomed Eppendorf tubes and weighed. Ten tubes with tissue from the frontal cortex (five left and five right), hippocampus (five left and five right), and brain stem (five left and five right), respectively, and six tubes with tissue from the hypothalamus (three left and three right) were collected and stored at −80°C until analysis.

Cytokines

For cytokine analysis, a 500-mM phenylmethylsulfonyl fluoride (PMSF) solution was prepared by dissolving 0.436 g PMSF in 5 ml dimethylsulfoxide. PMSF solution (80 µl) was added to a lysing solution that contained cell lysis buffer (19.8 ml) and factor 1 (80 µl) and factor 2 (40 µl) of a cell lysis kit (Bio-Rad, #171-304011). In total, 500 µl of the prepared lysis solution and a 5-mm tungsten bead were added to each sample tube (one tube per brain region). The samples were mechanically homogenized at room temperature for 4 min at 20 Hz using the TissueLyser 2 (Cat. No 85300, Qiagen). The homogenate was centrifuged at 4,500 × g for 20 min at 4°C (Heraeus Multifuge 3SR+ Centrifuge, Thermo Fisher Scientific, MA, USA). The supernatant was transferred to new Eppendorf tubes and stored at −20°C.

Cytokines in brain tissue were measured by a Milliplex MAP Porcine Cytokine and Chemokine Magnetic Bead Panel Immunology Multiplex Assay, including the cytokines interferon gamma (IFN-γ), tumor necrosis factor alpha (TNF-α), and interleukin 18 (IL-18) (PCYTMAG-23K; Merck, Norway). The rationale for including these cytokines was based on previous findings (6). Treatments were distributed randomly over the plates. The supernatant obtained from the homogenization of brain tissue was thawed for 10 min at room temperature, centrifuged at 4°C at 4,500 × g for 10 min and transferred to new Eppendorf tubes. The kit reagents were prepared according to the manufacturer's protocol. The thawed supernatant (50 µl) was transferred into new Eppendorf tubes and diluted by adding 49 µl assay buffer and 1 µl bovine serum albumin solution (20 %, Sigma-Aldrich, USA). In total, 25 µl of each standard or control was added to the appropriate wells using assay buffer for the 0-ng-ml^{-1} standard (background). In total, 25 µl of serum matrix solution was added to the background, standards, and control wells. In total, 25 µl of assay buffer and 25 µl of sample (neat) were transferred to the appropriate sample wells. In total, 25 µl of the mixed beads were transferred to each well, and the plate was incubated on a plate shaker at 2–6°C overnight. The well contents were removed after resting the plate on a hand-held magnet, and each well was washed three times with 200 µl of wash buffer. The plate was incubated for 2 h with 50 µl detection antibodies per well at room temperature. This step was followed by 30 min of incubation with 50 µl streptavidin–phycoerythrin and another washing step. The well content was suspended in sheath fluid, and the plate was run with the Luminex100 (Bio-Rad, Hercules, CA, USA) using the BioPlex Manager 6.0 software (Bio-Rad, Hercules, CA, USA). Sample wells with a bead count <20 were excluded from further analysis. For TNF-α, ca. 50% of the analyzed samples was below the lower limit of detection (LOD), and the calculated cytokine concentrations were consequently censored by the analysis software. Therefore, we used fluorescence intensity (FI) values for further analysis [see Nordgreen et al. (6) for further explanation]. Mean coefficient variation was 9.97% for IFN-γ (with all wells included, the range was 0–62.51), 9.50 for TNF-α (with all wells included, the range was 0–96.17), and 12.30 for IL-18 (with all wells included, the range was 0–78.0). The minimum detectable concentration was 0.042 ng ml^{-1} for IFN-γ, 0.006 for TNF-α, and 0.012 for IL-18. In order to correct for sample weight, the values for observed concentration (divided by two) and the values for fluorescence intensity were divided by the sample weight.

Monoamines, Tryptophan, and Kynurenine

A sensitive and selective high-performance liquid chromatography–electrospray ionization–tandem mass spectrometry (HPLC–ESI–MS/MS) method for quantification of several neurotransmitters, amino acids, and their metabolites was developed by co-author DD. These included TRP and its metabolite KYN, the three neurotransmitters dopamine (DA), serotonin (5-HT), and noradrenaline (NA), and their respective metabolites 3,4-dihydroxyphenyl-acetic acid (DOPAC), homovanillic acid (HVA), 5-hydroxyindoleacetic acid (5-HIAA), and 3-methoxy-4-hydroxyphenylglycol (MHPG). The pure compounds and their respective corresponding stable isotope-labeled standards were provided from CDN Isotopes (Quebec, Canada), Sigma-Aldrich (Darmstadt, Germany), and TRC (Toronto, Canada). All chemicals were of at least HPLC-grade and supplied by VWR International (Fontenay sous Bois, France). All samples were thawed on ice, and the respective corresponding isotope-labeled internal standards were added according to the weight/volume of each sample. The brain samples (sample size: 20–500 mg) were homogenized with acetonitrile 1:5 (v/w) on ice and then centrifuged at 12,000 × g for 30 min at 4°C. The plasma samples (sample size: 15 to 50 μl) were precipitated with acetonitrile 1:5 (v/v) after addition of the internal standard mixture. After being vortexed for 30 s and centrifuged at 12,000 × g for 15 min, 50 μl of plasma samples and, respectively, 100 μl of brain samples supernatants were subsequently transferred into a new 15-ml polypropylene centrifuge tube and evaporated to dryness at 40°C under a nitrogen stream in a water bath (Zymark Turbo Vap LV, Oregon, USA) (24, 25). The dry residue was reconstituted in 100 μl dilution solution of 10% methanol/water (v/v) with 0.1% formic acid and 0.05% ascorbic acid, filtered with Spin-X centrifuge tube filter, 0.22 μm (Costar, UT, USA), and transferred to a HPLC vial with insert (Agilent, Santa Clara, CA, USA). The HPLC–ESI–MS/MS system was performed using an Agilent 1100 setup consisting of a binary pump, degasser, and autosampler with thermostat (Agilent Technologies, Santa Clara, CA, USA) coupled to an API 4000 triple–quadrupole mass spectrometer (AB Sciex, Ontario, Canada) equipped with a turbo ion spray. The temperature of the autosampler was set at 5°C. Chromatographic separation was carried out on a reversed-phase Synergy–Fusion column, 100 mm × 2.1 mm, 2.5-μm particles (Phenomenex, CA, USA), with a Fusion-RP guard column. The column running temperature was 25°C. The mobile phase consisted of 0.1 % acetic acid in water (A) and acetonitrile/methanol (50% v/v) (B). The separated compounds were detected in positive and negative electrospray ionization-multiple reaction monitoring mode using the respective $[M+H]^+$ (protonated) and $[M-H]^-$ (deprotonated) ions in two separate run analyses, selecting one precursor ion to two product ion transitions for each compound. The positive ion transitions used for quantification were as follows: DA (m/z 154 > 137), DA-d4 (m/z 158 > 141), 5-HT (m/z 177 > 160), 5-HT-d4 (m/z 181 > 164), NA (m/z 170 > 107), NA-d6 (m/z 176 > 111), TRP (m/z 205 > 188), 13C-TRP (m/z 206 > 189), d5-TRP (210 > 192) (used for plasma samples only), KYN (m/z 209 > 192), and KYN-d4 (m/z 213 > 196). The negative ion transitions used for quantification were as follows: DOPAC (m/z 167 > 123); DOPAC-d5 (m/z 172 > 128); HVA (m/z 181 > 137), HVA-d5 (m/z 186 > 142), 5-HIAA (m/z 190 > 146), 5-HIAA-d5 (m/z 195 > 151), MHPG (m/z 183 > 150), and MHPG-d3 (m/z 186 > 150). The software used for controlling this equipment and for acquiring and processing the data was Analyst Version 1.7 (AB Sciex, Ontario, Canada). Since the analytes are endogenous components in biological matrices, the validation parameters (detection limit, linearity, precision, accuracy, recovery, and matrix effects) (26) were determined by spiking the brain homogenate and the plasma as matrices, with the corresponding stable isotope-labeled standard analogs of each compound as surrogate standard (27). Standard stock solutions were prepared in methanol at 1 mg ml^{-1}, except kynurenine, which was dissolved in dimethyl sulfoxide. Working solutions were prepared in dilution solution [10% methanol/water (v/v) with 0.1% formic acid and 0.05% ascorbic acid]. All the solutions were stored at −20°C. The calibration standards were prepared in dilution solution as surrogate matrix based on the correction factors calculated for each compound related to their respective matrix effects and recovery values (27). The linear ranges were as follows: 0–500 ng ml^{-1} for DA, 0–3,000 ng ml^{-1} for NA, 0–2,000 ng ml^{-1} for 5-HT, 0–250 ng ml^{-1} for KYN, 0–5,000 ng ml^{-1} for TRP, 0–250 ng ml^{-1} for DOPAC, 0–200 ng ml^{-1} for MHPG, 0–500 ng ml^{-1} for HVA, and 0–350 ng ml^{-1} for 5-HIAA, respectively, corresponding to 50 mg brain tissue scales, and 0–500 ng ml^{-1} for KYN and 0–15,000 ng ml^{-1} for TRP corresponding to 50 μl plasma. The calibration curves were constructed based on the peak area ratio of the analytes to internal standards vs. the nominal concentration ratio (analyte to internal standard). The concentration in each sample was calculated using the peak area ratio and linear regression analysis. The response for each compound was linear and gave a correlation coefficient of $r^2 \geq 0.99$. LOD was based on 3× signal-to-noise ratio, and a lower limit of quantification (LLOQ) was determined as the lowest concentration validated. LLOQs ranged between 1 and 10 ng ml^{-1} for all the compounds. Two quality control samples were used for each run of analysis and prepared in a real brain homogenate by spiking with a known concentration of each analyte in order to evaluate the inter-assay precision and accuracy of the method (94.41 ± 5.88–112.58 ± 5.73%). The extraction recoveries were between 50 and 95% for all analytes, except 5-HIAA with 20%. The use of the stable isotope-labeled internal standard is one of the approaches to correct for matrix effects and improve the accuracy and precision of the analytical method.

Statistical Analysis

The significance level for all analysis was set at $p < 0.05$. Standard deviations were used. Residuals were checked for normality and homogeneity of variance by visual inspection of plots. If they did not fulfill either of these criteria, raw data were transformed. Main effects are not presented when the interaction was in focus to answer the research question. *A priori* planned contrasts were used after running the main models as we had predefined the hypotheses to test [for further explanation, see Doncaster and Davey (28)].

Behavioral Data

Behavioral data were analyzed using mixed models in JMP Pro 14.3.0 (SAS, NC, USA). The removal of outliers did not improve the respective model fit (Akaike information criterion, AIC); thus, they were included in further analysis. For behavioral signs of sickness, the frequency of the respective behavioral pattern (lying lateral/sternal/alert, feeding, being active) was used as dependent variable, and the treatment (SS, SL, KS, KL), the hour after the injection (1, 2, 3, 4, 5, and 6 h), and the interaction of both were used as independent fixed effects. Pig nested in treatment was included as a random variable in all models. For planned comparisons between treatment groups at the different time points, Student's *t*-test was used after running the main model. We compared SL with SS to elucidate the effect of LPS on behavioral signs of sickness. In addition, the comparison of SL and KL should answer the question whether ketoprofen alleviates the effects of LPS. Furthermore, it was relevant to compare SS with KS in order to see whether ketoprofen has an effect even in pigs that are not sick.

For social behavior, the frequency and duration of each behavioral pattern performed and received (manipulation of tail/ear/other body parts, nosing of belly/flank or fighting and displacement) were skewed to the right, the model residuals did not confirm with the criteria listed in "Section Statistical analysis," and the raw data were therefore square root-transformed. The square root of frequency and duration of the respective behavior was used as dependent variable and treatment (SS, SL, KS, and KL) and day in relation to LPS injection (DAY0, DAY2, and DAY3) as fixed effect. The interaction between treatment and day was included in the model as the factor of most interest for testing our hypothesis of an effect of LPS and ketoprofen on the dependent variables. Pig nested in treatment was included as a random variable in all models. For planned comparisons, Student's *t*-test was used. In a first step, we compared SL with SS, SL with KL, and SS with KS within each day. If any of these pairwise comparisons were significant, we compared within-group differences between baseline and the day at which the significant treatment effect was found.

Physiological Data

Physiological data (cortisol, cytokines, tryptophan, kynurenine, monoamines) were analyzed using mixed models in JMP Pro 14.3.0 (SAS, NC, and USA). The removal of outliers did not improve the respective model fit (AIC); thus, they were included in further analysis.

For salivary cortisol, the square root of the observed concentration (ng ml^{-1}) was used as dependent variable and treatment (SS, SL, KS, and KL) and sampling time point in relation to i.v. injection (T0, T4, T24, T48, and T72) as fixed independent variables. The interaction between treatment and sampling time point was included in the model as the factor of most interest for testing our hypothesis of an effect of LPS and ketoprofen on the dependent variable. Pig nested in treatment was included as a random variable in the model. For planned comparisons of group means at the same time points, Student's *t*-tests was used on the LSmeans after running the full mixed model. We compared SL with SS, SL with KL, and SS with KS.

For brain cytokines, the square root of the observed concentration (ng g^{-1}) of IFN-γ and IL-18 and the FI of TNF-α were used as dependent variables. The treatment (SS, SL, KS, and KL) and the hemisphere (left, right) were fixed effects. The time span (TIME) between death of the respective pig until the last brain sample was collected and frozen was included as a covariate in all models. In case of nonsignificance, TIME was removed from the final model. Pig nested in treatment was included as a random variable in all models. For planned comparisons of treatments, Student's *t*-test was used. We compared SL with SS, SL with KL, and SS with KS. Analyses were run for each brain area separately as a difference between brain areas was not of interest.

For tryptophan and kynurenine, the observed concentration (ng ml^{-1} in plasma and ng g^{-1} in brain tissue) and the kynurenine–tryptophan ratio (calculated as KYN/TRP) were used as dependent variables. The ratios between KYN and TRP were calculated as a ratio between the mol mg^{-1} or mol ml^{-1} of each of the analytes included in the ratio. For modeling the plasma concentrations of the respective analyte, treatment (SS, SL, KS, and KL) and sampling time point (T0, T72) were used as independent variables. The interaction between treatment and sampling time point was included in the model as the factor of most interest for testing our hypothesis of an effect of LPS and ketoprofen on the dependent variable. For modeling brain concentrations, treatment (SS, SL, KS, and KL) and hemisphere (left, right) were used as independent variables. TIME from euthanasia until the brain tissue was frozen was included as covariate in all models and removed in case of non-significance. Pig nested in treatment was included as a random variable in all models. For planned comparisons of treatments, Student's *t*-test was used. We compared SL with SS, SL with KL, and SS with KS. Analyses were run for each brain area separately as a difference between brain areas was not of interest. For correlations between the concentrations in plasma and brain tissue at 72 h after the injection, Spearman rank coefficient was used. In total, 37 pigs were included in this analysis.

For brain monoamines, the observed concentration (ng g^{-1}) of the respective analyte (DA, NA, 5-HT) as well as the ratio between metabolite(s) and mother substance were used as dependent variables. Dopamine turnover [calculated as (DOPAC + HVA/DA)], serotonin turnover (calculated as 5-HIAA/5-HT), and noradrenaline turnover (calculated as MHPG/NA) were calculated as an index for the activity of the dopaminergic, serotonergic, and noradrenergic systems. High levels indicate a higher activity in the respective system. Ratios were calculated based on moles per milligram of the respective analytes. Treatment (SS, SL, KS, and KL) and hemisphere (left, right) were used as independent variables. TIME was included as covariate in all models and removed in case of nonsignificance. Pig nested in treatment was included as a random variable in all models. For planned comparisons of treatments, Student's *t*-test was used. We compared SL with SS, SL with KL, and SS with KS. Analyses were run for each brain area separately as a difference between brain areas was not of interest.

RESULTS

Behavioral Signs of Sickness

LPS had an effect on general activity, feeding, and lying behavior, and ketoprofen attenuated these effects (**Figure 1**). LPS-injected pigs (SL) were significantly less active [F(treatment × hour)$_{(15,240)} = 0.98$, $p = 0.47$] than saline-injected pigs (SS) at 3 h (planned comparison: $p = 0.017$), 4 h ($p = 0.002$), and 5 h ($p = 0.006$) after the challenge. Feeding was depressed at the same time points [$F_{(15,240)} = 1.45$, $p = 0.13$, planned comparison: 3 h: $p = 0.01$, 4 h: $p = 0.004$, 5 h: $p = 0.002$]. SL pigs compared to SS pigs lay more frequently with their heads up [$F_{(15,240)} = 3.01$, $p = 0.0002$] at 2 h ($p = 0.009$) and lay more sternally [$F_{(15,240)} = 1.01$, $p = 0.44$) at 4 h ($p = 0.017$) and 5 h ($p = 0.004$). LPS-injected pigs that received ketoprofen (KL) were significantly more active than untreated, LPS-injected pigs (SL) at 1 h (planned comparison: $p = 0.0025$), 4 h ($p = 0.0025$), 5 h ($p = 0.0003$), and 6 h ($p = 0.013$) after the challenge. This corresponds to the finding that SL pigs showed more sternal recumbency compared to KL pigs at 1 h ($p = 0.021$) and 5 h ($p = 0.007$) and were lying more on the side [$F_{(15,240)} = 1.55$, $p = 0.09$) at 3 h ($p = 0.034$), 5 h ($p = 0.0018$), and 6 h ($p = 0.039$) after the challenge.

Social Behavior

The frequency and duration of all performed and received behaviors observed are displayed in the **Supplementary Table A**. There was a considerable variation within each group for the duration of the experiment. LPS had an effect on the duration of performed ear manipulation [F(treatment × day)$_{(6,88)} = 1.49$, $p = 0.19$] but not on any other behavioral pattern observed (**Figure 2**). The LPS-injected pigs were manipulating

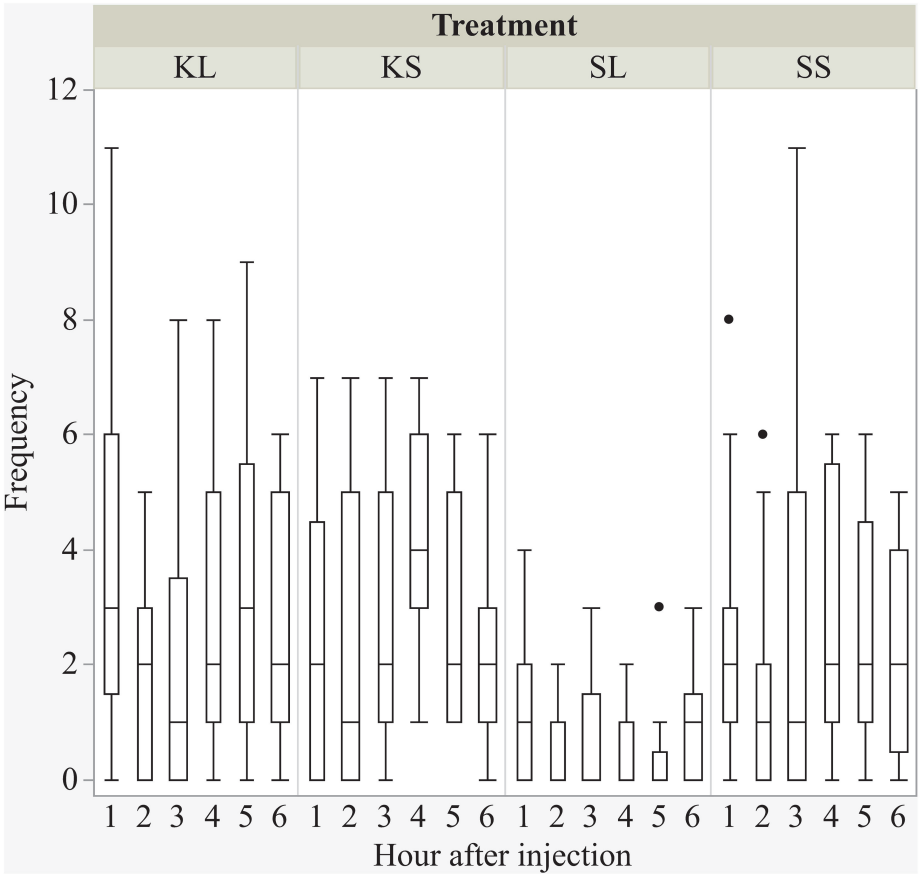

FIGURE 1 | Frequencies (per hour) of active behavior after injecting the pigs with ketoprofen–lipopolysaccharide (LPS), ketoprofen–saline, saline–LPS, and saline–saline. Round dots indicate outliers. $N = 13$ pigs per treatment.

the ears of their pen mates significantly longer (median = 118.82 s; min = 0 | max = 542.36 s) than the saline-injected pigs (37.38; 0 | 97.72) 2 days after the injection (planned comparison: $p = 0.022$). Ketoprofen had no effect on social behavior, neither in LPS-injected pigs nor in saline-injected controls. All results of the analysis of variance are displayed in the **Supplementary Table B**.

Cortisol

LPS activated the hypothalamic–pituitary–adrenal (HPA) axis and ketoprofen reduced this effect [F(treatment × time point)$_{(12,182.8)} = 4.65$, $p < 0.001$; **Figure 3**]. At 4 h after the injection, salivary cortisol was significantly higher in SL pigs (mean ± SD = 1.07 ± 0.59 ng ml^{-1}) compared to KL pigs (0.64 ± 0.68 ng ml^{-1}; planned comparison: $p < 0.001$) and SS pigs (0.42 ± 0.62 ng ml^{-1}; $p < 0.001$).

Cytokines

The descriptive results of all cytokine concentrations according to brain region are displayed in **Table 4**. LPS had no observed effect on the measured brain cytokines in the regions examined at 72 h after the injection. The measured concentration of IFN-γ did not differ between treatments and hemispheres in the frontal cortex ($n = 100$ samples), hippocampus ($n = 100$), hypothalamus

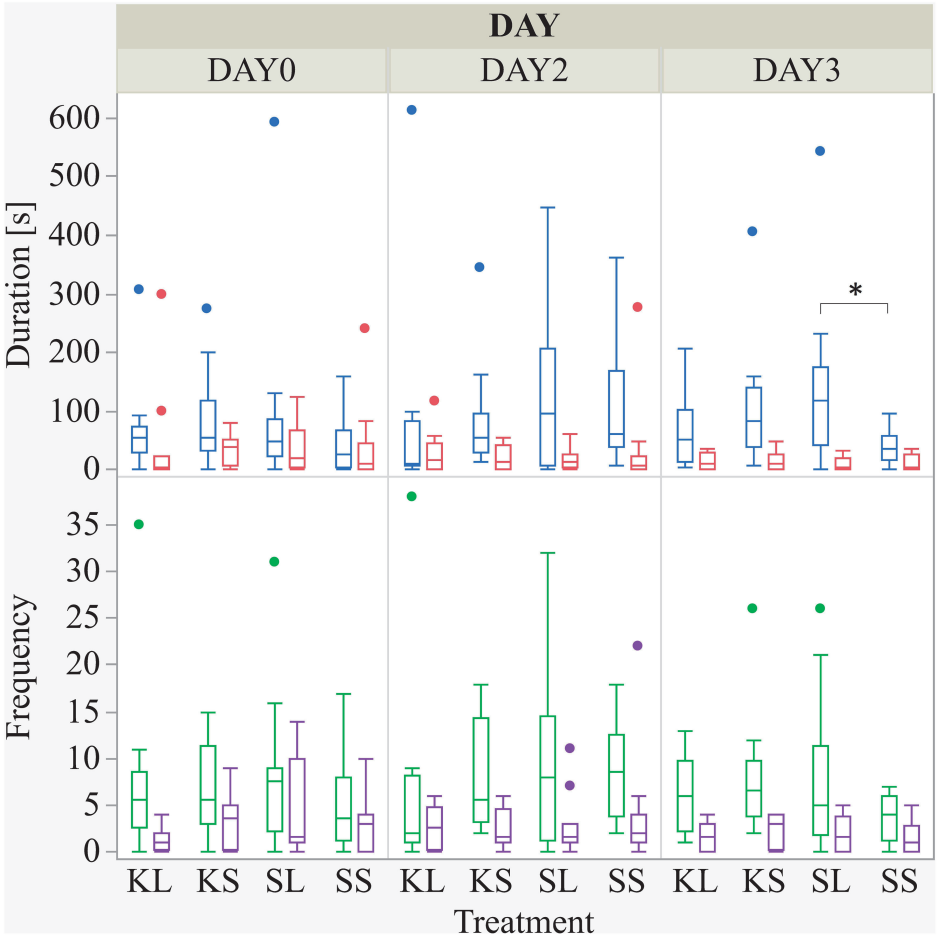

FIGURE 2 | Duration (s) and frequency of performed ear manipulation (blue and green boxplot, respectively) and tail manipulation (red and lilac) at baseline (DAY0) and at the first (DAY2) and second day (DAY3) after injecting the pigs with ketoprofen–lipopolysaccharide (LPS), ketoprofen–saline, saline–LPS, and saline–saline. Round dots indicate outliers. Significant differences of planned comparisons between treatments ($p < 0.05$) are marked with an asterisk.

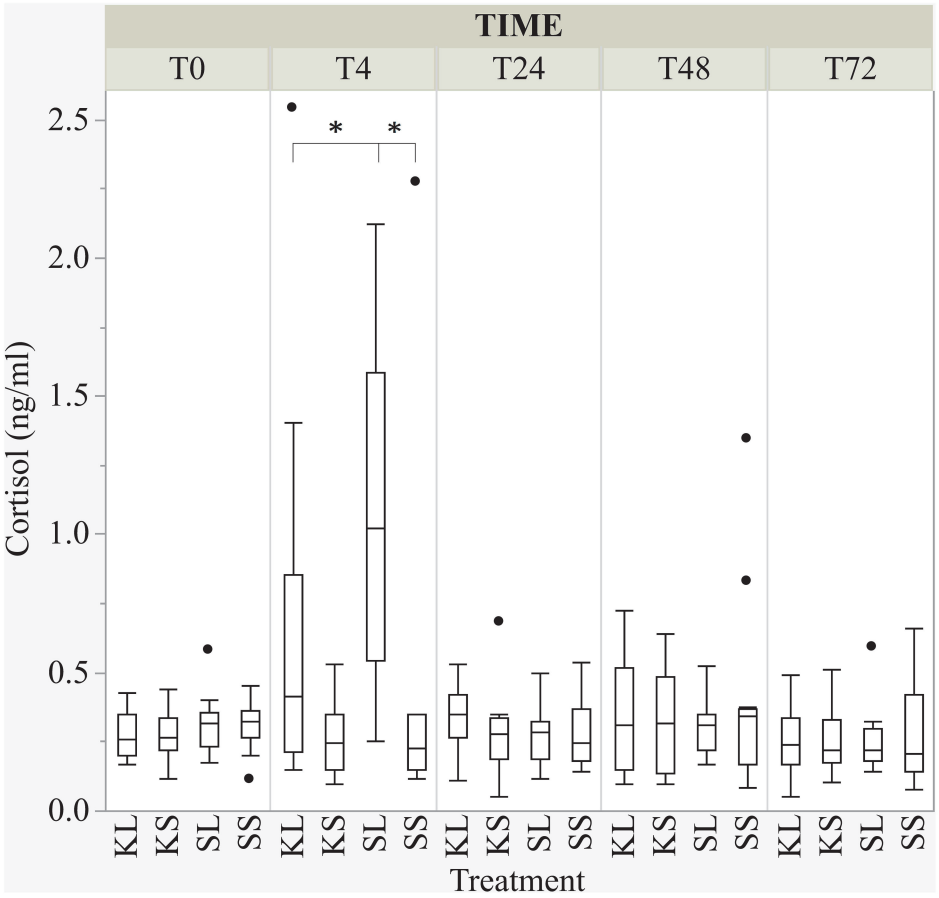

FIGURE 3 | The concentration of salivary cortisol (ng ml^{-1}) at baseline and at 4, 24, 48, and 72 h after injecting the pigs with ketoprofen–lipopolysaccharide (LPS), ketoprofen–saline, saline–LPS, and saline–saline. Round dots indicate outliers. Significant differences of planned comparisons between treatments ($p < 0.05$) are marked with an asterisk.

($n = 97$), and brain stem ($n = 104$). A hemisphere effect on TNF-α FI for the brain stem ($n = 104$) was seen [$F(\text{hemisphere})_{(1,51)} = 5.51$, $p = 0.02$]. The TNF-α FI levels were significantly higher in the right hemisphere (mean ± SD = 0.77 ± 0.26) compared to the left hemisphere (0.66 ± 0.25), although no treatment difference was observed. Neither treatment nor hemisphere had an effect on TNF-α FI levels in the frontal cortex ($n = 100$), hippocampus ($n = 100$), and hypothalamus ($n = 98$). Ketoprofen had an effect on IL-18 concentration in the brain stem ($n = 104$), [$F(\text{treatment})_{(3,48)} = 2.13$, $p = 0.11$]. SS pigs had higher concentrations (0.04 ± 0.01 ng g^{-1}) compared to KS pigs (0.03 ± 0.01 ng g^{-1}, planned comparison: $p = 0.018$). The concentration of IL-18 did not differ between treatments and hemispheres in the frontal cortex ($n = 100$), hippocampus ($n = 100$), and hypothalamus ($n = 98$). TIME had a significant effect on IFN-γ in the hypothalamus and brain stem and on TNF-α FI levels in the hypothalamus as reflected by decreasing concentrations with increasing time at collection post-mortem. All results of the analysis of variance (ANOVA) of IFN-γ, TNF-α, and IL-18 for treatment and hemisphere according to brain region are displayed in the **Supplementary Table C**.

Tryptophan and Kynurenine

Plasma

LPS had an effect on TRP concentrations in the plasma [$F(\text{treatment} \times \text{time point})_{(3,27.78)} = 2.32$, $p = 0.097$] (**Figure 4**). At 72 h after the injection, SL pigs had lower concentrations of TRP in their plasma (mean ± SD = 6,118.89 ± 1,451.04 ng ml^{-1})

TABLE 4 | Mean (± standard deviation) of concentrations (ng g^{-1}) of interferon gamma (IFN-γ) and interleukin 18 (IL-18) and fluorescence intensity (arbitrary units) of tumor necrosis factor alpha (TNF-α) in the frontal cortex, hippocampus, hypothalamus, and brain stem of pigs at 72 h after injecting the pigs with saline–saline (SS), saline–lipopolysaccharide (LPS) [SL], ketoprofen–saline (KS), and ketoprofen–LPS (KL).

Brain area	IFN-γ				TNF-α				IL-18			
	SS	SL	KS	KL	SS	SL	KS	KL	SS	SL	KS	KL
Frontal cortex	0.53 ± 0.68	0.37 ± 0.36	0.57 ± 0.78	0.40 ± 0.50	0.84 ± 0.51	0.79 ± 0.52	0.60 ± 0.26	0.78 ± 0.50	0.16 ± 0.06	0.14 ± 0.06	0.14 ± 0.04	0.15 ± 0.06
Hippocampus	0.32 ± 0.24	0.54 ± 0.66	0.33 ± 0.21	0.34 ± 0.23	0.65 ± 0.34	1.03 ± 1.37	0.90 ± 0.69	0.60 ± 0.40	0.04 ± 0.01	0.04 ± 0.01	0.04 ± 0.01	0.05 ± 0.02
Hypothalamus	0.24 ± 0.10	0.24 ± 0.08	0.25 ± 0.08	0.26 ± 0.08	1.46 ± 0.72	1.43 ± 0.46	1.38 ± 0.49	1.44 ± 0.65	0.04 ± 0.01	0.04 ± 0.01	0.04 ± 0.01	0.04 ± 0.01
Brain stem	0.16 ± 0.05	0.15 ± 0.05	0.15 ± 0.06	0.15 ± 0.05	0.75 ± 0.22	0.67 ± 0.20	0.79 ± 0.36	0.67 ± 0.23	0.04 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.01

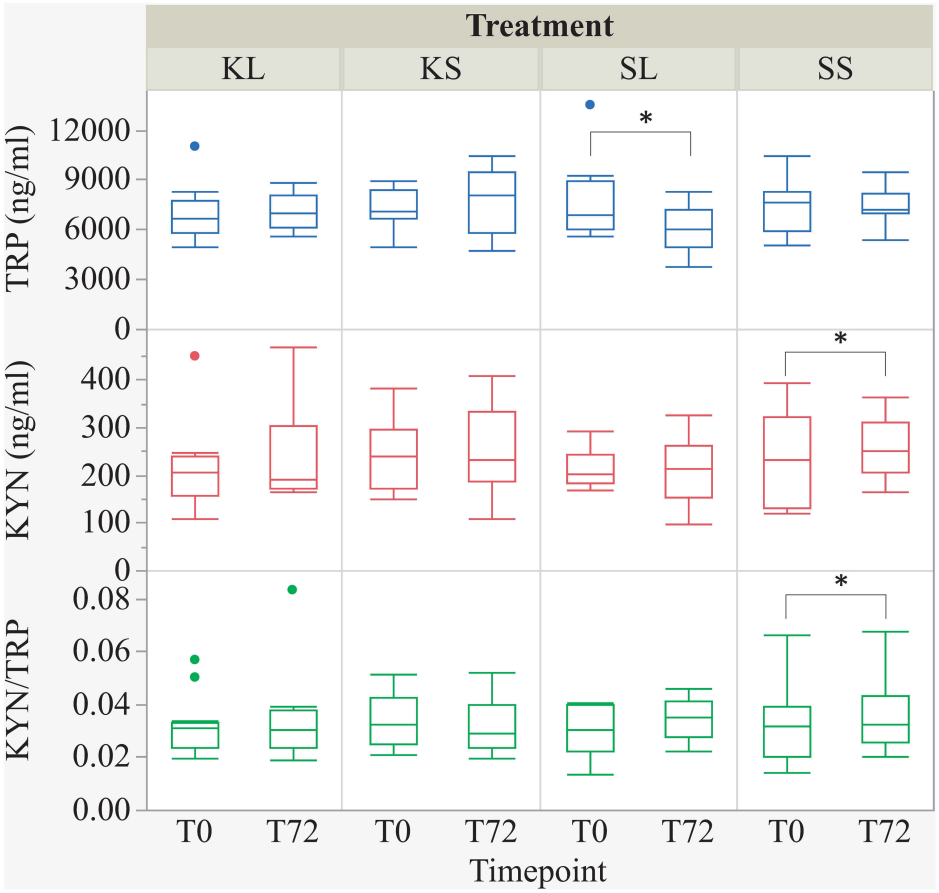

FIGURE 4 | Tryptophan (ng ml^{-1}), kynurenine (ng ml^{-1}), and kynurenine–tryptophan ratio in pig plasma at baseline and at 72 h after the injection with ketoprofen–lipopolysaccharide (LPS), ketoprofen–saline, saline–LPS, and saline–saline. Turnover rates were calculated based on moles per milligram of the respective analyte. Round dots indicate outliers. Significant differences of planned comparisons between sampling time points ($p < 0.05$) are marked with an asterisk.

compared to baseline (7,801.11 ± 2,453.08 ng ml^{-1}, planned comparison: $p = 0.035$). KYN concentrations [F(treatment × time point)$_{(3,29.37)} = 1.64$, $p = 0.20$] in SS pigs were significantly higher at 72 h after the injection (258.0 ± 65.31 ng ml^{-1}) compared to baseline (227.73 ± 91.46 ng ml^{-1}, $p = 0.026$). The kynurenine–tryptophan ratio [KYN/TRP, F(treatment × time point)$_{(3,27.28)} = 2.17$, $p = 0.11$] was lower at 72 h compared to baseline in SS pigs ($p = 0.044$).

The correlations between tryptophan in plasma and brain tissue were weak and positive (Spearman's rho, $\rho = 0.17$–0.32)

but significant for the hippocampus ($p = 0.007$) and brain stem ($p = 0.04$). The correlations between kynurenine in plasma and brain tissue were positive ($\rho = 0.23$–0.40) and significant for the frontal cortex ($p = 0.001$), hippocampus ($p = 0.006$), and hypothalamus ($p = 0.001$).

Brain Tissue

LPS injection had an effect on TRP and KYN concentrations in several brain areas measured at 72 h after administration (**Figure 5**). Lower concentrations of TRP were measured in the brain stem [$F(\text{treatment})_{(3,47.94)} = 1.90, p = 0.14$] and the frontal cortex [$F_{(3,45.41)} = 2.04$, $p = 0.12$] of LPS-injected pigs (SL) compared to saline-injected pigs (SS, planned comparisons: $p =$ 0.04, respectively) There were no differences between treatments and hemispheres in the hippocampus and the hypothalamus.

Lower concentrations of KYN were measured in the brain stem [$F_{(3,48.24)} = 2.33$, $p = 0.09$], frontal cortex [$F_{(3,47.4)} = 4.0$, $p = 0.01$], and hypothalamus [$F_{(3,47.11)} = 2.43$, $p = 0.08$] of SL pigs compared to SS pigs (planned comparison: $p = 0.035$, 0.003, 0.01). Moreover, the KYN/TRP ratio in the frontal cortex [$F_{(3,47.02)} = 1.95, p = 0.13$] and in the hypothalamus [$F_{(3,46.76)} =$ 2.21, $p = 0.10$] was significantly lower in SL pigs compared to that in SS pigs ($p = 0.037$, 0.017).

Ketoprofen had an effect on KYN concentrations in the brain stem of saline-injected pigs. Pigs that were treated with ketoprofen (KS) had lower concentrations than the untreated controls (SS) ($p = 0.02$). That finding was reflected by a significantly lower KYN/TRP ratio in the brain stem [$F_{(3,48.25)} = 1.81$, $p = 0.16$] of KS pigs compared to that of SS pigs ($p = 0.03$). There were no differences in KYN/TRP ratio between treatments and hemispheres in the hippocampus. The covariate TIME had a significant effect on the quantification of TRP and KYN concentrations in the frontal cortex and the hippocampus as reflected by decreasing concentrations with increasing time. The same effect was seen for TRP concentrations in the hypothalamus. All results of the analysis of variance are displayed in the **Supplementary Table D**.

Monoamines

The descriptive results of all monoamine concentrations according to brain region are displayed in **Table 5**. Highest concentrations of DA, NA, and 5-HT were found in the brain stem and the hypothalamus. LPS had an effect on DA concentrations [$F(\text{treatment})_{(3,47.06)} = 2.05$, $p = 0.12$; **Figure 6**] and respective turnover rate [$F_{(3,46.16)} = 2.28, p = 0.09$; **Figure 8**] in the hypothalamus measured at 72 h after the challenge. SL pigs had significantly lower DA concentrations compared to SS pigs (planned comparison: $p = 0.032$). This finding is reflected by a higher DA turnover of SL pigs compared to SS pigs ($p = 0.042$). There were no differences between treatments and hemispheres in the frontal cortex, hippocampus, and brain stem.

Ketoprofen attenuated the effects of LPS on serotonin (5-HT) concentrations in the hypothalamus [$F_{(3,47.14)} = 2.86$, $p = 0.047$; **Figure 6**], and 5-HT turnover rates tended to be affected [$F_{(3,47.79)} = 0.99$, $p = 0.41$, **Figure 8**]. SL pigs had significantly lower concentrations than KL pigs ($p = 0.005$) as reflected by higher turnover rates ($p = 0.13$). There were no differences between treatments and hemispheres in the frontal cortex, hippocampus, and brain stem.

We found a similar result for NA concentrations in the hippocampus [$F_{(3,47.8)} = 1.50$, $p = 0.23$; **Figure 7**]. SL pigs had significantly lower concentrations compared to KL pigs ($p = 0.04$), which was reflected by significantly higher NA turnover rates [$F_{(3,47.73)} = 2.96$, $p = 0.04$; **Figure 8**] in SL pigs compared to KL pigs ($p = 0.006$). NA concentrations in the hypothalamus [$F_{(3,46.97)} = 0.81$, $p = 0.49$] tended to be lower in SL pigs compared to SS pigs ($p = 0.13$). There were no differences between treatments and hemispheres in the frontal cortex and brain stem. The covariate TIME had a significant effect on DA, 5-HT, and NA concentrations in the brain stem as reflected by decreasing concentrations with increasing time. All results of the analysis of variance are displayed in the **Supplementary Tables E,F**.

DISCUSSION

Summary

LPS activated the HPA axis and elicited behavioral signs of sickness within 6 h after the injection as indicated by an increase in cortisol and decreased activity in LPS-injected pigs. Ketoprofen decreased the effect of LPS on cortisol release and alleviated behavioral signs of sickness. LPS had an effect on the duration of ear manipulation but not on any other behaviors. Controlled immune activation had no effect on the proinflammatory cytokines IFN-γ, TNF-α, and IL-18 measured at 72 h after the challenge in the frontal cortex, hippocampus, hypothalamus, and brain stem. The LPS-injected pigs had lower plasma concentrations of TRP at 72 h after the challenge compared to baseline. The correlations between TRP and KYN concentrations in plasma and brain tissue were weak and positive. The TRP and KYN concentrations were lower in the brain stem and frontal cortex of LPS-injected pigs compared to saline-injected pigs. The DA concentrations were lower in the hypothalamus of SL pigs compared to those in SS pigs. Ketoprofen attenuated the effects of LPS, resulting in lower concentrations of 5-HT in the hypothalamus and NA in the hippocampus of SL pigs compared to those in KL pigs.

Effect of LPS and Ketoprofen on Behavioral Signs of Sickness and Social Behavior

LPS depressed activity in challenged pigs. As predicted, ketoprofen attenuated this effect. This observation corroborates the findings of Johnson and von Borell (29) who showed that treatment with indomethacin, another NSAID, completely inhibited anorexia as well as reduction in activity in LPS-injected pigs (5 μg kg^{-1}, i.p.). Other studies showed that a pre-treatment of pigs with the NSAID meloxicam or flunixin significantly decreased the clinical symptoms induced by LPS (2–6 μg kg^{-1}, i.v.), including lethargy, skin flushing, vomiting, coughing, and labored breathing (13, 30).

Most studies of LPS effects focus on a relatively short time period (24 h) and rarely describe social behavior in detail. In

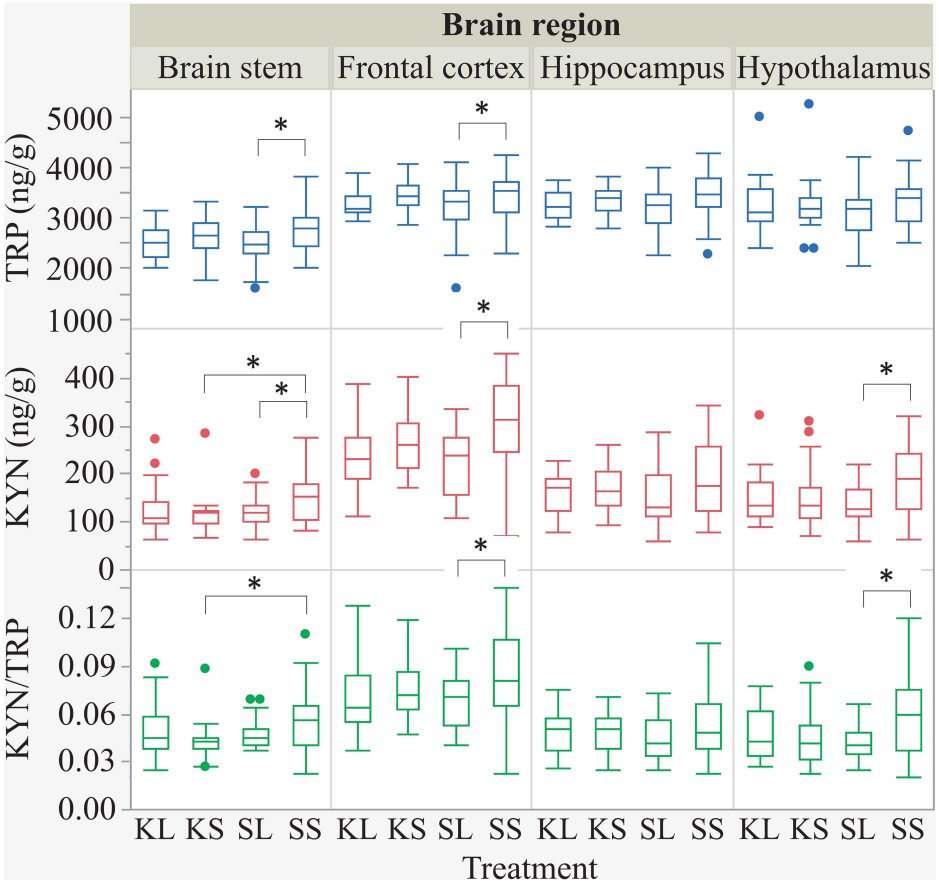

FIGURE 5 | Tryptophan (ng g^{-1}), kynurenine (ng g^{-1}), and kynurenine–tryptophan ratio in the brain stem, frontal cortex, hippocampus, and hypothalamus of pigs at 72 h after the injection with ketoprofen–lipopolysaccharide (LPS), ketoprofen–saline, saline–LPS, and saline–saline. Turnover rates were calculated based on moles per milligram of the respective analyte. Round dots indicate outliers. Significant differences of planned comparisons between treatments ($p < 0.05$) are marked with an asterisk.

TABLE 5 | Mean (± standard deviation) of concentrations (ng g^{-1}) of dopamine (DA), noradrenaline (NA), and serotonin (5-HT) as well as dopamine turnover (DOPAC + HVA/DA), noradrenaline turnover (MHPG/NA), and serotonin turnover (5-HIAA/5-HT) in the frontal cortex, hippocampus, hypothalamus, and brain stem of pigs at 72 h after the injection.

	Frontal cortex	Hippocampus	Hypothalamus	Brain stem
DA (ng g^{-1})	10.66 ± 8.45	7.03 ± 5.12	160.00 ± 73.59	157.52 ± 83.81
NA (ng g^{-1})	117.30 ± 33.15	131.61 ± 41.21	1,238.99 ± 416.00	529.42 ± 130.29
5-HT (ng g^{-1})	165.79 ± 37.53	164.85 ± 50.14	450.29 ± 164.43	623.84 ± 179.20
(DOPAC + HVA)/DA	4.11 ± 3.03	10.48 ± 28.02	2.01 ± 0.58	2.84 ± 0.81
MHPG/NA	0.11 ± 0.03	0.09 ± 0.03	0.03 ± 0.01	0.09 ± 0.02
5-HIAA/5-HT	0.22 ± 0.08	0.34 ± 0.09	0.38 ± 0.14	0.35 ± 0.09

The turnover rates were calculated based on moles per milligram of the respective analyte.

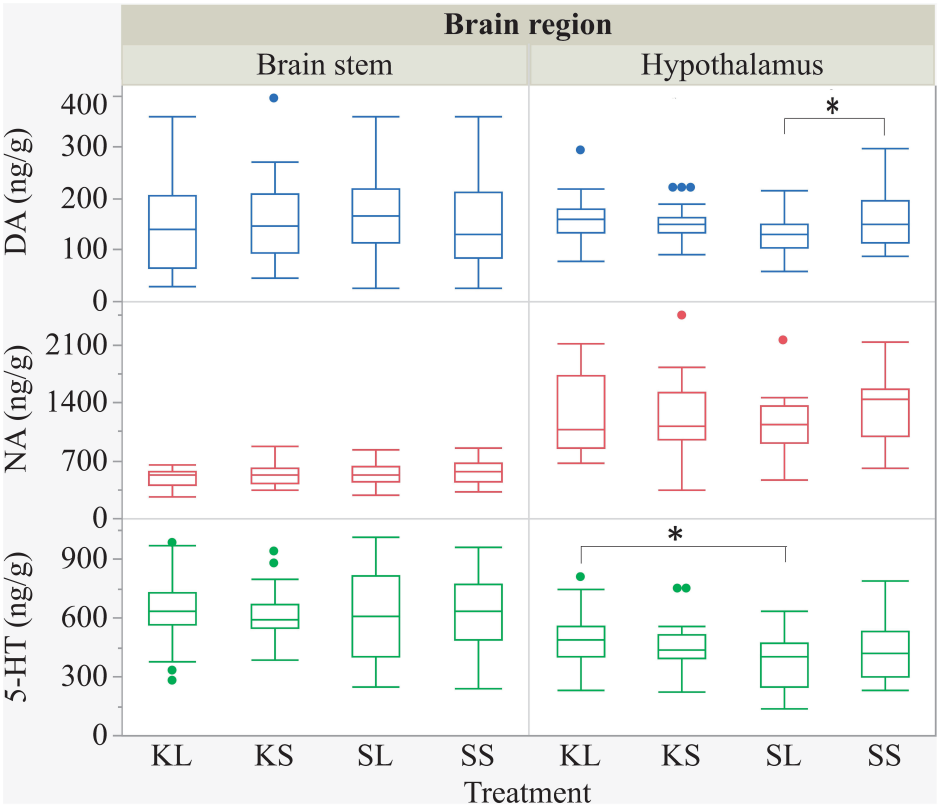

FIGURE 6 | Concentration (ng g^{-1}) of dopamine, noradrenaline, and serotonin in the brain stem and in the hypothalamus of pigs at 72 h after the injection with ketoprofen–lipopolysaccharide (LPS) (KL), ketoprofen–saline, saline–LPS, and saline–saline (SS). To improve the readability, two outliers were removed from the figure (one SS and one KL in the hypothalamus). Round dots indicate outliers. Significant differences of planned comparisons between treatments ($p < 0.05$) are marked with an asterisk.

rodents, it was shown that, when sickness behavior resolves, mice display depressive-like behaviors measured by increased immobility in the forced swim test and tail suspension test as well as decreased sucrose preference up to 48 h after the challenge (10, 31–33). In the present study, LPS seemed not to have a strong impact on social interactions, and pre-treatment with ketoprofen neither had an effect in LPS-injected pigs nor in saline-injected controls. The LPS-injected pigs manipulated the ears of their pen mates significantly longer than the saline-injected pigs 2 days after the injection. Tail manipulation happened for a very short duration of time, and the variation was very high; thus, any possible differences would not have been evident in this rather small sample size. There are indications that the LPS-treated pigs experienced a long-term shift in social motivation, resulting in more tail- and ear-directed behavior compared to controls 2 days after the challenge (5). The relationship between sickness and altered social behavior was also found in the field where pigs diagnosed with respiratory diseases tended to perform more ear and tail biting than controls in the days before they were diagnosed as sick (34). There is a correlation between nosing the tail and tail biting and between nosing the ear and ear biting (35). Thus, our findings of more ear manipulation can be interpreted as a higher probability of damaging behavior. Nevertheless, severe tail, ear, and flank biting that result in wounds was not observed in the present study. This could be interpreted as an acute and short-lived immune activation that is handled well by pigs kept in the conditions that our pigs were kept in. These were rather optimal (small group sizes, low stocking density, organic enrichment) regarding other known risk factors for damaging behaviors. It has been reported that environmental enrichment modulates different aspects of the immune system and the acute phase response (36, 37). Moreover, the pigs were familiar with each other as they were kept in stable groups from birth on. Regrouping, which is common in commercial pig production, causes stress and activates the immune system as

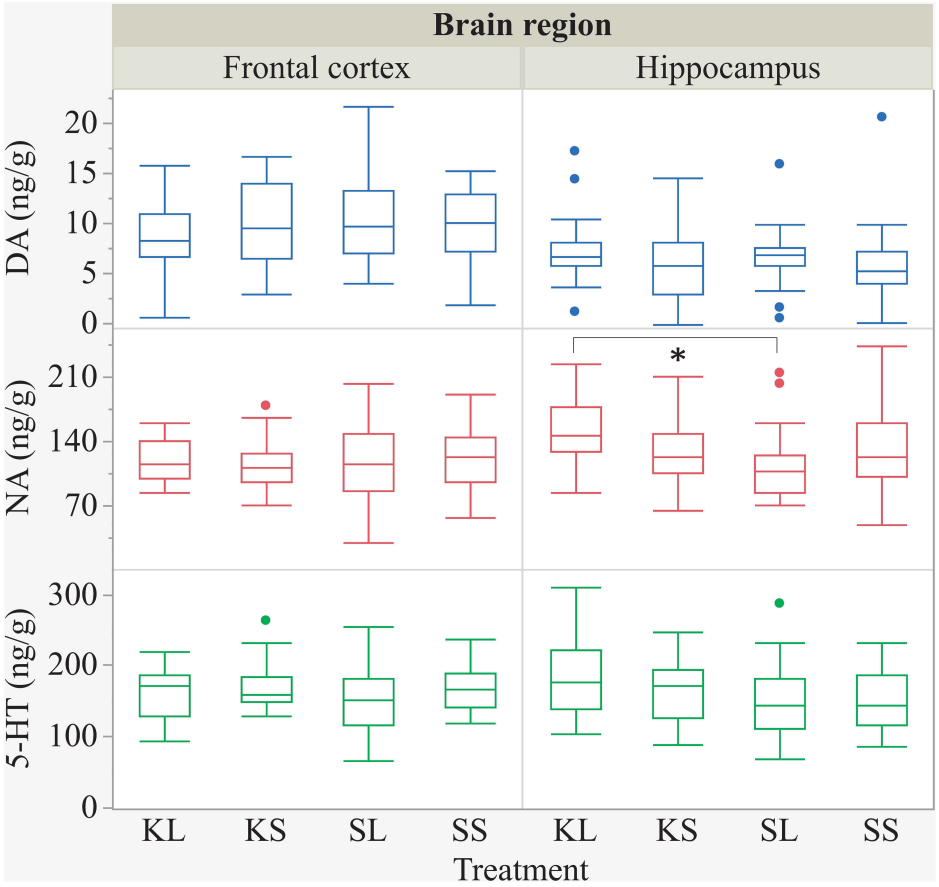

FIGURE 7 | Concentration (ng g^{-1}) of dopamine, noradrenaline, and serotonin in the frontal cortex and the hippocampus of pigs at 72 h after the injection with ketoprofen–lipopolysaccharide (LPS), ketoprofen–saline (KS), saline–LPS (SL), and saline–saline. To improve the readability, two outliers were removed from the figure (one KS in the frontal cortex and one SL in the hippocampus). Round dots indicate outliers. Significant differences of planned comparisons between treatments ($p < 0.05$) are marked with an asterisk.

shown by elevated salivary cortisol (38) and induced leukocyte mobilization (39).

Effect of LPS and Ketoprofen on HPA Axis Activity

LPS activates the HPA axis *via* prostaglandin (PGE2) release by endothelial cells as well as *via* a release of proinflammatory cytokines by macrophages. Salivary and plasma cortisols are highly correlated, and the values are around 10 times lower in saliva than in blood (40–42). In the present study, we collected saliva by letting pigs chew on a cotton pad. This is a non-invasive procedure compared to snaring the pigs for blood sampling which, in turn, has an effect on cortisol release by the adrenal glands. The significant elevation of salivary cortisol at 4 h after the challenge corroborates other studies measuring cortisol in plasma after intravenous (six) and intraperitoneal (20) LPS injection in pigs. The LPS-injected pigs that received a pre-treatment with ketoprofen showed a lower increase in salivary cortisol than SL pigs, but the concentrations were still higher than in SS pigs. Thus, a dose of 1.2 μg kg^{-1} LPS is sufficient to activate the HPA axis in 11- to 12-week-old pigs.

Effect of LPS and Ketoprofen on Brain Cytokines

The rationale for including IFN-γ and TNF-α in the present study was mainly based on previous findings (6) and the relevance of these cytokines for kynurenine metabolism through induction of IDO (see "Section Effect of LPS and ketoprofen on plasma

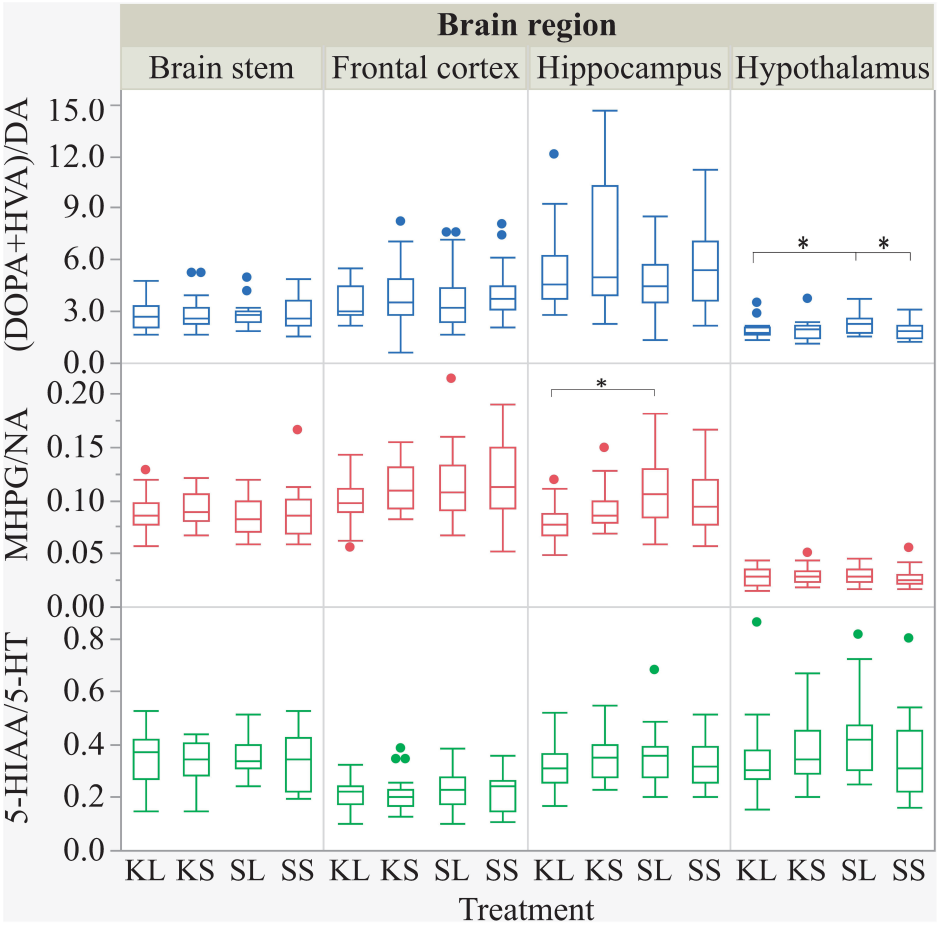

FIGURE 8 | Dopamine turnover, noradrenaline turnover, and serotonin turnover in the brain stem, frontal cortex, hippocampus, and hypothalamus of pigs at 72 h after the injection with ketoprofen–lipopolysaccharide (LPS) (KL), ketoprofen–saline (KS), saline–LPS (SL), and saline–saline (SS). The turnover rates were calculated based on moles per milligram of the respective analyte. To improve the readability, seven outliers were removed from the figure (two KS, two SS, and two SL in the hippocampus and one KL in the frontal cortex). Round dots indicate outliers. Significant differences of planned comparisons between treatments ($p < 0.05$) are marked with an asterisk.

and brain concentrations of tryptophan and kynurenine"). IL-18 is, in turn, involved in IFN-γ production *via* NK cell activation which might serve as a bridge between innate and adaptive immunity (43).

In the present study, we did not monitor the short-term effects of LPS on cytokines in the blood, as this has been extensively studied in pigs in the past (6, 9, 13, 14). Our focus was beyond the first 24 h after the challenge, as we wanted to see if there were longer-lasting physiological changes. The duration of effect on physiology is important for the relevance of the LPS model in the study of immune effects on mental health and damaging behavior in pigs. If the physiological and behavioral changes are short-lived, they may not teach us much about the mechanisms underlying mental illness. In mice, it was shown that TNF-α concentrations in the brain remained elevated for 10 months after systemic LPS administration (44).

In the present study, LPS had no observed effect on the proinflammatory cytokines IFN-γ, TNF-α, and IL-18 in any of the sampled brain areas 3 days after the challenge. This finding contradicts with a previous study, where higher concentrations of IFN-γ were found in the frontal cortex of LPS-injected pigs compared to saline-injected controls collected at the same

time point (6). The proinflammatory cytokines in the brain are produced by microglia, the resident immune cells of the CNS [reviewed by Smith et al. (45)] or transported from blood. A single injection with a low dose of LPS might not be enough to activate microglia or to elevate brain cytokines over a period of 3 days.

LPS induces cytokine production through binding to TLR-4 and the subsequent activation of transcription factor NFkappaB (14). All NSAIDs inhibit the production of cyclooxygenases; in addition, some NSAIDs are able to alter the expression of NFkappaB (13) and thereby reduce cytokine expression. Plasma cytokine concentrations are usually not affected by the use of NSAIDs (13, 14), and there are few studies on brain cytokines in pigs so far. It was shown in mice that the use of NSAIDs (indomethacin and ibuprofen) reversed the effect of LPS on behavior without changing the peripheral or central cytokine concentrations (46). The absence of an effect of LPS on brain cytokines in the present study made it impossible to test an effect of ketoprofen on this.

Effect of LPS and Ketoprofen on Plasma and Brain Concentrations of Tryptophan and Kynurenine

LPS influenced the tryptophan concentrations in plasma as well as the tryptophan and kynurenine concentrations in several brain areas. At 72 h, the LPS-injected pigs had lower plasma tryptophan concentrations compared to baseline, whereas the kynurenine concentrations were not affected. This corresponds to Melchior et al. (47), who demonstrated a depletion of tryptophan in plasma of pigs after experimentally induced lung inflammation over several days. In the short run, LPS caused a decrease in tryptophan and an increase in kynurenine serum concentrations in pigs measured at 3 h after the start of a 60-min LPS infusion (48). Wirthgen et al. (9) showed that tryptophan plasma concentrations were reduced for 24 h, whereas kynurenine concentrations were elevated for only 6 h after the LPS injection compared to the saline-injected pigs. It needs to be taken into account that SS pigs had a considerable baseline variation of plasma kynurenine concentrations, which might have led to a statistically significant but not biologically meaningful result.

In the present study, the effect of LPS on plasma tryptophan concentrations was not seen in pigs pre-treated with ketoprofen. This can be explained by the appetite-suppressant effect of IL-1, IL-6, IL-8, and TNF-α (49). The LPS-injected pigs showed depressed feeding and, in turn, reduced dietary uptake of tryptophan, which was reversed by ketoprofen. We found weak positive correlations between tryptophan concentrations in plasma and brain tissue. This effect was not seen in a study with mice where tryptophan concentrations were reduced in plasma but elevated in brain tissue at 24 h after the LPS injection (10).

The essential amino acid tryptophan is a peripheral precursor for the synthesis of the central neurotransmitter serotonin. Inflammation induces a shunt from serotonin to kynurenine metabolism of tryptophan mediated by the proinflammatory cytokines TNFα and IFNγ through an upregulation of the enzyme IDO. In the present study, we found lower tryptophan and kynurenine concentrations in the frontal cortex and brain stem as well as lower kynurenine concentrations in the hypothalamus of LPS-injected pigs compared to the saline-injected controls.

Ketoprofen had no influence on the effect of LPS on tryptophan and kynurenine concentrations in the sampled brain regions. There are few studies on tryptophan and kynurenine concentrations in the pig brain so far. In mice, the tryptophan concentrations in the hippocampus were found to be lower, and the kynurenine concentrations were higher 43 days after a chronic administration of LPS compared to the saline-treated controls (50). Kynurenine has been suggested by O'Connor et al. (10) to be essential for the development of depressive symptoms in rodents. As the changes in kynurenine concentrations last so shortly, subsequent changes in behavior might be caused by other mechanisms.

Effect of LPS and Ketoprofen on Brain Monoamines

LPS has an effect on central neurotransmitters, which is supposed to be mediated by different proinflammatory cytokines such as IL-1, IL-6, and TNF-α [reviewed by Dunn (51)]. The LPS-injected pigs had lower dopamine concentrations and higher turnover rates in their hypothalamus compared to the saline-injected controls at 72 h after the challenge. Similar alterations were found in genetically stress-susceptible pigs (52). Dopamine in the hypothalamus is produced by cell groups that are concerned with the control of prolactin release and the regulation of preganglionic sympathetic neurons in the spinal cord (53). Apart from its relevance for the reward system, dopamine plays a key role in response to stress (54–56), and the hypothalamus is, as a part of the HPA axis, one of the two major systems that respond to stress.

The intervention with ketoprofen alleviated the effects of LPS on serotonin in the hypothalamus and noradrenaline and its turnover rate in the hippocampus. The most important source of central noradrenaline, which is synthetized among the same pathways as dopamine, is the clusters of cell bodies in the locus coeruleus located in the pons that send branching axons, among others, to the hippocampus (57). The hippocampus plays a role in the control of the HPA response to stress and is itself an important target for glucocorticoids (58). The neurotransmitter serotonin derives from cell bodies in the raphe nuclei in the pons (part of the brain stem) that projects, among others, to the hypothalamus and the hippocampus (57) and has modulatory effects in almost all central nervous system integrative functions such as stress and fearfulness (59), aggression (60, 61), and mood. There is a close relationship between mood and state of arousal. In mice, lower concentrations of noradrenaline and serotonin in the prefrontal cortex (8) and lower levels of serotonin in the hippocampus of LPS-injected mice compared to controls were found at 24 h after the challenge (7). In both studies, depressive-like behaviors in forced swim and tail suspension tests were observed in LPS-injected mice. It needs to be taken into account that the focus of the present

study is on (negative) social behavior as there is no validated model for depressive-like behaviors in pigs. In rats, a pretreatment with the NSAID diclofenac completely prevented IFN-α-increased serotonin turnover in the prefrontal cortex and increased dopamine turnover in the hippocampus (62). A pretreatment with the NSAID indomethacin attenuated but did not abolished the increased extracellular levels of serotonin and noradrenaline in the hippocampus of rats within 6 h after the LPS challenge (63).

CONCLUSION

A controlled immune activation altered the behavior of the pigs in the 1st hours after the challenge but seemed not to have a strong impact on their social interactions in the subsequent 2 days. Central cytokine concentrations were not elevated 3 days after the challenge, but central monoamines were downregulated, and plasma tryptophan was depleted in LPS-injected pigs. Ketoprofen attenuated the effects of immune activation on behavioral signs of sickness, HPA axis activity, and central monoamines. LPS at a dose of 1.2 μg kg^{-1} seems not to be applicable as a model for long-term changes in the social behavior of pigs.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the NMBU IACUC and the food safety authorities (FOTS ID 15232).

AUTHOR CONTRIBUTIONS

Research idea and experimental design: JN, AMJ, and AV. Conduction of the experiment: CV, AMJ, JN, and BR. Behavioral observation: CV and JV supervised by AV and JN. Labwork: PP, DD, and CV. Data analysis: CV supervised by JN. Interpretation of data: all authors. Drafting the publication: CV. Revision of publication: all authors.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2020.617634/full#supplementary-material

REFERENCES

1. Fabrega HJ. Earliest phases in the evolution of sickness and healing. *Med Anthropol Q.* (1997) 11:26–55. doi: 10.1525/maq.1997.11.1.26
2. Konsman JP, Parnet P, Dantzer R. Cytokine-induced sickness behaviour: mechanisms and implications. *Trends Neurosci.* (2002) 25:154–9. doi: 10.1016/S0166-2236(00)02088-9
3. Fairbairn L, Kapetanovic R, Sester DP, Hume DA. The mononuclear phagocyte system of the pig as a model for understanding human innate immunity and disease. *J Leukocyte Biol.* (2011) 89:855–71. doi: 10.1189/jlb.1110607
4. Hart BL. Biological basis of the behavior of sick animals. *Neurosci Biobehav Rev.* (1988) 12:123–37. doi: 10.1016/S0149-7634(88)80004-6
5. Munsterhjelm C, Nordgreen J, Aae F, Heinonen M, Valros A, Janczak AM. Sick and grumpy: changes in social behaviour after a controlled immune stimulation in group-housed gilts. *Physiol. Behav.* (2019) 198:76–83. doi: 10.1016/j.physbeh.2018.09.018
6. Nordgreen J, Munsterhjelm C, Aae F, Popova A, Boysen P, Ranheim B, et al. The effect of lipopolysaccharide (LPS) on inflammatory markers in blood and brain and on behavior in individually-housed pigs. *Physiol. Behav.* (2018) 195:98–111. doi: 10.1016/j.physbeh.2018.07.013
7. Zhao X, Cao F, Liu Q, Li X, Xu G, Liu G, et al. Behavioral, inflammatory and neurochemical disturbances in LPS and UCMS-induced mouse models of depression. *Behav Brain Res.* (2019) 364:494–502. doi: 10.1016/j.bbr.2017.05.064
8. Zhu L, Wei T, Gao J, Chang X, He H, Miao M, et al. Salidroside attenuates lipopolysaccharide (LPS) induced serum cytokines and depressive-like behavior in mice. *Neurosci Lett.* (2015) 606:1–6. doi: 10.1016/j.neulet.2015.08.025
9. Wirthgen E, Tuchscherer M, Otten W, Domanska G, Wollenhaupt K, Tuchscherer A, et al. Activation of indoleamine 2,3-dioxygenase by LPS in a porcine model. *Innate Immunity.* (2013) 20:30–9. doi: 10.1177/1753425913481252
10. O'Connor JC, Lawson MA, André C, Moreau M, Lestage J, Castanon N, et al. Lipopolysaccharide-induced depressive-like behavior is mediated by indoleamine 2,3-dioxygenase activation in mice. *Mol Psychiatry.* (2009) 14:511. doi: 10.1038/sj.mp.4002148
11. Raison CL, Dantzer R, Kelley KW, Lawson MA, Woolwine BJ, Vogt G, et al. CSF concentrations of brain tryptophan and kynurenines during immune stimulation with IFN-α: relationship to CNS immune responses and depression. *Mol Psychiatry.* (2010) 15:393. doi: 10.1038/mp.2009.116
12. Vane JR, Botting RM. Mechanism of action of nonsteroidal anti-inflammatory drugs. *Am J Med.* (1998) 104(3 Suppl 1):2S−8S. doi: 10.1016/S0002-9343(97)00203-9
13. Peters SM, Yancy H, Deaver C, Jones YL, Kenyon E, Chiesa OA, et al. *In vivo* characterization of inflammatory biomarkers in swine and the impact of flunixin meglumine administration. *Vet Immunol Immunopathol.* (2012) 148:236–42. doi: 10.1016/j.vetimm.2012.05.001
14. Wyns H, Meyer E, Plessers E, Watteyn A, van Bergen T, Schauvliege S, et al. Modulation by gamithromycin and ketoprofen of *in vitro* and *in vivo* porcine lipopolysaccharide-induced inflammation. *Vet Immunol Immunopathol.* (2015) 168:211–22. doi: 10.1016/j.vetimm.2015.09.014
15. Thompson KA, Papich MG, Higgins B, Flanagan J, Christiansen EF, Harms CA. Ketoprofen pharmacokinetics of R- and S-isomers in juvenile loggerhead sea turtles (*Caretta caretta*) after single intravenous and single- and multidose intramuscular administration. *J Vet Pharmacol Ther.* (2018) 41:340–8. doi: 10.1111/jvp.12460
16. Mustonen K, Banting A, Raekallio M, Heinonen M, Peltoniemi OAT, Vainio O. Dose-response investigation of oral ketoprofen in pigs challenged with *Escherichia coli* endotoxin. *Vet Rec.* (2012) 171:70. doi: 10.1136/vr.100431
17. Salichs M, Sabaté D, Ciervo O, Homedes J. Comparison of the antipyretic efficacy of ketoprofen, acetylsalicylic acid, and paracetamol, orally administered to swine. *J Vet Pharmacol Ther.* (2012) 35:198–201. doi: 10.1111/j.1365-2885.2011.01314.x
18. Tegeder I, Pfeilschifter J, Geisslinger G. Cyclooxygenase-independent actions of cyclooxygenase inhibitors. *FASEB J.* (2001) 15:2057–72. doi: 10.1096/fj.01-0390rev
19. Ebdrup L, Krog JAN, Granfeldt A, Larsen PØ, Vestergaard C, Hokland M, et al. Leukocyte, plasma, and organ-associated cytokine profiles in an animal model of acute inflammation. *APMIS.* (2008) 116:352–60. doi: 10.1111/j.1600-0463.2008.00982.x

20. Webel DM, Finck BN, Baker DH, Johnson RW. Time course of increased plasma cytokines, cortisol, and urea nitrogen in pigs following intraperitoneal injection of lipopolysaccharide. *J Anim Sci.* (1997) 75:1514–20. doi: 10.2527/1997.7561514x
21. Moinard C, Mendl M, Nicol CJ, Green LE. A case control study of on-farm risk factors for tail biting in pigs. *Appl Anim Behav Sci.* (2003) 81:333–55. doi: 10.1016/S0168-1591(02)00276-9
22. Taylor NR, Parker RMA, Mendl M, Edwards SA, Main DCJ. Prevalence of risk factors for tail biting on commercial farms and intervention strategies. *Vet J.* (2012) 194:77–83. doi: 10.1016/j.tvjl.2012.03.004
23. Munsterhjelm C, Brunberg E, Heinonen M, Keeling L, Valros A. Stress measures in tail biters and bitten pigs in a matched case-control study. *Anim Welf.* (2013) 22:331–8. doi: 10.7120/09627286.22.3.331
24. Tong Q, Song J, Yang G, Fan L, Xiong W, Fang J. Simultaneous determination of tryptophan, kynurenine, kynurenic acid, xanthurenic acid and 5-hydroxytryptamine in human plasma by LC-MS/MS and its application to acute myocardial infarction monitoring. *Biomed Chromatogr.* (2018) 32:e4156. doi: 10.1002/bmc.4156
25. Zheng X, Kang A, Dai C, Liang Y, Xie T, Xie L, et al. Quantitative analysis of neurochemical panel in rat brain and plasma by liquid chromatography-tandem mass spectrometry. *Anal Chem.* (2012) 84:10044–51. doi: 10.1021/ac3025202
26. Fuertig R, Ceci A, Camus SM, Bezard E, Luippold AH, Hengerer B. LC-MS/MS-based quantification of kynurenine metabolites, tryptophan, monoamines and neopterin in plasma, cerebrospinal fluid and brain. *Bioanalysis.* (2016) 8:1903–17. doi: 10.4155/bio-2016-0111
27. Bergh MS-S, Bogen IL, Lundanes E, Øiestad ÅML. Validated methods for determination of neurotransmitters and metabolites in rodent brain tissue and extracellular fluid by reversed phase UHPLC-MS/MS. *J Chromatogr B.* (2016) 1028:120–9. doi: 10.1016/j.jchromb.2016.06.011
28. Doncaster CP, Davey AJH. *Analysis of Variance and Covariance: How to Choose and Construct Models for the Life Sciences*. Cambridge: Cambridge University Press (2007).
29. Johnson RW, von Borell E. Lipopolysaccharide-induced sickness behavior in pigs is inhibited by pretreatment with indomethacin. *J Anim Sci.* (1994) 72:309–14. doi: 10.2527/1994.722309x
30. Friton GM, Schmidt H, Schrödl W. Clinical and anti-inflammatory effects of treating endotoxin-challenged pigs with meloxicam. *Vet Rec.* (2006) 159:552. doi: 10.1136/vr.159.17.552
31. Frenois F, Moreau M, O'Connor J, Lawson M, Micon C, Lestage J, et al. Lipopolysaccharide induces delayed FosB/DeltaFosB immunostaining within the mouse extended amygdala, hippocampus and hypothalamus, that parallel the expression of depressive-like behavior. *Psychoneuroendocrinology.* (2007) 32:516–31. doi: 10.1016/j.psyneuen.2007.03.005
32. Ge L, Liu L, Liu H, Liu S, Xue H, Wang X, et al. Resveratrol abrogates lipopolysaccharide-induced depressive-like behavior, neuroinflammatory response, and CREB/BDNF signaling in mice. *Eur J Pharmacol.* (2015) 768:49–57. doi: 10.1016/j.ejphar.2015.10.026
33. Sulakhiya K, Keshavlal GP, Bezbaruah BB, Dwivedi S, Gurjar SS, Munde N, et al. Lipopolysaccharide induced anxiety- and depressive-like behaviour in mice are prevented by chronic pre-treatment of esculetin. *Neurosci Lett.* (2016) 611:106–11. doi: 10.1016/j.neulet.2015.11.031
34. Munsterhjelm C, Nordgreen J, Aae F, Heinonen M, Olstad K, Aasmundstad T, et al. To be blamed or pitied? The effect of illness on social behavior, cytokine levels and feed intake in undocked boars. *Physiol Behav.* (2017) 179:298–307. doi: 10.1016/j.physbeh.2017.06.024
35. Camerlink I, Turner SP. The pig's nose and its role in dominance relationships and harmful behaviour. *Appl Anim Behav Sci.* (2013) 145:84–91. doi: 10.1016/j.applanim.2013.02.008
36. Manciocco A, Sensi M, Moscati L, Battistacci L, Laviola G, Brambilla G, et al. Longitudinal effects of environmental enrichment on behaviour and physiology of pigs reared on an intensive-stock farm. *Ital J Anim Sci.* (2011) 10:e52. doi: 10.4081/ijas.2011.e52
37. Reimert I, Rodenburg TB, Ursinus WW, Kemp B, Bolhuis JE. Selection based on indirect genetic effects for growth, environmental enrichment and coping style affect the immune status of pigs. *PLOS One.* (2014) 9:e108700. doi: 10.1371/journal.pone.0108700
38. Coutellier L, Arnould C, Boissy A, Orgeur P, Prunier A, Veissier I, et al. Pig's responses to repeated social regrouping and relocation during the growing-finishing period. *Appl Anim Behav Sci.* (2007) 105:102–14. doi: 10.1016/j.applanim.2006.05.007
39. Bacou E, Haurogné K, Mignot G, Allard M, De Beaurepaire L, Marchand J, et al. Acute social stress-induced immunomodulation in pigs high and low responders to ACTH. *Physiol Behav.* (2017) 169:1–8. doi: 10.1016/j.physbeh.2016.11.012
40. Cook NJ, Schaefer AL, Lepage P, Jones SM. Salivary vs. serum cortisol for the assessment of adrenal activity in swine. *Can J Anim Sci.* (1996) 76:329–35. doi: 10.4141/cjas96-049
41. Merlot E, Mounier AM, Prunier A. Endocrine response of gilts to various common stressors: a comparison of indicators and methods of analysis. *Physiol Behav.* (2011) 102:259–65. doi: 10.1016/j.physbeh.2010.11.009
42. Schönreiter S, Huber H, Lohmüller V, Zanella AJ, Unshelm J, Henke J, et al. Salivary cortisol as a stress parameter in piglets. *Tierarztl Prax Ausg G Grosstiere Nutztiere.* (1999) 27:175–9.
43. Senju H, Kumagai A, Nakamura Y, Yamaguchi H, Nakatomi K, Fukami S, et al. Effect of IL-18 on the expansion and phenotype of human natural killer cells: application to cancer immunotherapy. *Int J Biol Sci.* (2018) 14:331–40. doi: 10.7150/ijbs.22809
44. Qin L, Wu X, Block ML, Liu Y, Breese GR, Hong JS, et al. Systemic LPS causes chronic neuroinflammation and progressive neurodegeneration. *Glia.* (2007) 55:453–62. doi: 10.1002/glia.20467
45. Smith JA, Das A, Ray SK, Banik NL. Role of pro-inflammatory cytokines released from microglia in neurodegenerative diseases. *Brain Res Bull.* (2012) 87:10–20. doi: 10.1016/j.brainresbull.2011.10.004
46. Teeling JL, Cunningham C, Newman TA, Perry VH. The effect of non-steroidal anti-inflammatory agents on behavioural changes and cytokine production following systemic inflammation: implications for a role of COX-1. *Brain Behav Immunity.* (2010) 24:409–19. doi: 10.1016/j.bbi.2009.11.006
47. Melchior D, Séve B, Le Floc'h N. Chronic lung inflammation affects plasma amino acid concentrations in pigs. *J Anim Sci.* (2004) 82:1091–9. doi: 10.2527/2004.8241091x
48. Tesch T, Bannert E, Kluess J, Frahm J, Hüther L, Kersten S, et al. 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.* (2018) 102:241–51. doi: 10.1111/jpn.12684
49. Plata-Salamán CR. Anorexia during acute and chronic disease. *Nutrition.* (1996) 12:69–78. doi: 10.1016/S0899-9007(96)90702-9
50. Rodrigues FTS, de Souza MRM, Lima CNdC, da Silva FER, Costa DVdS, dos Santos CC, et al. Major depression model induced by repeated and intermittent lipopolysaccharide administration: long-lasting behavioral, neuroimmune and neuroprogressive alterations. *J Psychiatr Res.* (2018) 107:57–67. doi: 10.1016/j.jpsychires.2018.10.003
51. Dunn AJ, Swiergiel AH, Beaurepaire Rd. Cytokines as mediators of depression: what can we learn from animal studies? *Neurosci Biobehav Rev.* (2005) 29:891–909. doi: 10.1016/j.neubiorev.2005.03.023
52. Adeola O, Ball RO, House JD, O'Brien PJ. Regional brain neurotransmitter concentrations in stress-susceptible pigs. *J Anim Sci.* (1993) 71:968–74. doi: 10.2527/1993.714968x
53. Kandel RE, Schwartz HJ, Jessell TM. *Principles of Neural Science*. 4th ed. New York, NY: McGraw-Hill (2000).
54. Chang C-h, Grace AA. Amygdala β-noradrenergic receptors modulate delayed downregulation of dopamine activity following restraint. *J Neurosci.* (2013) 33:1441–50. doi: 10.1523/JNEUROSCI.2420-12.2013
55. Rougé-Pont F, Piazza PV, Kharouby M, Le Moal M, Simon H. Higher and longer stress-induced increase in dopamine concentrations in the nucleus accumbens of animals predisposed to amphetamine self-administration. A microdialysis study. *Brain Res.* (1993) 602:169–74. doi: 10.1016/0006-8993(93)90260-T
56. Valenti O, Lodge DJ, Grace AA. Aversive stimuli alter ventral tegmental area dopamine neuron activity via a common action in the ventral hippocampus. *J Neurosci.* (2011) 31:4280. doi: 10.1523/JNEUROSCI.5310-10.2011
57. Rang HP, Ritter JM, Flower RJ, Henderson G. *Rang & Dale's Pharmacology*. 8th ed. London: Elsevier (2016).

58. Liu HH, Payne HR, Wang B, Brady ST. Gender differences in response of hippocampus to chronic glucocorticoid stress: role of glutamate receptors. *J Neurosci Res.* (2006) 83:775–86. doi: 10.1002/jnr.20782
59. Arroyo L, Carreras R, Valent D, Peña R, Mainau E, Velarde A, et al. Effect of handling on neurotransmitter profile in pig brain according to fear related behaviour. *Physiol Behav.* (2016) 167:374–81. doi: 10.1016/j.physbeh.2016.10.005
60. Ferrari PF, Van Erp AMM, Tornatzky W, Miczek KA. Accumbal dopamine and serotonin in anticipation of the next aggressive episode in rats. *Eur J Neurosci.* (2003) 17:371–8. doi: 10.1046/j.1460-9568.2003.02447.x
61. Van Erp AMM, Miczek KA. Aggressive behavior, increased accumbal dopamine, and decreased cortical serotonin in rats. *J Neurosci.* (2000) 20:9320–5. doi: 10.1523/JNEUROSCI.20-24-09320.2000
62. De La Garza R, Asnis GM. The non-steroidal anti-inflammatory drug diclofenac sodium attenuates IFN-α induced alterations to monoamine turnover in prefrontal cortex and hippocampus. *Brain Res.* (2003) 977:70–9. doi: 10.1016/S0006-8993(03)02757-4
63. Linthorst ACE, Flachskamm C, Holsboer F, Reul JMHM. Activation of serotonergic and noradrenergic neurotransmission in the rat hippocampus after peripheral administration of bacterial endotoxin: Involvement of the cyclo-oxygenase pathway. *Neuroscience.* (1996) 72:989–97. doi: 10.1016/0306-4522(95)00604-4

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Supplementary Table A: Frequency and duration [s] of all performed and received behaviours observed during 72 h after injection with ketoprofen-LPS (KL), ketoprofen-saline (KS), saline-LPS (SL) and saline-saline (SS).

			KL			KS			SL			SS		
			DAY0	**DAY2**	**DAY3**	**DAY0**	**DAY2**	**DAY3**	**DAY0**	**DAY2**	**DAY3**	**DAY0**	**DAY2**	**DAY3**
Tail mani-pulation	per-formed	Duration, median (min|max)	4.94 (0| 298.68)	16.12 (0|117)	10.46 (0|37.68)	38.88 (0|79.76)	14.26 (0|55.2)	10.58 (0| 48.08)	20.92 (0| 125.8)	13.78 (0|62.64)	3.9 (0|31.64)	11.1 (0|240.4)	6.54 (0| 276.48)	5.8 (0|35.88)
		Frequency, median (min|max)	1 (0|4)	2.5 (0|6)	1.5 (0|4)	3.5 (0|9)	1.5 (0|6)	3 (0|4)	1.5 (0|14)	1.5 (0|11)	1.5 (0|5)	3 (0|10)	2 (0|22)	1 (0|5)
	received	Duration, median (min|max)	27.72 (7.56| 146.32)	19.94 (0|84.48)	16.76 (0|63.2)	11.72 (0|76.72)	17.94 (0|39.84)	4.44 (0| 37.76)	10.26 (0| 319.92)	15.48 (0|88.52)	29.42 (0|70.88)	33.96 (4.84| 133.68)	21.22 (0| 74.84)	14.82 (0|59.68)
		Frequency, median (min|max)	3 (1|10)	2.5 (0|10)	2.5 (0|5)	1 (0|10)	2.5 (0|7)	1 (0|5)	2.5 (0|8)	2.5 (0|8)	3 (0|7)	3 (1|10)	2.5 (0|9)	2.5 (0|5)
Ear mani-pulation	per-formed	Duration, median (min|max)	55.4 (0|306.8)	9.76 (0| 612.36)	50.92 (4.04| 207)	56.42 (0| 274.24)	54.2 (13.56| 344.48)	84.22 (6.32| 405.48)	49.74 (0| 592.24)	95.56 (0| 447.92)	118.82 (0|160.24)	27.88 (0| 160.24)	62.16 (7.4| 362.28)	37.38 (0|97.72)
		Frequency, median (min|max)	5.5 (0|35)	2 (0|38)	6 (1|13)	5.5 (0|15)	5.5 (2|18)	6.5 (2|18)	7.5 (0|31)	8 (0|32)	5 (0|26)	3.5 (0|17)	8.5 (2|18)	4 (0|7)
	received	Duration, median (min|max)	66.1 (7.32| 196.68)	50.48 (8| 208.76)	71.6 (0| 180.08)	71.02 (19.88| 190.12)	55.88 (19.6| 346.64)	101.6 (5.68| 281.24)	37.78 (0| 199.12)	60.68 (27.72| 232.6)	39.06 (4.16| 147.2)	46.78 (20.16| 137.44)	52.68 (11.04|28 6.88)	83.6 (18.24| 165.48)
		Frequency, median (min|max)	6 (1|19)	5.5 (2|12)	5 (0|13)	7.5 (2|15)	7.5 (3|14)	8.5 (2|15)	4 (0|14)	8 (3|11)	5 (1|9)	6.5 (2|17)	5.5 (2|18)	7.5 (2|22)

Mani-pulation of other body parts	per-formed	Duration, median (min|max)	73.18 (7.76| 149.36)	62.48 (10.04| 174.52)	79.04 (11.96| 271.04)	97.96 (10.8| 311.88)	107.52 (37.76| 229.64)	151.36 (12.24| 230.44)	61.84 (4.96| 223.6)	51.12 (6.4| 630.76)	63.96 (14.84| 510.36)	66.86 (2.88| 378.83)	68.52 (2.8| 370.44)	69.34 (12.96| 302.24)
		Frequency, median (min|max)	8.5 (1|19)	9 (2|16)	10.5 (3|24)	10 (1|27)	9.5 (4|18)	9.5 (3|22)	8.5 (0|28)	8.5 (1|65)	7.5 (1|35)	5.5 (0|29)	8 (1|18)	7 (1|16)
	received	Duration, median (min|max)	112.4 (22.52| 331)	93.54 (0| 266.16)	80.86 (6.44| 164.6)	77.7 (18.72| 157.36)	79.22 (28.64| 295.96)	102.84 (38.44 |216.52)	90.78 (0| 232.8)	69.5 (10.72| 229.32	59.9 (0| 282.72)	102 (23.44| 275.6)	82.22 (2.2| 276.52)	95.12 (13| 487.72)
		Frequency, median (min|max)	8.5 (3|23)	9 (0|27)	7.5 (2|14)	9 (3|20)	8 (4|17)	8.5 (4|15)	9 (0|19)	7.5 (2|15)	8 (0|24)	9.5 (3|17)	8.5 (1|32)	8 (3|28)
Fighting	per-formed	Duration, median (min|max)	12 (0|57)	35.2 (0|68.16)	19.24 (0|44.24)	30.38 (7|89.36)	27.93 (0|91.56)	27.3 (0| 172.52)	21.08 (2|74)	34.08 (0|65.44)	12.14 (0| 139.52)	13 (0|82.92)	50.28 (0| 299.36)	17.64 (0|76.2)
		Frequency, median (min|max)	3 (0|13)	7.5 (0|16)	5.5 (0|14)	6 (1|12)	6 (0|21)	5.5 (0|27)	5 (1|16)	5 (0|12)	3 (0|33)	3 (0|12)	6.5 (0|31)	4 (0|13)
	received	Duration, median (min|max)	19.2 (1.8| 83.96)	16.64 (5.58| 121.96)	27.36 (2.96| 84.24)	20.16 (0| 146.68)	46.98 (0|129)	38.16 (8.84| 99.44)	36.2 (6.12| 114.96)	33.9 (8.36| 59.16)	20.32 (5.84| 130.16)	15.92 (0|65.96)	36.82 (0| 89.92)	28.7 (0|72.16)
		Frequency, median (min|max)	4.5 (1|15)	4.5 (1|21)	9.5 (2|15)	6 (0|14)	11 (0|23)	8 (1|16)	9 (2|19)	5.5 (2|19)	5 (1|25)	4.5 (0|17)	5 (0|18)	6 (0|16)
Displace-ment	per-formed	Duration, median (min|max)	0 (0|9.08)	0 (0|15)	1 (0|17.36)	0 (0|19.12)	0 (0|20.24)	0 (0| 20.88)	0 (0|13.2)	0 (0|13.64)	0 (0|9.2)	0 (0|24.16)	0 (0|18.12)	0 (0|7.88)
		Frequency, median (min|max)	0 (0|2)	0 (0|5)	0.5 (0|4)	0 (0|5)	0 (0|6)	0 (0|4)	0 (0|3)	0 (0|4)	0 (0|3)	0 (0|6)	0 (0|5)	0 (0|2)
	received	Duration, median (min|max)	0.9 (0|9.36)	0 (0|27)	0 (0|7.84)	0 (0|23.12)	1.6 (0|17.4)	0 (0| 19.56)	0 (0|10.8)	0 (0|13.48)	0 (0|6.441)	0 (0|14.24)	0 (0|8.44)	0 (0|10.68)
		Frequency, median (min|max)	0.5 (0|2)	0 (0|4)	0 (0|2)	0 (0|6)	0.5 (0|4)	0 (0|4)	0 (0|3)	0 (0|4)	0 (0|1)	0.5 (0|3)	0 (0|3)	0 (0|2)

Flank nosing	per-formed	Duration, median (min|max)	4.5 (0|52)	3.9 (0|25)	3 (0|45)	5.5 (0|56)	8.84 (0|49)	0 (0|112)	10.38 (0|41)	2 (0|41)	0 (0|120)	9 (0|30)	1.5 (0| 167.96)	10.5 (0|96)
		Frequency, median (min|max)	1 (0|4)	1 (0|2)	1 (0|5)	1 (0|8)	1 (0|6)	0 (0|10)	1 (0|5)	0.5 (0|6)	0 (0|7)	2 (0|3)	0.5 (0|5)	1 (0|6)
	received	Duration, median (min|max)	7.28 (0|44.44)	3.42 (0| 138.08)	8.64 (0| 103.04)	10.22 (0|65.4)	1.6 (0|76.16)	0.12 (0| 47.08)	2.34 (0| 41.56)	2.28 (0|24.64)	2.52 (0|609.6)	2.12 (0| 214.96)	3.22 (0|244)	0 (0|166.2)
		Frequency, median (min|max)	1.5 (0|7)	1 (0|3)	1 (0|6)	1 (0|5)	0.62 (0|4)	0.12 (0|5)	1 (0|6)	0.62 (0|3)	1 (0|6)	1 (0|4)	0.5 (0|7)	0 (0|7)
Belly nosing	per-formed	Duration, median (min|max)	4.48 (0|268)	0 (0|136)	2 (0|591)	3.56 (0|306)	0 (0|74)	1.08 (0|268)	0 (0| 17.84)	0 (0|116)	0 (0|201)	0 (0|321)	0.5 (0|248)	0 (0|239)
		Frequency, median (min|max)	1 (0|8)	0 (0|5)	1 (0|5)	1 (0|4)	0 (0|4)	1 (0|4)	0 (0|2)	0 (0|13)	0 (0|7)	0 (0|5)	0.5 (0|3)	0 (0|11)
	received	Duration, median (min|max)	3.26 (0| 171.04)	0 (0| 314.12)	1.04 (0|82.44)	2.78 (0| 200.16)	1.48 (0| 105.52)	0 (0| 684.68)	2.2 (0| 152.64)	2.72 (0| 350.88)	0.54 (0|53.28)	0 (0| 644.64)	0 (0| 151.92)	0 (0|273.4)
		Frequency, median (min|max)	1 (0|5)	0 (0|4)	1 (0|6)	1 (0|4)	0.5 (0|6)	0 (0|10)	0.5 (0|5)	0.5 (0|5)	0.5 (0|2)	0 (0|11)	0 (0|7)	0 (0|7)

Supplementary Table B: Results of the analysis of variance (ANOVA) of duration and frequency of performed and received behaviour for the interaction of treatment and day.

Behaviour		**ANOVA**	**F(treatment*day)**	**p-value**
Tail manipulation	performed	Duration	$F_{6,87.58} = 0.23$	0.97
		Frequency	$F_{6,88} = 0.83$	0.55
	received	Duration	$F_{6,88} = 0.70$	0.65
		Frequency	$F_{6,88} = 0.83$	0.55
Ear manipulation	performed	Duration	$F_{6,88} = 1.49$	0.19
		Frequency	$F_{6,88} = 1.38$	0.23
	received	Duration	$F_{6,88} = 0.48$	0.82
		Frequency	$F_{6,88} = 1.74$	0.12
Flank nosing	performed	Duration	$F_{6,88} = 0.20$	0.98
		Frequency	$F_{6,88} = 0.35$	0.91
	received	Duration	$F_{6,88} = 0.85$	0.54
		Frequency	$F_{6,88} = 0.39$	0.88
Belly nosing	performed	Duration	$F_{6,88} = 0.33$	0.92
		Frequency	$F_{6,88} = 0.54$	0.78
	received	Duration	$F_{6,88.57} = 0.72$	0.63
		Frequency	$F_{6,88.59} = 0.26$	0.95
Manipulation of other body parts	performed	Duration	$F_{6,88} = 0.36$	0.90
		Frequency	$F_{6,88} = 0.10$	1.00
	received	Duration	$F_{6,88} = 0.77$	0.60
		Frequency	$F_{6,88} = 0.12$	0.99
Fighting	performed	Duration	$F_{6,88} = 0.86$	0.53
		Frequency	$F_{6,88} = 0.54$	0.78
	received	Duration	$F_{6,88} = 0.42$	0.87
		Frequency	$F_{6,88} = 0.38$	0.89
Displacement	performed	Duration	$F_{6,88} = 1.00$	0.43
		Frequency	$F_{6,88} = 0.87$	0.52
	received	Duration	$F_{6,88} = 0.78$	0.59
		Frequency	$F_{6,88} = 0.95$	0.46

Supplementary Table C: Results of the analysis of variance (ANOVA) of interferon gamma (IFN-γ), tumor necrosis factor alpha (TNF-α) and interleukin 18 (IL-18) for treatment and hemisphere according to brain region.

Brain region	**ANOVA**	**IFN-γ**		**TNF-α**		**IL-18**	
		F-ratio	**p-value**	**F-ratio**	**p-value**	**F-ratio**	**p-value**
Frontal cortex	Treatment	$F_{(3,48.67)} = 0.34$	0.79	$F_{(3,46,.6)} = 0.98$	0.41	$F_{(3,48,32)} = 0.45$	0.72
	Hemisphere	$F_{(1,50.62)} = 0.0004$	0.98	$F_{(1,50.64)} = 2.60$	0.11	$F_{(1,48.57)} = 0.68$	0.41
Hippo-campus	Treatment	$F_{(3,48.75)} = 1.04$	0.38	$F_{(3,48.11)} = 1.32$	0.28	$F_{(3,48.22)} = 0.53$	0.66
	Hemisphere	$F_{1,50.49} = 0.91$	0.34	$F_{(1,50.29)} = 0.99$	0.33	$F_{(1,48.73)} = 0.12$	0.73
Hypo-thalamus	Treatment	$F_{(3,46)} = 0.24^{a}$	0.87	$F_{(3,48.31)} = 0.12^{a}$	0.95	$F_{(3,47.76)} = 0.93$	0.43
	Hemisphere	$F_{(1,48.64)} = 0.65^{a}$	0.42	$F_{(1,54.21)} = 0.18^{a}$	0.67	$F_{(1,47.62)} = 0.12$	0.73
Brain stem	Treatment	$F_{(3,47)} = 0.14^{a}$	0.94	$F_{(3,48)} = 0.91$	0.44	$F_{(3,48)} = 2.01$	0.13
	Hemisphere	$F_{(1,51)} = 3.23^{a}$	0.08	$F_{(1,51)} = 5.51$	0.02*	$F_{(1,51)} = 0.02$	0.88

Significant results ($p < 0.05$) are marked with *

[a]Covariate TIME included in the model

Supplementary Table D: Results of the analysis of variance (ANOVA) of tryptophan (TRP), kynurenine (KYN) and kynurenine-tryptophan ratio (KYN/TRY) for treatment and hemisphere according to brain region.

Brain region	**ANOVA**	**TRP**		**KYN**		**KYN/TRP**	
		F-ratio	**p-value**	**F-ratio**	**p-value**	**F-ratio**	**p-value**
Frontal cortex	Treatment Hemisphere	$F_{(3,45.41)} = 2.04^a$ $F_{(1,46.16)} = 0.12^a$	0.12 0.73	$F_{(3,47.4)} = 4.0^a$ $F_{(1,48.83)} = 2.8^a$	0.01* 0.10	$F_{(3,47.02)} = 1.95^a$ $F_{(1,48.63)} = 2.85^a$	0.13 0.10
Hippo-campus	Treatment Hemisphere	$F_{(3,46.52)} = 1.37^a$ $F_{(1,47.92)} = 3.86^a$	0.26 0.06	$F_{(3,46.99)} = 1.31^a$ $F_{(1,48.5)} = 0.40^a$	0.28 0.53	$F_{(3,47.17)} = 0.68^a$ $F_{(1,48.97)} = 1.25^a$	0.57 0.27
Hypo-thalamus	Treatment Hemisphere	$F_{(3,44.39)} = 0.73^a$ $F_{(1,45.82)} = 3.33^a$	0.54 0.07	$F_{(3,47.11)} = 2.43$ $F_{(1,45.51)} = 0.12$	0.08 0.73	$F_{(3,46.76)} = 2.21$ $F_{(1,44.83)} = 2.06$	0.10 0.16
Brain stem	Treatment Hemisphere	$F_{(3,47.94)} = 1.90$ $F_{(1,50.2)} = 0.33$	0.14 0.57	$F_{(3,48.24)} = 2.33$ $F_{(1,50.61)} = 1.58$	0.09 0.21	$F_{(3,48.25)} = 1.81$ $F_{(1,50.62)} = 0.94$	0.16 0.34

Significant results ($p < 0.05$) are marked with *

[a]Covariate TIME included in the model

Supplementary Table E: Results of the analysis of variance (ANOVA) of dopamine (DA), noradrenaline (NA), serotonin (5-HT) for treatment and hemisphere according to brain region.

Brain region	**ANOVA**	**DA**		**NA**		**5-HT**	
		F-ratio	**p-value**	**F-ratio**	**p-value**	**F-ratio**	**p-value**
Frontal cortex n = 103	Treatment Hemisphere	$F_{(3,48.53)} = 0.83$ $F_{(1,51.41)} = 0.89$	0.48 0.35	$F_{(3,47.25)} = 0.11$ $F_{(1,47.15)} = 0.11$	0.95 0.75	$F_{(3,47.32)} = 0.60$ $F_{(1,47.14)} = 1.40$	0.62 0.24
Hippo-campus n = 102	Treatment Hemisphere	$F_{(3,47.47)} = 0.97$ $F_{(1,49.69)} = 0.08$	0.42 0.78	$F_{(3,47.80)} = 1.50$ $F_{(1,48.37)} = 0.29$	0.23 0.59	$F_{(3,47.77)} = 1.07$ $F_{(1,48.41)} = 0.21$	0.37 0.65
Hypo-thalamus n = 93	Treatment Hemisphere	$F_{(3,47.06)} = 2.05$ $F_{(1,48.19)} = 0.003$	0.12 0.95	$F_{(3,46.97)} = 0.81$ $F_{(1,46.55)} = 2.20$	0.49 0.15	$F_{(3,47.14)} = 2.86$ $F_{(1,47.52)} = 3.98$	0.047* 0.05
Brain stem n = 104	Treatment Hemisphere	$F_{(3,47.34)} = 0.30^{a}$ $F_{(1,50.87)} = 2.27^{a}$	0.83 0.14	$F_{(3,47.24)} = 0.95^{a}$ $F_{(1,50.46)} = 0.99^{a}$	0.43 0.33	$F_{(3,47.13)} = 0.11^{a}$ $F_{(1,50.4)} = 3.61^{a}$	0.96 0.06

Significant results ($p < 0.05$) are marked with *

[a]Covariate TIME included in the model

Supplementary Table F: Results of the analysis of variance (ANOVA) of dopamine turnover (DOPAC+HVA/DA), noradrenaline turnover (MHPG/NA), serotonin turnover (5-HIAA/5-HT) for treatment and hemisphere according to brain region. Turnover rates were calculated based on mol mg^{-1} of the respective analyte.

Brain region	**ANOVA**	**(DOPAC+HVA/DA)**		**MHPG/NA**		**5-HIAA /5-HT**	
		F-ratio	**p-value**	**F-ratio**	**p-value**	**F-ratio**	**p-value**
Frontal cortex n = 103	Treatment Hemisphere	$F_{(3,47.9)} = 0.32$ $F_{(1,51.02)} = 0.56$	0.81 0.46	$F_{(3,48.76)} = 0.98$ $F_{(1,49.23)} = 0.0005$	0.41 0.98	$F_{(3,48.63)} = 0.21$ $F_{(1,50.08)} = 0.53$	0.89 0.47
Hippo-campus n = 102	Treatment Hemisphere	$F_{(3,48.94)} = 0.66$ $F_{(1,50.98)} = 0.05$	0.58 0.83	$F_{(3,47.73)} = 2.95$ $F_{(1,48.69)} = 5.46$	0.04* 0.02*	$F_{(3,46.58)} = 0.78$ $F_{(1,47.81)} = 1.65$	0.51 0.21
Hypo-thalamus n = 93	Treatment Hemisphere	$F_{(3,46.16)} = 2.28$ $F_{(1,46.26)} = 0.008$	0.09 0.92	$F_{(3,47.02)} = 0.30$ $F_{(1,47.01)} = 0.44$	0.83 0.51	$F_{(3,47.79)} = 0.99$ $F_{(1,47.22)} = 0.82$	0.41 0.37
Brain stem n = 104	Treatment Hemisphere	$F_{(3,47.84)} = 0.02$ $F_{(1,50.13)} = 1.58$	1.00 0.21	$F_{(3,48.16)} = 0.28$ $F_{(1,50.38)} = 0.004$	0.84 0.95	$F_{(3,48.1)} = 0.33$ $F_{(1,50.38)} = 1.02$	0.80 0.32

Significant results ($p < 0.05$) are marked with *

Paper II

Dynamics of Salivary Adenosine Deaminase, Haptoglobin, and Cortisol in Lipopolysaccharide-Challenged Growing Pigs

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Abstract

In commercial pig production, infectious and inflammatory conditions affect especially growing pigs. Lipopolysaccharide (LPS) is an important antigenic structure of Gram-negative bacteria and can be used to induce inflammation experimentally. As pigs are usually group-housed in commercial conditions, it is difficult to recognize diseased pigs particularly at an early stage. Acute phase proteins such as haptoglobin (Hp) are known indicators of an activated innate immune system whereas adenosine deaminase (ADA) is a relatively novel inflammatory biomarker in pigs. Both parameters are potential indicators of an inflammatory condition and are measurable in saliva. Compared with blood sampling, saliva sampling is less stressful procedure that is rapid, non-invasive and easy to perform both at group and at individual level.

In this blinded randomized clinical trial, 32 female pigs at their post-weaning phase were allocated to one of four treatments comprising two injections of the following substance combinations: saline-saline (SS), ketoprofen-saline (KS), saline-LPS (SL), and ketoprofen-LPS (KL). The first substance

was administered intramuscularly one hour before the second substance that was given through an ear vein catheter. Saliva was collected prior to injections (baseline) and at 4, 24, 48, and 72 hours post-injection in all groups for determination of ADA, Hp, and cortisol concentrations. A multivariate model was applied to describe the dynamics of each biomarker and pairwise relationships between ADA, Hp, and cortisol response from baseline to four hours post-injection within the SL group were studied with Spearman correlations.

Four hours post-injection, a significant increase in the SL group was seen in the studied biomarkers compared to baseline and all other time points (pairwise comparisons, $p < 0.01$ for all), as predicted. Ketoprofen alleviated the LPS effect. We found a significant positive correlation between ADA and Hp within the SL group ($r = 0.86$, $p < 0.05$). The primary and novel findings of the present study are the response of ADA to LPS, its time course and alleviation by ketoprofen. Our results support the evidence that ADA and Hp are promising inflammatory biomarkers in pigs. However, we suggest further studies to be conducted in commercial settings with larger sample sizes.

Introduction

In growing pigs, infectious and inflammatory diseases are common (1–3), and especially gastrointestinal (GI) diseases of bacterial origin affect pigs worldwide (4). In commercial pig production, growing pigs are housed in groups of varying sizes, potentially hindering the detection of sick individuals by herd employees. Sub-clinical illness poses a risk for disease transmission and can result in a reduced performance of pigs (5).

Lipopolysaccharide (LPS), known also as endotoxin, is an important antigenic structure of the cell wall in Gram-negative bacteria (6). It can be used experimentally to induce a systemic inflammation (7). When entering the bloodstream, LPS binds to LPS binding protein (LBP) and the LPS-LPB complex interacts with sentinel cells (6). Consequently, innate immune system is activated (6) followed by an

47 acute inflammatory response (6,8) accompanied by sickness behavior (9). The key mediators during
48 the inflammatory process are pro-inflammatory cytokines that trigger acute-phase protein (APP)
49 production in the liver (8).

50 Haptoglobin (Hp) is an important APP in pigs (10,11). As other APPs, it is primarily synthetized in the
51 liver (8) yet some evidence about local Hp production in salivary gland exists (12). Its serum
52 concentrations are known to increase in pigs suffering from infectious diseases (13–15) or acute
53 inflammatory processes (14,15). Adenosine deaminase (ADA) is an enzyme involved in purine
54 metabolism and is also linked to immune development (16). ADA is expressed in most tissues at some
55 levels (17), thus the expression is highest in lymphoid organs (16) indicating the role of ADA in
56 immune activation. It has been proposed as a potential inflammatory biomarker in pigs (18,19).
57 Cortisol, which is usually perceived as a stress biomarker, is an indicator of activation of the
58 hypothalamic-pituitary-adrenal (HPA) axis (20). Its release from the adrenal cortex happens within a
59 few minutes under various stressful situations (20), including LPS injection (21) after which it is spread
60 via bloodstream.

61 According to a recent review, non-infectious causes, including psychological stress, seem to result in
62 a pro-inflammatory cytokine response mimicking that seen after LPS challenge, albeit the magnitude
63 of the response appears to be lower than the response to endotoxin (22). This is supported by two pig
64 studies in which transportation stress (23) and feed deprivation (24) induced a slight increase (around
65 twofold) in serum Hp concentration. A recent study in sheep indicated that stress could also increase
66 salivary ADA concentration rapidly and transiently (25). The same study showed a minor positive
67 correlation between salivary ADA and cortisol, and a moderate positive correlation between ADA and
68 heart rate (25). To the authors' knowledge, neither the magnitude and time course of the ADA response
69 under a controlled immune challenge nor ADA's relation to Hp and cortisol in that setting have been
70 described previously. Former reports have primarily been either cross-sectional (18,19,26) or

longitudinal studies with sampling intervals of days or weeks (27–31) and conducted under farm conditions (19,28,31).

Ketoprofen is a commonly used nonsteroidal anti-inflammatory drug (NSAID) in veterinary medicine and has been established as a potent anti-inflammatory drug in pigs (32,33). NSAIDs target cyclo-oxygenase enzymes 1 and 2 (COX 1-2) and reduce pain, fever, and inflammation through inhibition of prostaglandin synthesis (34). Ketoprofen administration prior to LPS injection was recently shown to diminish the effect of LPS on cortisol and attenuated the behavioral signs of sickness in challenged pigs (35). An alleviating effect of an NSAID on an LPS-induced increase in one or more inflammatory biomarkers will strengthen the evidence to use those biomarkers in the evaluation of the pig health.

Blood sampling is stressful procedure and challenging to carry out for a large numbers of pigs. Less stressful sampling methods that are easily applicable to a group of animals under farm conditions (5,19,28) would therefore be of great value to improve pig herd health evaluation. Several biomarkers circulating in the bloodstream are detectable in saliva as well (19,20,30,36), and as saliva sampling is less stressful to the pigs, it is a good alternative to blood sampling. In order to minimize production losses, prevent disease outbreaks within herds, and restrain pathogen spread, it would be advantageous to detect problems at an early stage. Salivary Hp is elevated by systemic disease in pigs (18) and some evidence indicates that it is a more sensitive and specific biomarker for the detection of certain porcine diseases than serum Hp (36). It is also suitable for the detection of sub-clinical illness in pigs (28). Increasing evidence supports determination of a panel of biomarkers with different triggers (8,14,15,30) and time courses (20,29) instead of single ones. The reasoning is that measurement of several biomarkers improves diagnostic sensitivity (at least regarding APPs) (15) and they have the potential to represent different body regulatory systems (20) thus giving a more holistic picture of disease origin.

The aim of this study was to investigate the dynamics of salivary biomarkers of systemic inflammation in growing pigs exposed to LPS under experimental conditions and to test whether an NSAID could alleviate the effect of LPS. We predicted that salivary ADA, Hp, and cortisol would respond to LPS injection and that ketoprofen would alleviate the LPS effect. In addition, we aimed to describe the correlations between the responses of salivary 1) ADA and Hp, 2) ADA and cortisol, and 3) Hp and cortisol in LPS-challenged pigs.

Materials and Methods

Ethical statement

The Norwegian animal research authority approved the experiment (FOTS id 15232).

Animals and housing

The experiment took place in two blocks between 3 April and 11 May 2018 at the Livestock Production Research Center of the Norwegian University of Life Sciences (NMBU), campus Ås. Thirty-two female pigs (Norwegian Landrace), henceforth referred to as experimental pigs, were used in the study and comprised a subset of the pigs investigated by Veit et al. (35). All pigs were kept in one room and group-housed in pens containing four experimental and two companion male pigs in order to increase the stocking density up to 1.3 m^2 per pig. Pigs had visual and limited tactile contact with other pigs in the adjoining pen. The lying area with a solid concrete floor comprised half of the pen (2.4 m x 1.6 m), and the other half of the pen was slatted. Each pen had three nipple drinkers and pigs were fed pellets ad libitum (IDEAL S Die Ekstra, produced by Norgesfôr, Mysen, Norway) at an animal-to-feeding place ratio of 3:1. The staff provided two handfuls of wood shavings and a handful of grass silage per pen on the lying area twice per day. Additionally, one handful of grass silage was placed in a rack. Each pen was equipped with a water sprinkler, which turned on every 10 min for 20 s. Lights were on

between 6 am and 10 pm and the room was dimmed with night-lights during the night. Average ambient temperature in the unit was set to 20˚C.

Experimental procedures

At the beginning of the study, experimental pigs were aged 68-85 days (median 83 days) and weight of the experimental pigs ranged from 16.3 to 50.7 kg (median 41 kg). They were randomly allocated in each pen to one of four treatments comprising four substance combinations: saline-saline (SS), ketoprofen-saline (KS), saline-LPS (SL), and ketoprofen-LPS (KL). The numbers of pigs per treatment were nine for SS and KL, and seven for KS and SL. Pigs were weighed on the day before treatment in order to calculate the correct substance doses. The LPS dose used was determined according to previous research (21,37) and for ketoprofen, the dosing was according to Fosse et al. (38). Ketoprofen (Romefen vet 100 mg/ml, Ceva Santé Animale, France) or saline were administered intramuscularly (i.m.) in the trapezius muscle. LPS (Serotype 0111: B4 of *Escherichia coli* dissolved in 0.9% sterile saline to a concentration of 100 µg/ml, produced by Sigma, Germany) or saline (sodium chloride 9 mg/ml) were administered intravenously (iv) through an ear vein catheter on average 61 ± 16 min after the first substance. The ear vein catheter was used only for injection, and removed immediately afterwards. The ketoprofen dose was 6 mg/kg, and the LPS dose 1.2 µg/kg. The pigs injected with LPS were observed closely outside the pen in the hours after injection in order to detect possible overreaction.

Repeated saliva samples were collected from individual pigs before any substance administration (baseline) and at 4, 24, 48, and 72 h after the iv injection. All baseline saliva samples were taken during morning hours (between 08:30 and 10:45). Each pig chewed on a dental cotton pad suspended on a dental cord until it was moistened (modified from (39)). Each pad was fixed within the upper part of a 10 ml sampling tube and centrifuged for 5 min at 1000 × g to extract the saliva. Saliva was pipetted to

2 ml Eppendorf tubes and stored on dry ice until it was moved to a -80˚C freezer at the end of each sampling day.

Salivary ADA, Hp, and cortisol measurements

Salivary ADA and Hp were measured in collaboration with a Spanish laboratory (Department of Animal Medicine and Surgery, Faculty of Veterinary Medicine, University of Murcia, Spain). A commercial automatized assay (Adenosine-Glutamate Dehydrogenase, BioSystems S.A., Barcelona, Spain) was used for ADA quantification according to the manufacturer's instructions. The method of the assay is based on the measurement of the decrease in absorbance (OD) per minute of a coupled reaction initially catalyzed by ADA (OD/min x 3333 = U/L). The reaction is measured at 340 nm. The levels of ADA activity were calculated according to the manufacturer's instructions. Salivary Hp concentration was quantified by using an in-house time-resolved immunofluorometric assay, previously validated by Gutiérrez et al. (40). The assay is a non-competitive sandwich immunoassay based on the fluorescence of lanthanide chelate labels that provides a minimal background, lack of any sample interference, and an in-house highly specific monoclonal antibody against porcine Hp.. Salivary cortisol concentration was measured using an enzyme immunoassay kit (DetectX®, Catalogue Number K0033-H5W, Arbor Assays, MI, USA) according to the manufacturer´s protocol. Processing of saliva samples prior to cortisol analysis and the protocol itself are described in detail elsewhere (35).

Statistical analysis

SPSS (IBM SPSS Statistics 25) was used for statistical analysis of the data. Pig was used as experimental unit in all statistical analyses. In all statistical analyses, p-values below 0.05 were considered as significant and p-values of $0.5 \leq 0.1$ as tendency. Data normality was tested visually and with a Shapiro-Wilk test. No biomarker studied met the normal distribution criteria, and therefore, results are presented as median with range (see Table 1 in Results section).

To ensure normality of residuals and homogeneity of variance, all parameters were root-transformed prior to the statistical analysis. A multivariate approach was used to test the effect of LPS and ketoprofen on salivary ADA, Hp, and cortisol. Individual pigs were included as subjects and saliva sampling time point (0-72 h) as repeated measures. Saliva sampling time point and treatment and their interaction were added as fixed factors. Pre-planned pairwise comparisons were performed for all treatments at time point t4 and between different time points for the SL group using a Bonferroni correction. For the cortisol model, one pig belonging to the SS group was discarded from the analysis because it had exceptionally high salivary cortisol concentration at time point t4.

Nonparametric Spearman correlation was used to investigate whether ADA, Hp, and cortisol responses correlate between baseline (t0) and four hours post-injection (t4). For this purpose, new outcome variables for each biomarker were generated for each individual in the SL group by calculating the difference in measured concentrations between time points t4 and t0.

Results

Dynamics of salivary biomarkers

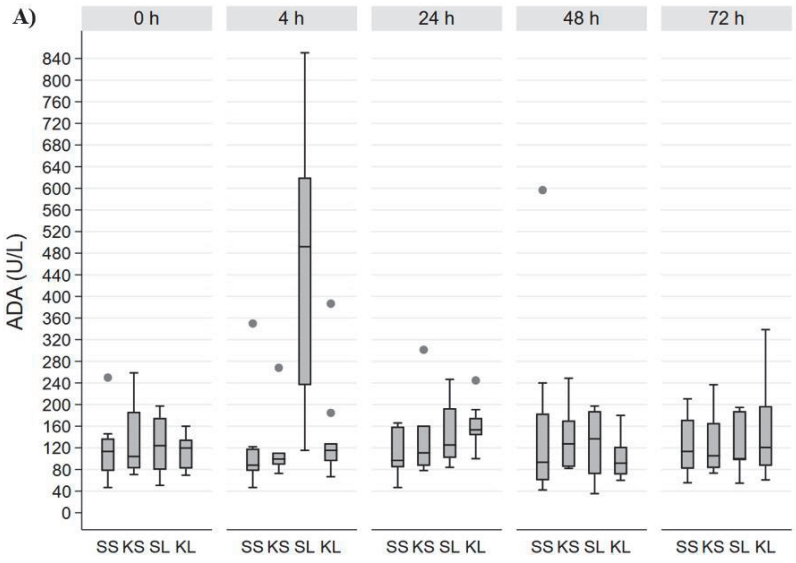
Descriptive results of salivary ADA, Hp, and cortisol measurements by sampling time point are shown in Table 1. Altogether seven ADA, seven Hp, and five cortisol samples had to be discarded from the analyses due to erroneous interpretation of the tube labelling in the laboratory.

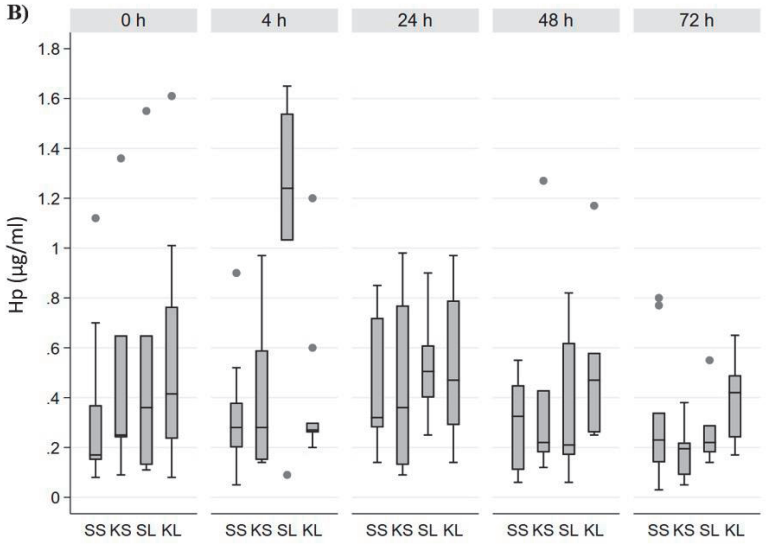
Table 1. Median (min - max) values of adenosine deaminase (ADA), haptoglobin (Hp), and cortisol in saliva across 32 experimental pigs at different time points. p.i. = post-injection.

Biomarker	Baseline n = 31	4 h p.i. n = 31	24 h p.i. n = 30	48 h p.i. n = 27	72 h p.i. n = 29
ADA, U/L	118.0	115.3	137.0	112.7	108.7

	(46.7 – 258.6)	(46.7 – 850.6)	(46.7-301.3)	(35.3 – 596.6)	(54.7 – 338.6)
Hp, µg/ml	0.33	0.31	0.45	0.37	0.23
	(0.08-1.61)	(0.05-1.65)	(0.09-0.98)	(0.06-1.27)	(0.03-0.80)
Cortisol, ng/ml	0.32	0.33	0.30	0.22	0.20
	(0.11-0.45)	(0.10-2.28)	(0.11-0.69)	(0.08-1.35)	(0.08-0.48)

Raw values of each biomarker separated by time point and groups are shown in Figure 1.





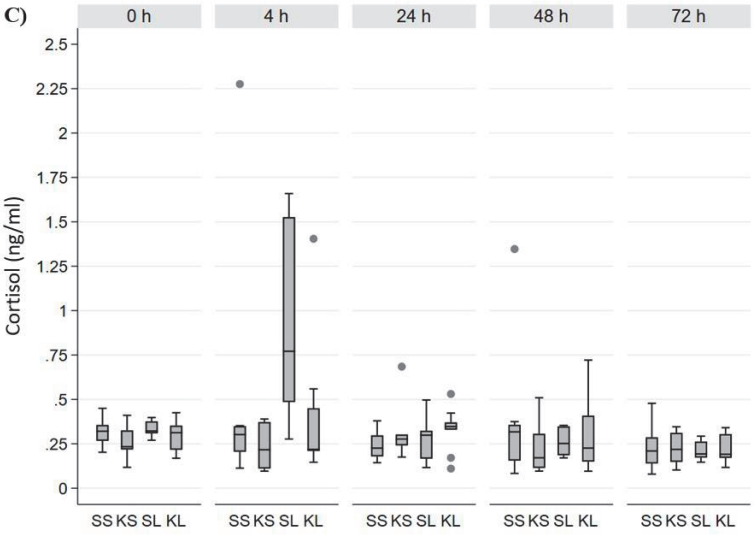


Figure 1. Raw values of salivary A) adenosine deaminase (ADA), B) haptoglobin (Hp), and C) cortisol across 32 experimental pigs. SS = saline-saline (control), KS = ketoprofen-saline, SL = saline-LPS, KL = ketoprofen-LPS.

There was a significant interaction between time point and treatment for ADA ($F_{12, 58} = 2.8$, $p = 0.01$). ADA was clearly increased in SL four hours post-injection compared with other treatment groups (pairwise comparisons, $p < 0.01$ for all). Moreover, ADA within the SL group at t4 was significantly increased relative to baseline and all other time points (pairwise comparisons, $p < 0.001$ for all). For Hp, the interaction between time point and treatment was not significant ($F_{12, 55.6} = 1.7$, $p = 0.10$). Overall, Hp concentration tended to be increased in the SL group compared with the SS group (pairwise comparisons, $p = 0.06$). However, four hours post-injection Hp was significantly increased in the SL group compared with t48 and t72 (pairwise comparisons, $p < 0.01$ for both), but not with baseline or t24.

Cortisol showed a response similar to ADA and Hp, with a significant interaction between time point and treatment ($F_{12, 68.4} = 1.9$, $p = 0.04$). A significant increase in salivary cortisol concentration occurred in SL four hours post-injection compared with SS and KS (pairwise comparison, $p < 0.01$ for both), and it tended to be higher than KL (pairwise comparison, $p = 0.05$). In the SL group at t4, salivary cortisol was significantly increased relative to baseline and all other time points (pairwise comparisons, $p < 0.01$ for all).

Correlations between salivary biomarkers

Across all experimental pigs, ADA, Hp, and cortisol did not correlate (Spearman correlation, $p > 0.05$ for all) at baseline. The response values calculated between baseline and four hours post-injection in the SL group showed a significant correlation for ADA and Hp ($r = 0.86$, $p < 0.05$). Although no significant correlations were found between ADA and cortisol or between Hp and cortisol, the correlation coefficients were moderate for both ($r = 0.64$ and $r = 0.57$, respectively).

Discussion

As predicted, LPS injection resulted in an increase in salivary ADA and Hp as well as in salivary cortisol. A significant elevation in all studied biomarkers occurred at four hours post-injection in LPS-challenged pigs. In other treatment groups, including the KL group, their concentrations remained relatively stable during the study period. Based on this, pre-treatment with ketoprofen was able to alleviate the LPS effect. The response of ADA and Hp showed positive significant correlations in the SL group indicative of their parallel dynamics under the influence of bacterial LPS.

Increased salivary ADA concentrations have been reported in pigs suffering from clinically evident infectious and inflammatory conditions (19) and in stressed sheep (25). Salivary ADA is also known to be influenced by sex (26,31), production stage, and breed (31) in pigs. Several pig studies investigated concentrations of Hp induced by viral (14,15,28,36,41–43) or bacterial (14,15,36,42,43) pathogens or by trauma (14). As for ADA, production stage is known to influence salivary Hp concentrations of healthy pigs (10, 24). In this experiment, Hp concentration in saliva varied strongly between individual pigs: 0.08-1.61 µg/ml at baseline. For serum, high inter-individual variation has been reported previously (44–46). As a strong positive correlation between serum and salivary Hp concentrations exists (28), serum and salivary Hp dynamics are comparable to each other. Moreover, we observed high Hp concentration in one pig per treatment group at baseline. All of these pigs were among the pigs of lowest weight within the respective groups. Even though we did not examine the study pigs clinically, all experimental pigs appeared healthy both prior to and during the experiment. However, an underlying illness cannot be ruled out.

Administration of *E. coli* LPS mimics an endotoxemic state that is known to induce a systemic inflammatory response (47). The pigs in this study were a subset of those in Veit et al. (35), where the behavioral signs of illness were reported. While the clinical onset of acute inflammatory response was not confirmed (for details, see (35)), an earlier report with the same *E. coli* strain and LPS dose indicates a strong activation of the innate immune system already one hour after LPS injection (21). The rapid

235 increase in the concentrations of all biomarkers in this paper is in line with this. The concentrations of
236 all biomarkers returned to baseline levels by 24 hours post-injection. To the best knowledge of the
237 authors, previous pig studies have not investigated either short-term dynamics of salivary ADA, or
238 ADA concentrations of pigs under a controlled immune challenge. Previous research has shown that
239 when triggered by an infectious agent serum Hp remains high for several days in pigs (15,29,48,49).
240 Heegaard et al. (15) reported differing dynamics of serum Hp regarding the disease causative agent,
241 including bacterial, viral, and parasitic ones, and compared with aseptic inflammation. In the present
242 study, the rapid decline in salivary Hp might have been caused by the use of a single low-dose LPS,
243 which was synthetically purified and therefore was likely to be eliminated from the body faster than
244 LPS during natural infection. Escribano et al. (29) reported that after LPS treatment, salivary Hp
245 increased by threefold and remained high throughout the seven-day study period in growing pigs.
246 Notably, in contrast to our study, they used a different *E. coli* strain (O55:B5), the dose of LPS injected
247 was around 30 times higher, and it was administered repeatedly (29). As predicted, salivary cortisol of
248 LPS-injected pigs not pre-treated with ketoprofen peaked at four hours after injection, confirming the
249 findings of others measuring cortisol from saliva and serum (21,35,50,51). These results are in line
250 with those of Escribano et al. (29) and Nordgreen et al. (21), who reported that salivary and plasma
251 cortisol was elevated for only a short period of time following LPS challenge.

252 Our results indicated that ketoprofen was able to inhibit the effect of LPS, when given intramuscularly
253 one hour prior to LPS injection. Others have shown that orally administered ketoprofen had a similar
254 effect on the intravenously administered *E. coli* endotoxin in pigs (32). The appropriate dose of oral
255 ketoprofen was set at 2 mg/kg (32), which is a third of the dose administered to KS and KL pigs in the
256 present study. Mustonen et al. (52) reported that the effect of oral ketoprofen was seen immediately
257 after its administration and that the effect lasted for approximately seven hours. Moreover, the

bioavailability of oral and intramuscularly administered ketoprofen has been reported to be similar (33).

Due to clear response to LPS seen in all biomarkers at the same time point in the SL group, we wanted to test whether the response values (between baseline and four hours post-injection) were correlated. A significant correlation was found only between ADA and Hp. Gutiérrez et al. (26) reported a significant positive correlation between salivary ADA and Hp in healthy finishing pigs. In addition, their study population contained both female and male pigs (26) and therefore the comparison between the results of these two studies is not straightforward. Neither ADA nor Hp correlated with cortisol in the SL group. Contreras-Aguilar et al. (25) reported a significant correlation between salivary ADA and cortisol concentrations in sheep caused by either shearing stress or being frightened by a dog. Although we found no significant correlations between these, the correlation coefficients were at least moderate compared with Contreras-Aguilar et al. (25), who reported low correlation coefficients (0.34 and 0.19, respectively). Escribano et al. (53) did not detect a correlation between salivary Hp and cortisol in pigs exposed to a psychosocial stressor, whereas Hp showed significant positive correlations between C-reactive protein, immunoglobulin-A, and chromogranin A.

Similar to cortisol (54), Hp follows a circadian rhythm (55). However, the effect of daytime on Hp concentrations seems to be less important than on cortisol concentrations (55). To the best of our knowledge, no circadian pattern has been reported for ADA. In the present study, saliva samples were taken in morning hours to minimize the potential bias caused by natural variation in salivary biomarkers due to circadian rhythm. Furthermore, the experimental pigs were kept for the entire study period in the same room in which they were born, and with their siblings, to avoid any unnecessary stress caused by moving them to a different room or re-grouping them.

Sanchez et al. (31) showed a significant interaction effect of gender and production phase on salivary Hp: males had higher concentrations before the finishing phase, whereas females had higher values after the post-weaning phase (31). In this study, all pigs were females at their post-weaning phase. Therefore, the effect of those factors on the studied salivary biomarkers did not bias the results. However, further studies including both genders as well as pigs at different stages of production should be conducted to investigate the dynamics of salivary biomarkers more thoroughly. In addition to sex, some evidence of breed effects on salivary ADA and Hp exists (31). In the present study, all experimental pigs were of the same breed. This should be taken into account when extrapolating these results to other studies, especially if pigs of various breeds are involved.

Haptoglobin is a widely studied APP in pigs, and it has been investigated in combination with ADA (19,31) and cortisol (29,30). Escribano et al. (29) concluded that the measurement of salivary Hp and cortisol could be used as a non-invasive, simple, and practical tool to evaluate the combined response to endotoxemia in pigs. Therefore, we decided to include it as the most promising APP to be combined with the other biomarkers.

Conclusions

All three biomarkers studied – ADA, Hp, and cortisol – were indicative of a systemic inflammatory response following LPS endotoxemia. The salivary concentration of those biomarkers increased following a similar pattern, and ketoprofen was able to alleviate the effect of LPS. The results indicate that the selected salivary parameters, are indicative of systemic inflammation in pigs at an early stage. Primary and novel findings of the study are the response of ADA to LPS, its time course, and alleviation by ketoprofen. Further studies should investigate the underlying mechanisms contributing to ADA dynamics; for Hp and cortisol they are already quite well known. Moreover, the usefulness of these biomarkers should be validated in a larger sample and in commercial conditions.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author contributions

VS drafted the manuscript, prepared the raw data, performed statistical analyses, and assisted in sample collection. CV processed the manuscript together with VS and participated in planning the experiment and sample collection. JN planned the experiment and performed sample collection. AV participated in experiment planning and statistical analyses. JN, MH, and SJ were actively involved in the manuscript writing process together with all other authors.

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References

1. Jensen VF, Emborg HD, Aarestrup FM. Indications and patterns of therapeutic use of antimicrobial agents in the Danish pig production from 2002 to 2008. J Vet Pharmacol Ther (2012) 35(1):33–46. doi: 10.1111/j.1365-2885.2011.01291.x

2. van Rennings L, von Münchhausen C, Ottilie H, Hartmann M, Merle R, Honscha W, et al. Cross-Sectional Study on Antibiotic Usage in Pigs in Germany. PLoS One (2015) 10(3):e0119114. doi: 10.1371/journal.pone.0119114

3. Lekagul A, Tangcharoensathien V, Yeung S. Patterns of antibiotic use in global pig production: A systematic review. Vet Anim Sci (2019) 7:100058. doi: 10.1016/j.vas.2019.100058

4. VanderWaal K, Deen J. Global trends in infectious diseases of swine. Proc Natl Acad Sci USA (2018) 115(45):11495-11500. doi: 10.1073/pnas.1806068115

5. Goecke NB, Kobberø M, Kusk TK, Hjulsager CK, Pedersen KS, Kristensen CS, et al. Objective pathogen monitoring in nursery and finisher pigs by monthly laboratory diagnostic testing. Porc Heal Manag (2020) 6:23. doi: 10.1186/s40813-020-00161-3

6. Mayeux PR. Pathobiology of lipopolysaccharide. J Toxicol Environ Health (1997) 51(5):415-35. doi: 10.1080/00984109708984034

7. Seemann S, Zohles F, Lupp A. Comprehensive comparison of three different animal models for systemic inflammation. J Biomed Sci (2017) 24(1):1–17. doi: 10.1186/s12929-017-0370-8

8. Murata H, Shimada N, Yoshioka M. Current research on acute phase proteins in veterinary diagnosis: An overview. Vet J (2004) 168(1): 28-40. doi: 10.1016/S1090-0233(03)00119-9

9. Hart BL. Biological basis of the behavior of sick animals. Neurosci Biobehav Rev (1988) 12(2):123-37. doi: 10.1016/s0149-7634(88)80004-6.

10. Eckersall PD, Bell R. Acute phase proteins: Biomarkers of infection and inflammation in veterinary medicine. Vet J (2010) 185(1):23–7. doi: 10.1016/j.tvjl.2010.04.009

11. Pradeep M. Application of acute phase proteins as biomarkers in modern veterinary practice. Ind J Vet Anim Sci (2014) 43(1):1–13. Corpus ID: 74029853

12. Gutiérrez AM, Yelamos J, Pallarés FJ, Gómez-Laguna J, Cerón JJ. Local identification of porcine haptoglobin in salivary gland and diaphragmatic muscle tissues. Histol Histopathol (2012) 27(2):187-96. doi: 10.14670/HH-27.187

13. Gutiérrez AM, Cerón JJ, Fuentes P, Montes A, Martínez-Subiela S. Longitudinal analysis of acute-phase proteins in saliva in pig farms with different health status. Anim An Int J Anim Biosci (2012) 6(2):321-6. doi: 10.1017/S1751731111001662

14. Parra MD, Fuentes P, Tecles F, Martínez-Subiela S, Martínez JS, Muñoz A, et al. Porcine acute phase protein concentrations in different diseases in field conditions. J Vet Med Ser B Infect Dis Vet Public Heal (2006) 53(10):488-93. doi: 10.1111/j.1439-0450.2006.01002.x

15. Heegaard PMH, Stockmarr A, Piñeiro M, Carpintero R, Lampreave F, Campbell FM, et al. Optimal combinations of acute phase proteins for detecting infectious disease in pigs. Vet Res (2011) 42(1):1–13. doi: 10.1186/1297-9716-42-50

16. Bradford KL, Moretti FA, Carbonaro-Sarracino DA, Gaspar HB, Kohn DB. Adenosine Deaminase (ADA) -Deficient Severe Combined Immune Deficiency (SCID): Molecular Pathogenesis and Clinical Manifestations. J Clin Immunol (2017) 37(7):626-637. doi: 10.1007/s10875-017-0433-3

17. Widar J, Ansay M. Adenosine deaminase in the pig: tissue-specific patterns and expression of the silent ADA0 allele in nucleated cells. Anim Blood Groups Biochem Genet (1975) 6(2):109-16. doi: 10.1111/j.1365-2052.1975.tb01357.x

18. Gutiérrez AM, Nöbauer K, Soler L, Razzazi-Fazeli E, Gemeiner M, Cerón JJ, et al. Detection of potential markers for systemic disease in saliva of pigs by proteomics: A pilot study. Vet Immunol Immunopathol (2013) 151(1–2):73–82. doi: 10.1016/j.vetimm.2012.10.007

19. Gutiérrez AM, De La Cruz-Sánchez E, Montes A, Sotillo J, Gutiérrez-Panizo C, Fuentes P, et al. Easy and non-invasive disease detection in pigs by adenosine deaminase activity determinations in saliva. PLoS One (2017) 12(6):e0179299. doi: 10.1371/journal.pone.0179299

20. Martínez-Miró S, Tecles F, Ramón M, Escribano D, Hernández F, Madrid J, et al. Causes, consequences and biomarkers of stress in swine: an update. BMC Vet Res (2016) 12(1):171. doi: 10.1186/s12917-016-0791-8

21. Nordgreen J, Munsterhjelm C, Aae F, Popova A, Boysen P, Ranheim B, et al. The effect of lipopolysaccharide (LPS) on inflammatory markers in blood and brain and on behavior in individually-housed pigs. Physiol Behav (2018) 195:98-111. doi: 10.1016/j.physbeh.2018.07.013

22. Nordgreen J, Edwards SA, Boyle LA, Bolhuis JE, Veit C, Sayyari A, et al. A Proposed Role for Pro-Inflammatory Cytokines in Damaging Behavior in Pigs. Front Vet Sci (2020). doi: 10.3389/fvets.2020.00646

23. Piñeiro M, Piñeiro C, Carpintero R, Morales J, Campbell FM, Eckersall PD, et al. Characterisation of the pig acute phase protein response to road transport. Vet J (2007) 173(3):669-74. doi: 10.1016/j.tvjl.2006.02.006

24. Piñeiro C, Piñeiro M, Morales J, Carpintero R, Campbell FM, Eckersall PD, et al. Pig acute-phase protein levels after stress induced by changes in the pattern of food administration. Animal (2007) 1(1):133-9. doi: 10.1017/S1751731107283909

25. Contreras-Aguilar MD, Escribano D, Quiles A, López-Arjona M, Cerón JJ, Martínez-Subiela S, et al. Evaluation of new biomarkers of stress in saliva of sheep. Animal (2019) 13(6):1278-1286. doi: 10.1017/S1751731118002707

26. Gutiérrez AM, Montes A, Gutiérrez-Panizo C, Fuentes P, De La Cruz-Sánchez E. Gender influence on the salivary protein profile of finishing pigs. J Proteomics (2018) 178:107-113. doi: 10.1016/j.jprot.2017.11.023

27. Martín de la Fuente AJ, Carpintero R, Rodríguez Ferri EF, Álava MA, Lampreave F, Gutiérrez Martín CB. Acute-phase protein response in pigs experimentally infected with Haemophilus parasuis. Comp Immunol Microbiol Infect Dis (2010) 33(6):455–65. doi: 10.1016/j.cimid.2008.11.001

28. Gutiérrez AM, Cerón JJ, Fuentes P, Montes A, Martínez-Subiela S. Longitudinal analysis of acute-phase proteins in saliva in pig farms with different health status. Animal (2012) 6(2):321–6. doi: 10.1017/S1751731111001662

29. Escribano D, Campos PHRF, Gutiérrez AM, Le Floc'h N, Cerón JJ, Merlot E. Effect of repeated administration of lipopolysaccharide on inflammatory and stress markers in saliva of growing pigs. Vet J (2014) 200(3):393-7. doi: 10.1016/j.tvjl.2014.04.007

30. Ott S, Soler L, Moons CPH, Kashiha MA, Bahr C, Vandermeulen J, et al. Different stressors elicit different responses in the salivary biomarkers cortisol, haptoglobin, and chromogranin A in pigs. Res Vet Sci (2014) 97(1):124-8. doi: 10.1016/j.rvsc.2014.06.002

31. Sánchez J, García A, Ruiz JM, Montes AM, Cabezas-Herrera J, Ros-Lara S, et al. Porcine Breed, Sex, and Production Stage Influence the Levels of Health Status Biomarkers in Saliva Samples. Front Vet Sci (2019) 6:32. doi: 10.3389/fvets.2019.00032

32. Mustonen K, Banting A, Raekallio M, Heinonen M, Peltoniemi OAT, Vainio O. Dose-response investigation of oral ketoprofen in pigs challenged with Escherichia coli endotoxin. Vet Rec (2012) 171(3):70. doi: 10.1136/vr.100431

33. Raekallio MR, Mustonen KM, Heinonen ML, Peltoniemi OAT, Säkkinen MS, Peltoniemi SM, et al. Evaluation of bioequivalence after oral, intramuscular, and intravenous administration of racemic ketoprofen in pigs. Am J Vet Res (2008) 69(1):108-13. doi: 10.2460/ajvr.69.1.108

34. Vane JR, Botting RM. Mechanism of action of nonsteroidal anti-inflammatory drugs. Am J Med (1998) 104(3). doi: 10.1016/S0002-9343(97)00203-9

35. Veit C, Janczak AM, Ranheim B, Vas J, Valros A, Sandercock DA, et al. The Effect of LPS and Ketoprofen on Cytokines, Brain Monoamines, and Social Behavior in Group-Housed Pigs. Front Vet Sci (2021). doi: 10.3389/fvets.2020.617634l

36. Gutiérrez AM, Martínez-Subiela S, Soler L, Pallarés FJ, Cerón JJ. Use of saliva for haptoglobin and C-reactive protein quantifications in porcine respiratory and reproductive syndrome affected pigs in field conditions. Vet Immunol Immunopathol (2009) 132(2):218–23. doi: 10.1016/j.vetimm.2009.06.013

37. Munsterhjelm C, Nordgreen J, Aae F, Heinonen M, Valros A, Janczak AM. Sick and grumpy: Changes in social behaviour after a controlled immune stimulation in group-housed gilts. Physiol Behav (2019) 198:76–83. doi: 10.1016/j.physbeh.2018.09.018

38. Fosse TK, Toutain PL, Spadavecchia C, Haga HA, Horsberg TE, Ranheim B. Ketoprofen in piglets: Enantioselective pharmacokinetics, pharmacodynamics and PK/PD modelling. J Vet Pharmacol Ther (2011) 34(4):338-49. doi: 10.1111/j.1365-2885.2010.01236.x

39. Munsterhjelm C, Brunberg E, Heinonen M, Keeling L, Valros A. Stress measures in tail biters and bitten pigs in a matched case-control study. Anim Welf (2013) 22(3):331–8. doi: https://doi.org/10.7120/09627286.22.3.331

40. Gutiérrez AM, Martínez-Subiela S, Cerón JJ. Evaluation of an immunoassay for determination of haptoglobin concentration in various biological specimens from swine. Am J Vet Res (2009) 70(6):691-6. doi: 10.2460/ajvr.70.6.691

41. Grau-Roma L, Heegaard PMH, Hjulsager CK, Sibila M, Kristensen CS, Allepuz A, et al. Pig-major acute phase protein and haptoglobin serum concentrations correlate with PCV2 viremia and the clinical course of postweaning multisystemic wasting syndrome. Vet Microbiol (2009) 138(1-2):53-61. doi: 10.1016/j.vetmic.2009.03.005

42. Pomorska-Mól M, Markowska-Daniel I, Kwit K, Stępniewska K, Pejsak Z. C-reactive protein,

haptoglobin, serum amyloid A and pig major acute phase protein response in pigs simultaneously infected with H1N1 swine influenza virus and Pasteurella multocida. BMC Vet Res (2013) 9(1):14. doi: 10.1186/1746-6148-9-14

43. Pomorska-Mól M, Markowska-Daniel I, Kwit K, Urbaniak K, Pejsak Z. Correlation between serum acute phase proteins, lung pathology, and disease severity in pigs experimentally co-infected with H3N2 swine influenza virus and bordetella bronchiseptica. Bull Vet Inst Pulawy (2015) 59(1):1–7. doi: 10.1515/bvip-2015-0001

44. Hennig-Pauka I, Menzel A, Boehme TR, Schierbaum H, Ganter M, Schulz J. Haptoglobin and C-Reactive Protein-Non-specific Markers for Nursery Conditions in Swine. Front Vet Sci (2019) 6:92. doi: 10.3389/fvets.2019.00092

45. Petersen HH, Dideriksen D, Christiansen BM, Nielsen JP. Serum haptoglobin concentration as a marker of clicinal signs in finishing pigs. Vet Rec (2002) 151(3):85-9. doi: 10.1136/vr.151.3.85

46. Piñeiro C, Piñeiro M, Morales J, Andrés M, Lorenzo E, Pozo M del, et al. Pig-MAP and haptoglobin concentration reference values in swine from commercial farms. Vet J (2009) 179(1):78-84. doi: 10.1016/j.tvjl.2007.08.010

47. Tuchscherer M, Kanitz E, Otten W, Tuchscherer A. Effects of prenatal stress on cellular and humoral immune responses in neonatal pigs. Vet Immunol Immunopathol (2002) 86(3–4):195–203. doi: 10.1016/S0165-2427(02)00035-1

48. Sorensen NS, Tegtmeier C, Andresen LO, Piñeiro M, Toussaint MJM, Campbell FM, et al. The porcine acute phase protein response to acute clinical and subclinical experimental infection with Streptococcus suis. Vet Immunol Immunopathol (2006) 113(1-2):157-68. doi: 10.1016/j.vetimm.2006.04.008

49. Pomorska-Mól M, Markowska-Daniel I, Kwit K, Stepniewska K, Pejsak Z. Kinetics of the response of four positive acute phase proteins in pigs experimentally infected with toxigenic Pasteurella multocida. Vet Microbiol (2011) 152(3-4):429-35. doi: 10.1016/j.vetmic.2011.05.032

50. Webel DM, Finck BN, Baker DH, Johnson RW. Time course of increased plasma cytokines, cortisol, and urea nitrogen in pigs following intraperitoneal injection of lipopolysaccharide. J Anim Sci (1997) 75(6):1514-1520. doi: 10.2527/1997.7561514x

51. Terenina E, Sautron V, Ydier C, Bazovkina D, Sevin-Pujol A, Gress L, et al. Time course study of the response to LPS targeting the pig immune gene networks. BMC Genomics (2017) 18(1):988. doi: 10.1186/s12864-017-4363-5

52. Mustonen K, Ala-Kurikka E, Orro T, Peltoniemi O, Raekallio M, Vainio O, et al. Oral ketoprofen is effective in the treatment of non-infectious lameness in sows. Vet J (2011) 190(1):55–9. doi: 10.1016/j.tvjl.2010.09.017

53. Escribano D, Gutiérrez AM, Tecles F, Cerón JJ. Changes in saliva biomarkers of stress and immunity in domestic pigs exposed to a psychosocial stressor. Res Vet Sci (2015) 102:38–44. doi: 10.1016/j.rvsc.2015.07.013

480 54. Ruis MAW, Te Brake JHA, Engel B, Ekkel ED, Buist WG, Blokhuis HJ, et al. The circadian
481 rhythm of salivary cortisol in growing pigs: Effects of age, gender, and stress. Physiol Behav
482 (1997) 62(3):623-30. doi: 10.1016/s0031-9384(97)00177-7

483 55. Gutiérrez AM, Escribano D, Fuentes M, Cerón JJ. Circadian pattern of acute phase proteins in
484 the saliva of growing pigs. Vet J 2013 May;196(2):167–70.
485 http://dx.doi.org/10.1016/j.tvjl.2012.10.003

486

Paper III

The use of social network analysis to describe the effect of immune activation on group dynamics in pigs

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Abstract

The immune system can influence social motivation with potentially dire consequences for group-housed production animals, such as pigs. The aim of this study was to test the effect of a controlled immune activation in group-housed pigs, through an injection with lipopolysaccharide (LPS) and an intervention with ketoprofen on centrality parameters at the individual level. In addition, we wanted to test the effect of time relative to the injection on general network parameters in order to get a better understanding of changes in social network structures at the group level. Fifty-two female pigs (11-12 weeks) were allocated to four treatments,

comprising two injections: ketoprofen-LPS (KL), ketoprofen-saline (KS), saline-LPS (SL) and saline-saline (SS). Social behaviour with a focus on damaging behaviour was observed continuously in 10 x 15 min bouts between 8 am and 5 pm one day before (baseline) and two subsequent days after injection. Activity was scan-sampled every 5 min for 6 h after the last injection in the pen. Saliva samples were taken for cortisol analysis at baseline and at 4, 24, 48, 72 h after the injections. A controlled immune activation affected centrality parameters for ear manipulation networks at the individual level. LPS-injected pigs had a lower *in-degree centrality,* thus, received less interactions, two days after the challenge. Treatment effects on tail manipulation and fighting networks were not observed at the individual level. For networks of manipulation of other body parts *in-degree centrality* was positively correlated with cortisol response at 4 h and lying behaviour in the first 6 h after the challenge in LPS-injected pigs. Thus, the stronger the pigs reacted to the LPS, the more interactions they received in the subsequent days. The time in relation to injection affected general network parameters for ear manipulation and fighting networks at the group level. For ear manipulation networks *in-degree centralisation* was higher on the days following injection, thus, certain individuals in the pen received more interactions than the rest of the group compared to baseline. For fighting networks *betweenness* was significantly higher at baseline compared to one day after injection, indicating that network connectivity increased after the challenge. Networks of tail manipulation and manipulation of other body parts did not change on the days after injection at the group level. Social network analysis is a method that can potentially provide important insights into the effects of sickness on social behaviour in group-housed pigs.

Keywords

Social behaviour, Sickness behaviour, Lipopolysaccharide, Cortisol, Sus scrofa

Implications

Damaging behaviour is a major welfare problem in pig husbandry and has been linked to poor health. We were able to detect changes in social interactions in response to a controlled immune activation both on individual and group level using social network analysis.

Introduction

When animals become sick, pro-inflammatory cytokines can alter social motivation so that they can withdraw, conserve resources and recover (reviewed by Nordgreen et al., 2020). In intensive pig production systems, animals housed in close confinement cannot withdraw from their pen mates when they experience a bout of illness, and this might influence their social interactions (Veit et al., 2021). There are indications that poor health status is positively correlated with damaging behaviours (Moinard et al., 2003; Taylor et al., 2012). In particular, it was shown that pigs diagnosed with respiratory diseases tended to perform more ear- and tail biting than controls in the days prior to disease outbreak (Munsterhjelm et al., 2017). These so-called damaging behaviours are supposed to spread either actively due to social learning (Blackshaw, 1981) or passively through animals encountering a wounded tail or ear (Fraser, 1987). Damaging behaviours have an unpredictable appearance and rapid spread, as well as a sporadic occurrence which makes them difficult to study (reviewed by D'Eath et al., 2014). So far, most studies of damaging behaviours focus either on pen-level data (Larsen et al., 2019; Li et al., 2020) or dyadic interactions (Brunberg et al., 2011; Zonderland et al., 2011; Munsterhjelm et al., 2016), or related indicators, such as tail posture (Zonderland et al., 2009; Lahrmann et al., 2018). Except for studies on the relationship between indirect genetic effects

74 for growth rate and biting behaviour (Camerlink et al., 2015), studies that take the
75 whole group of animals into account are lacking.

76 Social network analysis (SNA) provides standardised mathematical methods for
77 calculating measures of sociality across levels of social organisation and has become
78 an increasingly common tool for studying animal behaviour (reviewed by Makagon et
79 al., 2012). SNA is widely used in different fields (e.g. primatology, behavioural
80 ecology, epidemiology) and across many species but most extensively in wildlife
81 research (Stanton and Mann, 2012; Aplin et al., 2013; Brent et al., 2013) and to a
82 much lesser extent in captive farm animals (Abeyesinghe et al., 2013; Boyland et al.,
83 2016). Previous studies in pigs have focused on agonistic behaviour such as the
84 description of general network properties (Büttner et al., 2015a) and individual
85 network position (Büttner et al., 2015b) across three mixing events. SNA is relevant
86 for animal welfare and farm management, Foister et al. (2018) were able to predict
87 long term aggression (3 weeks post-mixing) by calculating network properties at 24 h
88 after mixing. Studies on damaging behaviours such as ear- and tail-biting (Li et al.,
89 2018) are underrepresented even though SNA has the potential to shed light on
90 underlying social mechanisms and the spread of these behaviours. SNA variables of
91 particular interest in this context is *degree centrality* and *edge density*. *Degree*
92 *centrality* is measured at individual level and is determined by how many interactions
93 this pig has with others. *Edge density* is measured at group level and indicates how
94 well the members of the group are connected in terms of their interaction with each
95 other (Foister, 2019). After a controlled immune activation, pigs exhibited a shift in
96 social motivation and performed more ear and tail manipulation two days after the
97 challenge (Munsterhjelm et al., 2019). Based on these findings, we would expect a
98 higher *out-degree centrality* in ear and tail manipulation networks of challenged pigs

as well as a higher *edge density* in the networks on the days following a controlled immune activation.

Lipopolysaccharide (LPS) is a part of the cell wall of Gram-negative bacteria (e.g. E. Coli) and can be used to model aspects of sickness. LPS binds to toll-like receptors (TLRs) on several types of immune competent cells and activates the innate immune system within an hour after administration. As a first response, interleukin-1, interleukin-6, tumour necrosis factor α, interleukin-8, C-reactive protein and cortisol are released. The proinflammatory cytokines give rise to sickness behaviour and an increase in prostaglandin synthesis through the enzyme cyclooxygenase (COX) as well as a profound reduction in activity and increase in cortisol during the first 6 h after injection (Nordgreen et al., 2018; Veit et al., 2021). In rodents, depressive-like behaviour after overt sickness has been observed (O'Connor et al., 2009). In pigs, more ear and tail manipulation and changes in central cytokine and monoamine levels have been reported within two to three days after LPS-injection (Nordgreen et al., 2018; Munsterhjelm et al., 2019; Veit et al., 2021). Nonsteroidal anti-inflammatory drugs (NSAIDs) such as ketoprofen are able to lower the effect of LPS on cortisol release and attenuate behavioural signs of sickness (Veit et al., 2021). Non-selective NSAIDs inhibit COX1 and COX2 and thereby prostaglandin E_2 (PGE_2) synthesis (Thompson et al., 2018).

Due to relatively small group sizes (1-3 pigs), previous studies (Nordgreen et al., 2018; Munsterhjelm et al., 2019) were unable to fully mimic the housing conditions on farms, where pigs are kept in larger groups (6 and more), thus, the complexity of social interactions that could be studied were limited. In this study we therefore wanted to further our understanding of how pig social behaviour is influenced when one member of a larger group becomes ill and thereby changes its behaviour. To

achieve this, we used social network analysis to test the effect of a controlled immune activation and an intervention with ketoprofen on centrality parameters (e.g. *degree centrality*) on pig level. In addition, we wanted to test the effect of time relative to injection on general network parameters (e.g. *edge density*) in order to get a better understanding of changes in social network structures on pen level. We hypothesised that an injection with LPS affects the standing of an individual pig in a group of pen mates and that illness in one pig changes the group dynamics after recovery. We predicted that the centrality parameters in ear and tail manipulation networks of a challenged pig would be affected in a way that the number of interactions received (*in-degree*) decreases, whereas the number of interactions initiated (*out-degree*) increases (I). Moreover, we predicted that the number of interactions within a group (*edge density*) would increase the subsequent days after challenge (II). We applied this method to continuous observations of social behaviour that were gathered during a previous experiment (Veit et al., 2021).

Material & Methods

Animals and husbandry

The experiment took place between March 23th and May 15th 2018 at the Livestock Production Research Center of the Norwegian University of Life Sciences (NMBU), campus Ås. Seventy-eight undocked pigs aged between 11 and 12 weeks were used in two blocks (52 females and 26 castrated males). Animal caretakers selected 4 females and 2 males per litter as even in size as possible to be group-housed in the 13 pens they were born in at a stocking density of 1.3 m² per pig. The four female pigs in each pen were randomly allocated to one of four treatments each, so that all treatments were represented in all pens, resulting in 13 pigs per treatment. The male

pigs were companion pigs (CO) used to increase the stocking density and group size. Housing details are provided in Veit et al. (2021).

Experimental design

The description of the design is adapted from Veit et al. (2021). The pigs were allocated to four treatments comprising to injections: ketoprofen-LPS (KL), ketoprofen-saline (KS), saline-LPS (SL), saline-saline (SS), Fig. 1. Ketoprofen (6 mg kg^{-1}) or saline (similar volume of 0.9 %) was injected in the *trapezius* muscle. LPS (1.2 µg kg^{-1}, serotype 0111: B4 of Escherichia coli dissolved in 0.9 % sterile saline to a concentration of 100 µg ml^{-1} , produced by Sigma, Germany) or a similar volume of saline was administered intravenously through an ear vein catheter on average 60 ± 14 min afterwards. Saliva samples were taken by letting the pigs chew on a cotton pad at baseline and at 4, 24, 48, 72 h after the intravenous injection. Details about sampling procedures are described in Veit et al. (2021). One camera per pen (door ccd-camera, Smartprodukter, Ulsteinvik, Norway) was placed centrally on the ceiling above and the pigs were individually marked on the back. The Media Recorder system from Noldus (Wageningen, the Netherlands) was used to run video recordings of behaviour continuously throughout the experiment.

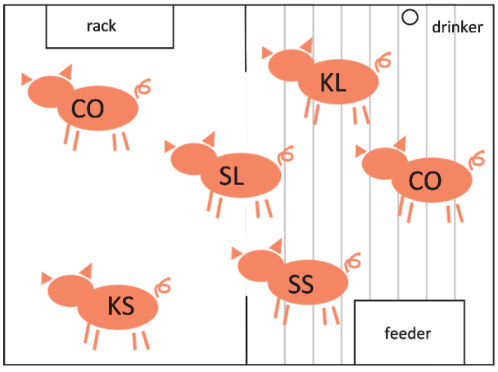


Figure 1: Pen design (shaded area = slatted flooring) and treatments of pigs (KL = ketoprofen-LPS, KS = ketoprofen-saline, SL = saline-LPS, SS = saline-saline and CO = companion).

Cortisol analysis

Cortisol concentration in saliva has been measured in a previous study (Veit et al., 2021). In brief, an enzyme immunoassay kit according to the manufacturer's protocol was used (DetectX®, Catalogue Number K0033-H5W, Arbor Assays, MI, USA). The optical density of each well was read with the Sunrise Absorbance Reader (Tecan Austria GmbH, Grödig/Salzburg, Austria) at 450 nm using the Magellan 6.4 software. Mean coefficient of variation varied between 4.69-7.63 %. Sensitivity was determined as 27.6 pg ml^{-1} and limit of detection was determined as 45.4 pg ml^{-1} according to manufacturer.

Video analysis

All behavioural video recordings have been analysed in a previous study (Veit et al., 2021) using the Observer XT 14.1 from Noldus (Wageningen, The Netherlands). The methods applied are described here for completeness. Behavioural signs of sickness were observed by instantaneous scan sampling (Altmann, 1974) every 5 min for 6 h after the injection of the last pig in the pen (DAY1). The observer was blinded to treatment. The frequency of lying lateral/ sternal/ alert and being active was included as a measure for the response strength to LPS. Social behaviour was observed at baseline (one day before injection), referred to as DAY0, and on the first and second day after injection (DAY2 and DAY3). Continuous observations of performers and receivers of social behaviour at certain intervals during the day was performed by one observer who was blinded to treatment and day of experiment. The sampling

scheme for DAY0, DAY2 and DAY3 was four 15 min intervals in the morning between 8:00 and 10:00 and six 15 min intervals in the afternoon between 14:00 and 17:00, (Fig. 2).

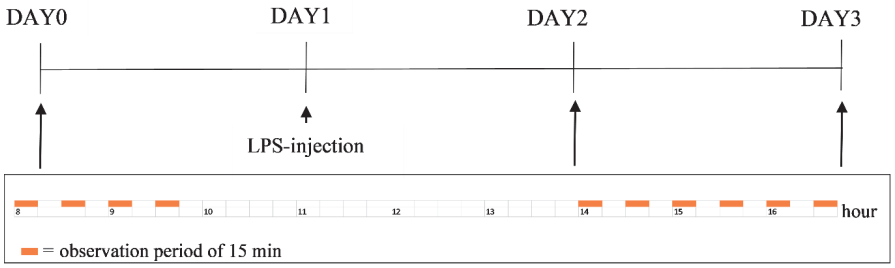


Figure 2: Schematic overview of the sampling scheme for observation of pig social behaviour over the 3-day experimental period.

The day of injection itself (DAY1) was not of interest for observation of social behaviour because it was interrupted due to more handling on that day. Only 12 out of 13 pens were included in analysis due to an inadequate quality of the video material from one pen (which was too brightly lit to identify back markings). The ethogram for the specific social behaviours performed is displayed in Table 1. Due to low frequencies, the behaviours flank nosing (4.9 % of all behaviour observed) and belly nosing (4.1 %) as well as displacement (2.1 %) were not used for further analyses.

Table 1: Ethogram for social behaviour in pigs

Behaviour	**Description**
Ear manipulation	Touching the ear of another pig with the snout, including taking the ear into the mouth
Tail manipulation	Touching the tail of another pig with the snout, including taking the tail into the mouth

Manipulation of other body parts	Touching body parts of another pig with the snout except for tail, ear, belly and flank region (e.g. head, legs, back), including taking the body parts into the mouth
Fighting	Biting, hitting, and knocking of another pig with the head. Includes chasing performed immediately after biting, hitting, knocking. Includes parallel pressing after knock, hit or bite. Pig that initiates the fight is the performer, pig that is being attacked is the recipient
Flank nosing	Touching the flank region (=upper part of the lateral side of the body from the beginning of the shoulder until the end of the body, except of tail) of another pig with the snout
Belly nosing	Repetitive up and down movements on the abdomen of another pig that is lying or standing
Displacement	Pushing away another pig without fighting (as defined above), results in active movement of the recipient and getting access to a resource (e.g. silage, lying space, drinker) for the performer

The package *igraph* in R 4.0.3 was used to construct networks and calculate network properties at pig and at pen level (Table 2). Centrality parameters were obtained via the *degree* and *eigen_centrality* functions and normalized by the pig with the highest value in the respective pen, thus, centrality was scaled between 0-1 (1 = most central pig in the pen). General network parameters were obtained via the *edge_density, centralization.degree, centralization.betweenness* and *centralization.evcent* functions. *Degree centralisation* was normalized by the most central pig in the respective pen. Codes for calculation of network parameters are given in the Supplementary Material S1.

Table 2: Social network parameters based on observations of social behaviour in pigs

Terminology	Description
Centrality parameters (pig level)	
Degree centrality	Number of direct interactions an individual has with other individuals of the group
In-degree	Number of interactions received by an individual
Out-degree	Number of interactions initiated by an individual
Eigenvector centrality	Takes the degree centrality of an individual, as well as the degree centrality of other individuals it is connected with, into account
General network parameters (pen level)	
Edge density	Amount of actual interactions between individuals divided by the total number of possible interactions in the group. An edge represents the interaction between two individuals.
Degree centralisation	The range or variability of the individuals' centrality values (0 indicates that all individuals in the network have equal centrality; 1 indicates maximum inequality)
In-degree	Description of whether certain individuals receive more interactions than the rest of the group
Out-degree	Description of whether certain individuals initiate more interactions than the rest of the group
Betweenness	Pens with high values contain individuals who connect other individuals that do not directly interact
Eigenvector	Pens with high values contain a small number of well-connected individuals, with the rest of the group being considerably less well connected

217

218 ***Statistical analysis***

219 JMP Pro 14.3.0 (SAS, NC, USA) was used to build mixed models for analysis of
220 network parameters. The significance level for all analysis was set at $p < 0.05$.
221 Residuals were checked for normality and homogeneity of variance by visual

inspection of plots. Main effects are not presented when the interaction was in focus to answer the research question. *A priori* planned contrasts were used after running the main models, as we had predefined assumptions (for further explanation see Doncaster and Davey, 2007). For centrality parameters, the calculated values of *degree centrality, in-degree centrality, out-degree centrality* and *eigenvector centrality* were used as dependent variables. The treatment (KL, KS, SL, SS), the day (DAY0, DAY2, DAY3) and the interaction of both were used as independent fixed effects. Pig nested in treatment was included as a random variable in all models. Companion pigs were not considered for analysis. For planned comparisons, Student's t-tests were used. In a first step, we compared SL with SS to elucidate the effect of LPS on centrality parameters. In addition, the comparison of SL and KL should answer the question whether ketoprofen alleviates the effects of LPS. Furthermore, it was relevant to compare SS with KS in order to see whether ketoprofen has an effect in pigs that were not challenged with LPS. If any of these pairwise comparisons were significant, we compared within-group differences between baseline and the day at which the significant treatment effect was found (Veit et al., 2021). For correlations between centrality parameters and cortisol concentrations at 4 h after injection, as well as general activity in the first 6 h after injection, Spearman rank coefficient was used. For general network parameters, the calculated values of *edge density, degree centralisation, in-degree centralisation, out-degree centralisation, betweenness* and *eigenvector* were used as dependent variables. The day (DAY0, DAY2, DAY3) was used as independent fixed effect and the pen was included as a random variable in all models. For planned comparisons, Student's t-tests were used. Codes for statistical models are given in the Supplementary Material S2.

Results

General description of the data set

LPS activated the HPA-axis as indicated by an increase in salivary cortisol at 4 h after injection and depressed activity within 6 h; ketoprofen alleviated this effect (reported in Veit et al., 2021). Pigs across all treatments and days manipulated mostly the ears (23.7 % of all behaviours observed) and other body parts (31.8 %) of their pen mates and were frequently involved in fights (24.4 %). Tail manipulation was shown to a much lesser extend (9.0 %). An overview of the different centrality parameters at pig level calculated by treatment and day are displayed in the Supplementary Table S1 and the results are described in detail in the following paragraph. An overview of the general network parameters calculated at pen level for all behaviours by day are displayed in the Supplementary Table S2 and the results are described subsequently.

Effect of treatment on centrality parameters (pig level)

The calculated centrality parameters were used to plot social networks for each pen and day (Fig. 3).

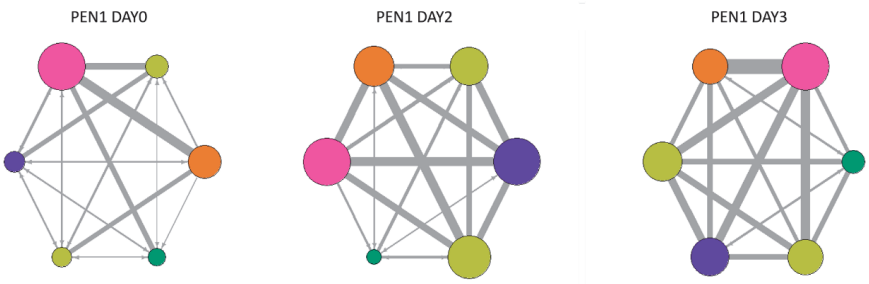
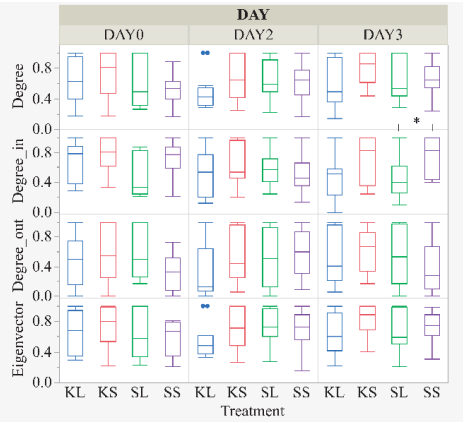


Figure 3: Example of a social network based on all interactions of pigs observed in pen 1 at baseline (DAY0) and on the first (DAY2) and second day (DAY3) after

injecting the pigs with ketoprofen-LPS (KL), ketoprofen-saline (KS), saline-LPS (SL) and saline-saline (SS). Nodes represent individuals in the pen, colour of the nodes indicate treatments (pink = SL, blue = KS, orange = KL, green = SS, yellow = CO) and size of the nodes represents *degree centrality*; edges represent interactions between individuals, arrows point from the actor to the receiver and thickness of the edges represents the frequency. In this example, the KS pig increases its *degree centrality* from baseline to DAY2 and DAY3 (but to a lesser extent than at DAY2), whereas the SL pig largely has unchanged *degree centrality*. The frequency of interactions within the network increases from baseline to DAY2 and DAY3. The pattern differed from pen to pen, and this illustration is meant as an aid in understanding.

LPS had a significant effect on centrality parameters of ear manipulation at an individual level ($F(\text{treatment*day})_{6,\ 87.53} = 1.82$, $p = 0.11$). SL pigs (median (min | max) = 0.40 (0.10 | 1.00)) had a significantly lower *in-degree centrality,* thus, received less interactions, compared to SS pigs (0.83 (0.40 | 1.00)) two days after injection (planned comparison: $p = 0.01$), Fig. 4a. A pre-treatment with ketoprofen did not alleviate this effect. A numerical difference between SL and SS pigs was present at baseline. Neither LPS nor ketoprofen had an effect on centrality parameters of tail manipulation (Fig. 4b), manipulation of other body parts (Fig. 4c) and fighting (Fig. 4d) and no clear patterns could be observed. *In-degree centrality* of SL pigs for manipulation of other body parts was positively and significantly correlated with salivary cortisol concentration at 4 h after injection (DAY2: Spearman's rho $\rho = 0.71$, $p = 0.009$) and lying laterally in the first 6 h after the challenge (DAY3: $\rho = 0.62$, $p = 0.03$). Thus, the stronger the pigs reacted to the LPS, the more interactions they received in the subsequent days.

a)



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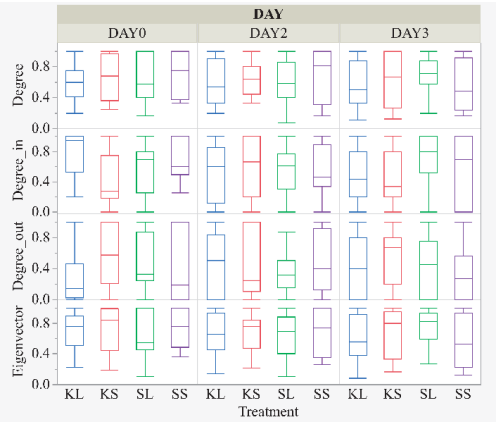
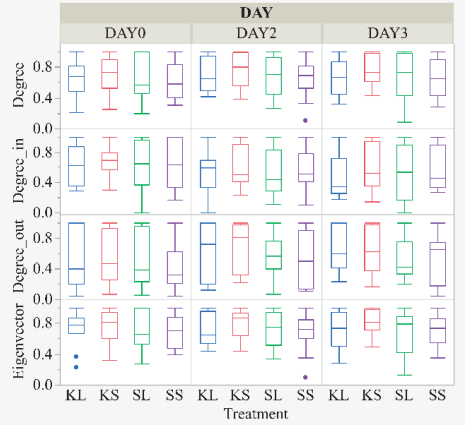


c)



d)

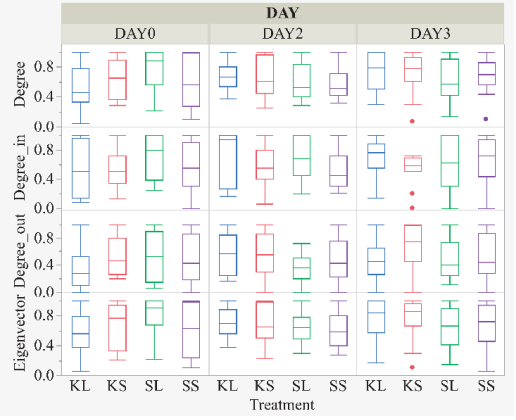


Figure 4: Centrality parameters calculated for ear manipulation (4a), tail manipulation (4b), manipulation of other body parts (4c) and fighting (4d) at baseline (DAY0) and at the first (DAY2) and second day (DAY3) after injecting pigs with ketoprofen-LPS (KL), ketoprofen-saline (KS), saline-LPS (SL) and saline-saline (SS). Round dots represent outliers. Significant differences of planned comparisons between treatments ($p < 0.05$) and within day are marked with an asterisk (*)

Effect of time relative to injection on general network parameters (pen level)

Day relative to injection had an effect on general network parameters of ear manipulation. On the first (median (min | max) = 0.41 (0.26 | 0.57)) and second day after injection (0.45 (0.28 | 0.67)) *in-degree centralisation* ($F(day)_{2,22} = 4.74$, $p = 0.02$) was significantly higher compared to baseline (0.33 (0.10 | 0.54), planned comparisons DAY0 vs. DAY2: $p = 0.04$, DAY0 vs. DAY3 $p = 0.007$), Fig. 5a. Thus, certain individuals in the pen received more interactions than the rest of the group. Day relative to injection had an effect on general network parameters of fighting. One day after injection, *betweenness* ($F(day)_{2,22} = 2.85$, $p = 0.08$) was significantly lower (0.15 (0.04 | 0.44)) compared to baseline (0.26 (0.07 | 0.48), planned comparison: $p = 0.03$), Fig. 5b, indicating that network connectivity increased following the injection. Day relative to injection had no effect on general network parameters of tail manipulation and manipulation of other body parts (Fig. 5).

a) b)

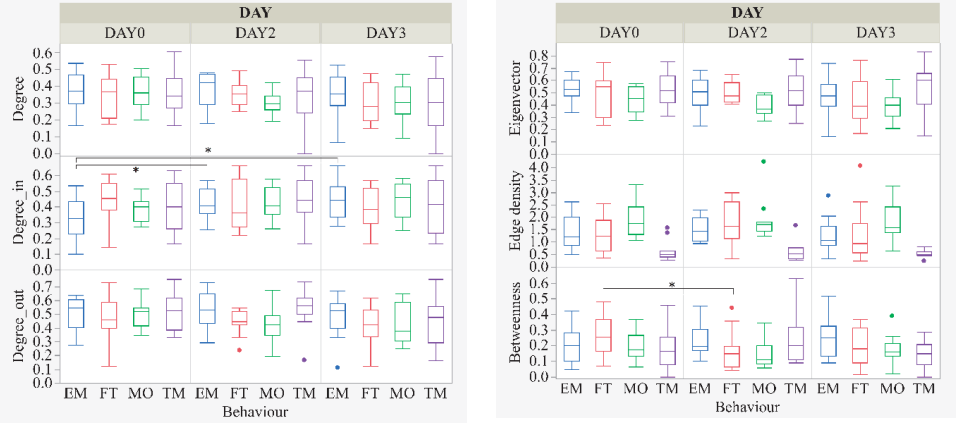


Figure 5: General network parameters (5a, 5b) calculated for ear manipulation (EM, blue boxplot), fighting (FT, red), manipulation of other body parts (MO, green) and tail manipulation (TM, lilac) over three days in pigs. Round dots represent outliers.

Significant differences of planned comparisons between days ($p < 0.05$) are marked with an asterisk (*)

Discussion

Summary

We were able to detect changes in social interactions in response to a controlled immune activation at both individual (pig) and group (pen) level using social network analysis. For ear manipulation networks, an injection with LPS resulted in a lower *in-degree centrality* two days after the challenge at pig level, meaning that the ears of LPS-injected pigs were manipulated to a lesser extent compared to saline-injected pigs. Treatment effects on tail manipulation and fighting networks were not observed. Ketoprofen seemed not to have an impact on centrality parameters at pig level. For networks of manipulation of other body parts, *in-degree centrality* was positively correlated with cortisol response and lying behaviour in the first 6 h after the challenge in LPS-injected pigs. This finding indicates that the stronger the pigs reacted to the challenge, the more manipulations were directed towards them on the following days. At the pen level, a higher *in-degree centralisation* for ear manipulation networks in the two subsequent days after injection compared to baseline was found, thus, certain individuals were more frequently manipulated than the rest of the group. For fighting networks, *betweenness* was significantly higher at baseline compared to one day after injection, indicating that network connectivity increased following the injection. Time relative to injection had no effect on general network parameters of tail manipulation and manipulation of other body parts.

General aspects

The proportions of different social behaviours observed in this study were similar to other studies in group-housed pigs. Bolhuis et al. (2006) found that manipulative oral behaviour directed at pen mates in 15- and 19-week-old pigs mainly consisted of manipulating other body parts (58 % of total observations on manipulative behaviour) and ear biting (30 %) whereas belly nosing (8 %) and tail biting (4 %) were observed less frequently (fighting was not included). Also, Van de Meer et al. (2017) showed that oral manipulation in 20- and 23-week-old pigs was directed mainly towards other body parts and ears, while to a lesser extent towards tails or belly. Slightly contradictory, Camerlink et al. (2013) observed that nosing between 8-week-old pigs consisted mainly of nose-to-nose contact, nosing the body and nosing the head while nosing the ears was rather uncommon, as was nosing the tails.

Pigs used in the present study were similar in age, and housed litter-wise in the same environment from birth. In commercial pig production, regrouping and rehousing are very common management procedures. The stable housing and social conditions in the present study might have had a general impact on network parameters. It has been shown that piglets in socialized pens showed a significantly lower *degree centrality*, *eigenvector centrality* and *clustering coefficient* compared to controls (Turner et al., 2020). We applied SNA to a rather small group of individuals (6) compared to the group sizes of previous studies (8 in Li et al., 2018, 6-29 in Büttner et al., 2015b, 15 in Foister et al., 2018, 12 in Turner et al., 2020), which limits the number of possible interactions within the group. Nevertheless, this is the first study that takes a variety of behavioural patterns into account when describing the effects of immune activation.

Effect of treatment on centrality parameters (pig level)

It was shown in rodents that when sickness behaviour resolves, mice display depressive-like behaviours measured by increased immobility in the forced swim test and tail suspension test at 24-28 h after LPS-challenge (Frenois et al., 2007, O'Connor et al., 2009, Ge et al., 2015, Zhu et al., 2015, Suliyaka et al., 2016, Zhao et al., 2019). It is these psychological aftereffects and their potential effect on social interactions that we wanted to investigate with the current experiment in pigs. Immune activation has been suggested as a major factor influencing social interactions in pigs, with outbreaks of damaging behaviors such as tail biting as a possible result (reviewed by Nordgreen et al., 2020). The shift in social motivation (seen as more tail and ear directed behaviour) was observed about 40 h after the signs of acute illness dissipated and was not accompanied by a similar increase in activity (Munsterhjelm et al., 2019). In boars, tail- and ear-biting tended to increase 0-2 weeks before clinical signs of respiratory infection were visible (Munsterhjelm et al., 2017), thus, behaviour changed already in a preclinical stage of illness. This could also be the case in the phase of recovery when clinical signs abate. Thus, pigs might feel irritable, which might increase the probability to become a biter. Irritability, emotional lability and short temper are reported side effects in humans undergoing cytokine therapy (Capuron et al., 2000, Constant et al., 2005, Denicoff et al., 1987, Renault et al., 1987).

In a previous study with the same pigs, we found that LPS-injected pigs manipulated the ears of their pen mates longer compared to saline-injected pigs on the second day after injection (Veit et al., 2021). In the present study, LPS-injected pigs received fewer ear manipulations two days after the challenge compared to saline-injected pigs. The previous results are based on the duration of the behaviour, whereas the

SNA is based on the frequencies of the respective behaviour. It appears logical, that pigs that perform longer ear manipulations are less likely a target for ear manipulations themselves. Thus, SNA provides a different perspective on the behavioural effect of LPS. It has been discussed that a “pre-damage” state (Fraser and Broom, 1997), in which pigs perform so called “tail-/ear-in-mouth behaviour” (Schrøder-Petersen et al., 2003, Diana et al., 2019) can develop into a “damage-state”. Thus, the gentle tail or ear manipulation we observed could be a precursor of more severe biting behaviour. Camerlink et al. (2013) found that nosing the tail correlated with tail biting and nosing an ear correlated with ear biting. Nevertheless, severe tail or ear lesions were not observed in the present study.

LPS activates the hypothalamic-pituitary-adrenal (HPA) axis as indicated by a peak in cortisol concentrations at 4 h after injection (Webel et al., 1997; Nordgreen et al., 2018). In the present study, the cortisol response and behavioural signs of sickness on the day of injection were correlated with centrality parameters calculated on the first and second day after the challenge. We found that the stronger the cortisol response at 4 h and the more frequently pigs were lying on their side in the first 6 h after the injection, the more these pigs were manipulated by their pen mates on the following days when they were recovered from the challenge. Exposure to stressors is commonly associated with increased HPA axis activity, and therefore, the response of cortisol is generally considered an indicator of stress (Dallman et al., 1987; Sapolsky et al., 2000). Stress has been found to significantly affect the physiology and behaviour of captive and wild populations, which can alter individual behaviour and overall network structure (Boogert et al., 2014). When it comes to interpreting manipulation of other body parts, Jensen and Wood Gush (1984) suggested a threatening function of "nose-to-nose" contact and associated “nose-to-

411 body" contact with individual recognition. Camerlink et al. (2013) found that nosing
412 other parts of the body was unrelated to damaging forms of interaction. It is therefore
413 not clear whether manipulation of other body parts can be interpreted as purely
414 affiliative social behaviour.

415 Ketoprofen reduces PGE_2 production in LPS-injected pigs and inhibits thereby a
416 fever response (Wyns et al., 2015). This is one possible pathway through which
417 ketoprofen can influence behaviour. Moreover, some NSAIDS are able to alter the
418 expression of NFkappaB and thereby reduce subsequent cytokine expression
419 (Peters et al., 2012), but whether ketoprofen works in this way is not known.
420 Ketoprofen alleviated the effect of LPS on sickness behaviour on the day of injection
421 but did not affect social network parameters in the subsequent days after the
422 challenge. However, the pigs that were injected with LPS and ketoprofen did not
423 change their behaviour in the same way that the pigs that received LPS without
424 ketoprofen did.

425 ***Effect of time on general network parameters (pen level)***

426 At pen level, high *degree centralisation* describes whether certain individuals initiate
427 or receive more interactions than the rest of the group. High *betweenness*
428 *centralisation* occurs where sub-groups within a pen interact only indirectly through a
429 small number of intermediary animals (Turner et al., 2020). In the present study, *in-*
430 *degree centralisation* was increased the days following injection for ear manipulation
431 networks, thus, the ears of certain pigs were manipulated more often than their pen
432 mates' compared to baseline. *Betweenness* was decreased for fighting networks one
433 day after injection, suggesting that interactions were more evenly spread across all
434 group members and no single individual was responsible for connecting a

fragmented network. *Edge density* increased only numerically for ear manipulation and fighting networks on the day after injection compared to baseline. *Edge density* indicates how well the members of the group are connected in terms of their interaction with each other and we expected it to increase in all networks the days after injection.

Behavioural changes can be seen in sick animals but also in their healthy social companions. LPS-injected pigs and pigs diagnosed with osteochondrosis received increased social attention by pen mates (Munsterhjelm et al., 2017, Munsterhjelm et al., 2019). On the other hand, LPS-injected pigs performed more tail and ear directed behaviour than their controls in the subsequent days after the challenge (Munsterhjelm et al., 2019). The increased interest in sick animals during the first hours seems logical, but the mechanisms behind the observed change in social behaviour in the following days need to be investigated in future research.

In other studies using social network analysis, the effect of mixing (Büttner et al., 2015a; b), feed-restriction (Cañon Jones et al., 2010) and higher stocking density (Cañon Jones et al., 2011) was tested at group level. In the present study, all treatments were represented in each pen, which hampers the interpretation of general network parameters. A change in group dynamics in the days following injection could be interpreted as a result of a behavioural change of one pig in the group (SL) or as a reaction to the handling of the group as a whole. Even so, the study provides insights on the effects of sickness on social behaviour, where there is still a paucity of scientific literature.

Conclusion

There might be long-lasting effects on social behaviour, both at individual and group level, when even just one individual in a group becomes ill. Changes were detected in ear manipulation and fighting, which together cover 48 % of observed social behaviours, thus, a significant part of social activity was affected. The results indicate that the pigs changed the way they directed social activities, and that the immune status of individuals affected these changes. This needs to be considered in studies of effects of health on behaviour when animals are kept in groups and shows a need for further studies on how individuals in a group should be managed when they become ill.

Ethics approval

The animal study was reviewed and approved by the NMBU IACUC and the food safety authorities (FOTS ID 15232).

Data and model availability statement

None of the data were deposited in an official repository. Data are made available upon request.

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Declaration of interest

None.

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References

Abeyesinghe, S.M., Drewe, J.A., Asher, L., Wathes, C.M., Collins, L.M., 2013. Do hens have friends? Applied Animal Behaviour Science 143, 61-66.

Altmann, J., 1974. Observational Study of Behavior: Sampling Methods. Behaviour 49, 227-266.

503 Aplin, L.M., Farine, D.R., Morand-Ferron, J., Cole, E.F., Cockburn, A., Sheldon, B.C., 2013.
504 Individual personalities predict social behaviour in wild networks of great tits (Parus major).
505 Ecology Letters 16, 1365-1372.
506 Blackshaw, J.K., 1981. Some behavioural deviations in weaned domestic pigs: persistent
507 inguinal nose thrusting, and tail and ear biting. Animal Science 33, 325-332.
508 Bolhuis, J.E., Schouten, W.G.P., Schrama, J.W., Wiegant, V.M., 2006. Effects of rearing and
509 housing environment on behaviour and performance of pigs with different coping
510 characteristics. Applied Animal Behaviour Science 101, 68-85.
511 Boogert, N.J., Farine, D.R., Spencer, K.A., 2014. Developmental stress predicts social
512 network position. Biology Letters 10, 20140561.
513 Boyland, N.K., Mlynski, D.T., James, R., Brent, L.J.N., Croft, D.P., 2016. The social network
514 structure of a dynamic group of dairy cows: From individual to group level patterns. Applied
515 Animal Behaviour Science 174, 1-10.
516 Brent, L.J.N., Heilbronner, S.R., Horvath, J.E., Gonzalez-Martinez, J., Ruiz-Lambides, A.,
517 Robinson, A.G., Skene, J.H.P., Platt, M.L., 2013. Genetic origins of social networks in rhesus
518 macaques. Scientific Reports 3, 1042.
519 Brunberg, E., Wallenbeck, A., Keeling, L.J., 2011. Tail biting in fattening pigs: Associations
520 between frequency of tail biting and other abnormal behaviours. Applied Animal Behaviour
521 Science 133, 18-25.
522 Büttner, K., Scheffler, K., Czycholl, I., Krieter, J., 2015a. Network characteristics and
523 development of social structure of agonistic behaviour in pigs across three repeated
524 rehousing and mixing events. Applied Animal Behaviour Science 168, 24-30.
525 Büttner, K., Scheffler, K., Czycholl, I., Krieter, J., 2015b. Social network analysis - centrality
526 parameters and individual network positions of agonistic behavior in pigs over three different
527 age levels. SpringerPlus 4, 185.
528 Camerlink, I., Turner, S.P., 2013. The pig's nose and its role in dominance relationships and
529 harmful behaviour. Applied Animal Behaviour Science 145, 84-91.

530 Camerlink, I., Ursinus, W.W., Bijma, P., Kemp, B., Bolhuis, J.E., 2015. Indirect Genetic
531 Effects for Growth Rate in Domestic Pigs Alter Aggressive and Manipulative Biting
532 Behaviour. Behavior Genetics, 45, 117-126.
533 Cañon Jones, H.A., Hansen, L.A., Noble, C., Damsgård, B., Broom, D.M., Pearce, G.P.,
534 2010. Social network analysis of behavioural interactions influencing fin damage
535 development in Atlantic salmon (Salmo salar) during feed-restriction. Applied Animal
536 Behaviour Science 127, 139-151.
537 Cañon Jones, H.A., Noble, C., Damsgård, B., Pearce, G.P., 2011. Social network analysis of
538 the behavioural interactions that influence the development of fin damage in Atlantic salmon
539 parr (Salmo salar) held at different stocking densities. Applied Animal Behaviour Science
540 133, 117-126.
541 Capuron, L., Ravaud, A., Dantzer, R., 2000. Early Depressive Symptoms in Cancer Patients
542 Receiving Interleukin 2 and/or Interferon Alfa-2b Therapy. Journal of Clinical Oncology 18,
543 2143-2151.
544 Constant, A., Castera, L., Dantzer, R., Couzigou, P., de Ledinghen, V., Demotes-Mainard, J.,
545 Henry, C., 2005. Mood alterations during interferon-alfa therapy in patients with chronic
546 hepatitis C: evidence for an overlap between manic/hypomanic and depressive symptoms.
547 The Journal of Clinical Psychiatry 66, 1050-1057.
548 Dallman, M.F., Akana, S.F., Cascio, C.S., Darlington, D.N., Jacobson, L., Levin, N., 1987.
549 Regulation of ACTH Secretion: Variations on a Theme of B, Proceedings of the 1986
550 Laurentian Hormone Conference, August 1986, Montebello, Quebec, Canada, pp. 113-173.
551 D'Eath, R.B., Arnott, G., Turner, S.P., Jensen, T., Lahrmann, H.P., Busch, M.E., Niemi, J.K.,
552 Lawrence, A.B., Sande, P., 2014. Injurious tail biting in pigs: how can it be controlled in
553 existing systems without tail docking? Animal 8, 1479-1497.
554 Denicoff, K.D., Rubinow, D.R., Papa, M.Z., Simpson, C., Seipp, C.A., Lotze, M.T., Chang,
555 A.E., Rosenstein, D., Rosenberg, S.A., 1987. The Neuropsychiatric Effects of Treatment with
556 Interleukin-2 and Lymphokine-Activated Killer Cells. Annals of Internal Medicine 107, 293-
557 300.

558 Diana, A., Carpentier, L., Piette, D., Boyle, L.A., Berckmans, D., Norton, T., 2019. An
559 ethogram of biter and bitten pigs during an ear biting event: first step in the development of a
560 Precision Livestock Farming tool. Applied Animal Behaviour Science 215, 26-36.
561 Doncaster, C.P., Davey, A.J.H., 2007. Analysis of Variance and Covariance: How to Choose
562 and Construct Models for the Life Sciences. Cambridge University Press, Cambridge, UK.
563 Foister, S., 2019. The application of social network analysis to understand acute and chronic
564 post-mixing aggression in commercially reared pigs. PhD thesis, The University of
565 Edinburgh, Edinburgh, UK.
566 Foister, S., Doeschl-Wilson, A., Roehe, R., Arnott, G., Boyle, L., Turner, S., 2018. Social
567 network properties predict chronic aggression in commercial pig systems. PLOS ONE 13,
568 e0205122.
569 Fraser, D., 1987. Attraction to blood as a factor in tail-biting by pigs. Applied Animal
570 Behaviour Science 17, 61-68.
571 Fraser, A.F., Broom, D.M., 1997. Farm animal behaviour and welfare. CAB International,
572 Wallingford, UK.
573 Frenois, F., Moreau, M., O'Connor, J., Lawson, M., Micon, C., Lestage, J., Kelley, K.W.,
574 Dantzer, R., Castanon, N., 2007. Lipopolysaccharide induces delayed FosB/DeltaFosB
575 immunostaining within the mouse extended amygdala, hippocampus and hypothalamus, that
576 parallel the expression of depressive-like behavior. Psychoneuroendocrinology 32, 516-531.
577 Ge, L., Liu, L., Liu, H., Liu, S., Xue, H., Wang, X., Yuan, L., Wang, Z., Liu, D., 2015.
578 Resveratrol abrogates lipopolysaccharide-induced depressive-like behavior,
579 neuroinflammatory response, and CREB/BDNF signaling in mice. European Journal of
580 Pharmacology 768, 49-57.
581 Jensen, P., Wood-Gush, D.G.M., 1984. Social interactions in a group of free-ranging sows.
582 Applied Animal Behaviour Science 12, 327-337.
583 Lahrmann, H.P., Hansen, C.F., D'Eath, R., Busch, M.E., Forkman, B., 2018. Tail posture
584 predicts tail biting outbreaks at pen level in weaner pigs. Applied Animal Behaviour Science
585 200, 29-35.

586 Larsen, M.L.V., Andersen, H.M.L., Pedersen, L.J., 2019. Changes in activity and object
587 manipulation before tail damage in finisher pigs as an early detector of tail biting. Animal 13,
588 1037-1044.
589 Li, Y., Zhang, H., Johnston, L.J., Martin, W., 2018. Understanding Tail-Biting in Pigs through
590 Social Network Analysis. Animals 8, 13.
591 Li, Y.Z., Johnston, L.J., Dawkins, M.S., 2020. Utilization of Optical Flow Algorithms to Monitor
592 Development of Tail Biting Outbreaks in Pigs. Animals 10, 323.
593 Makagon, M.M., McCowan, B., Mench, J.A., 2012. How can social network analysis
594 contribute to social behavior research in applied ethology? Applied Animal Behaviour
595 Science 138, 152-161.
596 Moinard, C., Mendl, M., Nicol, C.J., Green, L.E., 2003. A case control study of on-farm risk
597 factors for tail biting in pigs. Applied Animal Behaviour Science 81, 333-355.
598 Munsterhjelm, C., Heinonen, M., Valros, A., 2016. Can tail-in-mouth behaviour in weaned
599 piglets be predicted by behaviour and performance? Applied Animal Behaviour Science 184,
600 16-24.
601 Munsterhjelm, C., Nordgreen, J., Aae, F., Heinonen, M., Olstad, K., Aasmundstad, T.,
602 Janczak, A.M., Valros, A., 2017. To be blamed or pitied? The effect of illness on social
603 behavior, cytokine levels and feed intake in undocked boars. Physiology & Behavior 179,
604 298-307.
605 Munsterhjelm, C., Nordgreen, J., Aae, F., Heinonen, M., Valros, A., Janczak, A.M., 2019.
606 Sick and grumpy: Changes in social behaviour after a controlled immune stimulation in
607 group-housed gilts. Physiology & Behavior 198, 76-83.
608 Nordgreen, J., Edwards, S.A., Boyle, L.A., Bolhuis, J.E., Veit, C., Sayyari, A., Marin, D.E.,
609 Dimitrov, I., Janczak, A.M., Valros, A., 2020. A Proposed Role for Pro-Inflammatory
610 Cytokines in Damaging Behavior in Pigs. Frontiers in Veterinary Sciences 7, 646-646.
611 Nordgreen, J., Munsterhjelm, C., Aae, F., Popova, A., Boysen, P., Ranheim, B., Heinonen,
612 M., Raszplewicz, J., Piepponen, P., Lervik, A., Valros, A., Janczak, A.M., 2018. The effect of

lipopolysaccharide (LPS) on inflammatory markers in blood and brain and on behavior in individually-housed pigs. Physiology & Behavior 195, 98-111.

O'Connor, J.C., Lawson, M.A., André, C., Moreau, M., Lestage, J., Castanon, N., Kelley, K.W., Dantzer, R., 2009. Lipopolysaccharide-induced depressive-like behavior is mediated by indoleamine 2,3-dioxygenase activation in mice. Molecular Psychiatry 14, 511.

Peters, S.M., Yancy, H., Deaver, C., Jones, Y.L., Kenyon, E., Chiesa, O.A., Esparza, J., Screven, R., Lancaster, V., Stubbs, J.T., Yang, M., Wiesenfeld, P.L., Myers, M.J., 2012. In vivo characterization of inflammatory biomarkers in swine and the impact of flunixin meglumine administration. Veterinary Immunology and Immunopathology 148, 236-242.

Renault, P.F., Hoofnagle, J.H., Park, Y., Mullen, K.D., Peters, M., Jones, D.B., Rustgi, V., Jones, E.A., 1987. Psychiatric Complications of Long-term Interferon Alfa Therapy. Archives of Internal Medicine 147, 1577-1580.

Sapolsky, R.M., Romero, L.M., Munck, A.U., 2000. How Do Glucocorticoids Influence Stress Responses? Integrating Permissive, Suppressive, Stimulatory, and Preparative Actions*. Endocrine Reviews 21, 55-89.

Schrøder-Petersen, D.L., Simonsen, H.B., Lawson, L.G., 2003. Tail-in-mouth behaviour among weaner pigs in relation to age, gender and group composition regarding gender. Acta Agriculturae Scandinavica Section a-Animal Science 53, 29-34.

Stanton, M.A., Mann, J., 2012. Early Social Networks Predict Survival in Wild Bottlenose Dolphins. PLOS ONE 7, e47508.

Sulakhiya, K., Keshavlal, G.P., Bezbaruah, B.B., Dwivedi, S., Gurjar, S.S., Munde, N., Jangra, A., Lahkar, M., Gogoi, R., 2016. Lipopolysaccharide induced anxiety- and depressive-like behaviour in mice are prevented by chronic pre-treatment of esculetin. Neuroscience Letters 611, 106-111.

Taylor, N.R., Parker, R.M.A., Mendl, M., Edwards, S.A., Main, D.C.J., 2012. Prevalence of risk factors for tail biting on commercial farms and intervention strategies. The Veterinary Journal 194, 77-83.

640 Thompson, K.A., Papich, M.G., Higgins, B., Flanagan, J., Christiansen, E.F., Harms, C.A.,
641 2018. Ketoprofen pharmacokinetics of R- and S-isomers in juvenile loggerhead sea turtles
642 (Caretta caretta) after single intravenous and single- and multidose intramuscular
643 administration. Journal of Veterinary Pharmacology and Therapeutics 41, 340-348.
644 Turner, S.P., Weller, J.E., Camerlink, I., Arnott, G., Choi, T., Doeschl-Wilson, A., Farish, M.,
645 Foister, S., 2020. Play fighting social networks do not predict injuries from later aggression.
646 Scientific Reports 10, 15486.
647 Van der Meer, Y., Gerrits, W.J.J., Jansman, A.J.M., Kemp, B., Bolhuis, J.E., 2017. A link
648 between damaging behaviour in pigs, sanitary conditions, and dietary protein and amino acid
649 supply. PLOS ONE 12, e0174688.
650 Veit, C., Janczak, A.M., Ranheim, B., Vas, J., Valros, A., Sandercock, D.A., Piepponen, P.,
651 Dulgheriu, D., Nordgreen, J., 2021. The Effect of LPS and Ketoprofen on Cytokines, Brain
652 Monoamines, and Social Behavior in Group-Housed Pigs. Frontiers in Veterinary Sciences 7,
653 1096.
654 Webel, D.M., Finck, B.N., Baker, D.H., Johnson, R.W., 1997. Time course of increased
655 plasma cytokines, cortisol, and urea nitrogen in pigs following intraperitoneal injection of
656 lipopolysaccharide. Journal of Animal Science 75, 1514-1520.
657 Wyns, H., Meyer, E., Plessers, E., Watteyn, A., van Bergen, T., Schauvliege, S., De Baere,
658 S., Devreese, M., De Backer, P., Croubels, S., 2015. Modulation by gamithromycin and
659 ketoprofen of in vitro and in vivo porcine lipopolysaccharide-induced inflammation. Veterinary
660 Immunology and Immunopathology 168, 211-222.
661 Zhao, X., Cao, F., Liu, Q., Li, X., Xu, G., Liu, G., Zhang, Y., Yang, X., Yi, S., Xu, F., Fan, K.,
662 Ma, J., 2019. Behavioral, inflammatory and neurochemical disturbances in LPS and UCMS-
663 induced mouse models of depression. Behavioural Brain Research 364, 494-502.
664 Zhu, L., Wei, T., Gao, J., Chang, X., He, H., Miao, M., Yan, T., 2015. Salidroside attenuates
665 lipopolysaccharide (LPS) induced serum cytokines and depressive-like behavior in mice.
666 Neuroscience Letters 606, 1-6.

667 Zonderland, J.J., Kemp, B., Bracke, M.B.M., den Hartog, L.A., Spoolder, H.A.M., 2011.
668 Individual piglets' contribution to the development of tail biting. Animal 5, 601-607.
669 Zonderland, J.J., van Riel, J.W., Bracke, M.B.M., Kemp, B., den Hartog, L.A., Spoolder,
670 H.A.M., 2009. Tail posture predicts tail damage among weaned piglets. Applied Animal
671 Behaviour Science 121, 165-170.

672

The use of social network analysis to describe the effect of immune activation on group dynamics in pigs

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Supplementary Material S1

Formatting

#1 – Prior to applying function 'format_groups', the data is stored in dataframe format. This dataframe contains the interactions between individual pigs which provides the information required to create a network. This dataframe is split by the categorical variable "pen". This produces a list of dataframes, one for each pen. Once the dataframes are separated by pen, the pen variable is

no longer required and is removed (assigned “NULL”). The igraph function *graph.data.frame* is then applied to all dataframes in order to convert them to *graph* or *network* format in preparation for network analysis.

```
format_groups<-function(x){
  x$Pen<-NULL                    #Pen variable removed
  x<-graph.data.frame(x)         # Conversion to graph format
  return (x)
}
```

#2 – Certain network parameters can include additional information in the form of “weighting” the edges (interactions). In the case of this study, the frequency of interactions was used for weighting. Thus, each interaction had the weight of “1”. The igraph function *simplify* removes the multiple interactions, and replaces it with a single interaction, with the sum value of all prior edges.

```
weight.it<-function(x){
  E(x)$weight<-1     #Assigns edges a value of 1
  x<-simplify(x)     # Simplify removes multiple interactions
  return(x)          # Returns new weighted network format
}
```

#3 *myfunc* is required for certain outputs of network function. It transforms the outputs into a dataframe format, which allows smooth transference of the results from each network (pen) back into a single large dataframe of results.

```
myfunc<-function(arg1){
  x<-lapply(arg1, transform)
  x<-ldply(x)
}
```

Code for network parameters (pig level)

Function 4-7 are pre-existing functions from the *igraph* package, to calculate eigenvector and degree centrality. Functions 5-7 vary only in the calculation of either 'in', 'out' or 'total' interactions.

```
#4
eig<-function(arg1){
  scores<- eigen_centrality(arg1, directed=F, scale=F, weights = NULL)
  return(scores)
}

#5
degree.centrality<-function(x){
  y<-degree(x, v = V(x), mode = c( "total"), loops = FALSE, normalized = F)
  y<-myfunc(y)
  return(y)
}
```

```
#6
degree.centrality.in<-function(x){
  y<-degree(x, v = V(x), mode = c( "in"), loops = FALSE, normalized = F)
  y<-myfunc(y)
  return(y)
}

#7
degree.centrality.out<-function(x){
  y<-degree(x, v = V(x), mode = c( "out"), loops = FALSE, normalized = F)
  y<-myfunc(y)
  return(y)
}
```

#8. *Norm_by_max* - normalising individual degree centrality by the maximum centrality in the pen. This function was formulated to apply to networks that have multiple interactions, as the pre-existing options to normalise centrality measures is based on the theoretical maximum number of connections a binary network can have (one connection between two nodes).

```
norm_by_max<-function(x){
  z<-max(x$X_data)
  x$X_data<-x$X_data/z
```

```
  return(x)
}
```

As above. All pen level network measures are pre-existing functions from igraph manual.

Code for network parameters (pen level)

```
degree.cent<-function(x){
  y<-degree(x, v = V(x), mode = c( “all”), loops = FALSE, normalized = F)
  y<-myfunc(y)
  max<-max(y$X_data)
  max=max*6
  x<-  centralization.degree(x,  mode = c(“total”),loops = F, normalized = F) #changed to false
  cent=x$centralization
  norm=cent/max
  return(norm)
}

degree.cent.in<-function(x){
  y<-degree(x, v = V(x), mode = c( “in”), loops = FALSE, normalized = F)
  y<-myfunc(y)
  max<-max(y$X_data)
```

```
  max=max*6
  x<-  centralization.degree(x,  mode = c("in"),loops = F, normalized = F) #changed to false
  cent=x$centralization
  norm=cent/max
return(norm)
  }

degree.cent.out<-function(x){
  y<-degree(x, v = V(x), mode = c( "out"), loops = FALSE, normalized = F)
  y<-myfunc(y)
  max<-max(y$X_data)
  max=max*6
  x<-  centralization.degree(x,  mode = c("out"),loops = F, normalized = F) #changed to false
  cent=x$centralization
  norm=cent/max
  return(norm)
}

p.eig.cent<-function(arg1){
  x<- lapply(arg1, centralization.evcent, directed = FALSE, scale = TRUE, options = igraph.arpack.default, normalized = TRUE)
  x<- lapply(x,"[[","centralization")
```

```
  x=lapply(x, myfunc)
  x<-ldply(x)
  colnames(x)[1] <- “Pen”
  colnames(x)[2] <- “value”
  x$metric<-“Eigenvector”
  return (x)
}

p.between.cent<-function(arg1){
  x<- lapply(arg1, centralization.betweenness, directed = TRUE, nobigint = TRUE, normalized = TRUE)
  x<- lapply(x,”[[“,”centralization”)
  x=lapply(x, myfunc)
  x<-ldply(x)
  colnames(x)[1] <- “Pen”
  colnames(x)[2] <- “value”
  x$metric<-“Betweeness”
  return (x)
}
```

Supplementary Material S2

Code for statistical models (pig level)

```
Fit Model(
        Y( :Degree_cent, :Degree.in, :Degree.out, :Eigenvector ),
        Effects( :Treatment, :DAY, :DAY * :Treatment, :ID[:Treatment] & Random ),
        Personality( "Standard Least Squares" ),
        Emphasis( "Effect Screening" ),
        Method( "REML" )
)
```

Pig (=ID) is a random effect and nested in treatment. The other main effects and their interaction are fixed, categorical effects.

Code for statistical models (pen level)

```
Fit Model(
        Y(      :degree_cent,
                :degree_cent_in,
                :degree_cent_out,
                :Betweeness,
                :edge_density,
                :Eigenvector,
        ),
        Effects( :DAY, :Pen & Random ),
        Personality( "Standard Least Squares" ),
        Emphasis( "Effect Screening" ),
        Method( "REML" )
)
```

Pen is random, day is a fixed categorical effect.

Supplementary Table S1: Median (25-75 % - quartile) of centrality parameters (pig level) for all behaviours included presented by treatment and day

		Ear manipulation			Tail manipulation			Manipulation of other body parts			Fighting		
		DAY0	DAY2	DAY3	DAY0	DAY2	DAY3	DAY0	DAY2	DAY3	DAY0	DAY2	DAY3
Degree centrality													
	KL	0.62 (0.40-0.95)	0.43 (0.32-0.55)	0.49 (0.36-0.94)	0.60 (0.41-0.75)	0.54 (0.33-0.91)	0.50 (0.33-0.88)	0.68 (0.49-0.81)	0.65 (0.49-0.95)	0.68 (0.45-0.87)	0.46 (0.34-0.77)	0.66 (0.54-0.80)	0.79 (0.51-1.00)
	KS	0.82 (0.47-1.00)	0.65 (0.42-1.00)	0.85 (0.62-1.00)	0.68 (0.36-0.96)	0.64 (0.44-0.80)	0.67 (0.27-1.00)	0.73 (0.53-0.90)	0.80 (0.57-0.99)	0.74 (0.62-0.98)	0.65 (0.37-0.89)	0.60 (0.45-0.96)	0.78 (0.61-0.93)
	SL	0.50 (0.32-1.00)	0.60 (0.50-0.91)	0.54 (0.44-1.00)	0.57 (0.40-1.00)	0.58 (0.40-0.85)	0.71 (0.58-0.88)	0.58 (0.46-1.00)	0.71 (0.45-0.93)	0.73 (0.43-0.98)	0.88 (0.56-1.00)	0.53 (0.40-0.84)	0.57 (0.43-0.90)
	SS	0.54 (0.40-0.62)	0.65 (0.45-0.78)	0.65 (0.55-0.82)	0.75 (0.38-1.00)	0.82 (0.30-1.00)	0.49 (0.24-0.92)	0.58 (0.41-0.83)	0.69 (0.53-0.81)	0.65 (0.43-0.90)	0.56 (0.27-0.99)	0.51 (0.42-0.71)	0.70 (0.56-0.86)
In-degree centrality													
	KL	0.78 (0.37-0.88)	0.54 (0.20-0.78)	0.52 (0.23-0.58)	0.95 (0.53-1.00)	0.60 (0.11-0.86)	0.43 (0.20-0.80)	0.63 (0.36-0.88)	0.59 (0.33-0.69)	0.35 (0.26-0.72)	0.50 (0.14-0.96)	0.93 (0.26-1.00)	0.76 (0.55-0.88)
	KS	0.81 (0.62-1.00)	0.54 (0.46-0.97)	0.83 (0.36-1.00)	0.27 (0.18-0.75)	0.67 (0.20-1.00)	0.33 (0.20-0.80)	0.69 (0.57-0.80)	0.50 (0.42-0.91)	0.52 (0.36-0.94)	0.50 (0.33-0.72)	0.54 (0.39-0.80)	0.58 (0.50-0.69)
	SL	0.33 (0.25-0.83)	0.58 (0.42-0.72)	0.40 (0.26-0.62)	0.70 (0.25-0.80)	0.61 (0.30-0.77)	0.80 (0.51-1.00)	0.65 (0.37-0.97)	0.43 (0.29-0.83)	0.54 (0.17-0.90)	0.79 (0.39-1.00)	0.68 (0.44-1.00)	0.62 (0.30-1.00)
	SS	0.77 (0.60-0.88)	0.46 (0.36-0.66)	0.83 (0.44-1.00)	0.60 (0.50-1.00)	0.46 (0.33-0.90)	0.70 (0.00-1.00)	0.64 (0.33-1.00)	0.51 (0.41-0.78)	0.46 (0.34-0.90)	0.55 (0.30-0.90)	0.44 (0.31-0.72)	0.71 (0.43-0.94)

Out-degree centrality													
	KL	0.55 (0.16-0.75)	0.13 (0.06-0.64)	0.40 (0.22-0.96)	0.14 (0.02-0.46)	0.50 (0.00-0.83)	0.40 (0.00-0.80)	0.40 (0.20-1.00)	0.72 (0.20-1.00)	0.60 (0.41-1.00)	0.28 (0.10-0.53)	0.56 (0.26-0.84)	0.45 (0.26-0.66)
	KS	0.54 (0.25-1.00)	0.44 (0.24-0.96)	0.67 (0.34-0.87)	0.58 (0.20-1.00)	0.25 (0.10-1.00)	0.67 (0.20-0.80)	0.47 (0.26-0.93)	0.82 (0.32-0.98)	0.62 (0.38-0.98)	0.46 (0.27-0.80)	0.56 (0.30-0.85)	0.74 (0.46-0.98)
	SL	0.55 (0.26-1.00)	0.51 (0.12-0.93)	0.54 (0.16-0.98)	0.33 (0.25-0.88)	0.32 (0.15-0.50)	0.45 (0.00-0.75)	0.39 (0.23-0.95)	0.56 (0.40-0.77)	0.42 (0.34-0.76)	0.53 (0.15-0.90)	0.37 (0.20-0.50)	0.40 (0.25-0.73)
	SS	0.32 (0.08-0.52)	0.60 (0.30-0.87)	0.28 (0.10-0.67)	0.19 (0.00-1.00)	0.40 (0.13-0.92)	0.27 (0.00-0.56)	0.39 (0.23-0.95)	0.56 (0.40-0.77)	0.42 (0.34-0.76)	0.43 (0.19-0.85)	0.43 (0.23-0.76)	0.44 (0.27-0.88)
Eigen-vector													
	KL	0.68 (0.35-0.95)	0.49 (0.37-0.61)	0.61 (0.42-0.92)	0.76 (0.51-0.89)	0.66 (0.46-0.94)	0.56 (0.38-0.91)	0.32 (0.21-0.62)	0.50 (0.12-0.90)	0.66 (0.18-0.75)	0.56 (0.38-0.79)	0.70 (0.56-0.88)	0.84 (0.58-1.00)
	KS	0.81 (0.54-0.99)	0.71 (0.49-1.00)	0.89 (0.69-1.00)	0.84 (0.45-0.98)	0.76 (0.48-0.85)	0.80 (0.34-0.95)	0.78 (0.67-0.86)	0.65 (0.54-0.96)	0.74 (0.51-0.94)	0.76 (0.33-0.94)	0.66 (0.50-0.98)	0.86 (0.67-0.96)
	SL	0.58 (0.34-1.00)	0.72 (0.60-0.97)	0.60 (0.50-0.99)	0.55 (0.46-1.00)	0.70 (0.40-0.88)	0.82 (0.59-0.94)	0.81 (0.61-0.95)	0.87 (0.63-0.94)	0.81 (0.72-0.98)	0.90 (0.67-1.00)	0.64 (0.49-0.78)	0.67 (0.41-0.90)
	SS	0.68 (0.36-0.79)	0.73 (0.56-0.89)	0.75 (0.62-0.90)	0.76 (0.48-1.00)	0.74 (0.35-1.00)	0.53 (0.23-0.93)	0.66 (0.53-1.00)	0.75 (0.52-0.94)	0.79 (0.42-0.90)	0.63 (0.24-0.99)	0.59 (0.40-0.81)	0.73 (0.46-0.94)

Abbreviations: DAY0 = baseline; DAY2 = first day after injection; DAY3 = second day after injection; KL = ketoprofen-lipopolysaccharide; KS = ketoprofen-saline; SL = saline-lipopolysaccharide; SS = saline-saline

Supplementary Table S2: Median (25 | 75 % - quartile) of general network parameters (pen level) for all pig behaviours included presented by day

	Ear manipulation			Tail manipulation			Manipulation of other body parts			Fighting		
	DAY0	DAY2	DAY3	DAY0	DAY2	DAY3	DAY0	DAY2	DAY3	DAY0	DAY2	DAY3
Degree centralisation	0.37 (0.29| 0.47)	0.42 (0.29| 0.47)	0.36 (0.29| 0.46)	0.34 (0.27| 0.44)	0.37 (0.24| 0.45)	0.31 (0.17| 0.45)	0.36 (0.29| 0.46)	0.29 (0.26| 0.34)	0.31 (0.24| 0.39)	0.37 (0.21| 0.44)	0.35 (0.29| 0.40)	0.28 (0.19| 0.42)
In-degree centralisation	0.33 (0.23| 0.44)	0.41 (0.36| 0.51)	0.45 (0.34| 0.53)	0.40 (0.26| 0.55)	0.44 (0.37| 0.57)	0.42 (0.23| 0.57)	0.40 (0.31| 0.43)	0.41 (0.35| 0.53)	0.46 (0.34| 0.55)	0.46 (0.38| 0.55)	0.36 (0.27| 0.58)	0.38 (0.29| 0.52)
Out-degree centralisation	0.54 (0.41| 0.61)	0.53 (0.44| 0.65)	0.52 (0.40| 0.58)	0.53 (0.39| 0.62)	0.56 (0.51| 0.62)	0.48 (0.29| 0.56)	0.52 (0.42| 0.54)	0.42 (0.35| 0.49)	0.38 (0.31| 0.59)	0.46 (0.40| 0.59)	0.45 (0.42| 0.52)	0.42 (0.34| 0.54)
Betweenness	0.20 (0.10| 0.29)	0.20 (0.17| 0.31)	0.25 (0.14| 0.33)	0.16 (0.08| 0.26)	0.20 (0.11| 0.32)	0.15 (0.08| 0.21)	0.18 (0.13| 0.27)	0.11 (0.09| 0.20)	0.16 (0.14| 0.22)	0.26 (0.16| 0.37)	0.15 (0.06| 0.20)	0.18 (0.09| 0.32)
Eigenvector	0.52 (0.47| 0.61)	0.51 (0.40| 0.61)	0.47 (0.39| 0.57)	0.52 (0.42| 0.63)	0.52 (0.40| 0.64)	0.60 (0.41| 0.66)	0.46 (0.34| 0.55)	0.37 (0.33| 0.48)	0.40 (0.31| 0.46)	0.55 (0.30| 0.59)	0.47 (0.43| 0.58)	0.39 (0.29| (0.59)
Edge density	1.20 (0.88| 2.01)	1.43 (1.03| 1.98)	1.08 (0.88| 1.65)	0.52 (0.40| 0.62)	0.55 (0.34| 0.77)	0.50 (0.47| 0.60)	1.75 (1.33| 2.42)	1.70 (1.46| 1.80)	1.58 (1.38| 2.43)	1.23 (0.65| 1.88)	1.65 (1.16| 2.61)	0.93 (0.57| 1.74)

Abbreviations: DAY0 = baseline; DAY2 = first day after injection; DAY3 = second day after injection

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