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Neuroendocrine modulation of immune functions by repeated anticipation in domestic pigs

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1 Introduction

To improve animal welfare in pig husbandry it is necessary to expand our knowledge of affective states and their physiological effects. The aim of our project was to induce positive or negative mood by repeated anticipation of a positive, a negative or an unpredictable stimulus and to investigate the physiological consequences.

Measuring heart rate and its variability has become widely used to assess emotions and stress in farm animals (von Borell et al., 2007). In addition to these physiological parameters, emotional states may modulate immune functions. Therefore, the present study focused on possible changes in immunocompetence that may or may not accompany changes in mood (Hucklebridge et al., 2000; Mittwoch-Jaffe et al., 1995).

Studies of our own group showed that in pigs negative emotional stimuli, such as isolation of piglets, may lead to changes in gene expression in the limbic system and to suppressed immune functions (Kanitz et al., 2004 and 2009). In contrast, repeated positive emotional stimuli induced by cognitive enrichment, led to an increased T-lymphocyte proliferation and improved wound healing (Ernst et al., 2006).

2 Materials and methods

The experiment consisted of 16 replications. For each replication, six female 5-week old pigs, which were all siblings, were housed in individual pens in one room with olfactory and acoustic contact to each other. The animals were handled for a week to adapt to the procedures such as belt application for heart beat measurement. This was followed by a conditioning week and three measuring weeks.

Three of these piglets were conditioned six times a day to a sound that announced a positive stimulus (food), a negative stimulus (air-puff) or a random choice of food or air-puff. This was supposed to induce positive, negative or conflicting anticipation, respectively. The other three piglets obtained positive, negative or no stimuli without conditioning and served as controls. The daily amount of food was equal in all groups. The pigs with the negative stimulus received food in three portions outside the measuring periods. Within four weeks, we expected changes in mood and consequently physiological effects.

To analyse whether the different treatments have different effects on the physiological parameters, blood samples were collected at the beginning and at the end of the experiment. Furthermore, saliva was collected during the handling and measuring period. Heart activity

was measured throughout the experiment using the Polar S810i ® device (Polar Elektro Oy, Finland). At the end of the experiment, the animals were sacrificed and specific parts of the brain and the adrenal glands were isolated.

Neurotransmitter concentrations were determined by HPLC in the nucleus accumbens, the ventral tegmentum and the prefrontal cortex. Salivary cortisol was analysed throughout the experiment by ELISA. Adrenocorticotrophic hormone (ACTH) and immunoglobulin (IgA, IgG, IgM) concentrations were measured in plasma using ELISAs. Adrenal glands were cryosected and histochemically stained to count cells in cortex and medulla and measure their respective areas. Glucocorticoid receptor (GR) mRNA expression in hypothalamus, hippocampus and amygdala and corticotrophin-releasing hormone (CRH) mRNA expression in hypothalamus and amygdala were analysed by real-time RT-PCR. Lymphocyte transformation tests were performed on isolated peripheral blood mononuclear cells stimulated by staphylococcus enterotoxin B (SEB) to assess lymphocyte proliferation.

The statistical analyses were generated using SAS for Windows, Version 9.2 (SAS Institute Inc., 2009, Cary, NC, USA). Least-squares means and their standard errors were calculated for each fixed effect (replication, treatment, day) and their interactions using the Tukey-Kramer procedure. Results were considered as significant if $p < 0.05$.

3 Results

Heart rate analyses at the end of the experiment showed an increase of heart rate during the sound in positively anticipating pigs compared to pigs with negative ($p < 0.01$) or conflicting anticipation ($p < 0.01$). Also, positively anticipating pigs had an increased basal heart rate compared to the control group ($p < 0.05$) and to the same group at the beginning of the experiment ($p < 0.01$). Neurotransmitter analysis revealed an increased serotonin turnover in the ventral tegmentum compared to the group with conflicting anticipation ($p < 0.05$).

Salivary cortisol increased slightly but non-significantly during the handling period and significantly decreased towards the end of the experiment (Figure 1A). Plasma ACTH significantly decreased over the experimental period (Figure 1B). None of these values exceeded basal values. The analyses of the adrenal glands showed no significant differences between the different treatments (data not shown). Real-time RT-PCR of GR and CRH mRNA expression revealed no significant differences between the treatments (data not shown).

IgM levels significantly increased over the experimental period in all groups except for the positively anticipating pigs and the control group (Figure 2A) while IgA and IgG concentrations significantly increased in all groups (data not shown).

Mitogen-induced T-cell proliferation was similar in all groups at the beginning of the experiment, while at the end of the experiment positively anticipating pigs showed a lower T-cell proliferation than the control animals (Figure 2B).

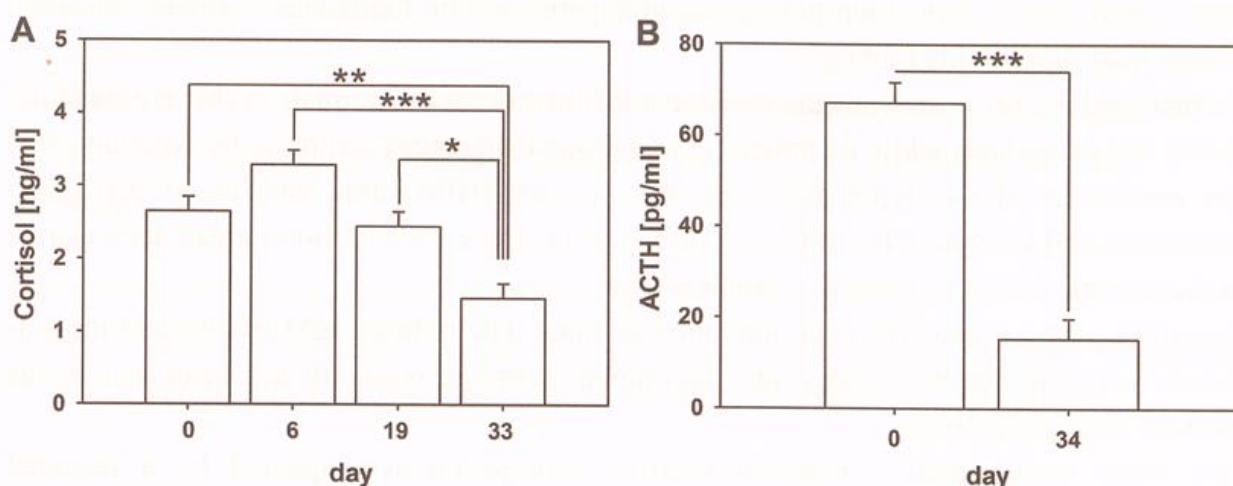


Figure 1

Stress parameters. (A) Salivary cortisol concentrations during the handling period (day 0-6) and during the measuring period (day 19-33). (B) Plasma ACTH concentrations at the beginning (day 0) and at the end of the experiment (day 34). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

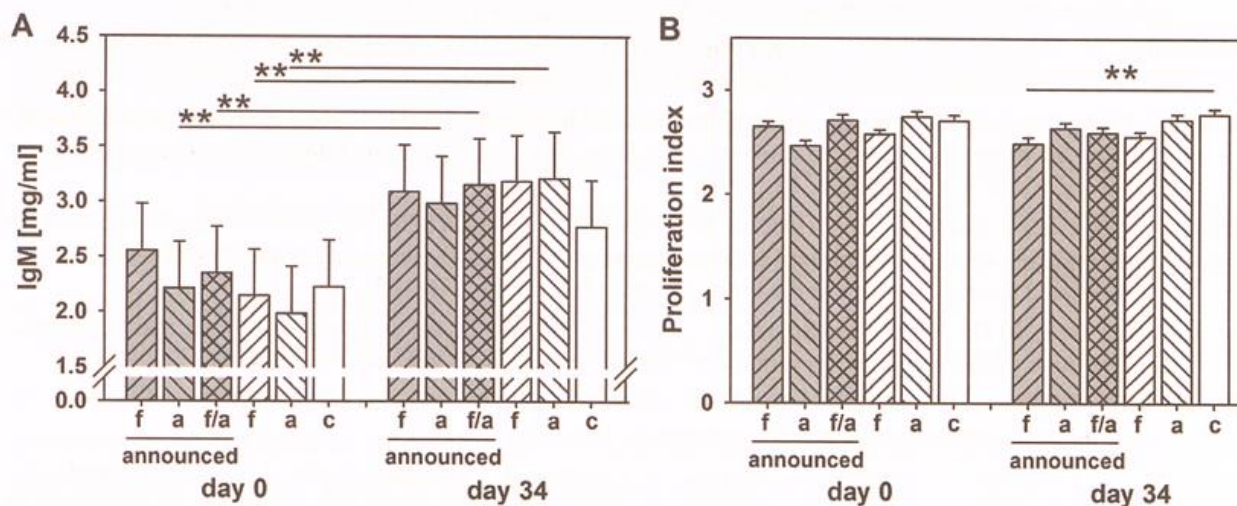


Figure 2

Immune parameters. (A) Plasma IgM concentrations at the beginning and the end of the experiment. (B) Proliferation index determined by lymphocyte transformation test with SEB stimulation. Groups: f = food, a = air-puff, f/a = food or air-puff, c = control. Stimuli were “announced” by a sound in three groups. ** $p < 0.01$

4 Discussion

Treatment-related effects were only found in the positively anticipating group. Missing treatment effects in the negatively anticipating pigs suggest that air-puffs are probably not perceived as strong aversive stimuli.

Heart rate measurements may indicate stress and well-being in farm animals (von Borell et al., 2007). Although we expected an age-dependent decrease of the basal heart rate during the experiment (Kuwahara et al., 1986), we found this for all groups except for the positively anticipating pigs. This increase indicates an activation of the sympathetic nervous system

which is also seen in this group during the anticipation of the food. The increased serotonin turnover may support this finding.

Cortisol and ACTH measurements indicate a habituation of the animals to the experimental housing and procedures while treatment-related group differences could not be detected. Also other parameters of the hypothalamic-pituitary-adrenal (HPA) axis such as adrenal gland composition and GR and CRH mRNA expression in stress-relevant brain areas demonstrate that the animals did not experience chronic stress.

Nevertheless, we found effects on immune functions. The reduced IgM levels and mitogen-induced T-cell proliferation suggest that the immune response is slightly compromised for the positively anticipating pigs.

This study implies that a repeated positive anticipation accompanied by a repeated sympathetic activation without an involvement of the HPA axis may induce a state of arousal that may affect immune functions.

5 Literature

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Toll-like receptors in rainbow trout (*Oncorhynchus mykiss*): Structure of TLR2 and regulation of factors contributing to TLR signaling during infection

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1 Introduction

TLRs (Toll-like receptors) are transmembrane receptors, sensing the presence of pathogens through binding their PAMPs (pathogen-associated molecular pattern). Thirteen distinct TLRs are found in mammals (TLR1-13). Genes encoding TLR6 and 10 have to date not been found in fish. However, pisces encode sets of other TLR receptors (TLRs 14, 21, 22, 23) which are not matched by any mammalian orthologs (reviewed by Rebl et al., 2010). TLR signalling generally requires formation of the Myddosome, a helical oligomer consisting of a layer of six MyD88 factors (myeloid differentiation primary response gene 88), sequentially topped by four IRAK4 (Interleukin 1 receptor-associated kinase 4) and four IRAK2 molecules (Lin et al., 2010). Autophosphorylation of IRAK4 activates via several downstream factors eventually the NF- κ B transcription factor complex to trigger the immune response (reviewed by Kawai & Akira, 2010 and Huxford & Ghosh, 2009).

TLR2 is the only mammalian TLR homolog that has not been described from rainbow trout so far. The mammalian factor is responsible for the recognition of bacterial membrane proteins like lipoproteins and lipopeptides (Jin & Lee, 2008) which are found in more than 400 different proteins from all kind of bacteria (Babu et al., 2006). Functional assays in the common carp (*Cyprinus carpio carpio*) revealed that LTA (lipoteichoic acid), PGN (peptidoglycan) and Pam₃CSK₄ (a synthetic triacylated lipopeptide) all activate TLR2. Additionally TLR2 mRNA levels were elevated in carp macrophages 6h after stimulation with LTA and with PGN (Ribeiro et al., 2010). TLR2 mRNA concentration in head kidney was down regulated after infection of catfish (*Ictalurus punctatus*) with gram negative *Edwardsiella ictaluri* bacteria (Baoprasertkul et al., 2007).

We wanted to resolve the gene structure of the TLR2 from rainbow trout and to survey the expression of TLR receptors, of some downstream factors of the TLR signaling cascade and of important secreted immune factors after infecting these fish with *Aeromonas salmonicida* subsp. *salmonicida*.

2 Materials and methods

DNA and RNA extracted from spleen and muscle tissue from imported rainbow trout bred in Institute of Fisheries, Born served as templates for the structural gene analyses.

Homologous DNA sequence elements of mammalian and piscine TLR2 genes were used to derive degenerated primers to retrieving a first TLR2 cDNA fragment from trout.