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ABSTRACT

Exposure to psychosocial stress can have a profound impact on immune reactivity and health mediated by hypothalamic–pituitary–adrenal (HPA) axis activation. However, current knowledge regarding the mechanisms involved in cross-sensitization between stress and the immune system is limited. Here, we investigated the effects of a single social isolation followed by repeated oral *Escherichia coli* (*E. coli*) applications on cortisol, tumor necrosis factor- α (TNF- α), interleukin-6 (IL-6), haptoglobin and C-reactive protein (CRP) concentrations in the blood; on clinical signs of disease; and on mRNA expression of the glucocorticoid receptor (GR), mineralocorticoid receptor (MR), 11 β -hydroxysteroid dehydrogenase 1 and 2 (11 β -HSD1 and 11 β -HSD2), TNF- α and IL-6 in the hypothalamus, prefrontal cortex (PFC) and spleen of 7-, 21- and 35-day-old piglets. Additionally, the protein levels of splenic TNF- α and IL-6 were analyzed. Non-isolated, *E. coli*-challenged piglets served as a control. Social isolation for 4 h induced a rise in the plasma cortisol concentrations immediately after social treatment and after repeated *E. coli* applications in isolated compared to non-isolated piglets. The circulating TNF- α concentration was not affected by social treatment. Furthermore, previously isolated piglets showed a higher frequency of signs of disease in response to *E. coli* challenge than non-isolated piglets, while the haptoglobin and CRP concentrations did not significantly differ between social treatments. In the brain, 11 β -HSD1, 11 β -HSD2 and IL-6 mRNA expression in the hypothalamus and GR, and 11 β -HSD1 and 11 β -HSD2 mRNA expression in the PFC were higher in isolated, *E. coli*-challenged piglets than in the corresponding controls. Moreover, isolated piglets also displayed higher MR, 11 β -HSD1 and IL-6 mRNA expression levels and TNF- α concentrations in the spleen. The stress-induced alterations in the hypothalamus and spleen were particularly pronounced in younger piglets. The present findings may contribute to a better understanding of the complex interplay between early psychological stress and an increased risk of disease and might also have implications on aspects of the health and welfare of farm animals and humans.

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1. Introduction

Biological responses to stressful situations generally represent adaptive processes to protect the survival of an organism under conditions of adversity, threat or fear (McEwen, 2007). Here, cross-talk between the brain and the immune system is essential to achieve and maintain physiological homeostasis. However, studies in humans and in animal models have indicated that psychosocial stress can negatively influence the regulation of the neuroendocrine and immune system, resulting in physiological

imbalances and increased disease susceptibility (Bartolomucci, 2007; Segerstrom and Miller, 2004). In farm animals, there is also evidence that social factors trigger physiological and behavioral stress responses and thereby play a crucial role in mediating disease risk (Proudfoot and Habing, 2015). For example, it has been shown that social mixing stress in pigs suppresses the immune response to viral vaccination and consequently impairs protection against challenge with pseudorabies virus (De Groot et al., 2001). Another study revealed an impact of social rank on susceptibility to Aujeszky's disease virus, with lower morbidity and mortality in dominant pigs (Hessing et al., 1994).

Using social isolation as an established model to study psychosocial stress in pigs, our previous studies have indicated that

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repeated daily isolation (2 h daily from days 3–11 of age) leads to activation of the hypothalamic–pituitary adrenal (HPA) system with enhanced cortisol release and increased glucocorticoid receptor (GR) and cytokine expression in the brain (Kanitz et al., 2004). Furthermore, the repeated isolation paradigm has caused long-term effects on sickness behavior and the pro-inflammatory network in the periphery and in the brain following lipopolysaccharide (LPS) challenge (Tuchscherer et al., 2004, 2006). However, the mechanisms and neural circuitry underlying cross-sensitization between psychosocial stress and the immune system are incompletely understood, but glucocorticoids, the final products of HPA activation, may modulate the sensitization of inflammatory processes (Sorrells and Sapolsky, 2007). Thus, it has been shown that administration of exogenous glucocorticoids in rodents potentiates the peripheral and central pro-inflammatory response to an LPS challenge (Frank et al., 2010). At the cellular level, glucocorticoid actions are mediated via GR and mineralocorticoid receptor (MR) and by the presence of tissue-specific 11β -hydroxysteroid dehydrogenases (11β -HSDs; Holmes and Seckl, 2006; Reul and de Kloet, 1985). Additionally, it is also known that cytokines, such as tumor necrosis factor- α (TNF- α) and interleukin-6 (IL-6), have autocrine and paracrine functions within specific tissues, as well as endocrine signaling functions via their release into the blood with subsequent actions in the brain (Bilbo and Schwarz, 2012; Besedovsky and del Rey, 2011).

Furthermore, within the communication between neuroendocrine and immune systems, the spleen plays a crucial role in maintaining immune homeostasis during stressful situations, including pathogen exposure (Avitsur et al., 2009; Mebius and Kraal, 2005). In this context, increased expression of several immune-related transcripts appears to provide important mediators of physiological responses to stress (Maslanik et al., 2012; You et al., 2011).

In early life, domestic piglets are generally exposed to certain psychosocial stressors such as handling by humans, disruption of social relationships and abrupt weaning, which may influence the risk and severity of infections (Kanitz et al., 2002; Proudfoot and Habing, 2015). Moreover, the first weeks of life constitute a critical period for the offspring of farm animals, because the neonatal immune system is not fully developed and the intestinal gut flora is still precarious, making the neonates highly susceptible to enteric diseases (Grierson et al., 2007; Sinkora and Butler, 2009). Exposure to a single episode of social isolation (4 h) of piglets has been shown to cause activation of stress-related gene expression in various brain regions (Kanitz et al., 2009) and immune alterations characterized by the redistribution of circulating lymphocytes and decreases in pro-inflammatory cytokines (Tuchscherer et al., 2009). However, it is not known whether this short-term stressor is sufficient to sensitize adaptive responses to a natural bacterial infection. Yet, information concerning the role of psychosocial stress during early postnatal life in the risk of diseases is limited, but necessary for a better understanding of the biological bases of animal welfare and health.

Therefore, in the present study we hypothesized that a single 4-h social isolation procedure in domestic piglets sensitizes neuroendocrine and inflammatory responses to subsequent immune challenges both in the periphery and in the central level of piglets. Accordingly, we examined the effects of social isolation followed by a repeated oral *Escherichia coli* (*E. coli*) application on stress hormone and cytokine responses (cortisol, TNF- α , IL-6), acute phase proteins (haptoglobin; C reactive protein, CRP), clinical signs of disease and mRNA expression of genes that regulate glucocorticoid and inflammatory responses (GR, MR, 11β -HSD1, 11β -HSD2, TNF- α , IL-6) in the hypothalamus, prefrontal cortex (PFC) and spleen of 7-, 21- and 35-day-old piglets.

2. Materials and methods

All procedures involving animal handling and treatment were conducted in strict accordance with the German Animal Protection law and were approved by the relevant authorities (Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei, Mecklenburg-Vorpommern, Germany; LALLF M-V/TSD/7221.3-2.1-015/05).

2.1. Animals and experimental design

A total of 48 piglets were obtained from six German Landrace litters that were bred and raised in the experimental pig unit of the Leibniz Institute for Farm Animal Biology (Dummerstorf, Germany). After birth, the litter size was standardized to 8 piglets. Each piglet could be recognized by a tattoo number in the ear and a number painted on the back. During the suckling period, sows and their piglets were housed in a separate loose farrowing pen (6 m²) with a plastic floor covered with sawdust and a water-heated lying area for the piglets with a constant room temperature (28 ± 1 °C) and lighting program (12/12 h light/dark cycle, lights on at 06:00), and with unrestricted access to food and water. The health status of the pigs was checked continuously by visual inspection during the days prior to the experiments. None of the piglets showed any clinical signs of disease. Single social isolation of piglets was carried out as previously described by Kanitz et al. (2009). At 7, 21 or 35 days of age, 8 piglets from each litter were randomly allocated either to the social isolation treatment or to the non-isolated controls at an approximately equal sex ratio (i.e., two litters were used at each age: $n = 8$ per treatment and age group). The experimental piglets were separated from their mother and siblings once for 4 h in the morning (between 07:00 and 11:00) in separate test rooms located within the same experimental station. Here, each piglet was placed alone into a wooden box (68 × 75 × 65 cm) with sawdust on the floor and adequate air passage. The socially deprived piglets were kept under the same air and temperature conditions as in the farrowing pen. The control piglets remained undisturbed in the farrowing pen during this time.

2.2. Preparation and application of pathogenic *E. coli*

The porcine enterotoxigenic challenge strain *E. coli* Abbotstown (EcA; serotype O149:K91, hemolytic) was a kind gift from G. Baljer, Institute for Hygiene and Infectious Diseases of Animals, University of Giessen, Giessen, Germany. This EcA harbors the genes for F4ac and F6 fimbria, heat-stable enterotoxins ST-Ip and ST-II, and heat-labile enterotoxin LT-I (Schroeder et al., 2006). The strain was cultured in Luria Bertani (LB) broth for 18 h at 37 °C, centrifuged at 3000 × g for 10 min at 4 °C, and resuspended in 0.1% peptone water. Inocula of EcA containing 1.5×10^{10} to 2.5×10^{10} colony-forming units (CFU) in 3 ml peptone water per dose were freshly prepared before use. Inocula were orally administered to socially isolated and non-isolated piglets via a syringe without a pinhead immediately after the social isolation treatment (first *E. coli* challenge) and 21 h after the first application (second *E. coli* challenge). The schedule for the experimental treatments of piglets is summarized in Fig. 1.

2.3. Clinical signs of disease

After the first and second *E. coli* application, the behavior of the piglets was observed by scan sampling every hour for the subsequent 8 or 12 h, respectively, in their home pen to determine the presence of clinical signs of disease: (1) decreased attention

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