CHRONIC STRESS AND PITUITARY-ADRENAL FUNCTION IN FEMALE PIGS

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Proefschrift

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STELLINGEN

1. De chronische stress die veroorzaakt wordt door aangebonden huisvesting van varkens, leidt tot langdurige veranderingen op het niveau van de bijnierschors.
Dit proefschrift.

2. Beperking van visuele controle en sociale contacten met soortgenoten speelt een belangrijke rol bij de ontwikkeling van veranderingen in de functie van de bijnierschors bij aangebonden varkens.
Dit proefschrift.

3. De toename in endorfine-activiteit, waargenomen bij aangebonden varkens, kan worden gezien als een vorm van 'omgaan' met chronische stress.
Dit proefschrift.

4. De omstandigheden tijdens de vroege ontwikkeling van een individu zijn op latere leeftijd bepalend voor de gevoeligheid van de hypothalamus-hypofyse-bijnieras voor stimuli uit de omgeving.
M.J. Meaney et al., 1993. Cell. Mol. Neurobiol., 13: 321-347.

5. Het welzijn van een dier is evenredig met het bewust(-)zijn van mens én dier.
M.S. Dawkins, 1993. Through our eyes only? The search for animal consciousness. W.H. Freeman/Spektrum, Oxford.

6. De hardnekkigheid waarmee diverse handboeken *(McDonald, 1989; Hafez, 1993)* het interval 'begin bronst tot ovulatie' bij varkens omschrijven als zijnde constant, staat in schril contrast met de grote variatie in de duur van dit interval, zoals die door diverse auteurs (1,2) is beschreven.
1) F.A. Helmond et al., 1986. In: J.M. Screenan and M.G. Diskin (eds.), Embryonic mortality in farm animals. Martinus Nijhoff Publishers, The Hague, p.119-125. 2) N.M. Soede et al., 1994. J. Reprod. Fert., 101: 633-641.

7. In tegenstelling tot wat wel wordt geclaimd (1,2), zijn lymfocyten hoogstwaarschijnlijk niet in staat om via expressie van een eigen pro-opiomelanocortine-systeem endocriene functies uit te oefenen (3).
1) J.E. Blalock and E.M. Smith, 1985. Fed. Proc. 44:108-111. 2) B.L. Clark et al., 1993. Endocrinology 132: 983-988. 3) A.D. van Woudenberg et al., 1993. Endocrinology 133: 1922-1933.

8. Aangezien de Sm-eiwitten in de splicing-factor U1 snRNP essentiëel zijn voor associatie van U1-specifieke eiwitten, is het aannemelijk dat de Sm-eiwitten in andere snRNP's een vergelijkbare rol spelen.
R.L.H. Nelissen et al., 1994. EMBO J., 13: 4113-4125.

9. Modificatie-gerelateerd functieverlies van het prion-eiwit, dat noodzakelijk is voor normale synaptische signaaloverdracht, kan leiden tot neurodegeneratieve aandoeningen.
J. Collinge et al., 1994. Nature 370: 295-297.

10. De bezuinigingskeuzen van opeenvolgende kabinetten illustreren de tweeslachtigheid van het Nederlandse wetenschapsbeleid: naast de zogenaamde 'stimulansronde' handhaaft men een 'destructiecyclus'.

11. De aard van het commentaar van een 'referee' op een wetenschappelijk manuscript zegt soms meer over de 'referee' dan over de kwaliteit van het desbetreffende werk.

12. Het bedrijven van wetenschap vereist soms meer verbeeldingskracht dan kennis.

13. Het wissen van 'files' is helaas alleen mogelijk op de electronic highway.

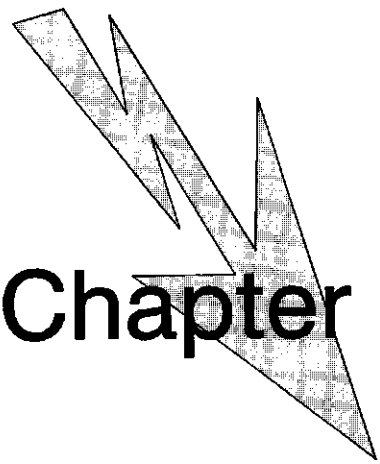
C.J.J.G. Janssens
Chronic stress and pituitary-adrenal function in female pigs
Wageningen, 13 december 1994

"Wie zich in wetenschappelijke problemen verdiept, heeft het niet gemakkelijk. Achter elk vraagstuk vindt hij een nieuwe complicatie; hij schilt een ui waarvan de rokken eindeloos zijn. Geen wonder dat zo iemand wel eens de tranen in de ogen springen."

Godfried Bomans

aan Rob
aan mijn moeder
en
ter nagedachtenis
aan mijn vader

Contents



Chapter 1

Chapter 1

General Introduction

Introduction

Charles Darwin used the term "struggle for life" in his evolution theory to indicate that life is not always easy. He recognized that organisms have to "struggle" in order to survive in a continuously changing and often threatening or dangerous environment. According to Darwin, this is the principle of natural selection, since only the fittest survive [1]. The struggle against pressures which derive from the physical and social environment may be considered as the pivot of the concept of stress [2,3].

Despite changes in environmental conditions many living species (especially mammals) have the ability to keep their internal milieu within fixed limits. This relative stability of the "milieu interieur" was first recognized by Claude Bernard [4] and termed "homeostasis" by Walter Cannon [5]. Homeostasis of physiological systems is secured by specific regulatory systems. Changes in environmental conditions may induce a deviation from certain physiological "set-points" (e.g., body temperature, blood pressure, blood glucose), which are mostly determined by the biological constitution of the organism. Generally, this will lead to activation of homeostatic mechanisms so that internal variables are adjusted towards their "set-point". Activation of regulatory systems in order to counteract disturbances in homeostasis is termed "adaptation".

Once the concept of internal homeostasis was established, research was focussed on the response of an individual, whose biological balance is threatened or disturbed by external factors in such a way that the capacity of homeostatic mechanisms is or may be exceeded. In this type of situations, which generally are termed "stressful", additional non-specific defence reactions (stress responses) are elicited. These consist of concerted behavioural, autonomic and endocrine responses serving to protect the organism against the (potentially) harmful situation.

The Concept of Stress: History

Walter Cannon [6] noticed that a variety of physical or emotional stimuli signalling threat to homeostasis induce not only behavioural but also physiological responses, in which the autonomic nervous system is instrumental. In particular, such stimuli lead to activation of the sympathetic branch of the autonomic nervous system and of the adrenal medulla. This results in enhanced release of catecholamines (adrenaline) into the blood. Circulating catecholamines induce, among other things, increased blood sugar, redistribution of blood from peripheral tissues to brain, heart and skeletal muscles, and increased blood coagulation. Cannon used the term "stress" to describe the status of the animal involved, and interpreted the physiological changes as responses preparing the animal for action. Since the action can be an emergency reaction like a fight or a flight, this response became known as "fight/flight reaction" or "defence reaction".

Selye [7,8] demonstrated with his experiments that a variety of noxious physical stimuli induce a characteristic triade of bodily responses, consisting of adrenal hypertrophy, thymolymphatic involution, and gastric mucosal lesions. Therefore, he conceptualized the stress response as a non-specific response of the body to any demand (usually noxious) or to any stimulus causing an alteration in homeostatic processes. The above-mentioned stereotyped reactions of the body appeared to be independent of the nature of the stressful stimulus, and Selye termed them "General Adaptation Syndrome" (GAS), a syndrome serving as a defence against the stressor [7]. During prolonged exposure to a stressful stimulus three phases of GAS can be distinguished: 1) the alarm reaction, characterized by the initial response of the individual to the stressor; 2) the stage of resistance or adaptation, in which the initial response has diminished or disappeared and the resistance to the stressor has increased; 3) the stage of exhaustion, in which the organism has become biologically incapable of coping with the demands of the continuing stimulus and in which the vulnerability for stressors has increased. During the exhaustion phase pathologies may develop, which may even lead to the death of the individual. In Selye's stress concept, activation of the pituitary-adrenocortical system, evidenced by enlargement of the adrenal cortex and increased blood glucocorticoid concentrations, plays a pivotal role [7].

While Selye emphasized mainly the physical nature of stressors, Mason recognized, in the late sixties, that adrenocortical responses are also induced by purely psychological conditions, and that the majority of physiological stressors

(e.g., bodily injury, disease etc.) also contain psychological components [9,10]. His observation that in fact psychological stimuli are among the most potent activators of the hypothalamic-pituitary-adrenal (HPA) axis led him to conclude that certain psychological components are the common denominator of stressors [10]. According to Mason, stress responses are not limited to activation of the pituitary-adrenocortical system, but rather the entire neuroendocrine system is involved and the pattern of the hormonal response differs from one type of stressor to another. The properties of the stimulus and the appraisal by the individual of it as stressful (signalled by emotions) determine the mode of behavioural and neuroendocrine stress responses that are relatively specific [10].

Around the same time, Weiss provided evidence illustrating the nature of psychological factors involved in stress responses. In experiments with rats, he demonstrated that the ability of the animal to predict or control (prevent) a physical stressor (electric shock), not only reduced the degree of arousal of the neuro-endocrine system but also the occurrence of pathophysiological changes (e.g., gastric erosions). Weiss defined the psychological factors modulating the impact of a stressor as controllability [11] and predictability [12,13]. Thus, according to Weiss, the perception of the aversive stimulus and consequently the degree of control sensed by the organism is the crucial variable determining response, rather than the physical parameters of the stimulus. The process of perceiving and handling of the stressor by the organism is referred to as "coping" [14].

Based on clinical observations and literature studies, Engel [15] postulated the existance of two coping strategies or modes of stress response: 1) the active defence strategy (referred to as "fight/flight" by Cannon) and, 2) a passive or "conservation/withdrawal" strategy (comparable with Selye's "non-specific" stress response). During the active defence strategy, contact with the environment is increased and energy supplies are used either to come into contact with the stressor (fight) or to escape from the stressful stimulus (flight). According to Engel, the sympathetic-adrenomedullary system is preferentially activated during this active mode of stress response, leading to increased levels of circulating glucose, lipids, and sex steroids and increased heart rate, cardiac output and arterial blood pressure. In contrast, the "conservation/withdrawal" mode of stress response serves to conserve energy and is characterized by immobility and suppression of environmentally directed activities. In Engel's view, this passive coping strategy is associated with activation of the hypothalamic-pituitary-adrenocortical system, increased vagus activity,

decreased plasma sex steroid levels and bradycardia [15].

The hypothesis of bimodal strategies was supported by Henry [16], who investigated effects of psychosocial stress in several species. According to his theory, the mode of coping strategy chosen depends on whether the animal perceives itself in control of the situation or not. This is consistent with the ideas of Weiss, mentioned earlier [11-13]. According to Henry, there is a close connection between social status and behavioural and/or physiological stress responses [16]. An individual will more likely chose a "fight/flight" response when it is in control of the situation (e.g., a dominant in a colony) and control is under threat. Under those conditions, the organism still has the ability to alter the situation through some behavioural response. However, with increasing perception that it cannot gain control by an active response and that helplessness may occur, there is a shift from active defence to a passive, non-aggressive, coping strategy. This coping strategy is preferentially chosen by subordinate individuals. In practice, combinations of both coping strategies will be seen.

The Concept of Stress: Current Status

After many decades of stress research, no one has succeeded in providing a stringent definition of the phenomenon stress. Stress may be seen as an integral part of an adaptive biological system [17]. The "stress cascade" consists of three major components: 1) the stimulus input (environmental event); 2) perception and appraisal of the stimulus as a stressor; 3) organization of the stress response (to re-establish physiological balance).

An important establishment is that a(n external) stimulus is a stressor, only when the individual perceives and evaluates it as a (potential) threat to its biological balance [17,18]. As emphasized by Kagan and Levi [19], in this stage the central nervous system (CNS) plays a crucial role in: 1) evaluation of a stimulus as a stressor (comparison of the new information with that already available), and 2) organization of the biological defence against the stressor. Whether a stimulus or a certain external event is indeed perceived as a stressor and leads to a condition of stress depends on various factors. As to that, not only the properties of the stressor itself (intensity, frequency, duration) are important, but also factors like genetic constitution (species, selection line, gender), prior experiences (history of the individual, learning, memory) and the physiological (normal or pathological) and psychological state of the organism at

that particular moment [17,19,20]. All of these factors do not only play an important role in the judgement of the perceived stimulus as a stressor, but also in the choice and organization of the biological defence (see Figure 1).

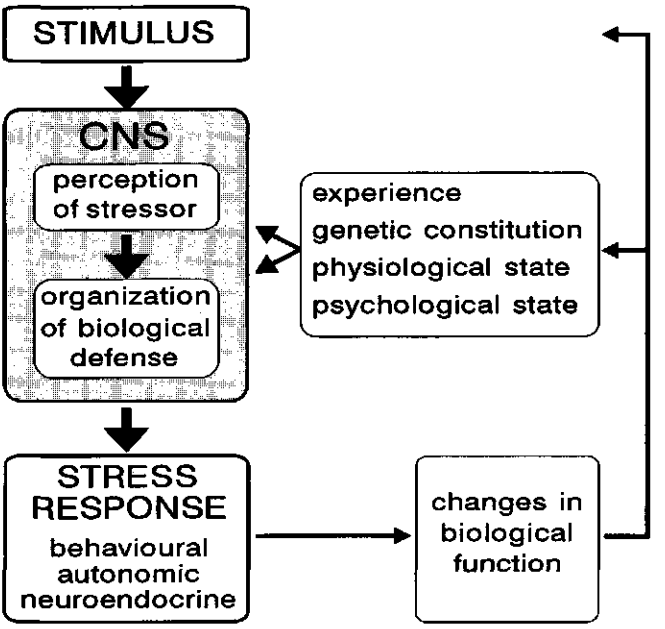


Figure 1. Schematic representation of the response to a potential stressor and factors that influence the response (adapted from Moberg [20]).

The purpose of stress responses is to successfully deal or cope with harmful or threatening situations and to eliminate the source of stress [17]. Generally speaking, three types of biological response systems can be activated by the organism in an attempt to withstand immediate threats to its homeostatic balance, namely behavioural, autonomic, and neuroendocrine systems. Behavioural responses may be used to approach the stressor or to withdraw from it, while autonomic and neuroendocrine changes may alter the physiological machinery, thereby providing the energy needed for such responses. Altered activity of these three biological response systems represents the non-specific component of the stress response. Its specific character is the result of interactions between the environment, the coping strategy, and the

characteristics of the stressor and of the homeostatic system(s) involved. These factors determine the magnitude and the temporal pattern of the behavioural and physiological reactions, which then represent the specific component of the stress response [21]. The stress response induces a change in biological function, thereby affecting future responses directly (by elimination of stressor or withdrawal) or indirectly (experience, change of physiological/psychological state) (see Figure 1).

Regulation of the Hypothalamic-Pituitary-Adrenal System

Since Selye's GAS, the hypothalamic-pituitary-adrenal (HPA) system has played a central role in all stress concepts. Along with the autonomic nervous system, the HPA axis is recognized as the main neuroendocrine effector system, which serves to counteract disturbances in the homeostatic state [2,3]. A schematic diagram of the HPA axis is presented in Figure 2.

The HPA system exhibits three characteristics: 1) it displays a circadian rhythm in basal activity (non-regulated activity), entrained by variables such as light-dark cycle, awake-sleep rhythm and food. In man and in diurnal animals, this rhythm is typified by elevated systemic concentrations of adreno-corticotropic hormone (ACTH) and glucocorticoids in the early morning. These concentrations decrease during the day and reach a nadir during the night. In nocturnal animals the opposite rhythm is displayed [22-24]; 2) the HPA system is activated in response to physical, but in particular to psychological stressors (regulated activity) [9,25]; and 3) both the circadian and the stress-induced activities of the HPA axis are regulated by feedback inhibition by adrenal corticosteroids [26,27]. In addition, the feedback sensitivity of the HPA axis changes in a circadian fashion [26,28].

As represented in Figure 2, four levels of organization in the anatomy of the HPA system can be distinguished: 1) the brain stem, the limbic system and the associated cortical areas of the brain, 2) the hypothalamus, 3) the anterior lobe of the pituitary (adenohypophysis), and 4) the adrenal cortex. When a stimulus is perceived which may be significant for survival, the limbic midbrain system induces an arousal reaction. In the limbic (forebrain) system, the novel situation is compared with stored information and the significance of the stimulus is estimated. If the outcome of this appraisal process is that the stimulus threatens the biological balance, the arousal state will be maintained and a decision with respect to the kind of biological action has to be taken.

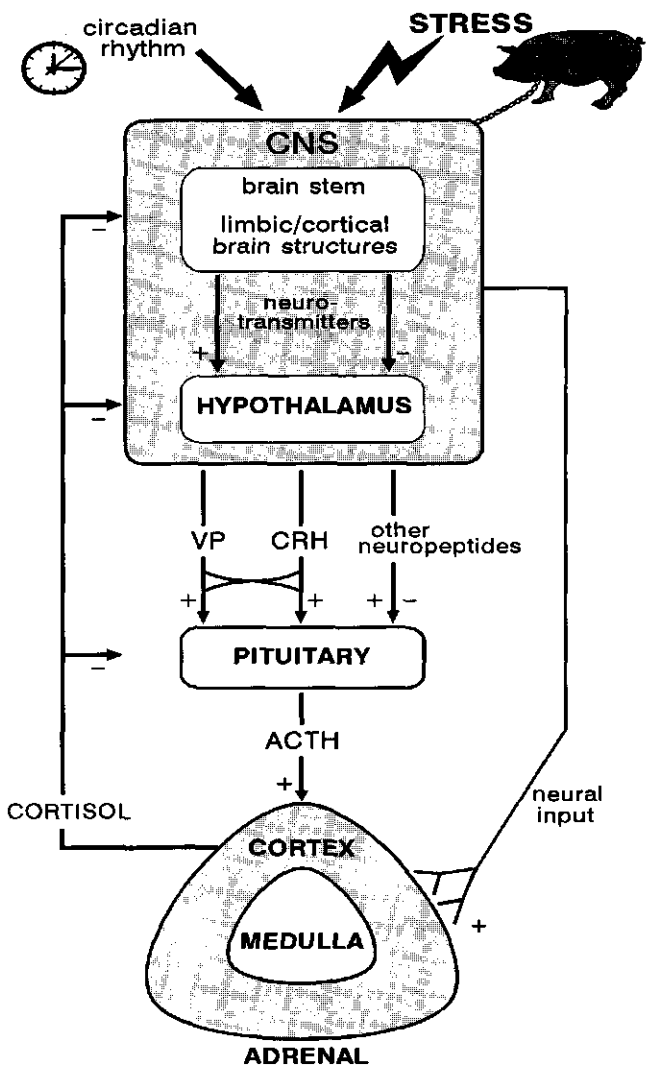


Figure 2. Schematic representation of the hypothalamic-pituitary-adrenocortical axis: The hypothalamus receives stimulatory and inhibitory input from several extrahypothalamic brain areas. The secretion of ACTH from the anterior pituitary is controlled by CRH, vasopressin and other hypothalamic peptides. ACTH stimulates synthesis and secretion of cortisol from the adrenal cortex, which exerts negative feedback on the system. The adrenal cortex also receives neural input, which can modulate the response of the cortex to ACTH [29].

The judgement of the behavioural response (active or passive) to be made takes place in the frontal lobe (limbic system). The amygdala plays a crucial role in the active behavioural "fight/flight" strategy, which is performed to remove the threat and thereby extinguish the arousal reaction. If it is unclear, however, which action must be taken (e.g., in novel or unpredictable situations, or when an adequate response is not possible), the "conservation/withdrawal" strategy is chosen, in which the (septo-)hippocampal system is thought to play an essential role. Characteristic for this strategy is the maintenance of a high level of arousal and alertness, as well as high activity of the HPA system [30].

HPA-activity is thought to be mediated and/or modulated by neurotransmitters such as noradrenaline, adrenaline, serotonin, acetylcholine, and γ-aminobutyric acid [31-35]. Opioid peptides, particularly β-endorphins, may also be important neuromodulators and/or hormones in this respect [36-38]. The effects of opioids on the HPA axis are thought to be mediated by altering the synthesis and/or release of hypothalamic releasing factors, release-inhibiting factors and/or other secretagogues, or by affecting neurotransmitters that can alter these factors [38]. Endogenous opioid systems may modulate basal activity as well as stress-induced activation of the HPA axis [39]. Although some investigators have found that opioids inhibit the activation of the HPA axis in response to stressors, others have found the opposite effect, probably as a consequence of differences in experimental conditions [38,39].

After perception and evaluation of a stimulus as a stressor by the CNS, signals are relayed from the limbic system to the endocrine hypothalamus inducing the release of corticotropin-releasing hormone (CRH). CRH is thought to play a key role in the body's integrated behavioural, autonomic, and neuroendocrine responses to stress. Its existence was demonstrated in the 50s [40,41] and in 1981 it was isolated from ovine hypothalami and characterized as a 41-amino-acid peptide [42]. CRH is generally considered to be the major factor responsible for ACTH release from the anterior pituitary. CRH-immunoreactive cells are widely distributed in the central nervous system [43], and in the periphery [44]. CRH directly involved in ACTH regulation is synthesized in parvocellular cells of the paraventricular nucleus (PVN) [45]. These cells project to the median eminence constituting the link between the CNS and the anterior pituitary gland. In the median eminence, CRH and several other neuropeptides [46] are released from nerve terminals into the hypophysial portal blood and transported to the capillairy bed of the anterior lobe of the pituitary.

In the pituitary, CRH evokes the secretion of 39-amino-acid peptide ACTH

and induces the synthesis of pro-opiomelanocortin (POMC), the ACTH precursor [47]. POMC contains sequences of several biologically active peptide hormones (e.g., ACTH, β-lipotropin, β-endorphin, α-MSH, γ-MSH). In addition to CRH, several other neuropeptides, such as vasopressin, oxytocin, angiotensin II, cholecystokinin, vasoactive intestinal peptide, endogenous opioids, and biogenic amines, are able to induce ACTH secretion from the anterior pituitary [48-50]. Among these substances, which may act to stimulate the pituitary in their own right, or potentiate the action of CRH [48,49,51], vasopressin is considered to be the most important peptidergic factor regulating ACTH secretion. After secretion into the systemic circulation, ACTH, in its turn, acts on adrenocortical cells of the zona fasciculata and reticularis to initiate synthesis and release of glucocorticoids. In addition, steroidogenesis is mediated by splanchnic nerve input to the adrenal, which may modulate cortical sensitivity to ACTH, probably in part by changes in blood flow [29] (see Figure 2). Glucocorticoids are 21-carbon steroids derived from cholesterol. In man and in the pig, cortisol is the predominantly secreted glucocorticoid, whereas in rodents it is corticosterone. Once secreted from the adrenal gland, most of the glucocorticoids (approximately 75-80%) bind with high affinity to a specific corticosteroid-binding α_2-globulin known either as corticosteroid binding globulin or transcortin, and an additional 15% binds to albumin. Only a small amount (5-10%) of the glucocorticoids is unbound. The free hormone is the biologically active form, which is taken up by target cells by passive diffusion [52]. Glucocorticoids stimulate catabolic processes and suppress anabolic processes in order to mobilize glucose from various storage sites. They increase blood glucose levels by stimulation of hepatic glyconeogenesis and inhibition of glucose uptake by peripheral tissues. Also lipolysis and protein catabolism are induced, thereby increasing the level of free fatty acids and the availability of amino acids as substrates for gluconeogenesis [53,54]. Glucocorticoids synergize with the sympathetic nervous system in increasing heart rate and blood pressure, as a means to deliver the mobilized energy substrates to the muscles more rapidly. Besides mobilization of energy substrates, they also inhibit many functions in the immune system [53], and processes such as growth and reproduction [55].

Long-term elevated levels of glucocorticoids are potentially harmful to the organism, as will be considered later. Therefore, it is important to "turn off" the HPA stress response, whether or not the stressor is eliminated. This is achieved by glucocorticoid feedback inhibition of ACTH secretion. Additional feedback loops include the inhibitory effects of ACTH, β-endorphin and CRH on the hypothalamic CRH neuron [56]. Circulating glucocorticoids control their own

synthesis and release by negative feedback at the level of the anterior pituitary, the PVN and extrahypothalamic regions, particularly the hippocampus [26,57-59]. Three major time frames of negative feedback mediated by cortisol can be distinguished [59]: 1) the fast feedback of cortisol (within minutes after stress exposure) on the multisynaptic control of ACTH release in the median eminence; 2) the intermediate feedback (minutes to hours after stress exposure) involving the gene mediated intracellular protein synthesis, probably prominent in steroid regulated neuronal communication in limbic structures; 3) the slow feedback (hours to days after stress exposure) that involves gene mediated blockade of CRH and vasopressin expression in the PVN and POMC expression in pituitary corticotropic cells. In the CNS there are two steroid receptor types involved in glucocorticoid feedback on HPA functioning with different localization and steroid affinity: 1) high-affinity ($K_d \sim 0.5$ nM), type I "mineralocorticoid receptors", which bind aldosterone, corticosterone, cortisol and deoxy-corticosterone equally *in vitro* and to a greater extent than dexamethasone. Type I receptors are predominantly localized in the extrahypothalamic limbic system, in particular the hippocampus, and possibly in certain brain stem motor nuclei [26,60]. These receptors are thought to control basal ACTH secretion by control of CRH and vasopressin expression in the PVN [61] and they are involved in the maintenance of circadian rhythmicity of the HPA axis; 2) low affinity ($K_d \sim 3.0$ nM), type II "glucocorticoid receptors", which preferentially bind dexamethasone > cortisol > corticosterone > deoxycorticosterone > aldosterone. Type II receptors are widely distributed throughout the brain (limbic system, PVN, supra-optic nucleus, cerebral cortex, most brain stem monoaminergic nuclei [26,60]) and have been postulated to exert major control of stress-induced ACTH secretion [60,61].

Chronic Stress

In order to investigate HPA functioning, a wide variety of acute stressors has been used, including cold, ether, footshock, toxic substrate and restraint [62]. The common end-point of the acute stress-induced activation of the HPA axis is an increase in circulating ACTH and consequently increased secretion of glucocorticoids, as described earlier. Most studies concerned with chronic stress have been performed in rats, generally using repeated exposures of the animals to the same stressful stimulus, e.g., cold, restraint or intermittent footshock [63-65]. There is ample evidence that chronic stress may lead to changes in

synthesis and storage of hormones at different levels of the HPA axis, as well as in sensitivity of each tissue to the secretagogues activating it [62,66,67]. Increases in adrenal weight and in the capacity of the adrenal gland to respond to ACTH have been demonstrated in chronically stressed rats [68]. In response to a novel stimulus chronically stressed animals may show greater pituitary-adrenocortical responses than control animals, despite elevated levels of plasma glucocorticoids [68-70]. This suggests the occurrence of a lowered sensitivity of the HPA axis to endogenous glucocorticoid feedback signals under these conditions, so that further responsiveness of the system is maintained [71]. Studies by Sapolsky et al. [72] have demonstrated that under conditions of chronic stress hippocampal glucocorticoid receptors are down-regulated, which may partly explain the insensitivity for glucocorticoid feedback. Several studies in the rat suggest that the release of CRH and AVP into the portal circulation is increased during chronic stress [73,74]. In addition, it has been reported that repeated activation of the HPA system leads to plastic changes in hypothalamic CRH neurons, resulting in increased AVP stores and increased AVP expression in CRH-containing vesicles in the median eminence, leading to an increased ratio of secreted AVP/CRH [73,75-77]. Since vasopressinergic stimulation of ACTH secretion is less sensitive to glucocorticoid feedback than CRH [78], increased AVP in the secreted cocktail will act to maintain pituitary responsiveness even when circulating glucocorticoid levels are quite high.

Hypersecretion of Glucocorticosteroids

Although biological stress responses help the animal to cope with the stressor they also have their price, since they involve the redirection of energy to those physiological activities that need highest priority. Long-term stress responses, therefore, may be biologically costly to the organism. For most "daily-life" stressors, the biological cost of coping is relatively small, because the change in biological function is capable of eliminating the threat. However, when the stressor is severe and persistent (chronic stress) or when the organism experiences a series of stressors, the change in biological function may represent a serious biological cost and may lead to the development of a pathological state [20]. Secretion of glucocorticoids, represents a major chemical response of the body to certain stressors, and plays an important role in the adaptation of the organism to the stressful situation. Glucocorticoid actions are essentially catabolic, they increase the availability of energy

substrates. This process involves the deferment of energy-consuming activities, which are not of immediate benefit for the stress response, e.g., growth and reproduction. Moreover, glucocorticoids suppress immune system activity. Munck and co-workers, however, proposed that in stress, elevated levels of glucocorticoids serve to suppress the body's normal defences against stress, and thus to prevent them from overreacting and causing damage to the organism [53]. Although elevated glucocorticoid levels in the acute phase may be an effective means of keeping check on a potentially dangerous endogenous activity, hypersecretion may be harmful to the individual if it continues for too long. Chronic hypersecretion of glucocorticosteroids is thought to contribute to e.g., hypertension, hyperlipidaemia, hypercholesterolaemia, muscle atrophy, impaired growth and tissue repair, reproductive failure and immunosuppression [54]. In addition, Sapolsky and his co-workers proposed that sustained exposure to glucocorticoids may initially down-regulate glucocorticoid receptors in the hippocampus and, ultimately, cause hippocampal neuron loss [72,79].

Tethered Housing of Female Pigs

Studies on the effects of stressors on HPA regulation are almost exclusively conducted in male (or ovariectomized female) animals, to avoid the confounding influence of the variations in neuroendocrine activity associated with the female reproductive cycle. As a consequence, information on the activity of the HPA axis during the oestrous cycle, and the relevance of stress-induced changes in HPA function for reproductive performance in female animals, is largely lacking. In the experiments described in this thesis, prolonged tethered housing of female pigs was used as a model for chronic stress. In this chronic restraint stress paradigm, pigs are tethered by a neck-chain connected to a 50-cm heavy gauge chain which is attached to the floor. Tethered housing of pigs is not unusual in modern pig breeding farms. This housing system, in which sows are tethered by a neck or breast tether, has been introduced in the past three decades when there was a change towards intensive housing of livestock. The most important advantages of this housing system are the lower costs as a result of saving on both space and labour. Since only little floor space is available for each animal, tethered housing largely impairs movements and performance of natural behaviour of pigs.

Tethering itself has been recognized as an acute stressor for the animals. When first tethered, the animals fiercely resist, scream loudly and try to escape

by pulling and biting the chain. Besides this breakout behaviour [80], physiological reactions such as an increased heart rate and increased plasma levels of ACTH, β-endorphin and cortisol, characteristic for acute stress, are also displayed. Several hours after this initial stress response, the sows seem to calm down and heart rate and hormone concentrations gradually return to pre-stress levels.

During prolonged tethered housing the animals are subjected chronically to this aversive housing situation, deprived of their main behavioural tools to exert control over their environment. As already emphazised by Weiss, loss of control is generally recognized as a common denominator of stressful conditions [11,12,81]. Along this line, it can be reasoned that long-term tethered housing imposes a condition of chronic stress on the animals. This contention is supported by observations that behavioural (development of stereotypies, i.e. invariant patterns of behaviour performed repeatedly and persistently, having no obvious goal or function) [80,82-84], reproductive [85,86] and cardiovascular disturbances [83] are frequent in tethered pigs.

This Thesis...

In this thesis, the effects of chronic stress on the (re)activity of the HPA axis are evaluated in cyclic nulliparous female pigs (=gilts). To this end, the basal as well as the challenge-induced activity of the HPA system were investigated under loose housing conditions ("non-stress", control) and during tethered housing (chronic stress) of gilts.

Evidence that hormones of the HPA axis (CRH, ACTH, glucocorticoids) may influence the activity of the hypothalamic-pituitary-gonadal axis at several levels exists [87,88]. The aim of the study, described in Chapter 2, was to gain more insight in the relation between HPA activity and reproductive hormones in the female pig and to investigate whether this is affected by chronic stress. In a longitudinal study, the plasma cortisol pattern throughout the 21-day oestrous cycle of gilts was determined in relation to the profiles of luteinizing hormone (LH), progesterone and prolactin. Hormone profiles of one oestrous cycle of loose housing were compared with profiles of one oestrous cycle after 3-6 weeks of tethered housing.

Chapter 2 revealed significantly elevated basal cortisol concentrations after 3-6 weeks of tethered housing. Based on these results, a subsequent study

(presented in Chapter 3) was performed, using a prolonged period of chronic stress (up to 18 weeks of tethered housing) to establish whether the hypercortisolaemia is of transient nature. We also investigated whether the effect of chronic stress on plasma cortisol concentrations depends on the time of day, since evidence that the adrenocortical response to stress varies in a circadian fashion is increasing [89]. Therefore, cortisol concentrations were measured in the morning and the early evening of the 24-hour adrenocortical rhythm.

The aim of the longitudinal study, presented in Chapter 4, was to investigate whether chronic stress (up to 20 weeks) induces changes in functioning of the adrenal cortex, which may underlie the hypercortisolaemia, described in Chapter 2 and Chapter 3. To this end, the reactivity of the adrenal cortex to exogenous ACTH was determined before and during tethered housing. Additionally, possible effects of factors other than restraint (social/housing conditions) on the adrenocortical response to ACTH were investigated.

In Chapter 5, the role of endogenous opioid mechanisms in pituitary-adrenocortical responsiveness to acute stress (nose-sling), before and after exposure of the gilts to chronic stress, was studied. Plasma ACTH and cortisol responses were monitored after 15 minutes of challenge with a nose-sling during loose housing and after 10-11 weeks of tethered housing. The involvement of endogenous opioid peptides in these responses was tested using the opioid receptor antagonist naloxone.

In Chapter 6, we investigated the effect of 10-13 weeks of tethered housing on pituitary-adrenocortical responsiveness to exogenous CRH and/or lysine vasopressin (LVP). Three experiments were conducted to evaluate the potency of CRH and LVP to stimulate ACTH and cortisol release in gilts and the effect of chronic stress on this release. In Experiment 1 and 2, a dose-response curve was made for ovine CRH and LVP. In Experiment 3, the pituitary-adrenocortical response to LVP and oCRH, administered singly or in combination, was determined in loose housed and in tethered gilts.

In Chapter 7, the major findings of the Chapters 2-6 are summarized and discussed.

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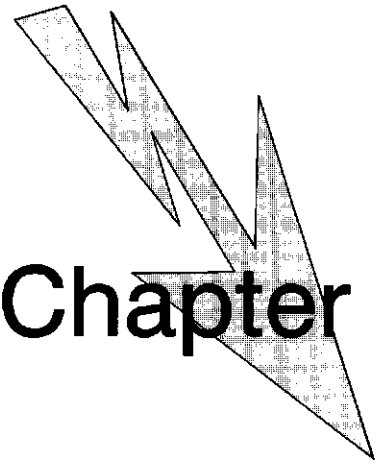

Chapter 2

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Chapter 2

Plasma levels of cortisol, prolactin, and reproductive hormones during the oestrous cycle in the pig: Effects of chronic stress

Summary

Plasma concentrations of cortisol were investigated in relation to those of luteinizing hormone (LH), progesterone, and prolactin throughout the oestrous cycle in the pig, before and during chronic restraint stress. After a period of loose housing in individual pens, gilts were tethered by a neck-chain (chronic stress). Blood samples for hormone determination were collected through a permanent jugular vein catheter at least twice daily. Plasma cortisol levels showed a marked rhythmicity throughout the oestrous cycle; significantly increased concentrations at 4 days prior to the LH peak, a small increase during the LH surge and rather stable levels during the luteal phase. A positive correlation was found between cortisol and prolactin levels. We suggest that these changes in cortisol and prolactin concentrations reflect alterations in the sensitivity of the pigs for "daily life stress" throughout the oestrous cycle. Three to 6 weeks of chronic stress significantly increased cortisol (10.7 ± 0.4 vs. 8.2 ± 0.4 ng/ml; $P<0.001$) and prolactin levels (10.2 ± 0.3 vs. 8.4 ± 0.3 ng/ml; mean ± SEM; $P<0.001$) throughout the oestrous cycle. Progesterone levels were significantly decreased (10.0 ± 0.3 vs. 10.8 ± 0.3 ng/ml; $P<0.05$), whereas neither LH nor the length of the oestrous cycle was affected by tethered housing. We conclude that these changes are due to chronic stress, since no such alterations in hormone levels were found in control gilts that were housed loose throughout the entire experiment.

Introduction

The concentration of glucocorticoid hormones in plasma is widely used as an indicator of stress [1]. Indeed, acute stressors invariably activate the hypothalamic-pituitary-adrenocortical (HPA) axis, and thereby lead to increased secretion of glucocorticoids from the adrenal cortex, both in males and females [2]. Studies on the effects of stressors on HPA regulation are almost exclusively conducted in male (or ovariectomized female) animals, to avoid possible variations in neuroendocrine activity associated with the female reproductive cycle. As a consequence, information on the activity of the HPA axis during the oestrous cycle in female animals is lacking.

Evidence exists, indicating considerable cross-talk between the HPA and the hypothalamic-pituitary-gonadal (HPG) axes [3,4]. It has been shown that hormones of the HPA axis (corticotropin-releasing hormone (CRH), adrenocorticotropic hormone (ACTH), glucocorticoids) can influence HPG activity at several levels [3-12]. Administration of glucocorticoids in particular can disrupt cyclicity in females depending on the phase of the reproductive cycle [7,11]. In addition, glucocorticoids are thought to play an important role in the adverse effects of stress on female reproductive functions [3-12].

In the pig, the secretory patterns of pituitary gonadotropins, prolactin and ovarian hormones during the 21-day oestrous cycle have been well characterized [13-19]. Concerning plasma cortisol levels in this respect, the available data indicate fluctuations during the oestrous cycle, suggesting the existence of one or more episodes of enhanced secretion [7,15]. Sofar, however, relationships between the secretory patterns of cortisol and those of reproductive hormones during the oestrous cycle have not been firmly established in the pig.

The purpose of the present study therefore was to determine the plasma cortisol pattern throughout the oestrous cycle of the pig in relation to the profiles of luteinizing hormone (LH), progesterone and prolactin, and in addition to investigate whether and how these hormonal profiles are affected by chronic stress. Tethered housing was used to induce chronic stress, since it restrains the animals and leads to persisting disturbances in behaviour [20-23] and in endocrine [24,25] and cardiovascular systems [22].

Materials and Methods

Experimental Design

The present study was conducted at the Animal Facilities of the Department of Human and Animal Physiology, Wageningen, The Netherlands. One experimental and one control group were run. For both groups the complete experiment was performed during a period of 3 months in which all of the gilts showed 4 complete oestrous cycles (cycle length approximately 21 days), labelled A,B,C and D in Figure 1.

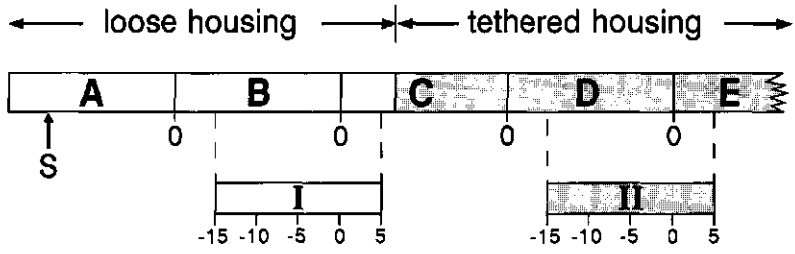

Figure 1. Experimental Design.
A,B,C,D,E, consecutive oestrous cycles; S, surgical implantation of jugular vein catheter; 0, day of LH surge; I, experimental oestrous cycle (from day −15 till day +5) before tethering (="non-stress" oestrous cycle); II, experimental oestrous cycle (from day −15 till day +5) during tethered housing (=chronic stress oestrous cycle). For the gilts of the control group a similar experimental design was employed except for tethering.

During the luteal phase of oestrous cycle A all gilts were surgically fitted with a permanent jugular vein catheter. Blood samples were collected daily at 1000 h and 1800 h throughout the experiment to determine plasma hormone levels. From 3 days before until 3 days after oestrus additional blood samples were collected at 0000, 0600, 1200 and 1400 h, in order to monitor the preovulatory LH surge (day 0) in gilts of the experimental group. During the luteal phase of oestrous cycle C, the gilts of the experimental group were tethered by a neck-chain (in the same individual pens) and kept tethered for a further period of 6 weeks.

To facilitate the analysis and the evaluation of interrelationships between

the several hormones and the effect of chronic stress on hormone profiles, the hormone profiles of the individual animals were synchronized with respect to the day of the LH surge (day 0). In that manner it was possible to eliminate the individual differences in oestrous cycle length. The experimental oestrous cycles I and II thus obtained ranged from 15 days prior to until 5 days after the LH surge (see Figure 1). To investigate the effect of chronic stress on hormone patterns throughout the oestrous cycle, the hormone patterns of oestrous cycle I (before tethering; "non-stress" oestrous cycle) were compared with those of oestrous cycle II (during tethered housing; chronic stress oestrous cycle). In gilts of the control group both oestrous cycles I and II represent "non-stress" oestrous cycles.

The experiments were approved by the Committee on Animal Care and Use of the Agricultural University of Wageningen.

Animals and Housing

Fourteen cyclic crossbred gilts (Great Yorkshire x British Landrace, Pig Improvement Company, United Kingdom), which had shown two or more normal oestrous cycles (18-22 days) prior to the experimental period were used. Ambient temperature ranged from 15 to 25°C and lights were on between 0730 h and 1900 h (and in addition between 0530 h and 0630 h, and between 2330 h and 0030 h for blood sampling around oestrus). Twice a day (at 0900 h and 1700 h) the animals were fed 1 kg of pelleted dry sow ration (12.2 MJ metabolizable energy per kg, 15.4% crude protein), which was delivered by hand. To prevent the animals from associating the presence of people with feeding, they were conditioned with a bell signal that always preceded the feeding. Water was available *ad libitum* through a nipple drinker. Oestrus detection was routinely carried out twice daily (0830 h and 1630 h) with a vasectomized boar, and by inspection of external oestrous signs. Gilts were considered to be in oestrus when showing a standing response to the boar and(or) showing vulval swelling and redness.

Experimental Group. Seven gilts (176 ± 18 kg body weight; mean ± SD) were housed loose in individual pens of 5.5 m^2. The floor was solid concrete, covered with straw, except for a 2.5 m^2 dunging area at the rear of the pens. After a 7-week period of loose housing, the gilts were tethered by the neck with a 50-cm heavy-gauge chain, connected to the fence at the front, at days 6-12 after the LH surge, i.e. during the luteal phase of oestrous cycle C (see Figure 1). They remained tethered in the same pens for a further period of 6 weeks.

Control Group. Seven gilts (109 ± 3 kg body weight; mean ± SD) were housed loose during the entire experiment in individual pens, similar to those used for the gilts of the experimental group. Due to cannula damage, two gilts of the control group could no longer be used for blood sampling at some stage during the experiment, and therefore did not yield data. These animals, however, remained in the housing system for the rest of the experiment.

Surgery

In order to allow repeated blood sampling without disturbing the gilts, an indwelling catheter was surgically implanted into the jugular vein as described previously [26]. All surgeries were carried out under sterile conditions and under general anaesthesia. In order to protect the cannula from damage, which was externalized between the scapulae, the gilts were equipped with a harness to which they had been habituated during the week before cannulation. The harness (23 cm x 20 cm, polyvinyl chloride with nylon; Bizon Chemie, The Netherlands) was fixed at the back of the animals with belts tied around the chest. From 3 days before surgery till 3 days after surgery, all gilts were treated once daily with antibiotics (0.2 ml/kg body weight Engemycine 10%, i.m. bolus injection; Mycofarm Nederland BV, The Netherlands), containing oxytetracycline (100 mg/ml). At least 10 days were allowed for the animals to recover from surgery and anaesthesia.

Cannula patency was maintained by flushing thrice weekly and filling the cannula with sterile heparinized physiological saline (25 IU/ml 0.9% saline; Leo Pharmaceutical Products, The Netherlands) when not in use.

Blood Collection and Analyses

Preceding the experimental oestrous cycle I, the gilts were often handled and habituated to the blood collection procedure which has been described previously [26]. Blood samples (approximately 10 ml) were collected in ice-cooled polypropylene tubes, containing 100 μl EDTA-solution (144 mg EDTA/ml saline; Titriplex®III, Merck Nederland BV, The Netherlands). They were immediately placed on ice and subsequently centrifuged at 2000 x *g* for 15 min at 4°C. Plasma was collected and stored at −20°C until hormone analysis. Plasma was assayed by validated immunoassay for cortisol [26] in 1000 h and 1800 h samples, and for progesterone [27] and prolactin [28] in 1000 h samples taken on each day of the oestrous cycles I and II. LH [13] was determined by radioimmunoassay in plasma samples taken around oestrus. Intra- and inter-assay coefficients of variation were 4.5 and 11.2%, 7.6 and 12.7%, 6.9 and

12.3%, and 13.8 and 15.6%, respectively, for cortisol, progesterone, prolactin and LH.

Analysis of Data and Statistics

The effects of oestrous cycle (oestrous cycle I vs. II), day of the oestrous cycle (according to the numbering in Figure 1), and their interaction on plasma concentrations of cortisol, LH, progesterone and prolactin were tested by an *F*-test using a split-plot model, procedure GLM [29]. In this model, the values of hormone levels within gilts were taken as repeated measurements and the analyses were performed separately for the control and experimental group:

$$Y_{ijk} = \mu + e_{1i} + C_j + D_k + CxD_{jk} + e_{2ijk}$$

where Y_{ijk} = hormone value of gilts i during oestrous cycle j at day k of the oestrous cycle; μ = overall mean; e_{1i} = error term 1, which represents the random effect of gilts (i = 1,..,7); C_j = fixed effect of oestrous cycle j (j = 1,2); D_k = fixed effect of day k of the oestrous cycle (k = 1,..,21); CxD_{jk} = the interaction effect between oestrous cycle j and day k of the oestrous cycle; e_{2ijk} = error term 2. The effect of oestrous cycle, day of the oestrous cycle and their interaction were tested against error term 2.

Oestrous cycle length (of oestrous cycles B and D in Figure 1) was determined as the interval between two consecutive preovulatory LH surges. Wilcoxon Matched Pairs Signed Ranks test (two-way) was used to determine the effect of tethered housing on oestrous cycle length. Pearson correlation coefficients between the different hormone concentrations were also calculated. Both tests were performed using the SPSS statistical package [30]. All results are expressed as the mean ± SEM.

Results

Oestrous Cycle Length

No significant difference was found between the length of the oestrous cycle before tethering (20.6 ± 0.4 days; oestrous cycle B in Figure 1) and after 2-5 weeks of tethered housing (21.4 ± 0.9 days; oestrous cycle D in Figure 1).

Also no significant effect of tethered housing was found on the time interval between the onset of oestrus and the LH surge. In oestrous cycle I of the experimental group, the LH surge occurred in 2 of 7 animals at the first day of standing oestrus, and in the remaining 5 animals at the second day of standing oestrus. In oestrous cycle II, the LH surge occurred in 1 of 7 animals at one day prior to standing oestrus, in 3 animals at the first, in 2 animals at the second, and in 1 animal at the third day of standing oestrus. Both in the loose housed and in the tethered-housed situation all gilts showed normal external oestrous signs and oestrous behaviour. In the gilts of the control group the length of oestrous cycle B (20.4 ± 0.7 days) was not significantly different from oestrous cycle D (20.4 ± 0.5 days).

Plasma Hormone Levels

Plasma levels of progesterone, cortisol and prolactin during oestrous cycle I and oestrous cycle II of gilts of the experimental and the control groups are shown in Figure 2 and Figure 3, respectively.

LH. Basal plasma LH levels (determined in the blood samples taken at 2-6 h intervals on days −3, −2, 2 and 3) were relatively constant. Approximately 10 hours after the onset of the LH surge maximal LH levels (day 0) were reached. Neither basal nor peak levels of LH were significantly affected by 3-6 weeks of tethered housing. Mean basal and mean peak levels of LH were 2.3 ± 1.7 ng/ml and 8.7 ± 0.8 ng/ml in oestrous cycle I and 2.2 ± 1.4 ng/ml and 7.7 ± 1.0 ng/ml in oestrous cycle II (Figure 2).

Progesterone. During the luteal phase of the oestrous cycle (days 2 until 5 and days −15 until −7 in Figure 2A) a rise in plasma progesterone levels was observed, reaching a maximum at 7 days prior to the LH surge. Thereafter the progesterone concentration rapidly declined and returned to basal (< 1 ng/ml) on day −3 (Figure 2A). Tethered housing significantly decreased mean plasma progesterone levels from 10.8 ± 0.3 ng/ml (oestrous cycle I) to 10.0 ± 0.3 ng/ml (oestrous cycle II) ($P<0.05$). The profile of progesterone throughout the oestrous cycle, however, remained unaltered ($P=0.135$).

In gilts of the control group the progesterone profile throughout the oestrous cycle was similar to that found in oestrous cycle I of the experimental group (Figure 3A). No significant difference was found between mean progesterone levels of oestrous cycle I (15.7 ± 0.8 ng/ml) and oestrous cycle II (14.0 ± 0.6 ng/ml), and there was no interaction between oestrous cycle and day of the oestrous cycle ($P=0.871$).

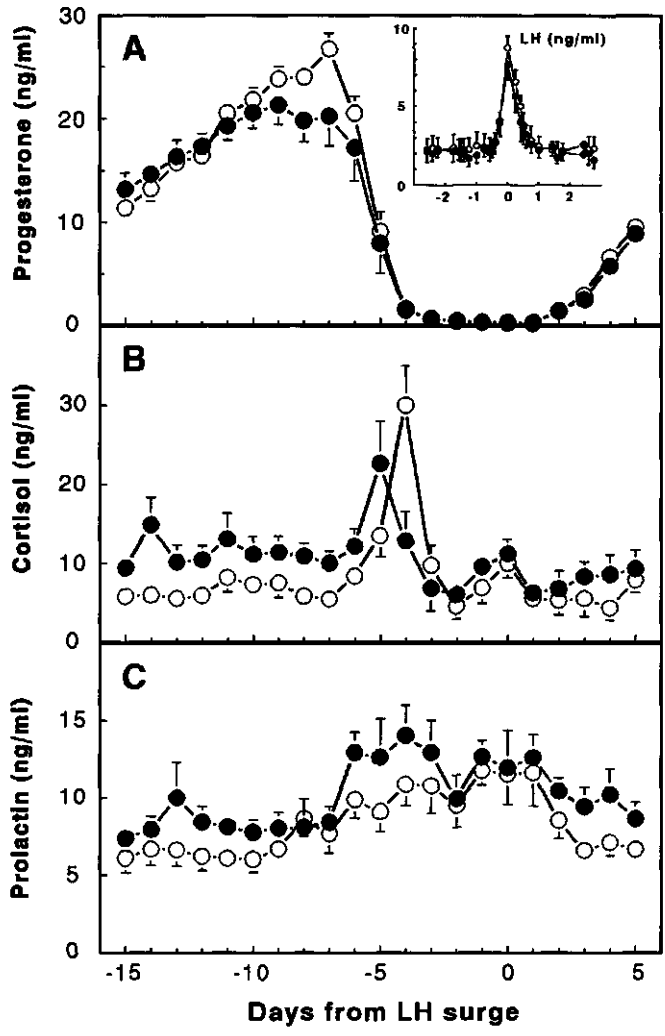

Figure 2. Plasma levels of progesterone (1000 h; Figure 2A), cortisol (average of 1000 h and 1800 h; Figure 2B) and prolactin (1000 h; Figure 2C) throughout oestrous cycle I (—○—; "non-stress" oestrous cycle) and oestrous cycle II (—●—; chronic stress oestrous cycle) in gilts of the experimental group (mean ± SEM; n = 7). The values are plotted by taking the day of the preovulatory LH surge as day 0 (Insert: plasma LH concentrations from day −2 through day 2).

Cortisol. The profile of daily mean cortisol levels (as determined from 1000 h and 1800 h values) during the oestrous cycle is shown in Figure 2B. A marked rhythmicity was observed both before and during tethered housing ($P<0.001$). During the luteal phase of the oestrous cycle, when progesterone levels were rising (days 2 until 5 and days -15 until -7 in Figure 2), rather stable plasma cortisol levels were found. A discrete cortisol peak, however, occurred during the early follicular phase, coinciding with the decline of plasma progesterone levels. In oestrous cycle I this cortisol peak was observed at 4 days prior to the plasma LH surge. A modest but consistent rise (although not statistically significant) occurred in both untethered and tethered animals during the late follicular phase at the time of the LH surge.

Tethered housing led to a significant increase in daily mean cortisol levels during oestrous cycle II (10.7 ± 0.4 ng/ml) as compared with pretethering levels (oestrous cycle I; 8.2 ± 0.4 ng/ml; $P<0.001$; Figure 2B). The chronic stress-induced hypercortisolemia was particularly evident during the luteal phase of the oestrous cycle (10.5 ± 0.6 ng/ml during oestrous cycle II vs. 6.2 ± 0.4 ng/ml during oestrous cycle I). A significant interaction was found between oestrous cycle and day of the oestrous cycle ($P<0.001$). This interaction was induced by the plasma cortisol peak, which occurred at day -5 of oestrous cycle II, whereas in oestrous cycle I it occurred at day -4.

A similar profile of cortisol concentrations throughout the oestrous cycle was found in the control gilts (see Figure 3B). In these gilts significantly increased cortisol levels ($P<0.001$) were found at day -4 of oestrous cycle I (28.3 ± 6.2 ng/ml) and at days -4 and 0 of oestrous cycle II (25.5 ± 3.5 ng/ml and 21.7 ± 3.0 ng/ml). No significant difference was found between daily mean cortisol concentrations, measured during oestrous cycles I and II (11.9 ± 0.5 ng/ml and 11.7 ± 0.5 ng/ml, respectively). In control gilts, there was no interaction between oestrous cycle and day of the oestrous cycle ($P=0.973$).

Prolactin. Figure 2C shows the profile of plasma prolactin (samples taken at 1000 h) during the oestrous cycle. Significantly increased prolactin levels were found during both the early (day -4) and the late (day -1 until day 1) follicular phase, compared with the luteal phase of the oestrous cycle when prolactin concentrations remained rather stable ($P<0.001$). The time interval between the first and the second prolactin peak ranged from 3 to 5 days in the individual animals. Tethered housing led to a significant increase in mean prolactin levels throughout the oestrous cycle from 8.4 ± 0.3 ng/ml to 10.2 ± 0.3 ng/ml ($P<0.001$).

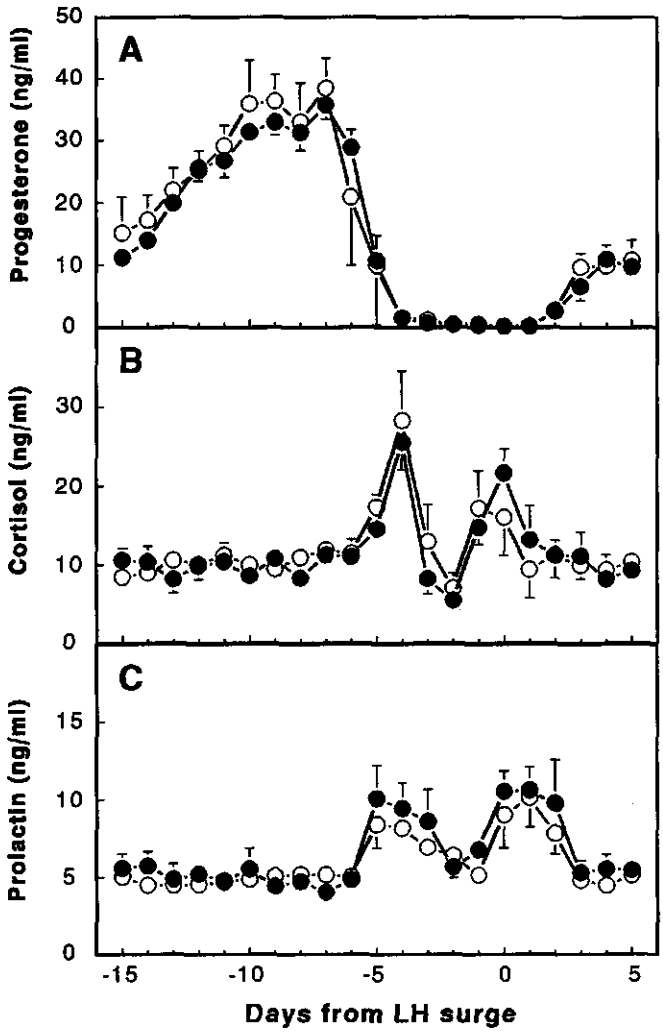

Figure 3. Plasma levels of progesterone (1000 h; Figure 3A), cortisol (average of 1000 h and 1800 h; Figure 3B) and prolactin (1000 h; Figure 3C) *throughout oestrous cycle I (—○—) and oestrous cycle II (—●—) in gilts of* the control group (mean ± SEM; n = 5). The values are plotted by taking the day of the preovulatory LH surge as day 0.

In the control gilts, in which the profile of plasma prolactin during the oestrous cycle was similar to that found in the gilts of the experimental group, mean prolactin levels throughout oestrous cycle I (6.0 ± 0.2 ng/ml) were not significantly different from levels measured during oestrous cycle II (6.5 ± 0.2 ng/ml). Both in gilts of the experimental group ($P = 0.993$) and control group ($P = 0.997$), corresponding prolactin profiles were observed for oestrous cycle I and oestrous cycle II.

Correlations between Hormones

Plasma cortisol and plasma prolactin showed similar profiles throughout the oestrous cycle with increased levels around day −4 and day 0. Both for the gilts of the experimental and the control group, a positive correlation was found between plasma prolactin and cortisol concentrations throughout the oestrous cycle (experimental group, $r = 0.238$, $P < 0.001$; control group, $r = 0.248$, $P < 0.001$). A negative correlation was found between prolactin and progesterone (experimental group, $r = -0.318$, $P < 0.001$; control group, $r = -0.427$, $P < 0.001$).

Discussion

The patterns of plasma cortisol, LH, progesterone and prolactin were investigated throughout the oestrous cycle of pigs during loose and tethered housing. The profiles of LH, progesterone and prolactin, which were observed during the "non-stress" control oestrous cycle in this study, are in accordance with data previously reported [13-19]. In addition, this study demonstrates that plasma cortisol levels also show cyclic variation throughout the oestrous cycle of the pig (similar to prolactin), with rather stable levels during the luteal phase and a cortisol peak in the early as well as in the late follicular phase of the oestrous cycle. McGuire *et al.* [15] also reported cyclic variations of cortisol during the oestrous cycle of the pig. They observed two broad peaks of plasma corticosteroids, one following the decrease of plasma progesterone and the second 7-14 days later, but there was a marked variation both within and between the animals. Hennessy *et al.* [7] did not find any relationship between plasma cortisol levels and progesterone concentrations or the stage of the oestrous cycle in the pig.

One possible explanation for the observed discrepancies between these data and ours may be the small number of animals used and infrequent blood

sampling (one blood sample per day) employed by Hennessy *et al.* [7] and McGuire *et al.* [15]. Additionally, plasma cortisol levels vary considerably within individual animals, due to pulsatility of pituitary ACTH secretion and slight changes in environmental conditions over time [31]. Therefore, we have used daily mean cortisol values, rather than values based on single plasma samples. Low sampling frequency may also partly explain why data on cortisol levels during the female cycle obtained in other species [32-35] have yielded conflicting results.

A better explanation for the discrepancies among the findings of the former studies [7,15] and the present study may relate to differences in the analysis of the data. In our studies, we have used the day of the LH surge to synchronize the hormone profiles of the animals, whereas other investigators used either oestrous signs [7] or the first day at which progesterone levels were undetectable [15]. We and others [13,17,18,27] have found that the interval between the onset of oestrus and the LH surge may vary from hours to about 2 days. Since our data indicate that the time interval between the cortisol peaks, in the early and late follicular phase, and the LH surge is quite stable, the LH surge appears to be a better criterion for the synchronization of hormone profiles than oestrous behaviour.

An interesting observation in our study was the positive correlation between plasma cortisol and prolactin levels by similar sequential profiles of these hormones throughout the oestrous cycle. Coinciding with the peaks of plasma cortisol and prolactin in the follicular phase of the oestrous cycle, elevated levels of plasma ACTH (although this was only determined in two animals) were observed. There is ample evidence, that the release of prolactin from the pituitary is stimulated during various forms of stress [36-41]. We suggest that the elevated plasma hormone levels during the early and late follicular phase of the oestrous cycle reflect an increased sensitivity of the animal to "daily life stressors" during those days.

Tethered housing did not induce an alteration in the rhythmicity of the hormones during the oestrous cycle. The only exception is the change in timing of the cortisol peak from 4 to 5 days before the LH surge. The basal cortisol and prolactin levels throughout the oestrous cycle were significantly increased during tethered housing. This is in accord with other findings [20-25] indicating that tethered housing, which in fact restrains the animal, induces chronic stress, and with the notion that chronic stress can lead to sustained elevation of corticosteroid levels [2,42]. Our observations (data not shown) that the plasma levels of ß-endorphin (a peptide hormone that is cosecreted with ACTH from the

pituitary corticotroph cell [43]) were not affected by tethered housing, suggest that the increase in cortisol was not caused by increased ACTH release from the pituitary. An alternative explanation may be an increase in the sensitivity of the adrenal cortex to ACTH. Indeed, there are several reports of elevated glucocorticoid levels that are not associated with significant increases in plasma ACTH [2,44,45]. There is increasing evidence that classical neurotransmitters or neuropeptides (e.g., CRH) may directly innervate the adrenal cortex, thereby controlling the secretory activity of the adrenal cortex, independent of pituitary ACTH release [44,46-48].

It has been reported in previous studies that tethered housing of pigs may induce reproductive disorders such as reduced rate of oestrus detection and reduced pregnancy rate [25,49]. Indeed we have found in the present study that 3-6 weeks of tethered housing induced a decrease of plasma progesterone concentrations during the oestrous cycle. No effects of 3-6 weeks of chronic stress, however, were found on plasma LH concentrations, oestrous behaviour or on the length of the oestrous cycle. Whether more prolonged tethered housing can disrupt LH secretion or ovarian function and thereby adversely affect reproductive performance in the pig needs to be investigated.

In summary, the present study demonstrates a coordinated rhythmicity of cortisol and prolactin during the oestrous cycle of the female pig. Three to six weeks of tethered housing (chronic restraint stress) increased plasma cortisol and prolactin levels and decreased progesterone levels throughout the oestrous cycle, whereas LH concentrations and length of the oestrous cycle were not affected.

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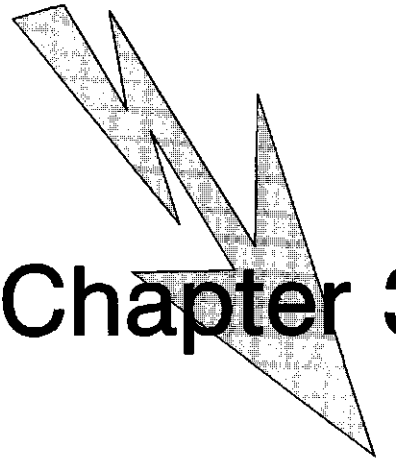
Chapter 3

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Domestic Animal Endocrinology (accepted for publication)

Chapter 3

The effect of chronic stress on plasma cortisol concentrations in cyclic female pigs depends on the time of day

Summary

The influence of tethered housing (a condition of chronic stress) on morning and evening basal plasma cortisol levels was investigated in a longitudinal study in cyclic female nulliparous pigs (gilts). After a period of loose housing in individual pens ("non-stress" oestrous cycles), six cannulated gilts were tethered by a neck-chain and tethered housed for a period of 20 weeks (chronic stress oestrous cycles). Blood was sampled twice daily (1000 h and 1800 h) for cortisol determination. Plasma cortisol levels showed a diurnal rhythm with significantly higher levels at 1000 h than at 1800 h. Tethered housing induced a significant increase in the 1800 h plasma cortisol concentrations during the first 3 oestrous cycles after tethering, whereas the 1000 h plasma cortisol concentrations did not change throughout the experimental period. During the period of increased 1800 h levels, cortisol was still released in a circadian fashion, albeit the rhythm was flattened. In control gilts, housed loose during the entire experimental period, plasma cortisol levels at 1000 h as well as at 1800 h remained unaltered, with 1000 h cortisol concentrations being significantly higher than 1800 h concentrations. Therefore, possible effects of the experimental procedure or age-related effects could be excluded. These data indicate that in tethered gilts the chronic stress-induced hypercortisolaemia is of transient nature, suggesting adaptive changes in regulation of the hypothalamic-pituitary-adrenocortical (HPA) axis. In addition, the data reveal circadian differences in the effect of chronic stress on HPA-function.

Introduction

Circadian fluctuations in circulating glucocorticoid levels have been reported for many species, including pigs. In diurnal species such as the human and the pig, plasma glucocorticoid levels are high in the early morning and then decline, reaching a nadir in the evening. In nocturnal animals an inverse pattern in the glucocorticoid level has been observed [1-7]. Circadian patterns of circulating cortisol are thought to result primarily from changes in plasma adrenocorticotropic hormone (ACTH) in response to circadian changes in corticotropin-releasing hormone (CRH) release [4,8-11]. Since the amplitude of the ACTH rhythm is relatively small compared to the cortisol rhythm, this suggests the involvement of other factors that mediate the glucocorticoid release, e.g., the sensitivity of the adrenal cortex [12,13], or neural input to the adrenal gland [14]. Indeed, it has been found in the rat that the sensitivity of the adrenal cortex to ACTH also varies in a circadian fashion [4,10,15-17].

Several reports have shown circadian differences in adrenocortical responses to acute stressors in a variety of species [14,18-22]. The effects of chronic stress on glucocorticoid secretion and circadian rhythmicity in the female pig have not been firmly established. Evidence exists that chronic stress can lead to changes in the regulation of the hypothalamic-pituitary-adrenal (HPA) axis [23,24]. We have found that 3-6 weeks of tethered housing, a condition of chronic stress, induces chronic hypercortisolaemia in cyclic gilts [25], indicating long-term changes in the regulation of the pituitary-adrenal axis during chronic stress. In a subsequent study we have shown that tethered housing of gilts is associated with an adaptational increase in adrenocortical steroidogenic capacity, which may underlie this hypercortisolaemia [26].

The purpose of the present study was to investigate the effect of chronic stress on adrenocortical function in cyclic female pigs, in the morning and the early evening of the 24-hour adrenocortical rhythm. Prolonged tethered housing was used to induce chronic stress, since it has been reported to lead to persisting disturbances in behaviour [27-29] and in endocrine [6,30] and cardiovascular systems [28].

Materials and Methods

Experimental Design

A longitudinal design was employed to investigate changes in basal plasma

cortisol levels induced by long-term tethered housing (chronic stress) in individual cyclic gilts. The complete experiment was performed during a period of 7 months in which all gilts showed 9 complete oestrous cycles (of circa 21 days each) (see Figure 1). The gilts were surgically fitted with a permanent jugular vein catheter for blood sampling (oestrous cycle A, Figure 1). One experimental and one control group were run. The gilts in the experimental group were first housed loose in individual pens (oestrous cycles A and EL1). Then they were tethered by a neck-chain (oestrous cycle B, Figure 1) and kept tethered for a period of 20 weeks.

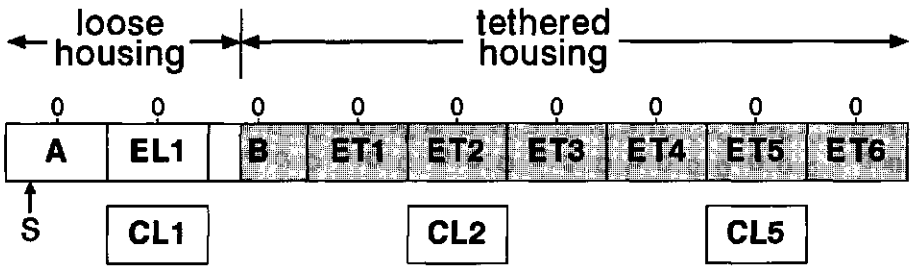

Figure 1. Experimental Design.
0, day of LH surge; S, surgical implantation of jugular vein catheter; T, tethering; A,EL1,B,ET1,ET2,ET3,ET4,ET5,ET6, consecutive oestrous cycles: A, oestrous cycle before tethering; EL1, experimental oestrous cycle before tethering (= control oestrous cycle); B, oestrous cycle of tethering; ET1 to ET6, 6 experimental oestrous cycles during tethered housing (= chronic stress oestrous cycles); CL1,CL2,CL5, experimental oestrous cycles of the gilts of the control group matching oestrous cycles EL1, ET2 and ET5, respectively.

Blood samples were collected twice daily at 1000 h and 1800 h during the entire experimental period. Plasma cortisol was determined by radioimmunoassay. Plasma LH was determined in the 1000 h and 1800 h samples taken around oestrus in order to localize the preovulatory LH surge for analysis. The day on which the highest LH value was found was defined as day 0. Cortisol profiles of the individual gilts were synchronized with respect to day 0. In that manner it was possible to overcome the individual differences in oestrous cycle length. For analyses of the data, estrous cycles were defined to range from day 10 before through day 10 after the LH surge (total 21 days). Basal plasma

cortisol levels of one oestrous cycle of loose housing (EL1, "non-stress" oestrous cycle) were compared with 6 oestrous cycles during tethered housing (ET1 to ET6, chronic stress oestrous cycles, Figure 1). During oestrous cycles EL1 and ET2, additional blood samples were collected around oestrus at 0000, 0600 and 1200 h. Control gilts were housed loose for the duration of the experiment under conditions that were otherwise similar to those applied for the experimental group, except for tethering. These animals were of the same age as the experimental group and experiments were performed in exactly the same season of the year (i.e. February-July). In the control gilts blood was sampled frequently during the entire experimental period. Cortisol was only determined in the 1000 h and 1800 h blood samples of 3 complete oestrous cycles CL1, CL2 and CL5, respectively, matching oestrous cycles EL1, ET2 and ET5 of the experimental group (Figure 1). The remaining samples were used for other determinations. For all gilts, oestrous signs were monitored during the entire experiment, and interventions (surgery and tethering) were performed during the luteal phase of the oestrous cycle.

The experiments were approved by the Committee on Animal Care and Use of the Agricultural University of Wageningen.

Animals and Housing

Twelve crossbred cyclic gilts (Great Yorkshire x British Landrace, Pig Improvement Company, United Kingdom) which had shown two or more normal oestrous cycles (18 to 22 days) were selected. The body weight of the gilts at the start of the experiment was 114.0 ± 6.9 kg (mean ± SD). The gilts were randomly assigned to control and experimental stress groups, with 6 gilts each.

All gilts were housed loose in individual pens of 5 to 6 m^2. The floor was solid concrete and covered with deep straw, except for a 2.5 m^2 dunging area at the rear of the pens. After two complete oestrous cycles of loose housing, the gilts of the experimental group were restrained by a neck-tether attached to a 50 cm heavy gauge chain connected to the fence at the front. They were housed tethered in the same pens without straw but with a small quantity of wood shavings placed in the area behind the gilts to help to keep them clean. A metal plate (1.1 m x 0.8 m; length x height) was placed beside the gilts as a partition to reduce floor space to 2.0 m x 0.6 m and thereby further restricted their movements.

For all housing conditions lights were on between 0730 h and 1900 h and ambient temperature ranged from 15 to 25°C. Twice a day (at 0900 h and 1700 h) the animals were fed 1 kg of pelleted dry sow ration (12.2 MJ

metabolizable energy per kg, 15.4% crude protein), which was delivered by hand. To prevent the gilts from associating the presence of people with feeding, they were conditioned with a bell signal that always preceded feeding. Water was available *ad libitum* through a nipple drinker. Oestrus detection was routinely done twice daily (at 0830 h and 1630 h) with a vasectomized boar and by external oestrous signs. Gilts were considered to be in oestrus when they showed a standing response to the boar and/or showing vulval swelling and redness.

Surgery

To allow repeated blood sampling without disturbing the gilts, they were surgically fitted with a permanent jugular vein catheter (polyvinyl chloride, 1.5 mm i.d., 2.1 mm o.d.; Rubber BV, The Netherlands) as described previously [26]. Surgery was performed under sterile conditions and under general anaesthesia either by i.v. metomidate-azaperone infusion (Hypnodil-Stresnil®; Janssen Pharmaceutica BV, The Netherlands) or inhalation of O_2/N_2O, enflurane (Ethrane®: Abott BV, The Netherlands). In order to protect the cannula, which was externalized in between the scapulae, from damage the gilts were equipped with a harness to which they had been habituated during the week before cannulation. The harness (23 cm x 20 cm, polyvinyl chloride with nylon; Bizon Chemie, The Netherlands) was fixed at the back of the animals with belts around the chest. At least 10 days were allowed for the gilts to recover from surgery and anaesthesia.

Cannula patency was maintained by flushing thrice weekly. When not in use, the cannulae were filled with sterile heparinized physiological saline (25 IU heparin/ml of 0.9% saline; Leo Pharmaceutical Products, The Netherlands).

Blood Sampling

Prior to the experimental period, all gilts were frequently handled and habituated to the blood collection procedure that has been described previously [26]. Blood samples (approximately 10 ml) were immediately transferred to ice-cooled polypropylene tubes, containing 100 μl of EDTA-solution (144 mg of EDTA/ml of saline; Titriplex®III, Merck Nederland BV, The Netherlands), immediately placed on ice and subsequently centrifuged at 2000 x *g* for 10 min at 4°C. Plasma was collected and stored at −20°C until hormone analysis was completed.

Radioimmunoassay of Plasma Hormones

Cortisol. Plasma concentrations of cortisol were measured in duplicate in unextracted 50 μl samples using a single-antibody radioimmunoassay (RIA) technique as previously described [26]. A specific rabbit antiserum (K7348; kindly donated by Prof. Dr. T.J. Benraad, Nijmegen, The Netherlands), raised against 11ß,17,21-trihydroxy-4-pregnene-3,20-dione-3-CMO-BSA (Cortisol-Bovine Serum Albumin-conjugate), was used. The main cross-reacting steroids were 21-desoxycortisol (72%), cortisone (59%), prednisolone (53%), 11-desoxycortisol (43%), corticosterone (10%), progesterone (2.3%), oestradiol-17ß, dexamethasone, and triamcinolone acetonide (all < 1%). Cortisol (H 5885; Sigma Chemical, St. Louis MO) was used as a standard and [1,2,6,7-^{3}H]cortisol (TRK407, specific activity 80.5 Ci/mmol, Amersham, U.K.) was used as the tracer. The antiserum was used in a final dilution of 1:96,250, which yielded approximately 30% specific binding of the labeled hormone after incubation. The sensitivity of the cortisol assay was 0.5 ng/ml at 90% B/B_0. The intra-assay coefficient of variation was 7.7%, and the inter-assay coefficient of variation was 11.6%.

LH. Plasma concentrations of LH were measured by a double-antibody RIA as previously described by Niswender *et al.* [31], using porcine LH (LER 786-3, potency 0.65 x NIH-LH-SI; obtained from Dr. L.E. Reichert Jr., New York, USA) as a standard and for radioiodination (specific activity 130.6 μCi/μg). Anti-ovine LH 614 IV (also obtained from Dr. L.E. Reichert) was used in a 1:180,000 final dilution that gave an initial binding of the labeled hormone of approximately 30%. Goat anti-rabbit immunoglobulin was used as the second antibody. The sensitivity of the assay was 0.7 ng/ml at 90% B/B_0. The intra-assay coefficient of variation was 13.8% and the inter-assay coefficient of variation was 15.6%.

Analysis of Data and Statistics

Due to cannula damage that occurred in the course of the experiment, one gilt of the control group did not yield sufficient blood samples and was therefore excluded from further analysis. This animal, however, remained in the housing system for the rest of the experiment.

All plasma cortisol data were normalized by log transformation before statistical analysis. To examine the effect of tethered housing on plasma cortisol concentrations (at 1000 h and 1800 h), data were analyzed by analysis of variance using the General Linear Models procedure of SAS [32], followed by Tukey's t *post-hoc* test. For the analysis of variance, oestrous cycle and day of the oestrous cycle (nested within oestrous cycle) were used as main factors.

Time was nested within day within oestrous cycle. Based on previous findings [25] the period of the oestrous cycle during which progesterone levels were rising (>1 ng/ml) was defined as luteal phase of the oestrous cycle. This period included days 10 until 7 before the LH surge and days 2 until 10 after the LH surge. Differences in mean plasma cortisol concentrations during the luteal phase of the oestrous cycle among gilts of the control (oestrous cycles CL1, CL2, and CL5) and the experimental group (oestrous cycles EL1, ET2, and ET5) were analyzed by Student's *t*-test (two-tailed) using SPSS statistical package [33]. Differences in ratio of 1000 h and 1800 h plasma cortisol levels measured during the luteal phase of those oestrous cycles were also analyzed by Student's *t*-test. In all analyses $P<0.05$ was used as the criterion for rejecting the null hypothesis.

Data are presented as mean ± SEM of the untransformed data, with significance symbols derived from statistical analysis of the log transformed data.

Results

Both during the loose housed and the subsequent tethered housed condition morning and evening basal plasma cortisol concentrations were measured during the entire oestrous cycle. Mean 1000 h and 1800 h plasma cortisol levels of oestrous cycle EL1 ("non-stress" oestrous cycle; loose housing) and oestrous cycles ET1 to ET6 (chronic stress oestrous cycles; tethered housing) are displayed in Figure 2. During loose housing plasma cortisol levels were significantly higher at 1000 h (17.9 ± 3.0 ng/ml) than at 1800 h (7.2 ± 1.5 ng/ml; $P<0.01$), reflecting a circadian rhythmicity in cortisol secretion. The difference in 1000 h and 1800 h cortisol concentrations was significant during the entire period of tethered housing ($P<0.05$). Tethered housing led to significantly increased 1800 h plasma cortisol levels during the first 3 oestrous cycles after tethering (ET1 to ET3) as compared with pretethering values (oestrous cycle EL1) in the same gilts ($P<0.05$; indicated by the asterisks). During the oestrous cycles ET4 to ET6, 1800 h cortisol levels were no longer significantly different from pretethering levels. No significant effects of long-term tethered housing were found on 1000 h basal plasma cortisol levels.

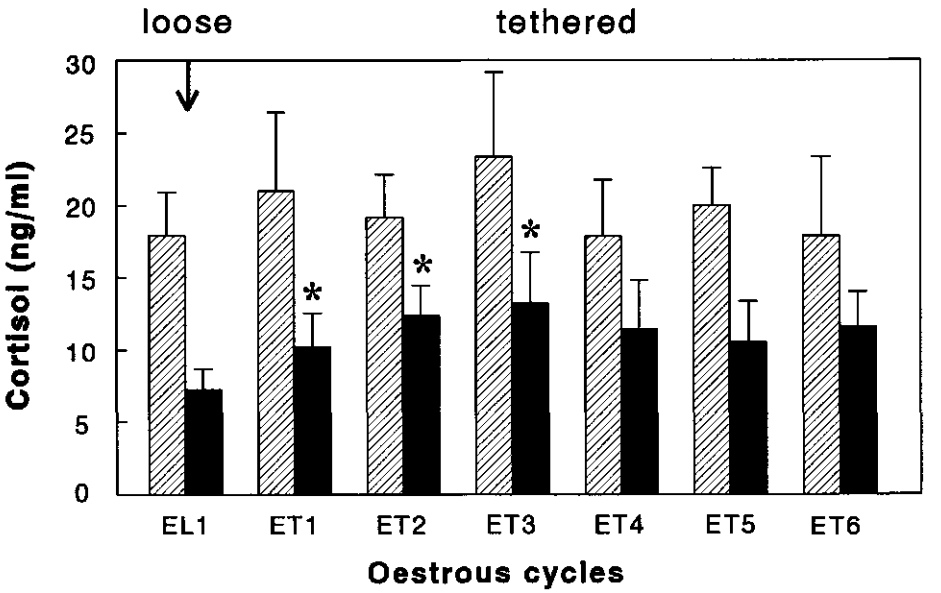

Figure 2. The effect of chronic stress on 1000 h and 1800 h plasma cortisol concentrations during the oestrous cycle of the pig.
Mean plasma cortisol concentrations per oestrous cycle during loose housing (EL1; "non-stress" oestrous cycle) and during 6 consecutive oestrous cycles during tethered housing (ET1 to ET6; chronic stress oestrous cycles) in gilts of the experimental group (mean + SEM; n=6). Hatched bars represent 1000 h cortisol concentrations and closed bars represent 1800 h concentrations. Asterisks indicate significant difference ($P<0.05$) when compared with loose housing at that particular time.

In the present study as well as in a previous experiment [25] cortisol levels remained rather stable during the luteal phase of the oestrous cycle compared with other parts of the oestrous cycle. As a result, the hypercortisolaemia, induced by tethered housing, was most evident during the luteal phase of the oestrous cycle. Therefore, this particular phase of the oestrous cycle was used to investigate possible effects of increased age, individual housing and blood sampling. Figure 3 shows the mean 1000 h (Figure 3A) and 1800 h (Figure 3B) plasma cortisol levels during the luteal phase (days 10 until 7 before the LH surge and days 2 until 10 after the LH surge) of 3 oestrous cycles of both the control gilts (oestrous cycles CL1, CL2, and CL5) and the gilts of the

experimental group (oestrous cycles EL1, ET2, and ET5). In all gilts 1000 h cortisol levels were significantly higher than 1800 h levels during the luteal phase of the oestrous cycle (oestrous cycle CL1, CL2, CL5, EL1 and ET5; $P<0.01$; oestrous cycle ET2; $P<0.05$), also reflecting a diurnal rhythm in cortisol secretion.

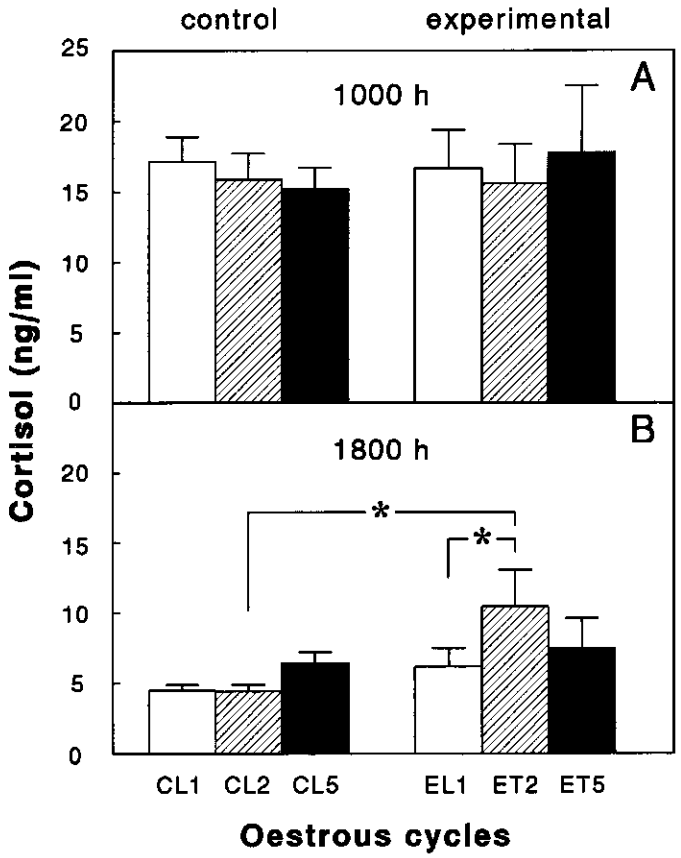

Figure 3. Plasma cortisol concentrations in loose and tethered housed gilts.
Mean plasma cortisol concentrations per luteal phase of the oestrous cycle (mean + SEM) (1000 h; Figure 3A; 1800 h; Figure 3B) of 3 oestrous cycles in gilts of the control group ($n=5$) and in gilts of the experimental group ($n=6$). Open bars represent mean (luteal) cortisol levels of oestrous cycles CL1 and EL1, hatched bars represent oestrous cycles CL2 and ET2, and closed bars represent oestrous cycles CL5 and ET5, respectively. Asterisks indicate significant difference ($P<0.05$).

Whereas significantly increased 1800 h plasma cortisol levels were found during the luteal phase of oestrous cycle ET2 in the tethered housed gilts compared with oestrous cycle EL1 (Figure 3B, hatched bar; $P<0.05$), 1800 h plasma cortisol levels in the gilts of the control group remained unaltered throughout the entire experimental period (Figure 3B). During the luteal phase of ET5, 1800 h cortisol concentrations were not significantly different from EL1 and ET2 (Figure 3B). Both in gilts of the experimental and control groups no significant changes were found in the 1000 h plasma cortisol levels throughout the whole experimental period (Figure 3A). The increased 1800 h plasma cortisol levels during oestrous cycle ET2 resulted in a significantly lower cortisol ratio (1000 h to 1800 h; 1.6 ± 0.2) as compared with oestrous cycle EL1 (2.8 ± 0.5;

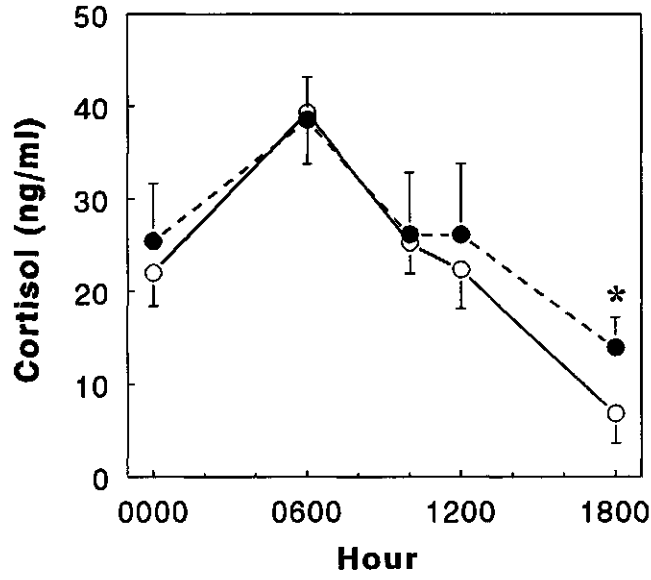

Figure 4. The effect of chronic stress on diurnal variation in plasma cortisol concentrations in gilts.
Plasma cortisol concentrations (mean ± SEM, n=6) at 0000, 0600, 1000, 1200, and 1800 h during oestrous cycle EL1 ("non-stress" oestrous cycle; —○—) and oestrous cycle ET2 (chronic stress oestrous cycle; ---●---) in gilts of the experimental group (average diurnal rhythm of 3 days: one day before the LH surge until one day after the LH surge). The asterisk indicates significant difference ($P<0.05$).

$P<0.05$), reflecting a flattening of the diurnal rhythm of cortisol secretion in this period. No significant difference was found between the ratio 1000 h/1800 h cortisol of oestrous cycle ET5 (2.3 ± 0.4) on the one hand, and oestrous cycle EL1 and ET2 on the other.

When plasma cortisol levels of oestrous cycles CL1, CL2 and CL5 (control group) were compared with oestrous cycles EL1, ET2, and ET5 (experimental group) respectively, significant difference ($P<0.05$; Student's t test) was found only between 1800 h plasma cortisol levels of oestrous cycles CL2 and ET2 (indicated by the asterisk in Figure 3B).

We investigated whether the increased evening cortisol levels during tethered housing reflected a shift in diurnal cortisol secretion instead of a flattening of the diurnal cortisol rhythm. Therefore, cortisol was determined in plasma samples taken around oestrus, when blood was sampled more frequently, during oestrous cycles EL1 and ET2 (when chronic stress-induced changes are most evident). Figure 4 shows the average diurnal cortisol profile of one day before the LH surge until one day after the LH surge. During this period of the oestrous cycle significantly ($P<0.05$) greater 1800 h plasma cortisol concentrations were found during oestrous cycle ET2 compared with EL1. Figure 4 demonstrates that tethered housing did not induce a shift in diurnal cortisol secretion, but that the diurnal rhythm was slightly flattened.

Discussion

Plasma cortisol concentrations were significantly greater in the morning (1000 h) than in the afternoon (1800 h), reflecting a circadian variation in adrenocortical activity, which is in accordance with data previously reported for the pig [1,2,5-7]. In the present study, tethered housing induced a significant increase in 1800 h plasma cortisol levels during the first 3 oestrous cycles after tethering, whereas 1000 h cortisol levels were not affected, indicating diurnal differences in the adrenocortical response to stress. After 11 weeks of tethered housing the hypercortisolaemia was no longer evident, suggesting adaptive changes in HPA-regulation attributed to chronic stress.

During tethered housing of pigs, the animals are largely restricted in their movements and behavioural performance, and experience a loss of control over their environment [34,35]. Tethered pigs often develop symptoms of chronic stress such as behavioural [27,29,35,36], reproductive [30] and cardiovascular [28] disturbances. Indeed, we observed that the gilts of the experimental group

performed stereotypies during tethered housing (although we did not quantify behavioural parameters in our study), whereas these behaviours were not displayed by the gilts of the control group. Long-term tethered housing thus imposes a condition of chronic stress on the pigs. It has been demonstrated in a variety of species that chronic or repeated stress can induce long-term changes in the regulation of the HPA axis. Repeated exposure to stressors can produce increases in adrenocortical function, as evidenced by increased basal plasma corticosteroid concentrations, or increased adrenal weight [23,24,37-40]. Indeed we have found in a previous study that exposure of gilts to 3-6 weeks of tethered housing results in sustained elevated basal plasma cortisol concentrations [25], indicating long-term changes in the regulation of the pituitary-adrenal axis. In the present study we found that the chronic stress-induced hypercortisolaemia was only present during the evening (in the 1800 h samples). This agrees with findings of Martí *et al.* [41], who reported increased corticosterone levels during the circadian trough in rats exposed to chronic intermittent immobilization stress. In contrast to our results, Becker and colleagues reported increased morning concentrations of cortisol in tethered pigs, whereas the evening cortisol levels remained unaltered [6]. The discrepancies between these data and ours may partly depend on the animals used for the experiments. Becker *et al.* [6] used ovariectomized gilts in their experiments, while we used intact cyclic gilts. The effect of ovarian steroids, such as oestradiol, has been suggested by Brann. In his model a stimulatory effect of oestradiol on ACTH secretion is suggested [42]. In addition or alternatively, differences in feeding patterns between both studies may explain the observed discrepancies. In the experiment described by Becker and co-workers [6], feeding (once daily) coincided with collection of the morning sample for cortisol determination. It can be reasoned that in tethered pigs, which are chronically deprived of their main behavioural tools to exert control over their environment, the impact of predictable events such as feeding had increased, leading to the elevated morning cortisol concentrations in that experiment.

In control gilts, blood was collected during 3 complete oestrous cycles but 1000 h and 1800 h plasma cortisol concentrations were measured only during the luteal phase of the oestrous cycle. During this particular phase of the oestrous cycle, when cortisol levels remain rather stable, chronic stress-induced hypercortisolaemia is most evident [25]. In gilts of the control group, which were housed loose in individual pens for the duration of the experiment, cortisol levels remained unaltered throughout the experimental period (oestrous cycles

CL1, CL2 and CL5). Therefore, the increase in 1800 h cortisol levels observed in tethered gilts is probably not related to effects induced by repeated manipulation (e.g., blood sampling), to individual housing per se, or to changes in adrenocortical function that are known to occur with increasing age [40,43,44]. Thus, we can conclude that the increased evening plasma cortisol levels observed in the tethered housed gilts, indeed resulted from the stress caused by tethered housing. In addition, Figure 4 demonstrates that the increase in 1800 h cortisol concentrations, which led to flattening of diurnal adrenocortical rhythmicity, cannot be attributed to a chronic stress-induced shift in diurnal cortisol secretion.

The exact mechanisms that are involved in the circadian variation in the adrenocortical response to chronic stress are still unclear. In the rat there is evidence suggesting that the sensitivity of the adrenal cortex to ACTH varies in a circadian fashion [4,10,15-17]. Armario *et al.* [39] showed an altered ACTH/corticosterone ratio in chronically stressed rats, which could be explained by a sensitisation of the adrenal cortex to endogenous ACTH released during stress. This accords with the results reported by Martí *et al.* [41], who showed that the increased corticosterone levels during chronic intermittent stress were not accompanied by changes in ACTH levels. Neither in the rat nor in the pig diurnal variation in the chronic stress-induced corticosteroid levels can be explained by changes in metabolic clearance rate of corticosteroids [5,41]. Although ACTH is generally considered the most important factor in the control of glucocorticoid secretion, it has been reported that neural inputs at the level of the adrenal gland can influence the sensitivity of the adrenal cortex to ACTH [12-14,45,46]. It has been suggested that endogenous CRH enhances the adrenal response to ACTH, possibly by a synergistic action of CRH and ACTH on adrenal blood flow [12,47]. Although this has only been described for the rat, neural induced changes in adrenocortical sensitivity may in part underlie the reduction in amplitude between morning and evening cortisol concentrations in gilts, occurring during tethered housing.

In conclusion, tethered housing induced a long-term increase in evening but not in morning plasma cortisol concentrations in cyclic gilts, resulting in a flattened diurnal rhythmicity of cortisol. Evening cortisol levels and diurnal adrenocortical rhythmicity were no longer significantly different from the loose housing condition after 11 weeks of chronic stress. The transient nature of the hypercortisolaemia may indicate development of adaptational changes in the HPA system (at the level of the adrenal cortex) during chronic restraint stress.

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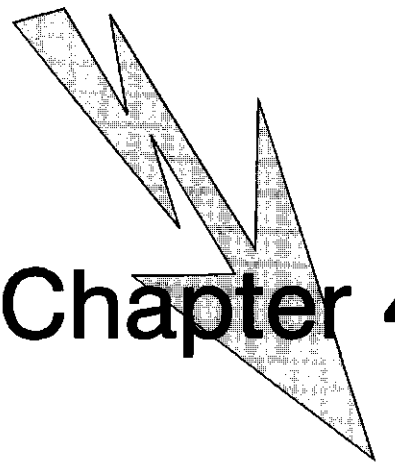

Chapter 4

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Chapter 4

Increased cortisol response to exogenous adrenocorticotropic hormone in chronically stressed pigs: Influence of housing conditions

Summary

In a longitudinal experiment, the influence of tethered housing (a condition of chronic stress) on the reactivity of the adrenal cortex to exogenous ACTH was investigated in gilts. To that end, the plasma cortisol response to synthetic ACTH (1-24; 10 μg/kg of BW; i.v. bolus injection via a permanent catheter) was determined before and after prolonged tethered housing. Two systems for tethered housing were used, one being more restrictive than the other with regard to possibilities for visual and tactile contacts with conspecifics and visual control over the environment. The ACTH treatment induced a marked, transient plasma cortisol response in all gilts studied, irrespective of their housing conditions. Long-term tethered housing increased the ACTH-induced cortisol response. Possible effects of the experimental procedure or age-related effects could be excluded, because in control gilts, which were housed loose during the entire experimental period, the cortisol response to ACTH remained unaltered. The chronic stress-induced increase in the ACTH-induced cortisol response was considerably more pronounced and persistent in gilts that were deprived of possibilities for social contacts with conspecifics and visual control over the environment than in gilts with such possibilities. These data indicate that in tethered gilts adaptational changes occur at the level of the adrenal cortex that affect the ACTH-induced adrenocortical response. In addition, not only physical restraint but also restriction of social contact and visual control play an important role in the development of these changes.

Introduction

Long-term tethered housing of female pigs in individual pens is not unusual in modern intensive pig breeding. This type of housing is considered stressful, because physical restraint largely impairs normal behaviour of the animals and thereby their control over the environment, and tethered pigs often develop behavioural and physiological disturbances [1]. However, factors in addition to physical restraint such as social restriction may contribute to the stressful character of the tethered housing condition [2]. Abundant literature indicates that chronic stress can lead to changes, at different levels of organization, in the regulation of the hypothalamic-pituitary-adrenal (HPA) axis [3,4]. Indeed, we have found that tethered housing induces chronic hypercortisolaemia in cyclic female pigs [5]. An increase in the corticosteroidogenic response to ACTH might well underlie this phenomenon.

The aim of the present study was to determine 1) whether tethered housing affects the adrenal cortisol response to ACTH, and 2) whether factors other than restraint, in particular possibilities to engage in visual and tactile contacts with other tethered pigs and to oversee the environment, play a role in the effects of tethered housing.

Materials and Methods

Experimental Design

A longitudinal design was used to investigate changes in adrenocortical steroidogenic capacity induced by long-term tethered housing (chronic stress) in individual cyclic gilts (see Figure 1). Two experimental and two control groups were used. The gilts in the experimental groups were first housed loose in individual pens for at least two complete oestrous cycles. Then they were tethered by a neck-chain for 15 or 20 weeks; the housing conditions of experimental Group 1 were more restrictive than those of Group 2 with regard to the possibilities for social interactions and the degree of visual isolation from the environment. Once during loose housing and twice during tethered housing (at 6 to 7 weeks and at the end of the experiment) the adrenocortical steroidogenic capacity of the gilts was challenged with i.v. administration of synthetic ACTH(1-24), and the subsequent cortisol response was monitored by radioimmunoassay of plasma. The control gilts (control Groups 1 and 2) were housed loose for the duration of the experiment under conditions that were

otherwise similar to those used for the respective experimental groups. In these groups, ACTH challenges were performed with time intervals approximately matching those used for the experimental groups. For all gilts, signs of oestrus were monitored during the experiment, and interventions (surgery, tethering, ACTH challenges) were performed during the luteal phase of the oestrous cycle, to prevent interference by changes in adrenocortical activity that are associated with the oestrous cycle [5].

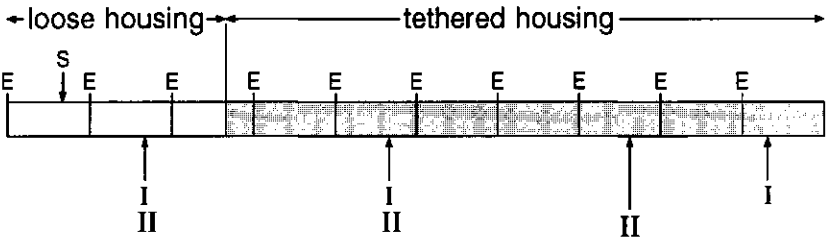

Figure 1. Experimental Design.
E, standing oestrus (oestrous cycle length 20.9 ± 0.2 days; mean ± SEM); S, surgical insertion of jugular vein catheter; I, ACTH challenges of experimental Group 1; II, ACTH challenges of experimental Group 2 and control Groups 1 and 2, which were treated similarly to the experimental Groups, except tethering.

Animals and Housing

Thirty healthy, cyclic, crossbred gilts (Great Yorkshire x British Landrace, Pig Improvement Company, U.K.) that had shown two or more normal oestrous cycles (20.9 ± 0.2 days; mean ± SEM) were used for this experiment. The body weight of the gilts at the beginning of the experiment was 114.2 ± 1.4 kg.

In all housing systems, lights were on between 0730 h and 1900 h, and ambient temperature ranged from 15 to 25°C. The gilts were fed 1 kg of a pelleted, nonlactating sow feed, delivered by hand twice a day (at 0900 h and 1700 h). To prevent the gilts from associating the presence of people with feeding, they were conditioned with a bell signal that preceded feeding. Water was available ad libitum through a nipple drinker. Oestrus detection was done routinely once daily (0830 h), and around oestrus twice daily (0830 h and

1630 h), with a vasectomized boar and by external signs of oestrus. Gilts were considered to be in oestrus when they showed a standing response to the boar and(or) vulvar swelling and redness.

Experimental Group 1. Six gilts were housed loose in individual pens (3.5 m x 1.4 m). The floor was solid concrete and covered with deep straw where the gilts stood, except for a dunging area (1.4 m x 1.4 m) at the rear of the pens. Six pens, separated by metal plates (3.5 m x 0.9 m; length x height), were placed in a single row in a closed room (11.6 m x 6.0 m). A vasectomized boar was housed in the same room, in an additional pen, that was separated from the nearest gilt by 1.4 m and two metal plates. Only limited tactile and visual contact between gilts in adjacent pens was possible through a 10-cm space under the metal partition. View of the environment was limited.

After two complete oestrous cycles, the gilts were tethered in the same pens without straw but with a small quantity of wood shavings placed in the area behind the gilts to help to keep them clean. The gilts were restrained by a neck tether attached to a 50-cm heavy-gauge chain connected to the floor. An extra metal plate (1.1 m x 0.8 m; length x height) was placed beside the gilts as a partition to reduce floor space to 2.0 m x 0.6 m, thereby further restricting their movements. The extra partition also prevented visual or tactile contact between gilts in adjacent pens, which were now separated by two metal plates, 0.8 m apart. The gilts were subjected to an ACTH challenge once during loose housing and after 6 and 20 weeks of tethered housing.

Control Group 1. Six gilts were housed individually in a similar room with pens similar to those used for the experimental Group 1 during the loose housing condition. As with experimental Group 1, a vasectomized boar was penned in the room with control Group 1. During the entire experiment, Group 1 controls were housed loose on deep straw. The ACTH challenges were performed three times with the same intervals as used for experimental Group 2 (see Figure 1).

Experimental Group 2. Twelve gilts were housed loose in individual pens (3.0 m x 2.0 m). The floor was solid concrete and covered with deep straw where the gilts stood, except for a slatted dunging area (1.0 m x 2.0 m) at the rear of the pens. The gilts were equally divided over two rooms, each with eight pens in two rows of four.

After two oestrous cycles, all 12 gilts were tethered by the neck with a 50-cm heavy-gauge chain in individual tether stalls, each 65 cm wide, and placed in a single row of 13 pens, divided by horizontal bars, in a closed 9.0-m x 5.0-m room. A small quantity of wood shavings was placed in the area behind the gilts

to keep them clean. Both before and during tethered housing, all gilts had a good view of the environment and auditory, olfactory, and visual contact was possible between the gilts. Tactile contact was possible between gilts in adjacent pens. An ACTH challenge was performed once during loose housing and after 7 and 15 weeks of tethered housing.

Control Group 2. Six gilts were housed loose during the experiment on deep straw in individual pens (3.0 m x 2.0 m) in one room, which was similar to the two rooms used for experimental Group 2 during loose housing. The ACTH challenges were conducted with the same time interval as in experimental Group 2. The vasectomized boar that was used for oestrus detection of experimental and control Groups 2 was housed in a separate room.

Surgery and Treatments

To collect serial blood samples, each gilt was surgically fitted with a permanent jugular vein catheter. Feed was withheld overnight before surgery. Surgery was performed during the luteal phase of the oestrous cycle (Figure 1), under sterile conditions and under general anaesthesia. The gilts were premedicated with an i.m. injection of 6 mg of azaperone/kg of BW (Stresnil®, Janssen Pharmaceutica BV, The Netherlands). After 30 min, general anaesthesia was induced and maintained either with i.v. metomidate-azaperone infusion (Hypnodil-Stresnil®, Janssen Pharmaceutica BV) or inhalation of O_2/N_2O, enflurane (Ethrane®, Abott BV, The Netherlands). The catheter (polyvinyl chloride, 1.5 mm i.d., 2.1 mm o.d.; Rubber BV, The Netherlands) was inserted into the vena jugularis externa toward the superior vena cava. The free end of the cannula was passed subcutaneously to the back of the gilt, where it was externalized between the scapulae. A one-way luer-lock stopcock (Vygon BV, The Netherlands) was secured to the end of the cannula so that a 10-ml syringe could be attached easily. To protect the cannula, the gilts were equipped with a harness to which they had been habituated during the week before cannulation. The harness (23 cm x 20 cm, polyvinyl chloride with nylon; Bizon Chemie, The Netherlands) was fixed at the back of the animals with belts around the chest. From 3 days before surgery until 3 days after surgery, all gilts were treated once daily with antibiotics (0.2 ml/kg of BW Engemycine®, i.m. bolus injection; Mycofarm Nederland BV, The Netherlands). At least 10 days were allowed for the animals to recover from surgery and anaesthesia.

Cannula patency was maintained by flushing the cannulae thrice weekly and filling them with sterile heparinized physiological saline (25 IU heparin/ml of 0.9% saline; Leo Pharmaceutical Products, The Netherlands) when not in use.

Adrenocorticotropin Hormone Challenge

Each gilt received 10 μg/kg of BW synthetic ACTH(1-24) (Organon Int., The Netherlands) i.v. through the catheter. The dose of ACTH induced the maximal plasma cortisol response in a dose-response study, performed in loose-housed cyclic gilts (data not shown). Before the ACTH treatment, four baseline blood samples were collected at 15-min intervals. At 1015 h, the ACTH(1-24) (dissolved in 1 ml sterile 0.9% saline solution) was administered as an i.v. bolus injection. At various times after the injection (15, 30, 45, 75, 105, 135, 165, 225, 285, and 345 min), blood was sampled for plasma cortisol determination. During the loose housed period, all gilts also were treated with an i.v. bolus injection of 1 ml of sterile 0.9% saline, to control for the effects of the infusion and blood sampling procedure on cortisol concentrations throughout the blood sampling period.

Blood Sampling

Before the experimental period, all gilts were frequently handled and habituated to the blood collection procedure. Before a blood sample was drawn, the luer-lock stopcock was disinfected with 70% ethyl alcohol, and the cannula was flushed with 4 ml of sterile saline (NPBI BV, The Netherlands) to remove possible blood clots. Then, approximately 10 ml of blood was collected using a sterile syringe (Becton Dickinson, Ireland). Subsequently, the cannula was filled with either sterile saline, when blood was sampled frequently, or with sterile heparinized saline (to maintain cannula patency). Blood samples were immediately transferred to ice-cooled polypropylene tubes containing 100 μl of EDTA-solution (144 mg of EDTA/ml of saline; Titriplex®III, Merck Nederland BV, The Netherlands), immediately placed on ice, and subsequently centrifuged at 2,000 x *g* for 10 min at 4°C. Plasma was collected and stored at −20°C until hormone analysis was completed.

Radioimmunoassay of Cortisol

Plasma concentrations of cortisol were measured in unextracted plasma samples using a single-antibody RIA technique. A rabbit antiserum (K7348; kindly donated by T.J. Benraad, Nijmegen, The Netherlands), raised against 11ß,17,21-trihydroxy-4-pregnene-3,20-dione-3-CMO-BSA (cortisol-bovine serum albumin-conjugate), was used. The main crossreacting steroids were 21-desoxycortisol (72%), cortisone (59%), prednisolone (53%), 11-desoxycortisol (43%), corticosterone (10%), progesterone (2.3%), oestradiol-17ß, dexamethasone, and triamcinolone acetonide (all < 1%). Cortisol (H 5885;

Sigma Chemical, St. Louis, MO) was used as a standard, and [1,2,6,7-^{3}H]cortisol (TRK407, specific activity 80.5 Ci/mmol, Amersham Int., Amersham, U.K.) was used as the tracer. The antiserum was used in a final dilution of 1:96,250, which yielded approximately 30% specific binding of the labeled hormone after incubation. All dilutions were made with PBS (pH 7.4) containing 0.1% of BSA (wt/vol).

The following reagents were added to each tube: 50 μl of standard (7.8 to 1000 pg cortisol/tube) or 50 μl plasma sample (10x diluted; unknowns or pools); 50 μl of cortisol-free porcine plasma (except tubes for total counts); 100 μl of antiserum (except tubes for nonspecific binding); 50 μl of [1,2,6,7-^{3}H]cortisol (10,000 dpm) and PBS-0.1%BSA buffer to bring the total volume to 550 μl/tube. Incubations were performed overnight at 4°C. Bound and free hormone were separated by precipitation with dextran (M, 60,000 to 90,000)-coated charcoal. After centrifugation (2,000 x *g*, 10 min at 4°C), the supernatant, which contained the bound hormone, was aspirated and counted for radioactivity in liquid scintillation cocktail (Ultima Gold, Packard Instrument Company, Meriden, CT). The counting data were evaluated using the computer program SECURIA (Packard). Standards were incubated in triplicate, pools and unknowns in duplicate. All plasma samples (50-μl sample volume, 10x diluted) that were assayed with less than 20% B/B_0 were reassayed (50-μl sample volume, 100x diluted) to bring them within a reliable portion of the standard curve. A pool of porcine plasma, containing 74.9 ± 7.1 ng cortisol/ml, assayed in various dilutions (n = 6), were parallel to the standard curve (range, 11 to 89% B/B_0). The sensitivity of the cortisol assay was 0.5 ng/ml at 90% B/B_0. The intraassay C.V. was 8.2%, and the interassay C.V. was 14.7%.

Analysis of Data and Statistics

Preliminary analysis of the cortisol data of each experimental and control group showed a significant interaction between treatment (saline vs three ACTH challenges) and time of sampling. Therefore, analyses of the effect of treatment on cortisol were carried out separately for each time point of sampling. The effect of treatment was tested by means of *F*-test using a split-plot model (GLM, [6]). The values for treatment within gilts were taken as repeated measurements:

$$Y_{ij} = \mu + e_{1i} + T_j + e_{2ij}$$

where Y_{ij} = cortisol value of gilt i at treatment j; μ = overall mean; e_{1i} = error

term 1, which represents the random effect of gilts (i = 1, .., n; n = number of gilts within the group); T_j = the effect of treatment j (j = 1, .., 4); and e_{2ij} = error term 2, which represents the random effect within gilts between treatments. The effect of treatment was tested against error term 2. Least squares mean differences were used for the pairwise comparisons between treatments following a significant *F*-test with an overall confidence level of 0.95. All statistical analyses were done separately for each group.

The curves for the cortisol responses to ACTH treatment were plotted for each gilt. A baseline was determined with linear regression using the plasma cortisol values of the four blood samples taken before ACTH injection and the cortisol values that reached preinjection concentrations after the cortisol response. The area under the curve was calculated as the area between the baseline and the cortisol response curve above the baseline. The peak height of the cortisol response was defined as the maximal plasma cortisol concentration minus the corresponding basal cortisol concentration. Differences in area under the curve and peak plasma cortisol response to three ACTH challenges were tested for significance with Student's *t*-test (paired data) using the Statistical Program System for the Social Sciences statistical package [7]. Results are given as mean ± SEM.

Results

Due to problems with cannula patency and cannula damage during the course of the experiment, a number of gilts could no longer be used for blood sampling at some stage during the experiment, and therefore did not yield data. These gilts, however, remained in the housing system for the rest of the experiment. The number of drop-outs in the control groups was greater than we expected. After completion of the experiment, data for six gilts of experimental Group 1, four gilts of control Group 1, nine gilts of experimental Group 2, and five gilts of control Group 2 were available for analysis.

The mean plasma cortisol responses of the experimental and control groups to i.v. infusion of 10 μg/kg of BW synthetic ACTH(1-24) or 1 ml of saline are shown in Figure 2. Saline administration had no effect on plasma cortisol concentrations. Regardless of housing conditions, ACTH induced a time-dependent increase in plasma cortisol concentrations in all gilts; the increase was highly significant compared with cortisol concentrations in the same gilts after infusion of saline. Considerable differences, especially with respect to the

peak height, in the adrenocortical response to ACTH were detected among gilts (see Table 1). Within control gilts, however, the cortisol response remained rather stable throughout the experimental period.

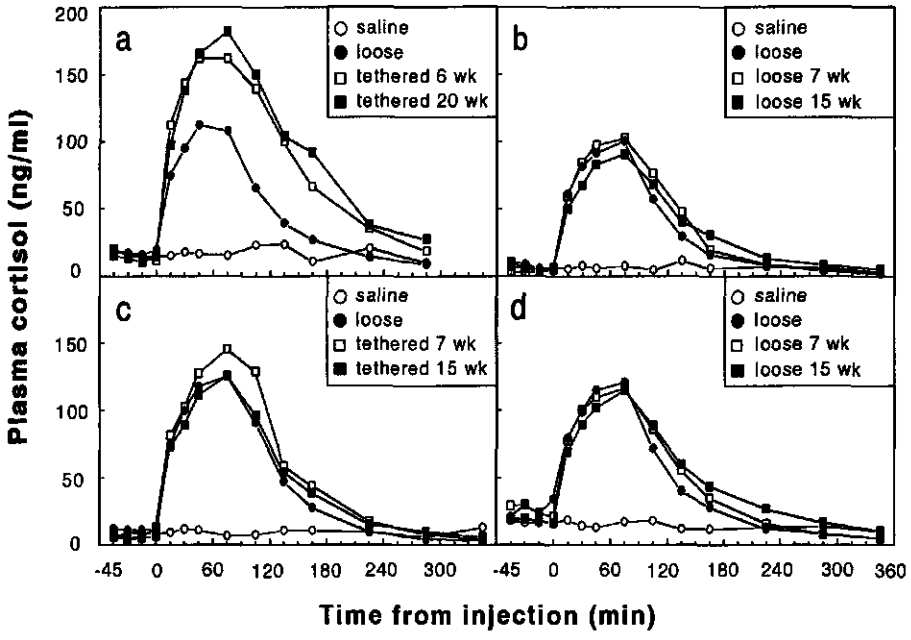

Figure 2. Mean plasma cortisol concentrations after i.v. injection of 10 μg/kg of BW of ACTH(1-24) (●; □; ■) or 1 ml of saline (○) in experimental Group 1 (Figure 2a; n=6), control Group 1 (Figure 2b; n=4; except for loose 15 weeks, n=2), experimental Group 2 (Figure 2c; n=9) and control Group 2 (Figure 2d; n=5).

Experimental Group 1 (Most Restricted Tethered Housing). After 6 and 20 weeks of tethered housing, the plasma cortisol response to ACTH was enhanced compared with pretethering values obtained for the same gilts (Figure 2a, Table 1). Also, the peak height (after 6 weeks, $P<0.05$; after 20 weeks, $P<0.05$) and the area under the curve (after 6 weeks, $P<0.01$; after 20 weeks, $P<0.05$) were increased. Further statistical analysis revealed significant differences between cortisol concentrations before and after 6 weeks of tethered housing at 135 min, and before and after 20 weeks of tethered

housing at 75, 135, 165, and 285 min after the ACTH infusion. No significant difference was found between the responses (peak height and area under the curve) measured after 6 and 20 weeks of tethered housing. Considerable variation was found between individual gilts in the increment of the response after tethering. In four of six gilts, 6 and 20 weeks of tethered housing increased the area under the curve by 100 to 200% and the peak height of the response by 30 to 150%. In the remaining two gilts, the area under the curve was increased by approximately 50% at 6 and 20 weeks of tethered housing compared with pretethering values. The peak height was increased in one of these gilts only after 20 weeks of tethered housing, and peak height remained unaltered throughout the experimental period in the other gilt (data not shown).

Control Group 1 (Loose Housing). In the control gilts, all three ACTH challenges yielded similar plasma cortisol responses (Figure 2b, Table 1), and no differences were found in area under the curve or peak height.

Table 1. Plasma cortisol response (peak height and area under the curve) to three consecutive ACTH challenges for the experimental and control groups.

Group	ACTH-induced cortisol response[a]		
	1	2	3
Experimental group 1, n=6			
Peak height, ng/ml	112.9[b] ± 16.4	162.5[c] ± 21.3	182.6[c] ± 24.8
AUC, arbitrary units	10,108[b] ± 1,237	21,102[c] ± 2,694	24,434[c] ± 4,996
Control group 1, n=4			
Peak height, ng/ml	94.8 ± 5.2	101.0 ± 11.6	84.7 ± 32.3[d]
AUC, arbitrary units	9,358 ± 717	11,456 ± 1,728	10,881 ± 5,123[d]
Experimental group 2, n=9			
Peak height, ng/ml	119.3[b] ± 5.8	142.7[c] ± 9.7	120.5[b] ± 10.5
AUC, arbitrary units	12,760[b] ± 698	17,225[c] ± 1,233	14,626[b] ± 1,439
Control group 2, n=5			
Peak height, ng/ml	96.5 ± 8.0	94.1 ± 7.6	95.6 ± 9.7
AUC, arbitrary units	10,151 ± 712	9,954 ± 898	11,583 ± 698

[a]1, 2, and 3 indicate three consecutive ACTH challenges.
[b,c]Means ± SEM within a row lacking a common superscript letter differ ($P<0.05$).
[d]n=2.

Experimental Group 2 (Least Restricted Tethered Housing). After 7 weeks of tethered housing, the cortisol response to ACTH was increased, compared with

pretethering conditions (area under the curve, $P<0.001$; peak height, $P<0.005$; Figure 2c; Table 1). Further analysis revealed significant differences at 105, 165, 225, and 285 min after ACTH administration. After 15 weeks of tethered housing, the response was not different from pretethering values (Figure 2c; Table 1).

Control Group 2 (Loose Housing). Peak height and area under the curve of the cortisol response did not change significantly during the experimental period (Figure 2d, Table 1). At 225 min after ACTH administration, cortisol concentrations were greater ($P<0.05$) at 15 weeks of loose housing, compared with the other challenges.

Discussion

In the present experiment, long-term tethered housing enhanced the cortisol response to exogenous ACTH in cyclic gilts, indicating a change in adrenocortical function. The increase was considerably more pronounced and persistent in gilts that were visually isolated from their environment and deprived of possibilities for social (visual, tactile) contacts with conspecifics than in gilts with such possibilities. These data indicate that in tethered gilts adaptational changes occur at the level of the adrenal cortex. Also, restriction of social contact and visual control over the environment play an important role in the development of these adrenal changes and may contribute to the stress experienced by the gilts.

Tethered housing largely impairs movements and behavioural performance of pigs. The pigs are thus deprived of their main behavioural tools to exert control over their environment [1]. Loss of control is generally recognized as a common denominator of stressful conditions [1,8]. Thus, it can be reasoned that long-term tethered housing imposes a condition of chronic stress on the pigs. This contention is supported by observations that behavioural [1,9-11], reproductive [12], and cardiovascular [13] disturbances are frequent in tethered pigs.

Abundant literature substantiates that chronic stress induces long-term changes in the regulation of the HPA axis in a variety of species [3,4,14,15]. In line with data reported by Becker et al. [16], we found that prolonged tethered housing of cyclic gilts results in a sustained increase in basal plasma cortisol concentrations [5]. Our observations that tethered housing of cyclic gilts increases the cortisol response to exogenous ACTH point to the adrenal cortex

as at least one of the sites in the HPA axis where stress-induced adaptational changes occur. These changes may underly the hypercortisolemia that occurs during tethered housing [5].

In the control gilts that were housed loose in individual pens for the duration of the experiment, the cortisol response remained unaltered. Therefore, the increase in adrenocortical response observed in tethered gilts cannot be attributed to effects induced by experimental procedures (e.g., repeated ACTH challenges, repeated blood sampling), to individual housing per se, or to changes in adrenocortical function that are known to occur with increasing age [17,18]. Moreover, possible effects of variations in sensitivity of the adrenal cortex occurring during the diurnal [19] and oestrous cycles [5] can be excluded, because the challenges were invariably performed during the midluteal phase of the oestrous cycle, when cortisol levels remain rather stable, at 1015 in the morning. Therefore, the change in the ACTH-induced cortisol response in the experimental gilts probably resulted from the stress caused by tethered housing.

In accord with other investigators [20,21], we found that the cortisol response to ACTH within gilts was consistent during the experimental period, indicating that adrenocortical reactivity is an individual characteristic. However, there were considerable differences in the responses between individual gilts. Such differences seem to be primarily of adrenal origin and probably cannot be attributed to changes in metabolic clearance rate of cortisol [20,22,23]. The interanimal differences in cortisol response were more pronounced during tethered housing than during loose housing; this may reflect differences in neuroendocrine susceptibility to the stressful situation [1,23,24].

The increase in the cortisol response during tethered housing was considerably more pronounced and persistent in the most restricted than in the least restricted, tethered group of gilts. These results indicate that housing factors other than physical restraint per se affected the response to chronic stress. Notably, in the most restricted group, closed partitions between the pens precluded tactile and visual contacts between the gilts and severely limited their visual range. Perhaps the relative lack of visual information from the environment reduced the predictability of environmental changes for these gilts and increased uncertainty. Low predictability or uncertainty are generally seen as characteristics of stressful situations [8,25]. In addition, there is ample evidence that the lack of social interactions with conspecifics can affect stress responses, especially with respect to the HPA axis [26]. Thus, both reduced visual control and social restriction appear to be important factors contributing to the adaptive changes in adrenocortical function observed in the present

experiment.

The exact mechanisms that are involved in the neuroendocrine regulation during chronic stress, which results in an increased corticosteroidogenic capacity, are still unknown. The findings of the present experiment indicate that the adrenal cortex is one of the sites of regulatory changes in the HPA axis during tethered housing. Although ACTH is generally considered to be the most important factor in the control of glucorticoid secretion from the adrenal cortex, there is increasing evidence that neural inputs at the level of the adrenal gland influence the sensitivity of the adrenal cortex to ACTH [27,28].

Implications

The results of this experiment indicate that tethered housing of pigs is associated with an adaptational increase in adrenocortical steroidogenic capacity and support the idea that it represents a chronic stressor for the gilts. In addition, housing factors, other than physical restraint per se, affect this response to chronic stress. Also, social interactions between gilts and visual control over the housing environment can mitigate stress-induced changes in endocrine regulation.

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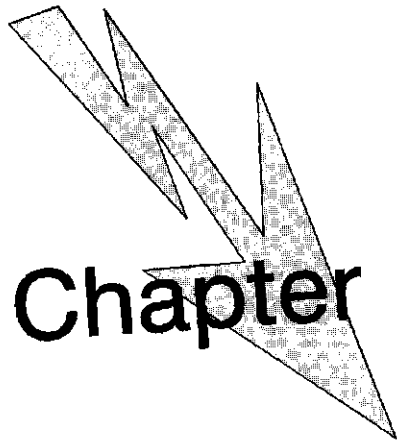
Chapter 5

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Chapter 5

Chronic stress increases the opioid-mediated inhibition of the pituitary-adrenocortical response to acute stress in pigs

Summary

The role of endogenous opioid mechanisms in the pituitary-adrenocortical response to acute stress was investigated in a longitudinal study in cyclic female pigs, before and after exposure to chronic stress (long-term tethered housing). Challenge of loose housed pigs with acute nose-sling stress for 15 min induced an activation of the hypothalamic-pituitary-adrenocortical (HPA) axis, evidenced by a transient increase in plasma ACTH (98 ± 12 pg/ml; peak height above basal; mean ± SEM) and cortisol (54 ± 3 ng/ml) concentrations. Pretreatment with the opioid receptor antagonist naloxone (0.5 mg/kg of body weight; i.v. bolus) increased the challenge-induced ACTH and cortisol responses to 244 ± 36 pg/ml and 65 ± 5 ng/ml, respectively. This indicates that during acute nose-sling stress endogenous opioid systems are activated that inhibit the pituitary-adrenocortical response. After exposure of the pigs to chronic stress (10-11 weeks of tethered housing) the challenge-induced ACTH response was attenuated, whereas the cortisol response remained unchanged, suggesting an increased adrenocortical sensitivity to circulating ACTH. In addition, pretreatment with naloxone induced a greater increment in the ACTH and cortisol responses in tethered pigs than in loose housed pigs. Since no such changes were found in control animals housed loose during the entire experimental period, this indicates that the impact of opioid systems had increased due to chronic stress. The increased impact of opioid systems during chronic stress may prevent excessive HPA responses to acute stressors, and thus may be of adaptive value.

Introduction

It is well established that the hypothalamic-pituitary-adrenocortical (HPA) axis is subject to inhibitory control of opioids in a variety of species, including pigs [1-4]. This opioid-mediated inhibition of the HPA axis is apparent under resting conditions, but is most evident during stress [5-9]. There is abundant literature substantiating that chronic stress can lead to changes in the regulation of the HPA axis [10,11]. Little is known, however, with respect to the relevance of opioid systems for HPA function during chronic stress.

In the present study we have examined the effect of chronic stress on the pituitary-adrenocortical response to challenge with an acute nose-sling stress in female pigs. The nose-sling procedure, which is used in veterinary practice for temporary immobilization of pigs, represents an acute stressor that activates both the HPA axis and endogenous opioid systems [7,9]. Activation of opioid systems may have an antinociceptive effect, as evidenced by an opioid-based reduction in vocalization and a transient naloxone-reversible hypoalgesia following nose-sling stress in pigs [7]. This effect may be related to the apparent antinociceptive effect of the nose "twitch" in horses, in which endogenous opioids are also involved [12].

Chronic stress was induced by long-term tethered housing of the pigs, since this housing condition has been shown to induce changes in pituitary-adrenocortical activity [13-16], and in autonomic regulatory systems [17]. Previous studies have demonstrated that tethered housing can also lead to development of behavioural disturbances, stereotypies [17-20]. These stereotypies can be blocked by naloxone [19], suggesting an increased activity of endogenous opioid systems under these conditions. On account of these findings, we investigated whether changes in opioid function that are induced by chronic stress affect the pituitary-adrenocortical response to acute nose-sling stress.

Materials and Methods

Experimental Design

To investigate the effect of chronic stress (tethered housing) on the pituitary-adrenocortical response to an acute stressor (nose-sling) in cyclic nulliparous female pigs (gilts), and the involvement of endogenous opioids in this response, a longitudinal experimental design was used with one experimental

and one control group of animals. All gilts were surgically fitted with a permanent jugular vein catheter for blood sampling. The pigs of the experimental group were housed loose in individual pens for an 11-week period. During the second phase of the experiment (11 weeks) they were housed tethered by a neck-chain (chronic stress). Twice during loose housing (3 weeks and 2 weeks prior to tethering) and twice during tethered housing (after 10 and 11 weeks of tethered housing) the animals were challenged with a nose-sling for 15 min, and the subsequent plasma ACTH and cortisol responses were monitored. Immediately preceding the nose-sling challenge, the gilts were i.v. treated with either physiological saline or naloxone, in order to investigate the role of endogenous opioid systems. The gilts of the control group were housed loose during the entire experiment under conditions that were otherwise similar to those used for the experimental group. In the control group, four nose-sling challenges were performed with time intervals matching those used for the experimental group. In all animals, oestrous signs were monitored during the experiment, and interventions (surgery, tethering, nose-sling) were performed during the luteal phase of the oestrous cycle, to prevent possible interference by changes in adrenocortical activity that are associated with the oestrous cycle [15].

The experiments were approved by the Committee on Animal Care and Use of the Agricultural University of Wageningen.

Animals and Housing

Eighteen healthy cyclic crossbred gilts (Great Yorkshire x British Landrace, Pig Improvement Company, United Kingdom) which had shown 2 or more normal oestrous cycles (oestrous cycle length 20.6 ± 1.3 days; mean ± SD) were selected for this study. The body weight of the pigs at the beginning of the experiment was 115 ± 10 kg (mean ± SD). All animals were housed in individual pens of approximately 5.5 m^2. The floor of the pens was solid concrete and covered with deep straw, except for a slatted dunging area (2.5 m^2) at the rear of each pen. The gilts of the control group ($n = 9$) were housed loose for the duration of the experiment (i.e. 22 weeks). The gilts of the experimental group ($n = 9$) were housed loose for a period of 11 weeks. Thereafter, they were tethered by the neck with a 50-cm heavy gauge chain in individual tether stalls, each 65 cm wide, placed in a single row in a closed 9.0 m x 5.0 m room. The floor was solid concrete, with a slatted dunging area at the rear of the stalls. A small quantity of wood shavings was placed in the area behind the gilts to keep them clean.

Lights were on between 0730 h and 1900 h and ambient temperature ranged from 15 to 25°C. Twice a day (0900 h and 1600 h), the pigs were fed 1 kg of a pelleted, dry sow feed, delivered by hand. To prevent them from associating the presence of people with feeding, they were conditioned with a bell signal that always preceded the delivery of food. Water was available *ad libitum* through a nipple drinker. Oestrus detection was routinely done once daily (1630 h) with a vasectomized boar and by external oestrous signs. Gilts were considered to be in oestrus when showing a standing response to the boar and(or) showing vulval swelling and redness.

Surgery

To allow for i.v. administration of drugs and repeated blood sampling without disturbing the gilts, all animals were surgically fitted with a permanent jugular vein catheter under general anaesthesia as has been described previously [16]. In order to protect the cannula the gilts were equipped with a harness to which they had been habituated during the week before cannulation. The harness (23 cm x 20 cm, polyvinyl chloride with nylon; Bizon Chemie, The Netherlands) was fixed at the back of the gilts with belts tied around the chest. From 3 days before surgery until 3 days postsurgery, all gilts were treated once daily with antibiotics (orally; 12 ml of T.S. Sol®, containing trimethoprim and sulphamethoxazol; Dopharma, The Netherlands). The animals were allowed at least 10 days to recover from surgery and anaesthesia.

Cannula patency was maintained by flushing thrice weekly and filling the cannula with sterile heparinized physiological saline (25 IU heparin/ml of 0.9% of NaCl; Leo Pharmaceutical Products, The Netherlands) when not in use.

Nose-sling Challenge

During the nose-sling procedure, which always started at 1015 h, the gilts were confined for 15 min by a rope tied around the upper jaw and attached to one of the bars of the pen. Immediately after application of the nose-sling, the gilts showed resistance by fiercely pulling the rope and screaming loudly, and their heart rate was markedly increased. After approximately 5 min the gilts became quiet and sedated, and often lay down. During the period of sedation heart rate decreased (Loyens, Janssens, Schouten, Helmond and Wiegant, unpublished results). Immediately after removal of the nose-sling the gilts became active again.

The gilts were pretreated immediately before the nose-sling was applied with an i.v. bolus injection of either 5 ml of sterile physiological saline (0.9%

NaCl; NPBI BV, The Netherlands) containing 0.5 mg/kg of body weight naloxone (an opioid receptor antagonist) or 5 ml of physiological saline (control), injected through the catheter. Subsequently the gilts were restrained for 15 min with a nose-sling. The order of pretreatment with saline or naloxone was randomized (with a 1-week interval between two subsequent nose-slings). Preceding the nose-sling, four baseline blood samples were collected at 15-min intervals for plasma ACTH and plasma cortisol determination. Blood was sampled immediately after termination of the stress at 15, 30, 45, 75, 135, 165, 225, 285 and 245 min after pretreatment.

Blood Sampling

Prior to the experiment, all gilts were frequently handled and adapted to the blood collection procedure, which has been described in more detail previously [16]. Blood samples (approximately 10 ml) were immediately transferred to ice-cooled polypropylene tubes containing 100 μl of EDTA-solution (144 mg of EDTA/ml of saline; Titriplex®III, Merck Nederland BV, The Netherlands). They were immediately mixed and placed on ice, and subsequently centrifuged at 2,000 x *g* for 10 min at 4°C. Plasma was collected and stored at −20°C until analysis of ACTH and cortisol.

Determination of Plasma Hormones

ACTH. Plasma concentrations of ACTH were determined by two-site immunoradiometric assay (IRMA), using a commercial testkit (Euro-Diagnostica BV, Apeldoorn, The Netherlands). The ACTH assay was performed in singular in 200 μl unextracted plasma, and all samples from an individual gilt were analyzed in the same assay. The two control samples provided with the kit yielded values within the limits specified by the manufacturer. Details of the method have been described by Copinschi [21]. The sensitivity of the assay was 0.8 pg/ml. The intra-assay coefficient of variation was 2.6% and the inter-assay coefficient of variation was 4.7%.

Cortisol. Plasma concentrations of cortisol were measured in duplicate in unextracted 50 μl samples according to a single-antibody radioimmunoassay (RIA) technique previously described by Janssens *et al.* [16]. The sensitivity of the assay was 0.5 ng/ml at the 90% B/B_0 level. The intra-assay coefficient of variation was 8.2% and the inter-assay coefficient of variation was 14.7%.

Analysis of Data and Statistics

Due to problems with cannulae patency several plasma samples, particularly

of some animals in the control group, were missing. From each gilt, however, samples of all time points were available from at least 3 nose-sling challenges.

The curves of the cortisol response to nose-sling challenge were plotted for each animal. A baseline was calculated for each curve with linear regression using the plasma cortisol values of the four blood samples taken before to saline or naloxone pretreatment and the cortisol values that reached prechallenge concentrations after the nose-sling procedure. The area under the curve (AUC) was calculated as the area between the baseline and the cortisol response curve above the baseline. The peak height of the cortisol response was defined as the maximal plasma cortisol concentration minus the corresponding basal cortisol value. The ACTH data were analyzed using the same method as described for cortisol.

Since no effects of repeated challenge and of the sequence of the pretreatments were found on the pituitary-adrenocortical response (Kruskal-Wallis, $P>0.05$), the ACTH or cortisol data with similar pretreatment during the same housing period were combined. Differences in peak height and AUC between saline and naloxone pretreatment, as well as differences between loose and tethered housing were tested for significance by Wilcoxon matched pairs signed-ranks test. The Mann-Witney U test was used to test for differences in the pituitary-adrenocortical responses between gilts of the experimental and the control group.

All statistical analyses were performed using the SPSS statistical package [22]. The criterion for statistical significance was $P<0.05$. Results are given as mean ± SEM.

Results

Experimental Group. Both during the loose housed and the subsequent tethered housed condition, gilts were challenged twice with a nose-sling procedure, once following pretreatment with saline and once following naloxone (0.5 mg/kg of body weight). The time course of the plasma ACTH and cortisol responses is shown in Figure 1. It should be noted that individual differences in the timing of the cortisol peak led to flattened curves of the mean cortisol responses, displayed in Figure 1. The differences in peak height and in AUC of the ACTH and cortisol responses that are represented in Table 1, however, were calculated per animal. In all animals, the nose-sling challenge induced a time-dependent increase over basal levels in plasma ACTH and cortisol (Figure1; left

panels, saline pretreatment). Highest plasma ACTH concentrations were found in the samples taken immediately after termination (i.e. 15 min after the beginning) of the acute stress, and ACTH returned to basal levels within approximately 1.5 hour after termination of the nose-sling challenge (Figure 1; upper panels, saline pretreatment). After 10-11 weeks of tethered housing the AUC of the ACTH response was significantly decreased ($P<0.05$) compared with values obtained in the same gilts during loose housing (Table 1). Peak height of the ACTH response was also reduced, although not to a statistically significant extend ($P<0.09$). Pretreatment of the gilts with naloxone significantly increased both peak height and AUC of the challenge-induced ACTH response compared with saline pretreatment (Figure 1, Table 1). This effect was found both during loose housing (peak height; $P<0.01$; AUC; $P<0.01$) and following the chronic stress of tethered housing (peak height; $P<0.01$; AUC; $P<0.01$). No differences were found in the response after naloxone pretreatment between loose and tethered housing.

Highest plasma cortisol concentrations were either observed in samples taken immediately or 15 min after termination of the nose-sling procedure and cortisol levels returned to basal in approximately 2 hours (Figure 1; lower panels, saline pretreatment). Neither peak height nor AUC of the plasma cortisol response were affected by tethered housing (Table 1). Following pretreatment of the gilts with naloxone the cortisol response was significantly increased compared with saline pretreatment, both during loose housing (peak height; $P<0.05$; AUC; $P<0.05$) and after 10-11 weeks of tethered housing (peak height; $P<0.05$; AUC; $P<0.05$). After exposure of the pigs to chronic stress, however, the cortisol response after naloxone pretreatment was significantly larger than during the loose housing condition (peak height; $P<0.05$; AUC; $P<0.05$).

Control group. A control group of gilts was loose housed during the entire experiment (22 weeks) and challenged with the nose-sling according to a schedule identical to that of the experimental pigs. Figure 2 shows the plasma ACTH and cortisol responses in these animals. During the first phase of the experiment (Figure 2; left panels; loose 1), in which housing conditions of the control group matched those of the experimental group, challenge-induced plasma ACTH and cortisol responses (after either pretreatment with saline or naloxone) were not significantly different ($P>0.05$) from those measured in the experimental group. In addition, no significant difference was found between the plasma ACTH (peak height; $P=0.35$; AUC; $P=0.25$) and cortisol (peak height; $P=0.75$; AUC; $P=0.92$) responses measured during the first (loose 1) and the

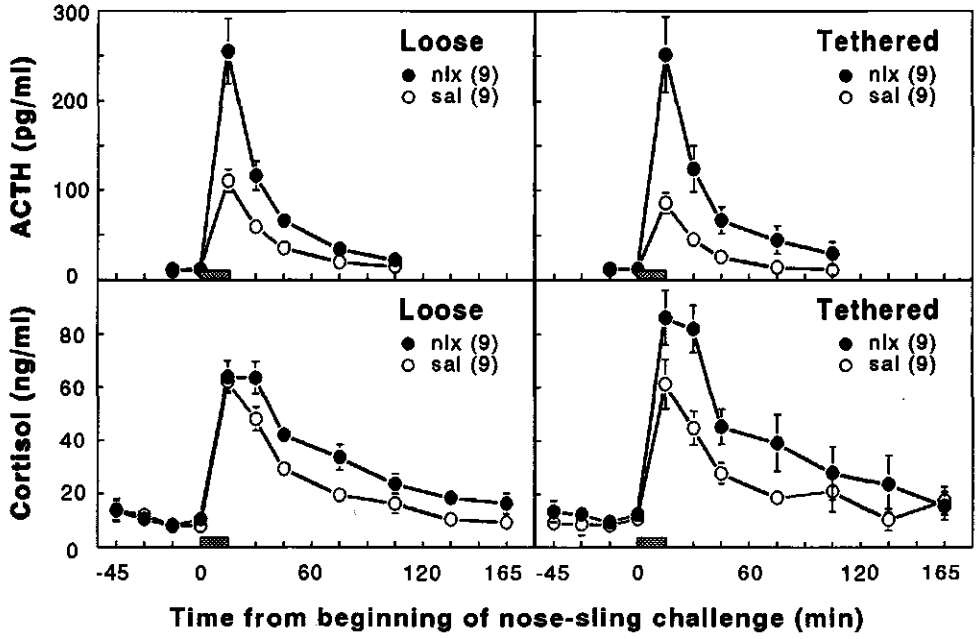

Figure 1. Plasma ACTH (upper panels) and cortisol (lower panels) responses to acute nose-sling stress in pigs of the experimental group during loose housing and after 10-11 weeks of tethered housing.
Pigs of the experimental group were challenged with an acute nose-sling stress for 15 min (hatched bar) before (left panels, loose) and after 10-11 weeks of tethered housing (right panels, tethered). At t = 0 min, immediately preceding the challenge, the pigs were pretreated (i.v.) with naloxone (0.5 mg/kg of body weight; ●) or with saline (5 ml; ○). Data are presented as mean ± SEM with the number of animals in each treatment group between parentheses.

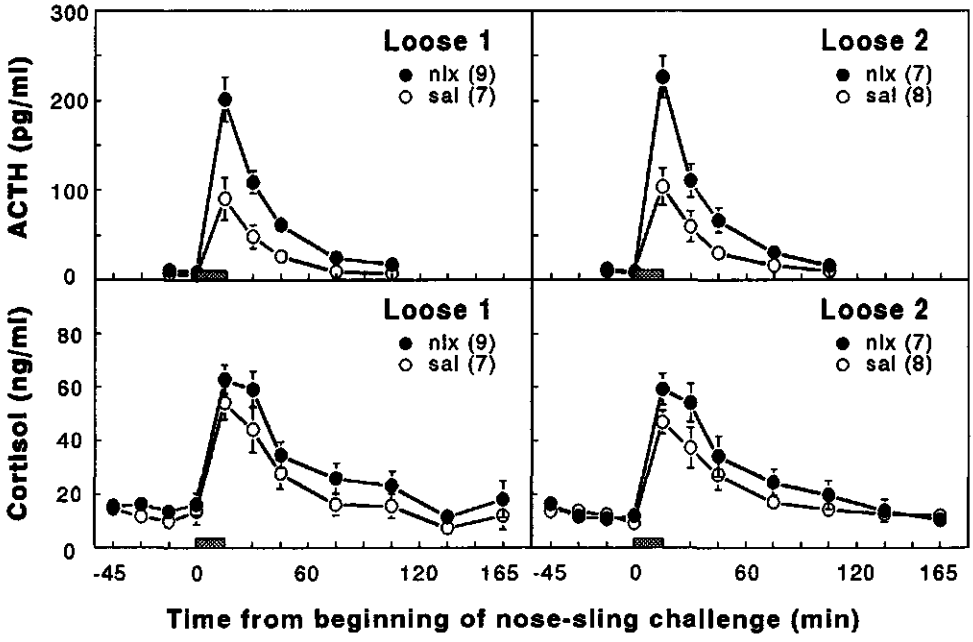

Figure 2. Plasma ACTH (upper panels) and cortisol (lower panels) responses to acute nose-sling stress in pigs of the control group during two periods of loose housing.

Pigs of the control group were challenged with an acute nose-sling stress for 15 min (hatched bar) during two phases of loose housing (loose 1, left panels; loose 2, right panels) matching the time intervals used for the experimental group, before and after 10-11 weeks of tethered housing, respectively. At t=0 min, immediately preceding the challenge, the pigs were pretreated (i.v.) with naloxone (0.5 mg/kg of body weight; ●) or with saline (5 ml; ○). Data are presented as mean ± SEM with the number of animals in each treatment group between parentheses.

second (loose 2) phase of loose housing (Figure 2; saline pretreatment; Table 2).

A significantly higher ACTH response was found after naloxone compared with saline pretreatment with respect to peak height (loose 1; $P<0.05$; loose 2; $P<0.05$) and AUC (loose 1; $P<0.05$; loose 2; $P<0.05$). Naloxone pretreatment had a similar effect on the cortisol response (peak height, loose 1; $P<0.05$; loose 2; $P<0.05$; and AUC, loose 1; $P<0.05$; loose 2; $P=0.17$). Both ACTH and cortisol responses in naloxone pretreated gilts were not significantly different between the loose 1 and loose 2 period (Table 2).

Discussion

In the present study, the effect of chronic stress and the involvement of opioids on the pituitary-adrenocortical response to challenge with an acute stressor (nose-sling) was established in female pigs. The challenge-induced activation of the HPA axis was enhanced and prolonged in animals pretreated with naloxone. This indicates that during the challenge endogenous opioid mechanisms are activated that mitigate the response. Exposure of the animals to chronic stress (long-term tethered housing) led to attenuation of the challenge-induced ACTH response, whereas the cortisol response remained unchanged. This suggests adaptive changes in HPA functioning at the pituitary or suprapituitary level as well as an increase in adrenocortical sensitivity to circulating ACTH. Interestingly, the inhibitory impact of opioid systems on the pituitary-adrenocortical response appeared to be increased after prolonged tethered housing, which may serve to prevent excessive HPA responses to acute stressors during chronic stress.

Exposure of pigs to the acute physical stress of the nose-sling procedure led to both resistance behaviour with marked vocalization and an increase in heart rate. Consistent with former studies, we found a challenge-induced stimulation of both the HPA axis and endogenous opioid systems that mitigated this response [7,9]. Our observations that, after a brief period of resistance, the pigs became quiet and sedated, combined with an opioid-based reduction in heart rate during nose-sling stress (Loyens, Janssens, Schouten, Helmond and Wiegant, unpublished results), support an antinociceptive effect of endogenous opioids [7].

There is abundant literature substantiating that chronic stress can lead to changes in HPA reactivity to further stressful stimulation [10,11]. Although divergent effects of chronic stress on HPA function have been reported in

Table 1. Plasma ACTH and cortisol responses to acute nose-sling challenge in pigs of the experimental group, before (loose housed) and after 10-11 weeks of chronic stress (tethered housed).

Housing	Pretreatment	N	Plasma ACTH		Plasma Cortisol	
			Peak height (pg/ml)	Area under the curve (arbitrary units)	Peak height (ng/ml)	Area under the curve (arbitrary units)
Loose	Saline	9	98^{a} ± 12	2,840^{a} ± 319	54.3^{a} ± 3.2	2,442^{a} ± 171
Loose	Naloxone	9	244^{b} ± 36	7,215^{b} ± 1,126	65.1^{b,d} ± 5.2	3,619^{b,d} ± 376
Tethered	Saline	9	74^{a} ± 12	1,997^{c} ± 332	58.4^{a,d} ± 6.5	2,208^{a,d} ± 304
Tethered	Naloxone	9	240^{b} ± 41	7,657^{b} ± 1,913	87.1^{c} ± 7.8	5,277^{c} ± 1,303

Data are presented as means ± SEM. Values in the same column followed by different superscript letters are significantly different from each other (Wilcoxon; $P<0.05$); N = number of gilts.

Table 2. Plasma ACTH and cortisol responses to acute nose-sling challenge in pigs loose housed during the entire experiment (control group).

Housing	Pretreatment	N	Plasma ACTH		Plasma Cortisol	
			Peak height (pg/ml)	Area under the curve (arbitrary units)	Peak height (ng/ml)	Area under the curve (arbitrary units)
Loose 1	Saline	7	85^{a} ± 23	2,412^{a} ± 705	45.5^{a} ± 5.5	1,856^{a} ± 283
Loose 1	Naloxone	9	188^{b} ± 27	6,015^{b} ± 852	53.2^{b} ± 4.8	2,679^{b} ± 326
Loose 2	Saline	8	95^{a} ± 21	2,904^{a} ± 881	38.9^{a} ± 5.0	1,564^{a} ± 306
Loose 2	Naloxone	7	218^{b} ± 23	6,781^{b} ± 992	49.2^{a,b} ± 4.8	2,754^{b} ± 608

Data are presented as means ± SEM. Values in the same column followed by different superscript letters are significantly different from each other (Wilcoxon; $P<0.05$); N = number of gilts.

literature, probably as a consequence of differences in experimental conditions, most of the studies report that the pituitary responsiveness to an acute stress stimulus is maintained or even enhanced after chronic stress [10,11,23]. In the present experiment, however, long-term tethered housing of the pigs resulted in a decrease of the challenge-induced ACTH response as compared with the loose housing situation. This effect cannot be attributed to a decreased secretory reserve of the corticotrope cells of the pituitary, since a significantly greater ACTH response was achieved after pretreatment with naloxone. It has been shown in rats that prolonged or repeated stress can lead to enhanced negative feedback by circulating corticosteroids [23,24], decreased sensitivity of the pituitary to CRH [24], decreased expression of mRNA for CRH [25], or changes in hypothalamic signals for ACTH secretion [26]. The reduced ACTH response during chronic stress therefore may reflect changes at pituitary and/or suprapituitary level and a variety of mechanisms underlying the effect can be considered but undoubtedly opioid systems are involved.

Notwithstanding the reduced ACTH response, the cortisol response remained unaffected by long-term tethered housing. This finding strongly suggests that the sensitivity of the adrenal cortex to circulating ACTH had increased due to chronic stress. This is consistent with results of our previous study, in which we demonstrated that prolonged tethered housing of pigs can lead to an enhanced plasma cortisol response to exogenous ACTH [16].

In line with literature reports from several species our data clearly illustrate that opioids are involved in the regulation of HPA function, since naloxone pretreatment led to an increase in the ACTH and cortisol responses to acute challenge. In animals exposed to the chronic stress of tethered housing, a greater increment of the challenge-induced ACTH and cortisol responses after naloxone pretreatment was found than in loose housed animals. No such changes were found in the animals of the control group that were housed loose throughout the entire experimental period. This indicates that in tethered animals the impact of endogenous opioid systems had increased as a result of chronic stress. The contention that chronic stress alters the activity of opioid systems is supported by other data in tethered pigs. It has been demonstrated that tethered pigs often develop stereotypies which can be antagonized by naloxone, suggesting that the activity of opioid systems had increased under these conditions [17-20,27]. In addition, the opioid-mediated inhibition of feeding-induced cardiovascular responses is increased during prolonged tethered housing [17]. Apparently, chronic stress leads to adaptive changes in opioid systems that mediate behavioural as well as physiological reactions [28].

The opioid-mediated inhibition of the pituitary-adrenocortical response to the challenge could arise at several levels of the HPA system [29,30]. Since our data revealed that naloxone induced parallel enhancement of the cortisol and the ACTH response, it seems likely that the effect on the cortisol response was secondary to that on the ACTH release. This contention is supported by data of Estienne *et al.* [3], who did not find an effect of naloxone in stimulating cortisol secretion after hypophysial stalk transsection in gilts. Therefore, they concluded that naloxone enhanced cortisol secretion principally by acting at suprapituitary level [3]. Siegel *et al.* [2] demonstrated that in rats with complete hypothalamic deafferentation, naloxone continued to raise plasma ACTH concentrations. This points to the hypothalamus as an important central locus of action of naloxone. Thus, challenge-induced activation of opioid systems possibly mitigate the HPA response most likely including opioid mechanisms at the level of the hypothalamus.

In conclusion, our results provide strong evidence to indicate that chronic stress increases the impact of endogenous opioid systems, which mitigate the pituitary-adrenocortical response to additional acute stress. This change in opioid function may protect the animal from excessive stress responses and thus be of adaptive value.

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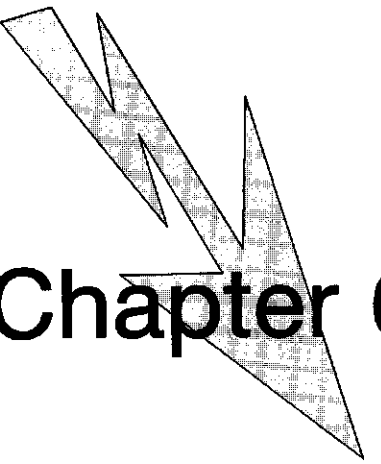

Chapter 6

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Chapter 6

Chronic stress and pituitary-adrenocortical responses to corticotropin-releasing hormone and vasopressin in female pigs

Summary

Effects of long-term tethered housing (a condition of chronic stress) on pituitary-adrenocortical responsiveness to exogenous corticotropin-releasing hormone (CRH) and lysine^{8}-vasopressin (LVP) were investigated in female pigs. Intravenous administration of CRH (dose range 10-440 pmol/kg body weight (BW)) or LVP (10-880 pmol/kg BW) elicited transient and dose-related increases in plasma concentrations of adrenocorticotropic hormone (ACTH) and cortisol. Comparison of the responses induced by the peptides indicated that CRH is a more potent ACTH secretagogue than LVP. LVP treatment produced a five-fold greater plasma cortisol/ACTH ratio than CRH, suggesting that in addition to stimulating pituitary ACTH release, it enhanced the ability of the adrenal cortex to secrete cortisol in response to ACTH. Whereas concomitant administration of 10 pmol CRH/kg BW and 20 pmol LVP/kg BW revealed an additive effect on ACTH release, synergism between both peptides was found with respect to their cortisol releasing effect. Ten to thirteen weeks of chronic stress did not significantly alter the absolute ACTH and cortisol responses to the two peptides. In tethered pigs, the cortisol/ACTH ratio after CRH treatment, calculated from the area under the curve, was two-fold of that in loose housed pigs. From these observations we conclude that after chronic stress the sensitivity of the adrenocortex to circulating ACTH was increased, whereas the sensitivity of the pituitary to CRH and/or LVP remained unaltered.

Introduction

A major component of the adaptive response to stress is the activation of the hypothalamic-pituitary-adrenocortical (HPA) axis. It is well documented that, among other hypothalamic substances, corticotropin-releasing hormone (CRH) and vasopressin play an important role in regulating pituitary-adrenal responses during stress [1-3]. Both peptides stimulate ACTH secretion from the anterior pituitary gland, thereby stimulating secretion of corticosteroids from the adrenal cortex. A synergism between CRH and vasopressin has been reported in a variety of species [1,4-6]. The relative potency of CRH and vasopressin to stimulate ACTH secretion appears to be species specific. While in man and in the rat [4,5] CRH is more potent than arginine^{8}-vasopressin (AVP), the opposite is documented for the sheep [7,8], and the two peptides are equipotent in the cow [9]. Recently, Minton and Parsons [10] demonstrated that in male pigs CRH is a more potent ACTH secretagogue than lysine^{8}-vasopressin (LVP), the naturally occurring form of vasopressin in the pig [11].

There is ample evidence that chronic stress can lead to increased synthesis and storage of hormones at different levels of the HPA axis [12-14]. Several studies in the rat suggest that the release of CRH and AVP into the portal circulation is increased during chronic stress [3,15,16]. It has been reported that chronic stress can also lead to changes in the ratio in which these peptide hormones are released from the median eminence [17-20]. In addition, stress-induced changes in the sensitivity of target cells to secretagogues of HPA activation have been reported [18,21,22]. The present study evaluates the effect of chronic stress, induced by long-term tethered housing [23], on the responsiveness of the pituitary-adrenocortical response elicited by CRH and LVP in female pigs.

Materials and Methods

Animals and Housing

Twenty-eight healthy cyclic crossbred gilts (93 to 125 kg of BW; Great Yorkshire x British Landrace, Pig Improvement Company, U.K.) that had shown two or more consecutive normal oestrous cycles (20.8 ± 1.9 days; mean ± SD) were used in this study. The pigs were housed loose in individual pens (approximately 5.5 m^2) throughout the experiments, except for Experiment 3, in which half of the animals were housed tethered. The floor was

solid concrete and covered with wood shavings, except for a slatted dunging area (2 m^2) at the rear of the pens. Lights were on between 0730 h and 1900 h, and ambient temperature ranged from 15 to 25°C. Twice a day (at 0800 h and 1600 h) the gilts were fed 1 kg of a pelleted, dry sow feed (12.2 MJ metabolizable energy per kg, 15.4% crude protein), delivered by hand. To prevent the gilts from associating the presence of people with feeding, they were conditioned with a bell signal that preceded feeding. Water was available *ad libitum* through a nipple drinker.

Surgery

In order to collect serial blood samples, the gilts were surgically fitted with a permanent jugular vein catheter (polyvinyl chloride, 1.5 mm i.d., 2.1 mm o.d.; Rubber BV, The Netherlands). Surgery, which has been described previously [23], was performed under sterile conditions and under general anaesthesia with inhalation of O_2/N_2O, enflurane (Ethrane®: Abott BV, The Netherlands). To protect the catheter, which was externalized between the scapulae, the animals were equipped with a harness to which they had been habituated during the week before cannulation. The harness (23 cm x 20 cm, polyvinyl chloride with nylon; Bizon Chemie, The Netherlands) was fixed at the back of the animals with belts around the chest. From three days before surgery until three days after surgery, all animals were treated once daily with antibiotics (orally; 12 ml of T.S. Sol®, containing trimethoprim and sulphamethoxazol; Dopharma, The Netherlands). At least 10 days were allowed for the pigs to recover from surgery and anaesthesia.

Catheter patency was maintained by flushing with saline thrice weekly. The catheters were filled with sterile heparinized physiological saline (25 IU heparin/ml of 0.9% saline; Leo Pharmaceutical Products, The Netherlands) when not in use.

Experimental Design

Three experiments were conducted to evaluate the ability of CRH and LVP to stimulate the secretion of ACTH and cortisol in female pigs, and to investigate whether their effects are influenced by chronic stress. In Experiment 1, a dose-response curve was made for CRH in six gilts which were housed loose. Doses of ovine CRH (oCRH) between 10-440 pmol/kg body weight (BW) were administered as an i.v. bolus and the corresponding plasma ACTH and cortisol responses were determined. In Experiment 2, a dose-response curve was made for LVP in six other gilts, using doses of LVP between

10-880 pmol/kg BW. In Experiment 3, doses of oCRH and LVP which evoked submaximal responses were used to investigate chronic stress-induced changes in pituitary responsiveness and the pituitary-adrenocortical responses to LVP and oCRH, administered singly or in combination, were determined in loose housed (control) and in tethered gilts (chronic stress). In all experiments, i.v. bolus injections of saline in the same animals were used as control.

Experiment 1: Dose-Response Curve of oCRH

Six gilts (106.8 ± 13.5 kg of BW; mean ± SD) were used to assess the dose-response curve with oCRH. They were housed loose in individual pens during the entire experimental period. Each pig received 0, 10, 20, 110, 220, and 440 pmol of synthetic oCRH/kg BW i.v. through the catheter. The sequence of the treatments was randomized and performed with an interval of at least two days between treatments. Before each infusion of oCRH, four baseline blood samples were collected at 15-min intervals. At 1015 h, 5 ml sterile 0.9% saline vehicle or oCRH (Sigma Chemical Co., St. Louis, MO; various doses dissolved in 5 ml sterile 0.9% saline solution) was administered as an i.v. bolus injection. Thereafter, approximately 5 ml of sterile saline was used to flush the peptide through the catheter to ensure that the entire mass of peptide entered the circulation. At various times after the injection (5, 10, 15, 30, 75, 105, 135, and 165 min), blood was sampled for plasma ACTH and cortisol determination.

Experiment 2: Dose-Response Curve of LVP

Six gilts (108.8 ± 2.9 kg of BW; mean ± SD) were used in the LVP dose-response curve experiment, which was performed in the same way as the dose-response study described in Experiment 1. At 1015 h, 5 ml sterile 0.9% saline vehicle or various doses of synthetic LVP (kindly donated by Organon Int., Oss, The Netherlands; 10, 20, 55, 110, 220, 440, and 880 pmol/kg BW dissolved in 5 ml sterile 0.9% saline solution) was administered as an i.v. bolus injection. An additional 5 ml of sterile saline was used to flush the peptide through the catheter. Due to catheter impatency, the doses of 10 pmol and 20 pmol LVP/kg BW could only be administered in three and two gilts, respectively. The intervals for sampling of plasma were identical to those in Experiment 1.

Experiment 3: Chronic Stress

Ten weeks prior to the experiment, 16 gilts (108.8 ± 7.9 kg of BW; mean ± SD) were randomly assigned to control and chronic stress groups,

containing eight pigs each. The animals of the control group were housed loose in individual pens during the entire experimental period, while the gilts of the chronic stress group were housed tethered. These gilts were tethered by a 50-cm heavy-gauge neck-chain in individual tether stalls, each 65 cm wide, and placed in a single row. After 8 weeks, all gilts were surgically cannulated. Two weeks after cannulation, the response of the pituitary and the adrenal cortex to treatment with LVP, oCRH, and a combination of both peptides was investigated in both groups.

The gilts were tested according to a randomized block design in which each animal received every treatment. The treatments used were: i.v. injection of 10 ml sterile 0.9% saline vehicle; 20 pmol LVP/kg BW + 5 ml saline; 10 pmol oCRH/kg BW + 5 ml saline; and 20 pmol LVP/kg BW + 10 pmol oCRH/kg BW (peptides were dissolved in 5 ml sterile 0.9% saline solution). These doses of peptides were administered as in Experiments 1 and 2. Blood samples for ACTH and cortisol determination were collected before treatment with 15-min intervals (three baseline samples) and at various times after treatment (5, 10, 15, 30, 45, 75, 105, and 165 min).

Blood Sampling

The procedure of blood collection has been described previously [23]. Before the experimental period, all animals were frequently handled and habituated to the blood collection procedure. Blood samples (approximately 10 ml) were immediately transferred to ice-cooled polypropylene tubes containing 100 μl of EDTA-solution (144 mg of EDTA/ml of saline; Titriplex®III, Merck Nederland BV, The Netherlands). They were immediately mixed and placed on ice, and subsequently centrifuged at 2,000 x *g* for 10 min at 4°C. Plasma was collected and stored at −20°C until hormone analysis.

Hormone Analyses

Plasma samples were analyzed for ACTH and cortisol by immunoassay. ACTH assay was performed in singular, cortisol assays in duplicate. All samples from an individual gilt were analyzed in the same assay.

ACTH. ACTH was measured in unextracted plasma using commercial two-site immunoradiometric assay (IRMA) testkits obtained from Euro-Diagnostica BV (The Netherlands). Details of the assay have been described by Copinschi [24]. The sensitivity of the ACTH assay was 0.8 pg/ml. The intra-assay coefficient of variation was 2.6% and the inter-assay coefficient of variation was 4.7%.

Cortisol. Plasma concentrations of cortisol were measured in unextracted 50 μl samples using a single-antibody radioimmunoassay (RIA) technique as described previously [23]. The sensitivity of the assay was 0.5 ng/ml at the 90% B/B_0 level. The intra-assay coefficient of variation was 7.8% and the inter-assay coefficient of variation was 12.3%.

Analysis of Data and Statistics

The curves of the plasma ACTH responses to all treatments were plotted for each animal. A baseline was determined for each curve with linear regression using the ACTH values of the blood samples taken before treatment (pre-injection values). The area under the curve (AUC) was calculated as the area between the ACTH baseline and the ACTH response curve above the baseline during the first 45 min after injection of the peptide(s). This particular time period was chosen in order to compare AUC data of all three experiments, since for the LVP dose-response curve only ACTH data of the first 45 min after injection were available. In this approach the cortisol/ACTH ratios calculated for the AUC were not substantially different from the cortisol/ACTH ratios calculated for the AUC of the entire sampling period (165 min; tested for all oCRH doses). The peak height of the ACTH response was defined as the maximal plasma ACTH concentration minus the corresponding basal ACTH value. The cortisol data were analyzed using the same method.

The Kruskal-Wallis test was used to assess whether treatment induced a significant effect on the pituitary-adrenocortical response. Thereafter, differences in peak height and AUC of the ACTH and cortisol responses to various doses of oCRH and LVP, and the combination of LVP and oCRH were tested for significance by Wilcoxon matched pairs signed-ranks test. The Mann-Witney U test was used to test for differences in the responses (peak height and AUC) between pigs of the chronic stress (tethered housed) and the control (loose housed) groups.

All statistical analyses were performed using the SPSS statistical package [25]. The criterion for statistical significance was set at $P<0.05$. Results are given as mean ± SEM.

RESULTS

Experiment 1: Dose-Response Curve of oCRH

Figure 1 shows the mean plasma ACTH and cortisol responses and their

time course following i.v. bolus injection of saline vehicle, or oCRH in doses of 10 to 440 pmol/kg BW. Saline administration had no effect on ACTH and cortisol concentrations. Regardless of the doses used in this experiment, oCRH induced a time-dependent increase over basal (pre-injection) levels in ACTH and cortisol concentrations in all animals; the increase was significant ($P<0.05$) compared with concentrations in the same animals after infusion of saline (Figure 1; Table 1).

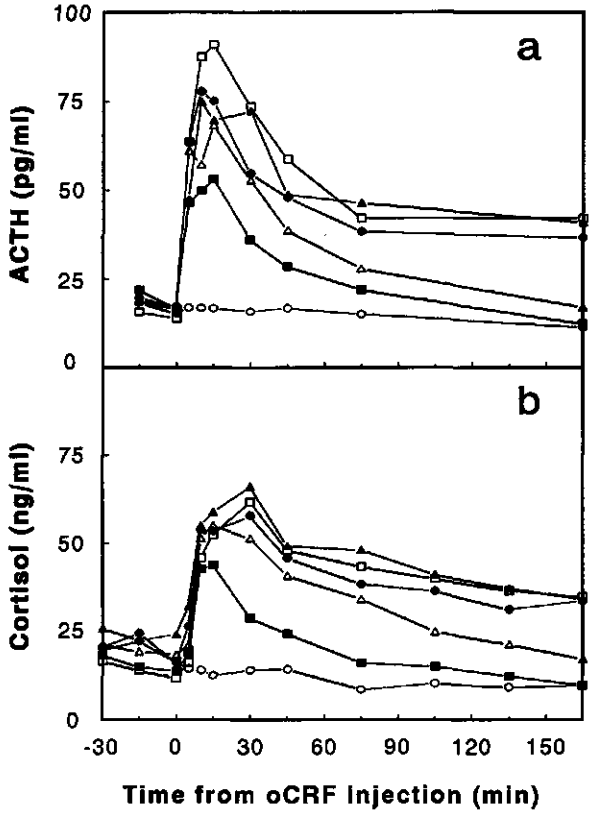

Figure 1. Mean plasma concentrations of ACTH (Figure 1a) and cortisol (Figure 1b) in cyclic gilts (n=6) before and after i.v. administration of 5 ml saline vehicle (○) or incremental doses of synthetic oCRH (■ 10; ▵ 20; ● 110; □ 220; ▲ 440 pmol oCRH/kg BW).

Highest ACTH concentrations were found in the samples taken at 5 to 30 min after oCRH injection. Maximal peak height (61.3 ± 12.2 pg/ml) and AUC (2088 ± 322 arbitrary units) of the ACTH response were induced at a dose of 110 pmol oCRH/kg BW and greater (see Table 1). ACTH returned to pre-injection levels within approximately 2.5 hours after injection of 10 pmol and 20 pmol oCRH/kg BW, whereas ACTH levels remained elevated throughout the sampling period after injection of oCRH doses of 110 pmol/kg BW and higher.

Peak plasma cortisol concentrations occurred at 10 to 45 min after injection. Similar to ACTH, cortisol concentrations after injection of low doses of oCRH (10 pmol and 20 pmol/kg BW) returned to basal levels within 2.5 hours, whereas cortisol levels remained elevated for at least 165 min after injection of high doses of oCRH. Cortisol responses (peak height, 40.7 ± 5.5 ng/ml; AUC, 1513 ± 217 arbitrary units) reached a plateau at oCRH doses of 110 pmol/kg BW and greater (see Table 1).

Cortisol/ACTH ratios of peak height and AUC remained rather stable (approximately 0.8) with increasing doses of oCRH (Table 1).

Experiment 2: Dose-Response Curve of LVP

Mean plasma ACTH and cortisol concentrations before and after LVP injection are illustrated in Figure 2. Highest ACTH concentrations were found in the samples taken at 5 to 15 min after LVP injection. Neither peak height nor AUC of ACTH appeared to have reached a plateau at the highest dose of LVP used. Thus, the maximum response of ACTH secretion in response to LVP cannot be defined from this study.

Peak plasma cortisol concentrations occurred at 10 to 45 min after injection. Peak height of cortisol did not increase significantly above the 220 pmol/kg BW dose of LVP (see Table 2). The lowest LVP doses (10 pmol and 20 pmol/kg BW) were only tested in three and two gilts, respectively, and therefore could not be included in the statistical analysis.

With increasing dose of LVP, cortisol/ACTH ratios of peak height and AUC significantly decreased from approximately 7 to 0.5. This decrease in cortisol/ACTH ratio was the result of a marked increase in ACTH response with increasing doses of LVP, whereas only a minor increase occurred in the corresponding cortisol levels.

Comparison of the pituitary-adrenocortical responses to equimolar doses (110 pmol and 220 pmol/kg BW) of oCRH and LVP, revealed significantly higher ACTH responses (peak height; $P<0.05$; AUC; $P<0.05$; Mann-Witney U test) after oCRH treatment as compared with LVP. In addition, oCRH induced a

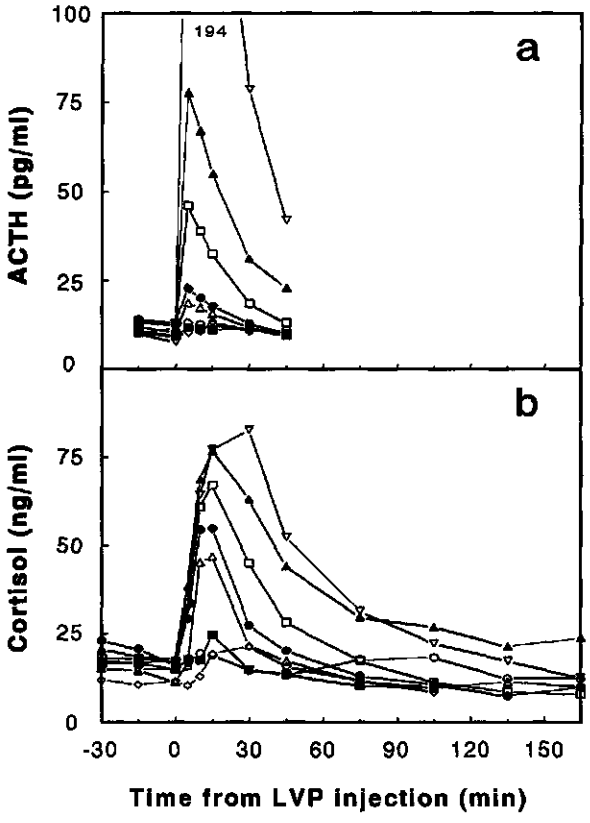

Figure 2. Mean plasma concentrations of ACTH (Figure 2a) and cortisol (Figure 2b) in cyclic gilts (n = 6) before and after i.v. administration of 5 ml saline vehicle (○) or incremental doses of synthetic LVP (◇ 10, n = 3; ■ 20, n = 2; ▵ 55; ● 110; □ 220; ▲ 440; ▽ 880 pmol LVP/kg BW).

significantly higher cortisol response than LVP with respect to the AUC ($P < 0.05$), while peak height was equal. Plasma cortisol/ACTH ratios after LVP treatment were approximately five-fold of those obtained with oCRH treatment (peak height; $P < 0.05$; AUC; $P < 0.05$; Mann-Witney U test).

Experiment 3: Chronic Stress

Loose and tethered gilts were challenged with saline vehicle, 10 pmol oCRH/kg BW, 20 pmol LVP/kg BW, and with a combination of both peptides. Mean plasma ACTH and cortisol responses to the challenges are presented in Table 3. Infusion of saline vehicle did not affect ACTH and cortisol levels. Treatment with LVP, oCRH, or LVP+oCRH induced a transient increase in ACTH and cortisol concentrations. In both housing groups, 10 pmol oCRH/kg BW induced significantly higher ACTH and cortisol responses (tested for peak height and AUC; $P<0.05$) than 20 pmol LVP/kg BW. No significant differences in ACTH and cortisol responses to LVP, oCRH, or LVP+oCRH were found between loose and tethered housed gilts.

Treatment with LVP+oCRH elicited significantly greater responses than either peptide alone (indicated by asterisks in Table 3). In animals of both housing groups, combined administration of the peptides produced a cortisol response (with respect to peak height) that was significantly greater than the sum of the responses to each of the peptides alone ($P<0.05$; Table 3), indicating synergism. Such a synergistic action of LVP and oCRH was also found with AUC as index for the cortisol response, albeit after pooling of the values of loose and tethered housed gilts. The effect of the two peptides on the ACTH response (with respect to peak height and AUC) was additive.

Analogous to experiments 1 and 2, the cortisol/ACTH ratio in loose housed gilts after LVP was approximately five-fold of that after oCRH treatment (peak height; 5.0 ± 1.9 vs. 0.9 ± 0.3; $P<0.05$; AUC; 4.8 ± 1.6 vs. 0.6 ± 0.2; $P<0.05$; Table 3). This appeared to be the case also in tethered animals (peak height; 2.9 ± 0.5 vs. 1.4 ± 0.3; $P<0.05$; AUC; 4.0 ± 1.2 vs. 1.2 ± 0.2; $P<0.05$). In tethered pigs, however, a greater cortisol/ACTH ratio was found after oCRH treatment (peak height; 1.4 ± 0.3 vs. 0.9 ± 0.3; $P<0.10$; AUC; 1.2 ± 0.2 vs. 0.6 ± 0.2; $P<0.05$) than in loose housed animals.

Discussion

In the present study, the influence of chronic stress on pituitary-adrenocortical responsiveness to oCRH and/or LVP was investigated in intact female pigs. Our results indicate that at low doses (<440 pmol/kg BW) oCRH is more potent in stimulating ACTH release than LVP. LVP, however, elicited a greater cortisol response than oCRH relatively to the ACTH response, pointing to a direct effect of this peptide on the adrenal cortex. The results of concomitant administration

Table 1. Plasma ACTH and cortisol responses to various doses of oCRH in cyclic gilts (n = 6).

oCRH	Plasma ACTH		Plasma Cortisol		Ratio Cortisol/ACTH	
Dose (pmol/kg BW)	Peak height (pg/ml)	AUC[1] (arbitrary units)	Peak height (ng/ml)	AUC (arbitrary units)	Peak height (ratio)	AUC (ratio)
0	0.9 ± 1.0	8 ± 11	−1.9 ± 2.0	9 ± 29	–	–
10	40.0^{a} ± 9.9	901^{a} ± 71	35.1^{a} ± 3.0	812^{a} ± 84	$1.1^{a,b}$ ± 0.3	0.9^{a} ± 0.1
20	$52.7^{a,b}$ ± 8.0	1604^{b} ± 212	$42.1^{a,b}$ ± 6.1	$1207^{b,c}$ ± 182	$0.8^{a,c,d}$ ± 0.1	0.8^{a} ± 0.1
110	$61.3^{b,c}$ ± 12.2	$2088^{b,c}$ ± 322	$40.7^{a,c}$ ± 5.5	1513^{d} ± 217	$0.9^{a,b,c}$ ± 0.3	$0.8^{a,b}$ ± 0.1
220	80.6^{c} ± 13.7	2644^{c} ± 420	53.6^{c} ± 8.1	$1585^{c,d}$ ± 260	$0.7^{b,e}$ ± 0.1	0.6^{b} ± 0.1
440	74.3^{c} ± 9.8	2092^{c} ± 245	$46.8^{b,c}$ ± 4.8	1579^{d} ± 180	$0.7^{d,e}$ ± 0.1	0.8^{a} ± 0.1

Data are presented as means ± SEM. Values in the same column with the same different superscript letter are not significantly different from each other (Wilcoxon; $P > 0.05$).

[1]AUC = Area under the curve for the first 45 min after injection.

Table 2. Plasma ACTH and cortisol responses to various doses of LVP in cyclic gilts (n = 6).

LVP	Plasma ACTH		Plasma Cortisol		Ratio Cortisol/ACTH	
Dose (pmol/kg BW)	Peak height (pg/ml)	AUC (arbitrary units)	Peak height (ng/ml)	AUC (arbitrary units)	Peak height (ratio)	AUC (ratio)
0	$-0.2^{a} \pm 1.0$	$-1^{a} \pm 12$	$3.4^{a} \pm 2.2$	$15^{a} \pm 35$	–	–
10[2]	$3.6^{a} \pm 1.9$	$48^{a} \pm 25$	$15.2^{a} \pm 6.2$	$331^{a} \pm 186$	$5.6^{a} \pm 2.2$	$8.0^{a} \pm 3.3$
20[2]	$2.7^{a} \pm 0.4$	$85^{a} \pm 10$	$20.3^{a} \pm 9.4$	$250^{a} \pm 64$	$7.1^{a} \pm 2.6$	$2.9^{a} \pm 0.4$
55	$8.2^{a} \pm 2.1$	$123^{a} \pm 34$	$42.3^{a} \pm 7.9$	$703^{a} \pm 101$	$6.6^{a} \pm 1.8$	$7.2^{a,b} \pm 1.6$
110	$12.1^{b} \pm 2.8$	$212^{b} \pm 43$	$47.6^{a,b} \pm 5.2$	$831^{a,b} \pm 121$	$4.6^{a} \pm 0.8$	$4.4^{a,c} \pm 0.7$
220	$33.7^{c} \pm 7.3$	$596^{c} \pm 123$	$54.8^{a,c} \pm 7.7$	$1431^{b,c} \pm 215$	$2.5^{a,b} \pm 1.1$	$3.8^{b,c} \pm 1.8$
440	$71.2^{c} \pm 17.4$	$1511^{c} \pm 491$	$63.9^{c} \pm 5.2$	$1880^{c,d} \pm 199$	$1.4^{b,c} \pm 0.6$	$2.0^{c} \pm 0.7$
880	$205.3^{d} \pm 47.2$	$4676^{d} \pm 1376$	$69.6^{b,c} \pm 6.4$	$2307^{d} \pm 150$	$0.5^{d} \pm 0.1$	$0.7^{d} \pm 0.2$

Data are presented as means ± SEM. Values in the same column with the same superscript letter are not significantly different from each other (Wilcoxon; $P > 0.05$).

[1]AUC = Area under the curve for the first 45 min after injection.

[2]Due to cannula impatency, the doses of 10 pmol and 20 pmol LVP/kg BW could only be administered in three and two gilts, respectively; the data of the 10 pmol and 20 pmol groups were not used for statistical analysis.

Table 3. Plasma ACTH and cortisol responses to infusion of saline, LVP, oCRH, and LVP+oCRH in loose and tethered housed gilts (n = 8 in each group).

Housing	Treatment	Plasma ACTH		Plasma Cortisol		Ratio Cortisol/ACTH	
		Peak height (pg/ml)	AUC[1] (arbitrary units)	Peak height (ng/ml)	AUC (arbitrary units)	Peak height (ratio)	AUC (ratio)
Loose	Saline	1.1 ± 0.8	4 ± 8	5.1 ± 1.9	19 ± 16	–	–
Loose	LVP[2]	8.0[a] ± 2.8	70[a] ± 23	11.9[a] ± 2.3	203[a] ± 57	5.0 ± 1.9	4.8 ± 1.6
Loose	oCRH[3]	30.5[b] ± 5.2	701[b] ± 95	21.3[b] ± 3.2	366[a] ± 66	0.9[a] ± 0.3	0.6[a] ± 0.2[4]
Loose	LVP[2] + oCRH[3]	39.3[b] ± 6.4	804[b] ± 128	40.7[c] ± 3.5 *	892[b] ± 115 **	1.3 ± 0.2	1.3 ± 0.3
Tethered	Saline	−0.8 ± 1.2	−2 ± 11	4.3 ± 3.0	14 ± 12	–	–
Tethered	LVP[2]	4.8[a] ± 1.3	72[a] ± 24	12.7[a] ± 3.3	197[a] ± 46	2.9 ± 0.5	4.0 ± 1.2
Tethered	oCRH[3]	22.0[b] ± 6.9	553[b] ± 147	23.7[b] ± 4.6	552[b] ± 104	1.4[a] ± 0.3	1.2[a] ± 0.2[4]
Tethered	LVP[2] + oCRH[3]	35.0[c] ± 5.8	773[c] ± 147	46.5[c] ± 7.8 *	908[c] ± 160 **	1.5[a] ± 0.3	1.3[a] ± 0.2

Data are presented as means ± SEM. Values in the same column within the same group (loose or tethered), with the same superscript letter are not significantly different from each other (Wilcoxon; $P>0.05$).

[1]AUC = Area under the curve for the first 45 min after injection.

[2]Dose administered: 20 pmol LVP/kg BW.

[3]Dose administered: 10 pmol oCRH/kg BW.

[4]Significant difference between loose and tethered gilts (Mann Witney U test; $P<0.05$).

*Significant difference between response to combined administration of LVP and oCRH, and the sum of the responses to each of the peptides (Wilcoxon; $P<0.05$).

**Significant difference between response to combined administration of LVP and oCRH, and the sum of the responses to each of the peptides after pooling of values of loose and tethered gilts (Wilcoxon; $P<0.05$).

of the two peptides revealed an additive effect on ACTH release, whereas synergism between both peptides was found for their cortisol releasing effect (Table 3), supporting this contention. Chronic stress did not significantly alter the absolute ACTH and cortisol responses to treatment with LVP, oCRH, or LVP + oCRH. In tethered animals, however, a significantly greater cortisol/ACTH ratio was found after oCRH treatment, compared with loose animals. This finding suggests that, as a result of chronic stress, the sensitivity of the adrenal cortex to circulating ACTH had increased.

The concerted action of hypothalamic CRH and vasopressin plays a crucial role in regulating ACTH release from the anterior pituitary gland [1,2,26]. Relative potencies of the two peptides to activate the pituitary-adrenal axis have been found to be species specific [4,5,7-10]. In the present study, we demonstrated that administration of oCRH or LVP can induce a transient and dose-related increase in plasma ACTH and cortisol concentrations in female pigs. Comparison of the responses to equimolar doses indicated that in (female) pigs CRH is a more potent ACTH secretagogue than LVP, which is in line with data reported by Minton and Parsons for male pigs [10]. The maximal peak height and AUC of the ACTH response with the highest dose of LVP (880 pmol/kg BW) appeared to be greater compared with the highest dose of oCRH (440 pmol/kg BW). We cannot exclude, however, that this effect was due to cardiovascular and other effects caused by a high dose of LVP injected. We therefore conclude that, with regard to the relative potencies of the two peptides in activating the pituitary-adrenocortical axis, the pig resembles man and rat [4,5].

In comparison with LVP, oCRH had a sustained effect (Figures 1 and 2) on ACTH and, consequently, on cortisol release. Based on data from literature [27,28], this can be explained by the fact that CRH, in contrast to vasopressin, not only induces secretion of ACTH from the readily releasable pool in the pituitary, but induces also synthesis and formation of ACTH. Differences in second messenger systems between both peptides may probably account for these differences in ACTH secretion: CRH activates adenylate cyclase, the enzyme that catalyses the synthesis of cAMP, whereas actions of vasopressin on corticotrophs are mediated via the phospho-inoside messenger system [1,8,29,30]. Alternatively, the different time course of the ACTH responses may be due to differences in plasma half-life of the peptides. In man, considerable differences have been reported between plasma half-life for oCRH (circa 1 hour) [31,32] and vasopressin (1 to 5 min) [33].

In the current study, LVP induced a significantly greater cortisol/ACTH ratio

than oCRH, both in loose and in tethered animals. Since both peptides were injected intravenously, they can have acted also at sites other than the pituitary. We therefore hypothesize that in the (female) pig, vasopressin can stimulate corticosteroidogenesis beyond that induced by vasopressin-stimulated ACTH release, possibly by a direct effect on the adrenal gland. In support of this hypothesis, there is increasing evidence in a variety of species, suggesting that vasopressin [34-38] as well as CRH [39] can act directly on the adrenal gland to stimulate glucocorticosteroid release. Brooks and Blakemore [36] showed that in dogs vasopressin increases plasma cortisol concentrations in the absence of an increase in ACTH, provided that background levels of ACTH are present.

It has been demonstrated in a variety of species [1,4-6,40] that vasopressin and CRH can act synergistically in stimulating ACTH secretion. In our study, no synergistic action of LVP and oCRH was found for their ACTH releasing effect, but the effect of both peptides was rather additive. This is in agreement with data reported by Minton and Parsons in pigs [10]. Unlike their findings, however, we found that concomitant administration of the peptides produced a significantly greater cortisol increase than the sum of the responses to each individual peptide. It can be speculated that this synergism between LVP and oCRH resulted from an LVP-induced increase in the sensitivity of the adrenal cortex to ACTH. The discrepancies between our findings and those of Minton and Parsons [10] may be explained by differences in animals and experimental design between their and our study, since they used tethered housed, castrated, male pigs that were used for experimentation shortly (4 days) after surgery and anaesthesia.

It is well established that chronic stress can induce changes at different levels of organization of the HPA axis [12-14]. Indeed, we have found previously that long-term tethered housing of female pigs induces a protracted hypercortisolaemia [41]. In the current study, no significant differences between loose and tethered pigs were found with respect to absolute responses of plasma ACTH and cortisol challenged by exogenous LVP, oCRH, or LVP+oCRH, indicating that the sensitivity of the pituitary for these hypothalamic secretagogues had not changed. The CRH-induced cortisol/ACTH ratio, however, displayed a two-fold increase in tethered animals compared with loose animals. In line with previous findings in tethered pigs (Janssens *et al.*, accepted for publication), these results indicate that the sensitivity of the adrenal cortex to circulating ACTH had increased as a result of chronic stress, and point to the adrenal cortex as a site where regulatory changes in HPA activity occur during chronic stress. This contention is supported by our observations that challenges

with synthetic ACTH-(1-24) revealed an enhanced adrenocortical steroidogenic capacity in tethered pigs [23]. From this and our previous findings, we conclude that chronic stress did not alter the responsiveness of the pituitary to LVP and/or CRH, whereas it increased the sensitivity of the adrenal cortex to circulating ACTH.

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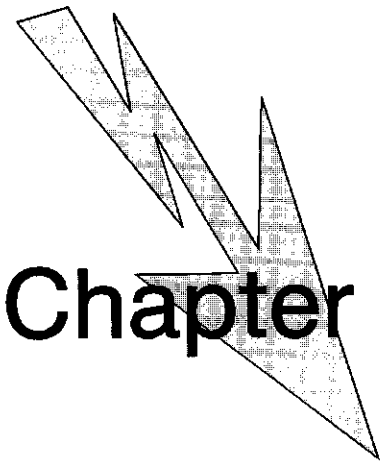
Chapter 7

Chapter 7

Summary & General Discussion

Introduction

The main purpose of the studies described in this thesis was to gain more insight in the regulation of the hypothalamic-pituitary-adrenocortical (HPA) system and the mechanisms underlying adaptation to chronic stress in female pigs. The function of the HPA axis, which coordinates multiple neuroendocrine and metabolic responses to stressors, has been subject of extensive basic and clinical research. HPA-activation by stressful stimuli results in an increase in circulating adrenocorticotropic hormone (ACTH) and consequently of glucocorticoid hormones. A brief review of stress and HPA functions is given in Chapter 1.

It has been demonstrated in a variety of species that exposure to chronic or repeated stress may induce long-term changes in the regulation of the HPA axis [1,2]. These changes may occur at the following levels: 1) extrahypothalamic centers modulating the activity of neurons in the paraventricular nucleus of the hypothalamus that secrete corticotropin-releasing hormone (CRH) and/or other ACTH secretagogues; 2) hypothalamic sites releasing ACTH secretagogues; 3) ACTH-secreting cells in the anterior lobe of the pituitary; 4) glucocorticoid secreting cells in the adrenal cortex.

This project was mainly focussed on the regulation of pituitary-adrenocortical function during chronic stress. Long-term restraint of female pigs by a neck-tether was used as a chronic stress paradigm. Previous studies have demonstrated that tethered pigs may develop behavioural, hormonal, and cardiovascular disturbances characteristic for chronic stress. In Chapters 2 and 3 of this thesis we showed that prolonged tethered housing leads to a sustained elevation of basal plasma cortisol concentrations. Chapters 4, 5 and 6 provide evidence that chronic stress may induce long-term changes at the level of the adrenal cortex, resulting in increased steroidogenic capacity and sensitivity for ACTH. No change was found in the sensitivity of the pituitary for CRH or

vasopressin. There was no indication for an increase in basal ACTH levels, but the ACTH response to a superimposed acute stress appeared to be reduced during chronic stress. The hypersecretion of cortisol may therefore well be a consequence of stress-induced changes in adrenocortical function. Moreover, chronic stress leads to an increase in the activity of opioid systems that inhibit pituitary-adrenal responses to additional acute stressors. These alterations in opioid systems may be of adaptive value in that they prevent excessive reactions of the pituitary-adrenal system during chronic stress. This may also underlie the transient nature of the hypercortisolaemia.

Pituitary-Adrenocortical Function after Chronic Stress

Hypercortisolaemia

There is ample evidence, particularly from studies in the rat, that repeated exposure to stressors may produce an increase in the activity of the adrenocortical system, as evidenced by increased circulating corticosteroid concentrations and/or adrenocortical hypertrophy and increased adrenal weight [1-4]. Indeed, in female pigs we found that chronic stress induces long-term elevated basal cortisol levels (Chapters 2 and 3). This hypercortisolaemia, which develops within the first weeks of tethered housing, is evident in every phase of the oestrous cycle of the pigs. It is particularly obvious during the luteal phase of the oestrous cycle when cortisol levels remain rather stable (Chapter 2). In addition, our data reveal circadian differences in the effect of chronic stress on HPA-regulation. Elevated cortisol concentrations are most pronounced in the evening, i.e. during the nadir of cortisol secretion, and lead to a flattened diurnal rhythm of cortisol secretion during chronic restraint stress (Chapter 3). These findings correspond with observations in rats and in man, that chronic stress-induced corticosteroid levels are increased during the trough of circadian adrenocortical activity [5-7]. Determination of cortisol over a 24-hour period demonstrated that elevated evening cortisol concentrations during chronic stress are not the result of a stress-induced shift in the phase of the cortisol rhythm (Chapter 3).

Chronic stress may conceivably cause "facilitation" of the HPA system, either by increasing its sensitivity to stimulus input or by decreasing its sensitivity to negative glucocorticoid feedback, or both, so that elevated corticosteroid concentrations are maintained throughout the period of stress [8].

It is well-known that ACTH has a trophic effect on the adrenal cortex. Prolonged ACTH stimulation (e.g., during chronic stress) may therefore lead to hypertrophic enlargement of the adrenal gland, resulting in increased output of glucocorticoids in response to this peptide with time. Furthermore, in rats it has been reported that chronic stress may result in a persistent decrease in feedback sensitivity to corticosteroids [9]. Akana and co-workers have suggested that there is a high sensitivity to glucocorticoid feedback during the trough of the circadian corticosteroid rhythm, accompanied by a diminished (or absent) circadian-dependent drive to CRH secretion in rats [10]. Moreover, they suggested that the circadian rise in plasma corticocosteroid levels is a result of stimulated CRH secretion as a consequence of diminished sensitivity to steroid feedback. One may speculate that the increase in evening, but not in morning plasma cortisol levels during chronic stress, described in Chapter 3, is in part the result of stress-induced changes in feedback sensitivity to circulating cortisol. In other words, the hypothalamus remains insensitive to cortisol, resulting in increased cortisol levels throughout the day.

Increased Sensitivity and Capacity of the Adrenal Cortex

We found that the chronic hypersecretion of cortisol in tethered pigs does not coincide with an increase in plasma levels of ß-endorphin (Chapter 2), a peptide co-secreted with ACTH from the pituitary corticotroph cell [11]. It may therefore be inferred that the hypercortisolaemia is not a reflection of a sustained increase in ACTH release from the pituitary, although basal plasma ACTH levels were not measured in those experiments. Several reports in rats, showing that elevated glucocorticoid levels during exposure to chronic stress are not necessarily associated with significant increases in plasma ACTH [1,6,12], support this contention.

Our data (Chapters 4, 5 and 6) provide evidence for stress-induced changes in HPA-regulation. In pigs tethered for a period of 10-11 weeks, the ACTH response to a superimposed stressor (acute nose-sling stress) appears to be reduced as compared with loose housing, whereas the cortisol response remains unaltered (Chapter 5). These data indicate that the sensitivity of the adrenal cortex to circulating ACTH increases as a result of chronic stress. This finding is supported by the study described in Chapter 6, in which the cortisol/ACTH ratio after CRH treatment in tethered pigs shows a two-fold increase when compared with loose housed pigs. Moreover, challenge with exogenous ACTH(1-24) reveals that the capacity of the adrenal cortex to secrete cortisol increases

during chronic stress (Chapter 4), which is in line with findings of other studies in pigs [13] and other species [14]. This increase in adrenocortical capacity may result from a hypertrophic enlargement of the adrenal gland. All in all, our observations point to the adrenal cortex as one of the dominant sites where functional changes occur during chronic stress.

We speculate that these changes in adrenocortical function may well underlie the hypercortisolaemia in tethered pigs (Chapters 2 and 3). Although ACTH is generally considered to be the most important factor in the control of glucocorticoid secretion from the adrenal cortex, there is increasing evidence that steroidogenesis is also under control of neural inputs at the level of the adrenal gland, which can modulate adrenocortical sensitivity to ACTH and thereby control the secretory activity of the adrenal cortex [12,15]. In rats it has been reported that locally secreted CRH is likely to mediate such an increase in adrenal sensitivity to ACTH by stimulating the blood flow through the adrenal gland [16,17], although, to our knowledge, no such mechanism has (yet) been demonstrated in the pig. In this context, it is interesting to note that a challenge with vasopressin in our study produced an approximately five-fold greater cortisol/ACTH ratio than a challenge with CRH (Chapter 6). This implies that in the pig, vasopressin can stimulate corticosteroidogenesis beyond that induced by vasopressin-stimulated ACTH release, possibly by a direct effect on the adrenal gland. In addition, our data indicate a synergism between vasopressin and CRH in their cortisol releasing effects, which may also result from a vasopressin-induced increase in the responsiveness of the adrenal cortex to ACTH.

Effects of Housing Conditions on Adrenocortical Capacity

It was interesting to find in our study that housing factors different from the physical restraint of tethered housing per se play a role in the development of adaptive changes in the steroidogenic capacity of the adrenal cortex during chronic stress. We showed that the increase in the cortisol response to challenge with exogenous ACTH(1-24) is considerably more pronounced and persistent in tethered pigs deprived of visual and tactile contacts with conspecifics and with very limited visual control over the environment, than in tethered pigs that have such possibilities, albeit to a limited degree (Chapter 4). The least restricted pigs were separated by horizontal bars, thus allowing social contacts with neighbouring pigs and visual control over the environment. In the most restricted pigs, closed partitions between the pens precluded social

interactions and severely limited their visual range. It seems likely that the relative lack of visual information from the environment reduces the predictability of environmental changes and increases uncertainty for those animals, thereby contributing to the stress experienced by the animals. As has been discussed in Chapter 1, low predictability or uncertainty is generally recognized as a characteristic of stressful situations [18,19]. Furthermore, it has been demonstrated that lack of social interactions with conspecifics may affect stress responses, especially with respect to the HPA axis [20]. Thus, both reduced visual control and social restriction are likely to be important factors contributing to the changes in adrenocortical function observed earlier. These findings underscore the notion that psychological factors are important activators of the HPA axis [21].

Sensitivity of the Pituitary

So far, effects of chronic stress on adrenocortical function have been emphasized. Studies in rats have provided evidence that chronic stress may also lead to changes in the sensitivity of the pituitary for hypothalamic peptides regulating ACTH secretion, possibly as a consequence of changes in expression and secretion of these secretagogues. Repeated activation of the HPA system may lead to plastic changes in hypothalamic CRH neurons, resulting in increased expression of vasopressin in CRH-containing neurons and increased vasopressinergic stores in vesicles in the median eminence, leading to an increased ratio of vasopressin/CRH that is secreted [22-26].

Hashimoto et al. [27] found that the pituitary-adrenocortical response to vasopressin was enhanced in rats that were chronically immobilized as compared with unstressed controls, whereas responses to exogenous CRH remained unaltered in these animals. They suggested that chronic stress caused a hypersensitivity of the pituitary to vasopressin. In pigs tethered for a 10 to 13-week period, the absolute ACTH and cortisol responses to exogenous CRH, vasopressin, or a combination of these two peptides (Chapter 6) are not significantly altered, as compared with loose housed control animals. The cortisol/ACTH ratio after CRH treatment, however, is significantly higher in tethered than in loose pigs.

In summary, chronic stress leads to an increase in the sensitivity and capacity of the adrenal cortex to circulating ACTH, whereas the sensitivity of the pituitary to stimulation with CRH and/or vasopressin in pigs remains unaltered. These findings again point to adaptive changes in adrenocortical

function as a consequence of stress, possibly mediated by mechanisms modulating adrenocortical sensitivity to ACTH.

Adaptation to Chronic Stress

Activation of stress systems results in behavioural and physiological changes which allow the organism to adapt. In general, adaptive responses to stress involve a redirection of both behaviour and energy [28]. Simultaneously, digestion and anabolic processes, such as growth, reproduction and immune function are suppressed [29]. It appears that the ability to regulate the stress response appropriately may be as important as the ability to initiate it. Containment of the stress response is crucial to avoid detrimental consequences of excessive mobilization of resources and behavioural responses.

As stated earlier, chronically stressed pigs develop changes in adrenocortical function so that further responsiveness of the adrenal system is maintained, despite elevated glucocorticoid levels. The hypersecretion of cortisol is maintained for at least three complete oestrous cycles after tethering (approximately 9 weeks) and thereafter it gradually disappears (Chapter 3). This suggests that adaptive changes occur during chronic stress affecting HPA-activity and leading to normalization of adrenocortical output. Nevertheless, the fact that both the sensitivity and the capacity of the adrenal cortex are increased during the same period (Chapters 4, 5, and 6), suggests that adrenocortical function has chronically changed. Moreover, the apparent adaptation of cortisol levels during chronic stress does not imply normalization of brain mechanisms controlling cortisol concentrations in response to challenges or stressors (Chapter 5). Presumably, adaptation consists in changing certain "set-points" in order to meet the new demands [30].

The mechanisms responsible for these adaptive changes during chronic stress likely include an increase in the activity of endogenous opioid systems. It has been demonstrated that tethered sows may develop stereotypies [31] associated with and dependent on the activation of opioid systems. Administration of naloxone, a specific opiate receptor antagonist, has shown to block or reduce the occurrence of this invariant behaviour [32-35]. Although we did not quantify behaviour in our experiments, we also observed performance of stereotypies such as bar or chain biting and sham chewing in tethered gilts. Brain opioid systems, however, are not only involved in the neurochemical control of behaviour, e.g., of stereotypies, but have also been implicated in the

regulation of the activity of several hormonal systems [36], including the HPA axis [37-40]. Thus, chronic stress of tethered housing leads to increased activity of endogenous opioid systems and may therefore affect the HPA axis by means of opioids. In view of this hypothesis, we subjected both tethered and loose housed female pigs to an acute nose-sling challenge. The enhanced and prolonged ACTH and cortisol responses to nose-sling challenge after pretreatment with naloxone (Chapter 5) point to an activation of both the HPA system and the endogenous opioid systems. This naloxone-dependent increment in ACTH and the cortisol responses are significantly greater in animals tethered for a 10 to 11-week period than in loose animals, indicating that the opioid-mediated suppression of the pituitary-adrenocortical response is increased during chronic stress (Chapter 5). It may be speculated that the gradual reduction of the hypercortisolaemia, observed during the same period of tethered housing is also a consequence of an increase in the impact of endogenous opioid systems. Based on our findings and on literature data, we postulate the hypothalamus to be a key central site for the mediation of this effect of opioids (Chapter 5). All in all, these data indicate that chronic stress of tethered housing leads to adaptive changes in opioid systems modifying behavioural as well as endocrine reactions.

Individual Differences in Pituitary-Adrenocortical Responses

A major focus of the stress and coping literature has been on individual differences in reactivity to stressors under challenging conditions. There is ample evidence that not all individuals of the same species experience the same situation as stressful, and, vice versa, that the same stressors do not necessarily result in identical behavioural and peripheral responses. As mentioned in the general introduction, differences between individuals in coping with stressful situations appear to be related to genetic constitution [41], as well as to prior (particularly: early life) experiences, and the actual physiological and psychological state of the organism [42-44]. Important in this respect is the way in which an individual estimates the situation and its possibilities to cope with the situation [30].

In the present project, we obtained evidence that pigs express individual variability in the (re)activity of the pituitary-adrenocortical system in adapting to the chronic stress of tethered housing. There are, for example, great differences in basal cortisol levels between individual pigs during tethered housing (Chapter 3), particularly with respect to the moment of disappearance of the

stress-induced hypercortisolaemia. In addition, as described in the experiment in Chapter 4, considerable differences are found between individual animals in their cortisol responses to challenge with exogenous ACTH. Within the same pigs, however, the cortisol response to repeated challenge with ACTH (measured in the loose housed control groups) is consistent during an 18-week period of loose housing. This is in good agreement with findings of others in pigs [45,46], indicating that the adrenocortical reactivity is an individual characteristic that remains stable in time. The interanimal differences in the adrenocortical reactivity pattern are particularly evident during exposure to chronic stress (Chapter 4). It is noteworthy, that the greatest individual differences are observed in animals that are tethered housed under the most restricted conditions (i.e. lacking possibilities for visual and tactile contacts with conspecifics and with very limited visual control over the environment).

It may be argued that variability among individuals in their ability to adapt to conditions inducing stress responses reflects differences in neuroendocrine susceptibility to the stressful situation and the degree of control sensed by the individuals [35,47,48]. Hessing [49] demonstrated that types of pigs can be identified based on individual behavioural characteristics and that those characteristics are related to different physiological strategies displayed in response to stressors. In addition, Schouten and collaborators not only showed individual differences in performance of stress-induced stereotypies between tethered pigs, but also in feeding-induced cardiovascular responses during prolonged tethered housing [50]. It has been suggested that these differences in behavioural and physiological reactions may represent individual coping characteristics of the animals involved [34].

Practical Implications

Consequences of Chronic Stress and Hypercortisolaemia

In the present study, prolonged tethered housing of female pigs was used as a chronic stress paradigm. Although this husbandry system, which is used in intensive pig farming, may be advantageous from an economical point of view, the well-being of the animals is lost sight of. Tethered pigs are housed in a barren environment with physical restraint and social restriction, which may have consequences for the animal's behavioural and physiological performance.

We showed that tethered pigs develop a hypercortisolaemia. Chronic

elevation of cortisol levels may have profound physiological consequences. As has been discussed in Chapter 1, chronic activation of the catabolic stress response may ultimately lead to various pathophysiological states. The systems responsible for reproduction, growth and immunity are directly linked to the stress system, and each is profoundly influenced by the effectors of the stress response. It has been reported that tethered housing of pigs may induce reproductive disorders, such as a reduced rate of oestrous detection (e.g., occurrence of silent oestrus) and a reduced pregnancy rate [51,52]. The present findings show that 3-6 weeks of tethered housing results in significantly decreased levels of plasma progesterone throughout the oestrous cycle (Chapter 2). No effects of 3-6 weeks of chronic stress were found on plasma LH concentrations, oestrous behaviour or on the length of the oestrous cycle. Nonetheless, it is well possible that more prolonged tethered housing may disrupt LH secretion or ovarian function and thereby adversely affect reproductive performance in the pig. Indeed, Helmond, Soede and Kemp recently showed that in tethered sows the duration of oestrous behaviour was significantly shorter as compared with loose housed sows (to be published). Moreover, the pulsatile LH release in that study seemed to be more "chaotic" in the tethered animals.

Besides suppression of reproductive performance, long-term activation of the HPA axis and consequently elevated cortisol levels may have metabolic (myopathy, fatigue, changes in glycemia) and cardiovascular consequences (hypertension). Furthermore, compromised growth and tissue repair, and peptic ulceration may occur, as well as immunosuppression [29]. In this respect, parallels have been drawn between the chronically stressed animal model and several human diseases. It has been demonstrated in clinical studies that some common psychiatric disorders, such as depression, panic disorder, anxiety and anorexia nervosa, may represent disorders occurring in response to stress [53]. Hypersecretion of glucocorticoids and resistance to glucocorticoid negative feedback have been shown to occur in people with anorexia nervosa [54,55] and major depression [55,56].

In addition to changes in HPA regulation, long-term exposure of pigs to tethered housing may lead to development of stereotypies [31-35,50] and persisting disturbances in the hormonal [52,57], cardiovascular [50], and immune systems. Von Borell and Ladewig showed that growth rate was reduced in tethered sows as compared with loose housed sows, despite the fact that both groups had similar food intake [46]. This reduction in growth rate indicates disturbed body function, possibly caused by stress related catabolic processes.

As described previously, we also found that chronic stress leads to an increase in the impact of endogenous opioid systems that mitigate HPA responses to additional acute stressors. Besides analgesic effects [58], activation of endogenous opioid systems may have a broader adaptive function. It has been demonstrated that rats which can turn off shock stimulation show no opioid activation, whereas yoked animals, which experience the same electric shock without being able to control its offset, give evidence of stress-activated opioids [59]. These data indicate that not the physically painful stimulation per se, but rather the psychological stress of its uncontrollability seems to be the key factor in opioid activation. By blunting the aversive impact of stressors, endogenous opioids enable the individual to deal more effectively with distressing environmental events. This contention is in line with the idea that stereotypies, displayed when loss of control is experienced and associated with activation of opioid systems, may serve a de-arousal purpose [47]. Schouten *et al.* [50] showed that the feeding-induced cardiovascular response is decreased and shortened in tethered pigs performing stereotypies, and that this effect is opioid-dependent. The adaptive alterations in opioid systems in tethered pigs may thus be a means of coping with the chronic stress conditions, thereby preventing excessive stress responses (e.g., of the hormonal and cardiovascular systems). However, all of the above mentioned adaptations in behavioural and physiological performance have to be recognized as symptoms of chronic stress. Therefore, they indicate that welfare and health of tethered pigs are compromised.

Concluding Remarks

The experiments described in this thesis provide evidence that long-term tethered housing of female pigs induces adaptive changes in adrenocortical function and in activity of endogenous opioid systems. Important in this respect is the finding that loss of control and predictability by factors, such as lack of (visual) information and restriction of social contact, play an important role in the development of these changes. It should be kept in mind, that the adaptive behavioural and physiological changes, observed in tethered pigs, are in fact the symptoms of their "struggle" in order to withstand the environmental demands, and thus indicate compromised welfare. Some of these changes may lead to increased vulnerability of the animals for diseases. Therefore, it is likely that the stress of tethered housing contributes to the high incidence of health problems observed in tethered sows.

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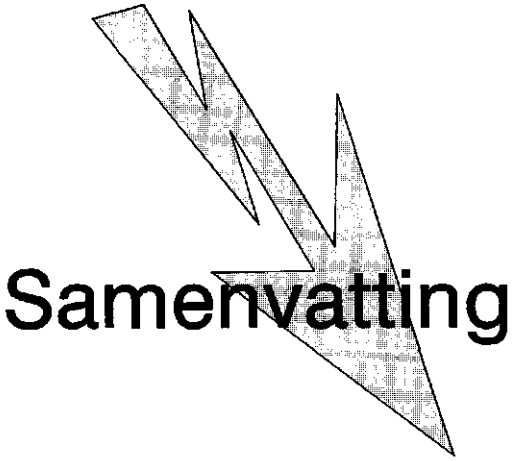

Samenvatting

Samenvatting

Inleiding

Het doel van het in dit proefschrift beschreven onderzoek was meer inzicht te krijgen in de regulatie van het hypothalamus-hypofyse-bijnier (HHB) systeem en de mechanismen die aan de adaptatie aan chronische stress in vrouwelijke varkens ten grondslag liggen. De HHB-as is het neuro-endocriene systeem dat er voor zorgt dat verstoringen van de homeostase in een organisme adequaat opgevangen worden. Activatie van de HHB-as door stressoren leidt onder meer tot afgifte van de hypothalame neuropeptiden corticotropin-releasing hormoon (CRH) en vasopressine, die op hun beurt de afgifte van adrenocorticotroop hormoon (ACTH) uit de hypofyse stimuleren. ACTH stimuleert de aanmaak en afgifte van cortisol uit de bijnierschors, een steroidhormoon dat als een belangrijke indicator van stress wordt gezien. Cortisol reguleert zijn eigen aanmaak en afgifte via een negatieve terugkoppeling op de hersenen en de hypofyse. Hoofdstuk 1 geeft een kort overzicht van stress en het HHB-systeem.

Dit projekt was voornamelijk gericht op onderzoek naar de invloed van chronische stress op de regulatie van het hypofyse-bijnier systeem, en daarvoor werd het langdurig aangebonden gehuisveste, vrouwelijke, varken als modelsysteem gebruikt. In dit, uit de intensieve veehouderij afkomstige huisvestingssysteem, worden varkens aangebonden met behulp van een nekbeugel, die via een korte ketting verankerd is aan de vloer. Op deze wijze worden de dieren naast elkaar gefixeerd op een beperkt vloeroppervlak. Hun mogelijkheden tot contact met soortgenoten zijn zeer beperkt en de dieren worden sterk belemmerd in het uitvoeren van hun natuurlijke gedragsrepertoire. Tijdens langdurig aangebonden huisvesting worden de dieren chronisch blootgesteld aan deze aversieve situatie, waarop zij geen invloed hebben. Dit kan leiden tot ontwikkeling van afwijkend gedrag (bijvoorbeeld stereotypieën) en hormonale en cardiovasculaire stoornissen. Deze veranderingen zijn indicatoren voor chronische stress en verstoord welzijn van de dieren.

Hypofyse-Bijnierfunctie tijdens Chronische Stress

Uit de experimenten, beschreven in de hoofdstukken 2 en 3, blijkt dat de chronische stress bij vrouwelijke varkens, die veroorzaakt wordt door aangebonden huisvesting, leidt tot langdurig verhoogde basale cortisolconcentraties in het bloedplasma. Deze hypercortisolaemie, die reeds binnen enkele weken na aanbinden aangetoond kan worden, is evident tijdens alle fasen van de bronstcyclus van de dieren en houdt gedurende tenminste drie bronstcycli (ca. 9 weken) aan (hoofdstuk 2). De stress-geïnduceerde toename in het basale cortisolniveau is het grootst op het einde van de dag, het moment waarop de cortisolconcentratie in het bloedplasma het laagst is (dal van het diurnale cortisolritme). Het diurnale ritme in cortisolconcentraties vervlakt, doordat het verschil tussen ochtend- en avondconcentraties kleiner wordt (hoofdstuk 3). Aangebonden huisvesting leidt dus tot langdurige veranderingen in het functioneren van het HHB-systeem. Deze toename in de cortisolafgifte gaat waarschijnlijk niet gepaard met een chronisch verhoogde afgifte van ACTH.

De experimenten, beschreven in de hoofdstukken 4, 5 en 6, tonen aan dat chronische stress leidt tot veranderingen op het niveau van de bijnier zelf. In varkens die gedurende een periode van 10 tot 11 weken staan aangebonden, is de ACTH-respons op een acute stressor (nose-sling stress) verlaagd, vergeleken met de respons vóór aanbinden, terwijl de cortisolrespons hetzelfde blijft (hoofdstuk 5). Dit wijst op een toegenomen gevoeligheid van de bijnierschors voor ACTH ten gevolge van chronische stress. Het feit dat de cortisol/ACTH ratio na toediening van CRH twee keer zo groot is in aangebonden als in loslopende dieren (hoofdstuk 6), bevestigt dit. Tevens blijkt uit challenge-testen met exogeen ACTH, dat de corticosteroidogene capaciteit van de bijnierschors significant toeneemt in dieren die langdurig zijn aangebonden, terwijl deze niet verandert in dieren die gedurende dezelfde periode los gehuisvest zijn (hoofdstuk 4). De capaciteit van de bijnierschors om cortisol te synthetiseren, wordt echter niet alleen door het aanbinden beïnvloed, maar ook door andere huisvestingsfactoren. In aangebonden varkens die door middel van massieve tussenschotten van elkaar zijn gescheiden, zijn de mogelijkheden tot sociaal contact met hun soortgenoten en de visuele controle over hun omgeving nog sterker verminderd dan bij dieren die van elkaar gescheiden zijn door horizontale stangen. Zij ontwikkelen hierdoor een sterkere en langer aanhoudende toename in de cortisolrespons op een ACTH-challenge in vergelijking met de minder geïsoleerde varkens.

Hoofdstuk 6 beschrijft een experiment, waarin onderzocht is in hoeverre chronische stress de gevoeligheid van de hypofyse voor de hypothalame peptiden CRH en vasopressine verandert. Er blijkt geen verschil te zijn tussen aangebonden en loslopende varkens met betrekking tot hun absolute ACTH- en cortisolresponsen op exogeen CRH, vasopressine, of een combinatie van beide peptiden. Opvallend is, dat vasopressine, ongeacht de huisvestingsconditie (los of aangebonden), een beduidend grotere cortisol/ACTH ratio teweeg brengt dan CRH. Blijkbaar induceert vasopressine in het varken meer afgifte van cortisol dan verklaard kan worden op basis van door vasopressine-gestimuleerde hypofysaire ACTH-afgifte, waarschijnlijk via een direct effect op de bijnier. Een door vasopressine teweeggebrachte toename in de gevoeligheid van de bijnierschors voor ACTH, zou tevens kunnen verklaren waarom de cortisolrespons na toediening van CRH + vasopressine groter is, dan de som van de cortisolresponsen op deze peptiden afzonderlijk.

Samenvattend, chronische stress leidt tot een toename in de gevoeligheid en de capaciteit van de bijnierschors voor ACTH, terwijl de gevoeligheid van de hypofyse voor CRH en vasopressine niet verandert. De veranderingen op het niveau van de bijnier liggen mogelijk ten grondslag aan de chronische stress-geïnduceerde hypercortisolaemie.

Endogene Opioide Systemen en Hypofyse-Bijnierfunctie

De hypersecretie van cortisol in aangebonden varkens verdwijnt geleidelijk na een periode van circa 9 weken. Tijdens deze periode hebben veel aangebonden varkens zogenaamde stereotypieën ontwikkeld, gedragspatronen, die weinig gevarieerd zijn, vaak herhaald worden en geen duidelijk aanwijsbare functie hebben. Deze stereotypieën kunnen worden geblokkeerd met naloxon, een specifieke opiaatreceptor antagonist, wat er op wijst dat tijdens chronische stress de activiteit van endogene opioide systemen is toegenomen. Langdurig aangebonden huisvesting leidt dus tot functionele veranderingen in opioide systemen, die daardoor mogelijk gevoeliger zijn geworden voor activatie. In hoeverre dat relevant is voor de HHB-functie tijdens chronische stress, is onderzocht in de studie beschreven in hoofdstuk 5. Hiertoe werden varkens zowel vóór (loslopend) als tijdens langdurig aangebonden huisvesting blootgesteld aan een acute stressor. Tijdens de "nose-sling" procedure, werden ze gedurende 15 minuten strak vastgezet met behulp van een koord dat in een lus om de bovenkaak werd gelegd. Deze procedure leidt tot activatie van de HHB-

as, hetgeen blijkt uit de ACTH- en cortisolresponsen. Voorbehandeling van de dieren met naloxon heeft een verhoging en verlenging van deze responsen tot gevolg, wat wijst op activatie van opioide systemen ten gevolge van de nose-sling stress. De ontremming van de ACTH- en cortisolresponsen na naloxon is groter in langdurig aangebonden dieren dan in loslopende dieren. Hieruit blijkt dat de impact van endogene opioide systemen, die waarschijnlijk op het niveau van de hypothalamus voor een demping zorgen van de hypofyse-bijnierrespons op een acute stressor, is toegenomen tijdens chronische stress. Mogelijk ligt dit mechanisme ook ten grondslag aan de geleidelijke verdwijning van de hyper-cortisolaemie, die vanaf circa 9 weken na aanbinden werd waargenomen (hoofdstuk 3). De adaptieve veranderingen in opioide systemen in aangebonden varkens kunnen worden gezien als een vorm van "coping" met de chronische stress condities, waardoor extreme, en daardoor schadelijke, responsen van hormonale, cardiovasculaire en andere systemen op stressoren worden tegengegaan.

Conclusies

De in dit proefschrift beschreven experimenten tonen duidelijk aan, dat langdurig aangebonden huisvesting van vrouwelijke varkens leidt tot adaptieve veranderingen in bijnierschorsfunctie (toegenomen steroidogene capaciteit en gevoeligheid voor ACTH), en in de activiteit van endogene opioide systemen die reacties van het HHB-systeem op additionele acute stressoren dempen. Belangrijk in dit opzicht is de bevinding, dat verminderde beheersbaarheid en voorspelbaarheid van de situatie door factoren zoals gebrek aan (visuele) informatie en sociaal contact met soortgenoten, een belangrijke rol spelen bij het tot stand komen van deze veranderingen. In feite zijn de adaptieve veranderingen in gedrag en fysiologie, die optreden in aangebonden varkens, de symptomen van hun pogingen om te overleven onder condities die verre van optimaal zijn. Zij duiden er dus op dat het welzijn van de dieren is aangetast. Sommige van deze veranderingen kunnen bovendien leiden tot een toegenomen vatbaarheid voor ziekten. Het is dan ook aannemelijk dat de chronische stress, waartoe langdurig aangebonden huisvesting leidt, bijdraagt tot de hoge frequentie van gezondheidsproblemen bij aangebonden zeugen in de praktijk van de veehouderij.

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Cecile

Curriculum Vitae

Cecilia Jozefina Johanna Gerarda Janssens werd geboren op 1 juli 1966 te Heerlen. In 1984 behaalde zij het Gymnasium-β diploma aan het Coriovallum College te Heerlen. In datzelfde jaar begon zij met haar studie biologie aan de Katholieke Universiteit Nijmegen. Tijdens de doctoraalfase deed zij hoofdvakken bij de Vakgroepen Dierfysiologie (KU Nijmegen) en Fysiologie-Microcirculatie (Rijksuniversiteit Limburg). In 1989 studeerde zij af in de Medisch Biologische richting (als eerste aan de KU Nijmegen) en tevens in de Fysiologisch-Biochemische richting.

Van 15 september 1989 tot 1 januari 1994 was zij als assistent in opleiding in dienst van de Vakgroep Fysiologie van Mens en Dier aan de Landbouwuniversiteit van Wageningen. Tijdens haar AiO-periode behaalde zij de artikel 9 bevoegdheid Proefdierkunde, en leverde zij een bijdrage aan het onderwijs voor de studierichtingen biologie, zoötechniek en humane voeding.

Naast de jaarlijkse symposia en bijeenkomsten in Nederland werden onderzoeksresultaten gepresenteerd tijdens de summer meetings van de Society for Veterinary Ethology, in mei 1990 (Montecatini Terme, Italië) en in augustus 1991 (Edinburgh, Schotland). In augustus 1992 leverde zij een bijdrage aan het "XXIII Congress of the International Society of Psychoneuroendocrinology" (Madison, Wisconsin, VS) en in mei 1993 aan de "Fourth International Conference on Pig Reproduction" (Columbia, Missouri, VS). Tevens bracht zij in juli 1990 een bezoek aan de werkgroep van Dr. B.A. Baldwin en Dr. R.F. Parrott, Institute of Animal Physiology and Genetics Research (Cambridge, Engeland) en in mei 1991 aan de werkgroep van Dr. C.R. Barb en Dr. R.R. Kraeling, Animal and Dairy Science Department, University of Georgia (Athens, VS).