Neuroendocrine adaptation to stress in pigs

CRH and vasopressin in the paraventricular nucleus

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Differences in coping strategy present at birth as well as housing conditions may influence autonomic and endocrine stress responses. In rodents, corticotropin-releasing hormone (CRH) and vasopressin (VP) signaling in the paraventricular nucleus (PVN) plays an important role in stress responses. Stress is known to induce expression of VP in PVN-CRH neurons, with the degree of VP expression relating to duration and intensity of the stress. Moreover, there is evidence that the activity of these systems is altered in stress-induced sensitization. This suggests that the functional state of CRH and VP systems, likely also in the pig PVN, could be used as a neurobiological index of stress vulnerability.
The aim of the thesis was to asses in pigs the effects of individual coping strategy (high and low resisting, HR and LR respectively), and rearing and housing conditions on the susceptibility of pigs to stress sensitization. Changes in CRH and VP expression in the PVN were studied in female and also male pigs with different coping strategies subjected to different housing conditions using immunocytochemistry and *in situ* hybridization techniques.
We found a clear sex difference in both CRH and VP peptide content in the PVN, i.e. male pigs showed higher VP and CRH peptide content compared to female pigs. This confirms and extends previous reports of the existence of a sexually dimorphic regulation of the HPA axis. The results of this study suggests that the housing conditions tested, although they clearly affected some behavioral and endocrine parameters, were not contrasting enough with respects to stress load to result in changes in the activity of the HPA axis at the hypothalamic level. Individual housing (IND), however, did affect the HPA axis, with a clear, coping strategy dependent difference on VP peptide and CRH mRNA, but not on CRH peptide levels. The PVN in IND HR pigs contained higher VP peptide levels compared to group housed (GRO) HR and IND LR gilts. Moreover, we found that the absolute number, but not the percentage of cells showing co-localization of VP and CRH peptide was increased in IND HR gilts compared to GRO HR gilts. We suggest that the higher levels of VP peptide in the medial region of the PVN in IND HR pigs plays a role in potentiating the actions of CRH to stimulate ACTH secretion from the anterior pituitary. Furthermore, IND LR gilts showed increased levels of CRH mRNA compared to GRO LR and IND HR gilts, which suggests that LR pigs react to chronic housing stress with an up-regulation of CRH transcription.
The experiments described in this thesis provide evidence that pigs characterized with different coping strategies at early age, differ as adults in the way they adapt to individual housing at the level of the PVN. The differences found in CRH and VP levels in the PVN support the differences in basal behavioral and physiological variables between these animals that became evident under chronic stress. Moreover, the changes in activity of CRH and VP observed are reminiscent to those observed in human psychopathology (e.g. depression), suggesting that these changes may be interpreted as (pre) pathological signs in the pig. This suggests that individual housing is not an optimal housing condition for both HR and LR pigs. Based on these results, we conclude that housing condition likely increases the susceptibility to stress in both HR and LR gilts. Selection of pigs for coping strategy to maintain common practice of individual housing of breeding pigs will therefore not benefit the welfare of the animals. To improve pig welfare and minimize the risk of stress-related diseases, the focus should be on optimizing the pigs' housing conditions.

Contents

Chapter 1

General introduction

Introduction

Homeostasis, the state of relative physiological stability in an organism is a prerequisite to survive. Despite changes in environmental conditions many living species (especially mammals) have the ability to maintain their homeostasis within fixed limits by means of a set of specific innate repertoire of counter regulatory behavioral and physiological mechanisms (control). When the individual innate and acquired repertoire of counter regulatory mechanisms is overridden by environmental or internal perturbations a state of stress is reached and the 'stress responsive systems' are activated. The 'stress system' consists of neuroanatomical and functional structures that produce the behavioral, physiological and biochemical changes directed towards maintaining homeostasis when it is threatened (Chrousos and Gold, 1992; Johnson et al., 1992). Loss of control is a common denominator of stressful conditions (Weiss, 1968). The activation of the 'stress system' in order to counteract disturbances in homeostasis is termed "adaptation".

Repeated exposure of an organism to one and the same controllable stressor, through the facilitation of those mechanisms that help terminate, escape or prevent the stress experience, results in the successive attenuation of the stress response to this and related kind of stressors. It will result in the successive facilitation of certain coping strategies, and therefore, in behavioral specialization. The repeated experience of controllability of stress is a prerequisite for the acquisition of behavioral strategies, which allow an individual to act and not simply react to a certain stressor. Therefore, the repeated, short-lasting activation of the neuroendocrine stress response caused by the exposure to mild controllable stressors is generally acknowledged as a positive proadaptive event. The long-lasting activation of the central stress responsive systems resulting from the experience of severe uncontrollable environmental or psychosocial challenges, however, is regarded as a fatal maladaptive response. Since stress responses involve a redirection of both behavior and energy to those activities that need highest priority, other functions like digestion and anabolic processes such as growth, reproduction and immune function are suppressed. As a consequence, intense, chronic, frequent or uncontrollable stress cannot be tolerated. The individual will experience adverse effects and this may eventually lead to stress-related disturbances or diseases.

The regulation of the HPA axis

Under normal conditions

As said, the constancy and maintenance of the internal milieu within limited boundaries or homeostasis is critical for survival of higher organisms. The preservation of this internal environment requires continuous adaptation to external or internal stimuli or stressors, involving behavioral, autonomic and endocrine changes necessary to anticipate, prevent and counteract disturbances. The neuroendocrine system that is assumed to have particular adaptive and homeostatic value during periods of stress is the hypothalamic-pituitary-adrenocortical (HPA) axis. In response to stressors, the central nervous system of mammalian species evokes physiological responses that ultimately result in the activation of the HPA axis, resulting in a rapid increase in circulating adrenocorticorticotropic hormone (ACTH) with the subsequent rise in glucocorticoids (corticosterone in rat, cortisol in human and pig). This mechanism is critical for successful adaptation (Dallman et al., 1992; Munck et al., 1984) of the animal to its environment. A schematic diagram of the HPA axis is presented in Figure 1.

ACTH is the principal regulator of cortisol synthesis and secretion. In turn, the secretion of ACTH appears to be regulated by a variety of hypothalamic peptides, of which corticotropin-releasing hormone (CRH) and vasopressin (VP) are the most important. In the rat, parvocellular neurons in the hypothalamic paraventricular nucleus (PVN) express the 41 amino acid peptide CRH (Vale et al., 1981), under the influence of the central circadian rhythm (Cascio et al., 1987; Dallman et al., 1987a). Most mammals exhibit a peak in CRH gene expression just before the onset of the normal period of wakefulness and a trough 6 hrs later (Watts and Swanson, 1989). The parvocellular cells in the PVN project axons to the portal capillary plexus in the external zone of the median eminence (Swanson and Kuyper, 1980; Antoni et al., 1983). About half of the parvocellular CRH neurons in rats also express the corticotropin releasing factor VP (Whitnall et al., 1985, Whitnall and Gainer, 1988). VP is a 9 amino-acid neuropeptide that, just like CRH, acts as a neurotransmitter, as well as a neurohormone. The pig is an unusual mammal inasmuch that it synthesizes lysine-vasopressin (LVP) instead of arginine-vasopressin (AVP) as found in rat and the human. The two compounds are closely related, differing only in a lysine instead of an arginine residue in position 8 of the molecule (summarized by Arsenijevic et al., 1994). CRH and VP secreted from axons of PVN neurons reach the anterior pituitary via portal capillaries, and synergistically stimulate the synthesis and release of ACTH from corticotroph cells (Aguilera, 1994, 1998). ACTH, in turn stimulates the synthesis and secretion of

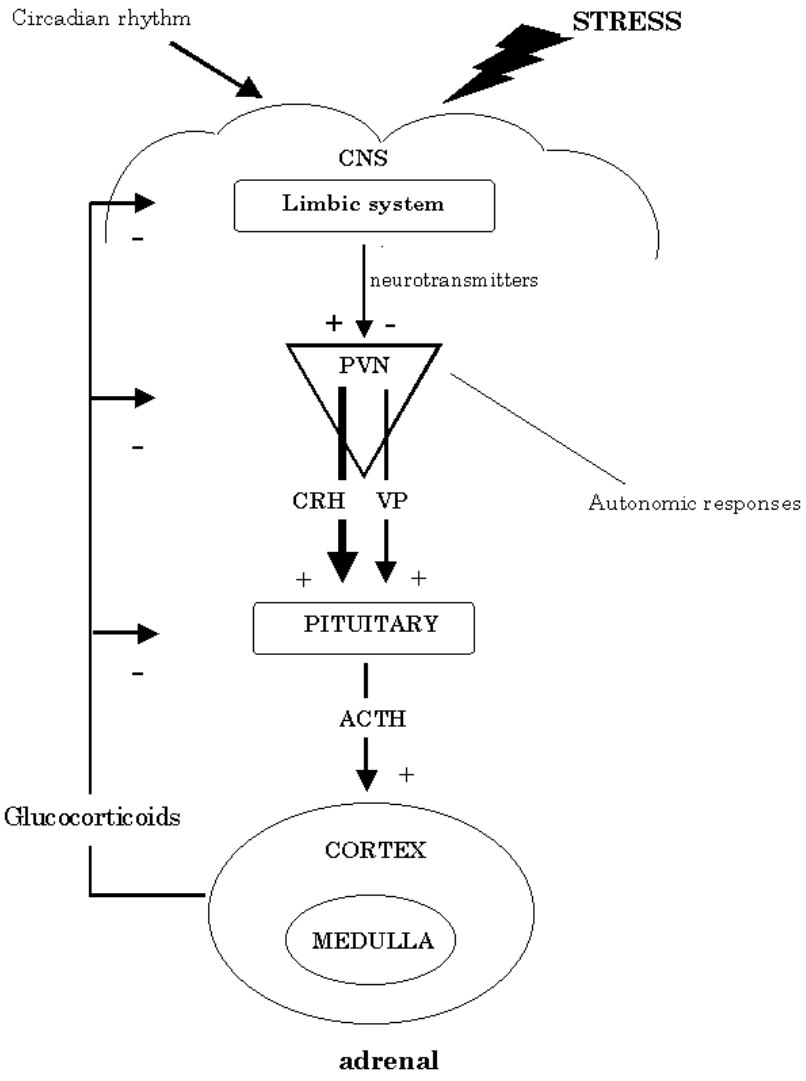

Figure 1. Diagram illustrating the integrated responses to stress emphasizing the activation of the hypothalamic-pituitry-adrenocortical axis and the feedback and regulatory effects of glucocorticoids. The paraventricular nucleus (PVN) in the hypothalamus receives stimulatory and inhibitory input from the limbic system. The secretion of adrenocorticotropin hormone (ACTH) from the anterior pituitary is mainly controlled by corticotropin-releasing hormone (CRH) and vasopressin (VP). ACTH stimulates synthesis and secretion of glucocorticoids from the adrenal cortex, which exert negative feedback on the system.

glucocorticoids from the adrenal cortex. Circulating glucocorticoids modulate energy utilization throughout the organism, affect cardiovascular tone, modulate many functions in the immune system, modulate many brain functions that play a role in adaptation to stress, and exert direct negative feedback on the hypothalamic neurosecretory cells and pituitary corticotrophs of the HPA axis, and indirect via the hippocampal glucocorticoid receptors. This feedback inhibition by circulating glucocorticoids is an important mechanism to control the activity of the HPA axis (Dallman et al., 1987a,b).

Under stress condition

Under stress conditions, CRH secretion is stimulated from the parvocellular neurons and VP expression and secretion in these neurons is increased as well (Kovacs and Sawchenko, 1996, Ma et al., 1997; Schmidt et al., 1997). VP is only a weak ACTH secretagogue on its own, but acts synergistically with CRH and is believed to play an important role in sustaining pituitary responsiveness during chronic stress in the rat (Antoni, 1993; Scaccianoce et al., 1991; Dallman, 1992; Aguilera, 1994, 1998; Aguilera and Rabadan-Diehl, 2000). It has been shown that there is a desensitization of the CRH transcription response, but not of the VP transcription response to repeated restraint, that is in fact increased (Aguilera, 1994, 1998). The up-regulation of VP in the PVN of the rat in the restraint stress paradigm therefore suggests that VP synthesis in the PVN may play an important role in activating the HPA axis under conditions of chronic stress.

Apart from the parvocellular neurons, another set of neurons in the rat PVN express VP. Magnocellular neurons also synthesize VP and release the hormone into the circulation, where it mediates antidiuresis, to conserve body water and protect osmolarity; and also vasoconstriction, to defend blood pressure against hypovolemia (Leng et al., 1999). In contrast to VP in parvocellular neurons, which is responsive to stress and changes plasma glucocorticoids (Aguilera, 1994), VP in the magnocellular neurons is regulated by osmotic changes. Nevertheless, under certain conditions VP of magnocellular origin could facilitate pituitary ACTH secretion (Aguilera, 1994; Amaya et al., 1999), and prolonged activation of the magnocellular VP system during chronic osmotic stimulation decreases pituitary ACTH responsiveness rather than increasing it (Aguilera, 1994).

Glucocorticoid negative feedback

Not only activation but also termination of the HPA stress response is critical for the survival of individuals, since prolonged glucocorticoid exposure can lead to muscle atrophy, hypertension, arterial disease, impairment of growth and

tissue repair, as well as immunosupression (Baxter and Tyrrell, 1987). Moreover, glucocorticoids exert negative feedback at the level of the anterior pituitary, the hypothalamus and the suprahypothalamic limbic structures such as the hippocampus and the amygdala (Plotsky et al., 1986; Dallman et al., 1987; Van Loon and Souza, 1987), to control the activity of the HPA axis. Thus far, two types of receptors for glucocorticoids have been identified in many species, including the rat, human and pig hippocampus. These receptors, the MR and GR, are characterized by different steroid specificity and neuroanatomical distribution (McEwen et al., 1982; Reul and De Kloet, 1985, 1986; Kanitz et al., 1998; Perreau et al., 1999; Weaver et al., 2000). Within the central nervous system (CNS), mineralocorticoid receptors (MR) are predominantly localized in the hippocampus and septum. The MR binds glucocorticoids with high affinity but low capacity, are operational when glucocorticoid levels are low, and regulate the diurnal fluctuations in circulating glucocorticoids under basal conditions. The glucocorticoid receptors (GR) are widely distributed in the brain (reviewed by Whitnall, 1993). They show relatively low affinity but high capacity to bind glucocorticoids. It is generally accepted that the GRs are primarily responsible for the inhibitory influence of glucocorticoids when the HPA axis is activated and glucocorticoid levels are high, for instance during stress (de Kloet and Sutano, 1989). Much of the repression of CRH and VP synthesis and release is directly or indirectly mediated by glucocorticoid-responsive circuits that project from the limbic system to the hypothalamus (Dallman et al., 1987a,b; Canny et al., 1989; Sapolsky et al., 1984, 1991).

The hippocampal MRs and GRs appear to be particularly sensitive to the suppressive action of elevated circulating glucocorticoids. Downregulation of these receptors is often associated with reduced sensitivity of the HPA axis to glucocorticoid suppression resulting in longer exposure of the organism to elevated glucocorticoids levels. Chronic down-regulation of hippocampal MR and GR receptors is thought to be a major pathophysiological mechanism of impaired HPA axis regulation in chronic stress, aging and affective disorders.

The paraventricular nucleus

The hypothalamic paraventricular nucleus (PVN) is located in the hypothalamus on both sides of the third ventricle and has an important role in endocrine and autonomic functions including the control of pituitary-adrenocortical activity in response to stress, body fluid homeostasis, milk ejection reflex and food intake (Kiss, 1988; Swanson et al., 1986). In the rat, the PVN contains two major types of neurons, parvocellular and magnocellular cells (reviewed by Van der Pol, 1982). Both the parvocellular and magnocellular neurons can be further subdivided

on the basis of peptide localization and content (see Swanson and Sawchenko, 1983, for a review), projection sites (Swanson and Kuypers, 1980), location in the nucleus and cell size (Kiss et al., 1991). In the rat, the following subregions of the PVN have been recognized: the anterior, medial and the posterior magnocellular subregions, the anterior, dorsal, lateral and medial parvocellular subregions, and the periventricular region. In the rat, neurons of the anterior and medial magnocellular subdivisions contain oxytocin (OT), and the posterior magnocellular subdivision contains vasopressin (VP). All three of these cell groups project to the posterior pituitary where OT and VP are released in the bloodstream (Swanson and Kuypers, 1980). The neurons from the lateral and dorsal parvocellular subregions, on the other hand, project to the brainstem and spinal cord and feature neurons mainly containing corticotropin-releasing hormone (CRH), VP and OT (Swanson, 1977, Swanson et al., 1981). Finally, neurons of the medial parvocellular subregion that contain CRH, VP (Swanson et al., 1983) and other releasing hormones (Brownstein et al., 1982) send axons to the median eminence, where they regulate the hormonal output from the anterior pituitary (Antoni et al., 1983). In addition, in the rat, co-expression of VP and CRH is found following stress (Whitnall, 1990; Tilders et al., 1993; De Goeij et al., 1992a,b), and also after colchicine treatment. Colchicine blocks fast axonal transport, which makes it possible to visualize existing CRH neurons and co-localization of VP and CRH in the discrete subsets of parvocellular CRH containing neurons.

The pig, the HPA axis and the paraventricular nucleus

Compared to rodents, only very few studies have been carried out in pigs to determine the precise neuroanatomical sites in the PVN at which neuropeptide expression occurs. For the pig PVN a similar subdivision based on neuron size and localization has been suggested (Seeger, 1987). Yet, the few available studies on the distribution of VP, OT and CRH neurons in the pig hypothalamus do not describe these cell populations in great detail (Watkins and Choy, 1977; Weindl et al., 1984; Van Eerdenburg et al., 1992; Vellucci and Parrott, 1997, 1998). Therefore in the present project we started to explore the localization of CRH, VP and OT neurons throughout the porcine hypothalamus, aiming to give more insight into the distribution of VP, CRH and OT neurons in the pig PVN.

Even fewer studies have been carried out on pigs to assess changes in neuropeptide synthesis that may occur following experimental manipulations, such as exposure to stress. So far it has been established that CRH is more potent than VP in stimulating ACTH release from the pituitary in the pig, a situation similar to that seen in the rat (Minton and Parsons, 1993; Abraham and Minton, 1996). In addition, it has been indicated that CRH mRNA is present in a “parvocellular

PVN" of non-stressed animals comparable to that seen in rats. Since in rodents CRH mRNA is up-regulated in response to an acute stress situation, it was investigated whether also in pigs up-regulation of CRH mRNA would occur when the animals are stressed. The few studies investigating possible effects of stress on CRH expression in the pig, however, did not show any changes whatsoever. First, an immunological challenge (intravenous injection of lypopolysaccharide endotoxin (LPS)) in pre-pubertal boars did not induce any alterations in CRH mRNA in the "parvocellular PVN" (Vellucci and Parrott, 1998). Also VP mRNA in the "parvocellular PVN" was not affected by this immunological challenge. Yet, this is not surprising since in the rat, too, up-regulation of CRH mRNA was not found after i.v. administration of LPS (Lee et al., 1995). In another study in which prepubertal boars were restraint with a nosesling for 15 minutes, it was demonstrated that VP mRNA was up-regulated in the "magnocellular PVN", but not in the "parvocellular PVN" (Vellucci and Parrott, 1997). It has to be emphasized that these two studies were the first attempts to investigate CRH and VP mRNA expression in the pig under basal and acute stress conditions. The small numbers of animals in some of the treatment groups and the small numbers of sections per animal examined might have influenced the absence of any effects.

Although little is known about the effects of stress on the expression of CRH and VP in the porcine brain, behavioral, autonomic and endocrine stress responses to some procedures and interventions routinely used in pig husbandry practice are well documented. It has been reported that pigs housed under intensive conditions in a barren and restricted environment behave more aggressively than pigs housed in an enriched environment, such as pens provided with substrate (Haskell et al., 1996), or pens with increased floor space and substrate (Beattie et al., 1995, 1996; Schouten, 1986). Moreover, pigs under tethered housing conditions experiencing chronic stress, develop behavioral stereotypies within weeks after tethering, and show changes in physiological regulation. Long-term tethered housing enhanced the cortisol response to exogenous ACTH in cyclic gilts, indicating a change in adrenocortical function (Janssens et al., 1995). In addition, tethered housing resulted in central adaptations, which reduced the ACTH response to stress (nose sling) (Janssens et al., 1995). Moreover, basal cortisol and prolactin were increased during tethered housing (Janssens et al., 1994).

Based on rodent studies, CRH and VP signaling in the brain play a pivotal role in behavioral, autonomic and endocrine stress-responses. Changes in the activity of the CRH and VP systems as a result from exposure to stressful events or chronic stress, may be part of the underlying mechanism. Therefore, the study of changes in CRH and VP following experimental manipulation in the pig is important not only to provide new knowledge about the neurobiology of stress in

this species, but also to gain more insight in the neurobiological basis of welfare of the domestic pig under current husbandry regimes.

Normal field practice in pig husbandry

Pig husbandry has developed from extensive husbandry systems in which pigs were practically kept in a wild state, into the modern indoor systems in which growing pigs are housed under intensive conditions in a barren and restricted environment. The two types of systems differ in more than one aspect from each other. Apart from the space restriction, it is now common practice in modern pig husbandry to wean piglets at 3 or 4 weeks of age, in order to increase the number of litters one sow can have during her (short) reproductive life. This separation of sows from the piglets is very early, considering that piglets left with their mother are spontaneously weaned between 14 and 17 weeks of age (Jensen, 1986). Early weaning can cause dramatic behavioral and physiological changes (Ladd et al., 2000; Meaney, 2001). Also, standard pig husbandry procedures include castration of the male piglets, teeth clipping, ear tattooing, and tail docking, all carried out at 3 days after birth. On top of that piglets may be mixed into new groups several times during their life to create fattening groups that are made up of individuals who are similar in breed, size and age. This mixing results in fighting of the pigs in order to settle social hierarchy positions.

It is common practice to house breeding sows individually in pens with fully or partly slatted floors without bedding. Since pigs are social animals, individual housing may be considered as chronic stress. Moreover, pigs are nest builders and the lack of bedding prevents the sows from performing their natural nest behavior, resulting in the development of abnormal agonistic behavior (Schouten, 1986). In addition, rooting is an essential behavior for pigs, which they cannot perform in a pen without bedding. It has been shown that both individual and group housing affect the behavior of pigs in many ways. Ruis et al. (2001a,b), recently showed that, compared to socially housed pigs, isolated gilts generally develop a higher state of fearfulness, and become more responsive to environmental changes. In addition, several studies have shown that growing pigs reared under barren conditions, for instance in the intensive pig husbandry, have disturbed development, develop abnormal agonistic behavior, and behave more aggressively than pigs reared in an enriched environment (pens with bedding available) (De Jonge et al, 1996; Shouten, 1986). Moreover, in a novel environment test, enriched housed piglets tended to have shorter latency to leave the pen and tended to spend less time in their home pen than barren housed piglets.
The above mentioned changes in behavior might result from the fact that the above summarized routine housing and interventions are uncontrollable and unpredictable

stressors for the pig. It has been shown that in particular uncontrollable and unpredicted stressors induce changes in a wide variety of behavioral and physiological parameters (Weiss, 1970; Cabib et al., 1994). The nature of these effects are thought to be determined by the quality, duration and amount of control and of the stressor (Cabib et al., 1994; Huether, 1996), as well as life history and genetic background of the stressed animal (Benus et al., 1991; Armario et al., 1995).

It can be concluded that certain housing conditions and interventions routinely used in pig husbandry are perceived as stressful by the animals. They may hamper the development of normal behavior patterns and have a negative impact on pig welfare.

Genetic background/ coping strategy

Individuals in a normal population show considerable variation in behavioral and endocrine responsiveness to cope with relevant environmental changes that threaten homeostasis (Koolhaas et al., 1999, review). Research has documented the existence of basically two different ways of coping with stressful situations. Firstly, active coping, resembling the fight/flight response initially described by Cannon (1915), which is characterized by a high activation of the sympathetic adrenal modullary system, and secondly, passive coping, resembling the conservation-withdrawal response (Engel and Schmale, 1972), characterized by an increase of the pituitary adrenal cortical system (Henry and Stephens, 1977). Intriguingly, each individual seems to have a preference for one or the other coping strategy, determined by genetic background and early life experience (Mendl and Paul, 1991). Several studies have shown that the two behavioral strategies are highly correlated with different physiological and neuroendocrine regulation (Von Holst, 1986; Bohus et al., 1987; Sapolsky, 1990; Schouten and Wiepkema, 1991). Numerous studies on individual vulnerability to stress-related diseases are based on the use of lines generated by genetic selection on these behavioral strategies (Korte et al., 1997; Schouten and Wiegant, 1997).

Also in pigs, two types of individual coping strategies have been observed in the behavioral and physiological response patterns in adults when stressed (Hessing et al., 1993). An indication for the behavioral strategy of pigs can be obtained in the first two weeks after birth by measuring the degree of resistance in a so-called “backtest”. In this test each pig is restrained on its back and its reaction is scored by counting the number of escape attempts. The piglets are tested twice with a one-week interval. Piglets are classified as “high-resisting” (HR) if they show more than two escape attempts in each of the tests. Piglets that show less than 2 escape attempts in each of the tests are classified as “ low-resisting” (LR). There

is also a group of piglets (20%) that are not consistent over both tests and classified as switchers or intermediates.

The classification of pigs in this test as HR or LR is to a certain extent predictive for the way pigs will respond to stressful events later in life. It has been shown that HR and LR animals differ in a variety of features such as the behavioral response to apomorphine (Bolhuis et al., 2000), basal cortisol concentration, aggression and hypothalamic-pituitary-adrenocortical reactivity (Hessing et al., 1994; Ruis et al., 2000; Geverink et al., 2002a,b). In addition, some behavioral, pathological and immunological characteristics of pigs were reported to depend on the interaction between backtest qualification and housing conditions (Schrama et al., 1997; Bolhuis et al., 2003). These results indicate that the backtest, apart from being a valuable tool for determination of coping strategies in early life, may also be a useful predictor for the vulnerability of an individual to stress-related disturbances and diseases.

Stress sensitization

A large body of evidence indicates that previously experienced stress can increase the responsivity of an individual to subsequent challenges and stressors (Antelman et al., 1980; Stam et al., 1996; Bruijnzeel et al., 2001, Jansen et al., 2003). This enhanced responsiveness of the HPA axis is called sensitization and can last for very long times, as evidenced by increased HPA responses following exposure to the same or other stimuli, weeks later (Schmidt et al., 2001). Stress-sensitization of the HPA axis has been demonstrated not only after chronic stress but also after a single exposure of adult rodents to emotional stimuli (Buwalda et al., 1999) immune activation (Schmidt et al., 1995, 2001) or psycho-stimulants (Schmidt et al., 1999, 2001). Moreover, it has been demonstrated that a single session of footshocks sensitizes the HPA axis to novel stressors. For instance, preshocked rats have an enhanced ACTH and corticosterone response to an open field test two to four weeks after the footshock session (Levine et al., 1973; van Dijken et al., 1993). In addition, it has been established that CRH immunoreactivity was increased in the PVN of pre-shocked rats as well, indicating that a previous stressful stimulus sensitized the responsiveness of CRH neurons to a relatively mild stressor (Bruijnzeel et al., 2001). Yet, stress sensitization is also associated with changes in VP, the peptide known to potentiate CRH-induced release of ACTH from the pituitary gland, in stress responsive neurons. It has been shown that during immune-induced HPA sensitization CRH neurons show a long-lasting increase in the production and release of VP (Schmidt et al., 1995, 1996). These data suggest that the functional state of CRH and VP signaling may be used as

neurobiological parameters for stress sensitization and vulnerability (Schmidt et al., 1997).

Thus, it has become clear that not only chronic or frequent stress, but even a single stress experience can alter behavioral, autonomic and endocrine responsitivity over longer periods of time. Since rearing and housing conditions and interventions routinely used in current pig husbandry are often stressful as mentioned previously, they may be potentially sensitizing. Yet, individual animals of the same species, including the pig, can differ in their mode of response and sensitivity to stress (coping strategy), and it is likely that they may also differ in susceptibility to long-term sensitization effects of stressful conditions and events.

HPA axis and depression

Depression is an illness intrinsically linked to both psychological and occasionally physical stressors. Stress paradigms in animals are often used to investigate the pathophysiology of human depression. The role of the HPA axis in human depression disorders may be of particular interest since an increase of baseline cortisol levels was found in patients with depression (Sachar et al., 1973). The reported hypercortisolism in patients with depression reflects a central rather than a peripheral disturbance. The suggested hyperactivity of the HPA axis was shown by the presence of high levels of CRH in the cerebrospinal fluid, an increased number of CRH expressing neurons in the PVN, a blunted ACTH response to an exogenous CRH stimulus, and an increased volume of the adrenal glands (reviewed by Scott and Dinan, 2002). Based on these results it is likely that the hypercortisolism seen in depression is a result of overproduction of CRH in the hypothalamus. Yet, VP, the other main ACTH secretagogue, is also thought to play a crucial role in the pathophysiology of affective disorders and their underlying neuroendocrine dys-regulation (Holsboer and Barden, 1996). Holsboer (1995) postulated that the mechanism underlying the increased HPA activity comprises weakened glucocorticoid receptor function in the hippocampus and hypothalamus, leading to enhanced synthesis and release of not only CRH, but also of VP. This was later confirmed in post mortem studies of depressed patients (Raadsheer et al., 1994, 1995; Purba et al., 1996).

The present project was designed to collect more information on the effects of environmental factors on the regulation of the HPA axis, in particular stress-adaptive changes in CRH neurons in the PVN, to yield new insights and tools to be used at the benefit of pig welfare. Yet, since stress paradigms in animals are often used to investigate the pathophysiology of human depression, insight in long-term consequences of stress in pigs may benefit man as well, in particular with respect to depression.

Scope of this thesis

The aim of the present thesis was to assess in pigs, to what extent individual coping strategy, rearing and housing conditions determine the susceptibility of the animals to stress sensitization. To this end, changes in the hypothalamic CRH and VP signaling and in particular the co-expression of these two neurohormones in the PVN, important for the regulation of endocrine responses were studied in more detail.

Chapter 2. When considering the functional state of CRH and VP systems as a neurobiological indicator for stress vulnerability, basic knowledge about the localization and distribution of CRH and VP in the pigs PVN is necessary. Whereas patterns of peptide expression in hypothalamic regions involved in the regulation of the activity of the HPA axis have been widely studied in rats, only a few studies dealt with the pig hypothalamus, and they do not describe the cell populations in great detail (Watkins and Choy, 1977; Weindl et al., 1984; Van Eerdenburg et al., 1992; Vellucci and Parrott, 1997, 1998). Therefore, the project was started with a detailed investigation of the localization and distribution of CRH, VP and OT peptide containing neurons in the porcine PVN. To this end, paraffin sections of the hypothalamus of just pre-pubertal animals were taken at regular intervals from rostral to caudal throughout the hypothalamus, immunocytochemically stained for VP, CRH or OT, and mapped using computer assisted image analysis. Based on the analysis a subdivision of the PVN into four lateral regions was proposed, each characterized by the presence of selective OT, VP and CRH and VP and OT expressing cells.

Chapter 3. After mapping the CRH, VP and OT peptide containing neurons in the porcine PVN it was possible to determine changes in CRH and VP peptide in the PVN following experimental manipulation. In this chapter the effect of enriched housing conditions was compared to standard barren housing. The effect of rearing and housing conditions and coping strategy on CRH and VP expression in both pre-pubertal male and female pigs were investigated by measuring the immunocytochemical staining for VP or CRH in specific PVN regions using computer assisted image analysis.

Chapter 4. Since pigs are socially living animals, individual housing may be considered as a long-term stressor and will lead to chronic stress (Janssens et al., 1995; Ruis et al., 2001a,b). In current pig husbandry it still is common practice to house breeding sows individually, in confinements such as stalls. Based on differences found between adult HR and LR breeding pigs in basal behavioral and physiological variables particularly expressed under conditions of chronic stress such as individual housing, we hypothesized differences in the regulation of CRH and VP at the level of the hypothalamus between adult pigs characterized as HR

and LR in the backtest in early life as well. To test this, we investigated the effects of individual confined housing (i.e. chronic stress) on the expression of CRH and VP peptide in the PVN in young adult female pigs (gilts) displaying different coping strategies by means of immunocytochemistry.

Chapter 5. Since CRH peptide in the PVN of the gilts did not differ significantly between housing conditions and/or coping strategy, the aim of the experiment presented in this chapter was to determine whether CRH transcription in the PVN was changed after individual housing for 5 months in these young adult female pigs (gilts) displaying different coping strategies. To this end, we developed a non-radioactive DIG-labeled in situ hybridization protocol using a specific pig CRH probe, and measured possible changes in mRNA presence using computer assisted image analysis.

Chapter 6. Under stress conditions in the rat, co-expression of VP has been found in a subset of CRH neurons. Also in humans, co-expression of VP in CRH neurons has been found, particularly in depressive patients. In chapters 2-5 we assumed that the organization of the hypothalamic CRH and VP neuro-secretory system resembles the one proposed for the rat. In this chapter we wanted to determine for definite that the changes in VP reported in chapter 4 represent actual co-localization of VP in CRH neurons, similar to the situation in rat. Therefore, the aim of the experiment presented in this chapter was to establish whether co-expression of VP in CRH neurons in the PVN of HR gilts occurs. Our first study in which we investigated the distribution of CRH neurons in the pig PVN showed an overlap between CRH and VP immunoreactive neurons in the medial region of the PVN (Chapter 2), yet whether CRH and VP were expressed in the same neuron in this region could not be determined. Furthermore, based on the results of the study described in chapter 4, an investigation to determine possible effects of housing condition on the number of CRH neurons and potential co-localization of CRH with VP in HR gilts specifically was carried out. To this end, we studied the co-expression of VP in CRH containing neurons by means of immunocytochemistry in 3 consecutive paraffin sections from the midrostrocaudal part of the pig PVN in which CRH neurons were known to be abundantly present.

Chapter 7. The results of the studies are summarized and discussed.

Chapter 2

The distribution of vasopressin, corticotropin-releasing hormone and oxytocin containing neurons in the paraventricular nucleus of the porcine hypothalamus

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Abstract

Immunocytochemical methods have been used to achieve a detailed peptide map of the porcine paraventricular nucleus (PVN) based on the distribution of corticotropin-releasing hormone (CRH), vasopressin (VP) and oxytocin (OT) peptide containing neurons. To this end, paraffin sections of the hypothalamus of pubertal animals were taken at regular intervals from rostral to caudal throughout the hypothalamus, immunocytochemically stained for VP, CRH or OT, and mapped using computer assisted image analysis. The pig PVN shows clear caudal to rostral heterogeneity for each of the three peptides. Based on the cellular distribution of the VP, CRH and OT, we found that the pig PVN can be divided into four regions from the midline ventricle wall and laterally. We can conclude that the pig shows a quite comparable anatomical localization of VP, CRH and OT as found in several other species. This may suggest that the hypothalamic CRH neuro-secretory system in the pig is regulated in a similar fashion as has been described for, for instance, the rat.

Introduction

Patterns of peptide expression in hypothalamic regions regulating hypothalamic-pituitary- adrenal (HPA) axis activity have been widely studied in rats with respect to vasopressin (VP) oxytocin (OT) and corticotropin-releasing hormone (CRH), but the few available studies on the distribution of VP, OT and CRH neurons in the pig hypothalamus do not describe these cell populations in great detail (Watkins and Choy, 1977; Weindl et al., 1984; Van Eerdenburg et al., 1992; Vellucci and Parrott, 1997; 1998).

In the rat, the following regions of the paraventricular nucleus (PVN) have been recognized based on cellular appearance (Kiss et al., 1991), peptide content (see Swanson and Sawchenko, 1983, for a review) and projection patterns (Swanson and Kuypers, 1980): the anterior, medial and the posterior magnocellular subdivisions, the anterior, dorsal, lateral and medial parvocellular subdivisions, and the periventricular region. Furthermore, it has been shown that these subdivisions of the PVN are divided into several smaller parts (Swanson and Sawchenko, 1983). For the pig PVN a similar subdivision based on neuron size and localization has been suggested (Seeger, 1987).

In the rat, neurons of the anterior and medial magnocellular subdivisions contain OT, the posterior magnocellular subdivision contains VP; all three of these cell groups issue to project to the posterior pituitary where OT and VP are released into the bloodstream (Swanson and Kuypers, 1980). The neurons from the lateral and dorsal parvocellular subdivisions on the other hand, project to the brainstem and spinal cord. The lateral and dorsal parvocellular subdivisions feature neurons

containing CRH, VP and OT and other releasing neurohormones (Swanson, 1977; Swanson et al., 1981). Finally, neurons of the medial parvocellular subdivision that contain CRH, VP (Swanson et al., 1983) and other releasing neurohormones (Brownstein et al., 1982) send axons to the median eminence, where they regulate the hormonal output from the anterior pituitary by timed release of these peptides (Antoni et al., 1983). In addition, in the rat, co-expression of VP and CRH is found following stress (Whitnall, 1990; Tilders et al., 1993; De Goeij et al., 1992a,b), and also after colchicine treatment in discrete subsets of parvocellular CRH containing neurons.

In the present study, we have mapped the CRH, VP and OT peptide containing neurons in the porcine PVN. To this end, paraffin sections of the hypothalamus of pubertal animals were taken at regular intervals from rostral to caudal throughout the hypothalamus, immunocytochemically stained for VP, CRH or OT, and mapped using computer assisted image analysis.

Materials and Methods

Animals and Tissue preparation

Pig brains (n = 5) were obtained from another experiment, in which pigs were reared under contrasting housing conditions, i.e. in standard/impoverished or slightly enriched pens. Three pigs from the standard condition and two pigs from the slightly enriched condition were used (Bolhuis et al., unpublished). Each pen ($7m^2$) had a solid area (70%) and a slatted dunning area. Enriched pens contained straw bedding as well. Pigs were housed in groups of ten from rearing to slaughter. On postnatal day three, boars were castrated routinely and litters were equated to 10 piglets. Pigs were fed commercial feed ad-lib and had free access to water from a nipple drinker. Two barrows and two gilts of the crossbred [Large White x (Duroc x English Landrace)] x Pietrain, and one gilt of the crossbred (Great York x Dutch Landrace) x Pietrain all raised in different pens were used.

The animals were sacrificed at the age of 26 weeks, which is just during pubertal onset, by means of electrocution. The brains were removed from the skull and immersed in freshly prepared 10 % paraformaldehyde (Merck; Darmstadt, Germany) in 0.1 M phosphate buffered solution (pH 7.2) for 14 days at 4°C. After 14 days, the hypothalamus was dissected and immersed in the same fixative for another week, dehydrated through series of graded ethanol, embedded in paraffin (Histowax©; (melting point 56-58°C), Klinipath B.V., Duiven, Holland) and stored at room temperature until sectioning. Serial 6 μm sections were cut, and selected sections taken at regular intervals (90 μm) were mounted on gelatin coated slides and stored at room temperature. For staining, sections were deparafinized with

xylene (Merck), and sections were hydrated with graded series of ethanol and washed in Tris-buffered saline (TBS; pH 7.4) for 5 minutes. Every 15th section (90 µm interval) was stained with cresyl violet (0.5% cresyl violet (BDH, Poole, UK) in acetic buffer pH 3.8) in order to identify the PVN in the hypothalamus before selecting sections for immunocytochemical staining.

Antibody cross-reactivity

A spot-blot experiment, based on the press-blotting procedure of Van der Sluis (1988), was performed in order to investigate possible cross-reactivity of the antisera raised against AVP (W3, Rudolf Magnus Institute, Utrecht) and OT (O2T; generous gift from Prof. R.M. Buijs, Netherlands Institute for Brain Research, Amsterdam, for more detail see: Buijs et al., 1989) with the peptides Arginin vasopressin (AVP), Lysine vasopressin (LVP) and Oxytocin (OT). Spots containing a step wise 1:4 dilution from 200 ng to 3.13 pg of the synthetic peptides AVP, LVP or OT (Sigma, Zwijndrecht, The Netherlands) were applied to gelatin coated nitrocellulose membranes in 2 µl spot buffer (10% v/v dimethylformamide (Merck), 1% glycerol (BDH), 2.5% nonidet P-40 (Sigma) in distilled water), and allowed to dry for 2 hr at room temperature. The peptide was fixed to the sheet by covering it overnight with filter-paper containing 5% paraformaldehyde in press-blot equipment to obtain a matrix comparable to that of fixed brain tissue. The sheets were subsequently washed in distilled water, Tris-HCl buffer (pH 7.4), and TBS containing 0.25% gelatin (Merck) and 0.5% Triton X-100 (BDH) ("Supermix", staining buffer).

For the immunochemical staining of the nitrocellulose membranes a procedure was used with either anti-AVP (W3, diluted 1:7500), or anti-OT antibody (O2T, diluted 1:8000) in staining buffer. For detection, a biotinylated goat-anti-rabbit antiserum (Vector Laboratories; Burlingame, CA), diluted 1:500 in staining buffer, was used as secondary antibody, followed by incubation in ABC-solution (Vector), diluted 1:1000. Staining was visualized (20 min room temperature) with filtered DAB-substrate prepared by dissolving 30 mg diaminobenzidine (DAB, 10 mg tablets (Sigma)) in 60 ml TBS with 1% imidazole (Merck) added for intensification of the end product, and activated by adding 0.03 % H_2O_2 (Sigma). Finally, the sheets were cleared by embedding them in oil between plastic sheets.

Pre-absorption with homologous antigen

Antibody specificity of staining was checked by pre-absorbing antisera with their homologous antigen (Van der Beek et al., 1992). The method used for

affinity absorption of antibodies was based on press-blotting procedures (Van der Sluis et al., 1988). Solid-phase absorption was performed with synthetic peptides covalently coupled to gelatin-coated nitrocellulose sheets. The following peptides were used: AVP (Sigma, 1.3 ng/ml; 1.95 ng/ml; 2.6 ng/ml), LVP (Sigma, 1.3 ng/ml), OT (Sigma, 1.25 ng/ml; 1.88 ng/ml; 2.5 ng/ml). Based on a slight cross-reactivity for OT (see results section), the AVP antiserum was also routinely pre-absorbed with OT (Sigma, 1.3 ng/ml; 1.95 ng/ml; 2.6 ng/ml). Diluted peptide solution was pipetted in 24 aliquots of 10 µl onto gelatin-coated nitrocellulose sheets and the press-blotting procedure was performed as described above. Nitrocellulose sheets of 22.4 cm^2 were used for absorption of 2.5 ml antiserum diluted for immunocytochemistry (W3, 1:7500; O2T, 1:8000). The antiserum was absorbed twice with peptide-coated nitrocellulose sheets in small sealed bags to allow optimal contact between antigen and antibody. The first absorption was performed 3-4 hrs and the second absorption with a fresh peptide-coated sheet for 1 hr, both at room temperature. The antisera were used for immunocytochemistry immediately after pre-absorption. Brain sectons were incubated with pre-absorbed antisera and the antibody was detected with GaR-bio, ABC and DAB as described below.

Pre-incubation with protein A

To prevent nonspecific, Fc-mediated staining that is normally seen with rabbit antibodies in the porcine hypothalamus, protein A (Sigma) was added to the diluted antiserum (AVP or OT), according to procedures described previously by Van der Beek et al. (1992). The antibody-protein A mix was subsequently used for immunocytochemical staining performed as described in the section "immunocytochemistry". The concentration of Protein A used was determined in a pilot experiment (dilutions 1:50, 1:100, 1:400). The 1:100 dilution yielded optimal results and was therefore used routinely for further immunostaining with the (preabsorbed) rabbit antisera (W3 and O2T).

Immunocytochemistry

Immunocytochemical staining for CRH, VP and OT was performed on three consecutive series of sections taken at 90 µm intervals throughout the region in which the PVN was present. This amounted to approximately 21 sections per peptide staining in each animal.

The monoclonal rat anti-CRH (PFU 83, IgG2a subclass) used was directed to the C-terminal part (amino acids 38-39) of rat/human CRH (generous gift from Prof. F.J.H. Tilders, Amsterdam, for more details see: Van Oers et al., 1989). After

deparafination and hydration of the slides, endogenous peroxidase activity was blocked with 0.5% H_2O_2 in methanol for ten minutes, followed by washing in TBS for 5 min. Sections remained in TBS until incubation. Incubations with PFU 83, diluted 1:80,000 in staining buffer (0.05 M Tris (Sigma), 0.5 M NaCl (Sigma), pH 7.4, containing 0.5%Triton-X-100 (BDH), and 0.25% gelatine (Merck)) were performed at room temperature overnight in moist boxes with a lid to prevent evaporation. The following day, the sections were washed in TBS (3 × 10 min) and incubated for 90 minutes at room temperature with a biotinylated goat-anti-rat IgG (H+L) serum (Vector) diluted 1:300 in staining buffer. Thereafter, sections were rinsed in TBS (3 × 10 min) and incubated for 2 hours with rabbit ABC-complex (Vector), diluted 1: 600 in staining buffer. The sections were subsequently washed in TBS. Staining was visualized (20 min room temperature) with filtered DAB-substrate prepared by dissolving 30 mg diaminobenzidine (DAB, 10 mg tablets (Sigma)) in 60 ml TBS with 1% imidazole (Merck) added for intensification of the end product, and activated by adding 0.03 % H_2O_2 (Sigma). Finally, the sections were washed, dehydrated in graded series of ethanol and coverslipped using DEPEX (DBH).

Rabbit antiserum “W3” (final dilution 1:6000), preabsorbed for OT as described above was used for immunocytochemical staining of VP, and rabbit antiserum “O2T” (final dilution 1:7500) for immunostaining of OT. Sections were incubated with the primary antibody, diluted in staining buffer, and kept at room temperature (26°C) overnight as described above. Subsequently the sections were incubated with biotinylated goat-anti-rabbit IgG serum (Vector), diluted 1:500 in staining buffer (90 min at room temperature), followed by rabbit-ABC complex diluted 1:1000 in staining buffer for 2 hours at room temperature. Between incubations, sections were thoroughly washed with TBS. The sections were subsequently incubated (20 min room temperature) in filtered DAB-substrate as described above and coverslipped.

Analysis

The localization and distribution of immuno-reactive cells was determined by microscopic examination at a magnification of 2.5 ×10.

To count the cells in each section, images of the PVN were captured with the assistance of an image analysis system (SION Image software program using a background correction). Thereafter images were imported into Adobe Illustrator (version 8.0), a drawing program in which schematic drawings can be easily made, since several different layers can be laid over one another in one file. The exact localization of the immuno-reactive neurons was determined with the help of the

adjacent Nissl-stained sections. Images of the VP, CRH and OT staining were imported into Adobe Illustrator 8.0 and only immunoreactive neurons in which cytoplasm was stained and a clear nucleus was present were marked and saved in an empty layer which was on top of the digitized staining. Subsequently, each peptide-staining layer showing the marked cells was imported into the Nissl-stained profile in Adobe Illustrator. The layers were lined out using clear neuroanatomical identification marks such as ventricle wall, blood vessels etc., to assure that each layer was positioned exactly on top of the Nissl staining. When the layers of VP, CRH and OT were put on top of each other, the complete distribution pattern could be examined. In order to compare the distribution pattern of the three peptides in different (sub)-regions of the PVN between animals, one animal was used to make a mold of every level and used as a template for the other animals used in this study. The different regions within the PVN at each level were defined with the main focus on the localization of CRH containing neurons, but also localization of VP and OT was taken into account. Subsequently, the VP, CRH and OT containing neurons in the PVN were counted in each of the levels 1 to 15 from rostral to caudal through the complete PVN.

Results

Antibody cross-reactivity and pre-incubation with homologous antigen

Since in the pig Lysine-vasopressin (LVP) predominates compared to Arginin-vasopressin (AVP) (Rao et al., 1991), a spot-blot experiment was performed to examine whether antiserum W3, which has originally been raised against AVP, also detects LVP. The antiserum clearly recognized LVP, albeit with slightly lower affinity than AVP (Figure 1, Table 1). In addition, some cross-reactivity of W3 with OT was only found in the spots containing the highest concentrations of OT, i.e. 50 ng and 200ng (Figure 1, Table 1). The O2T antiserum stained OT only and showed no cross-reaction with AVP or LVP.

When antiserum W3 was pre-absorpted with its homologous antigen, staining in the periventricular region of the PVN was still present, even when relatively high concentrations of AVP were used. When the VP antibody was additionally pre-absorpted with OT peptide, staining was completely abolished, indicating that OT specific antibodies were indeed present in the W3 antiserum. Therefore, W3 antiserum (final dilution 1:6000) was routinely pre-absorpted with OT (final concentration 2.08 ng/µl) to eliminate OT-staining and frozen until further use.

Staining of the PVN with antiserum O2T was completely abolished by pre-absorption with OT. Since the antiserum (final dilution 1:7500) showed no cross-reaction with LVP or AVP, it was used for OT immunocytochemistry without pre-absorption.

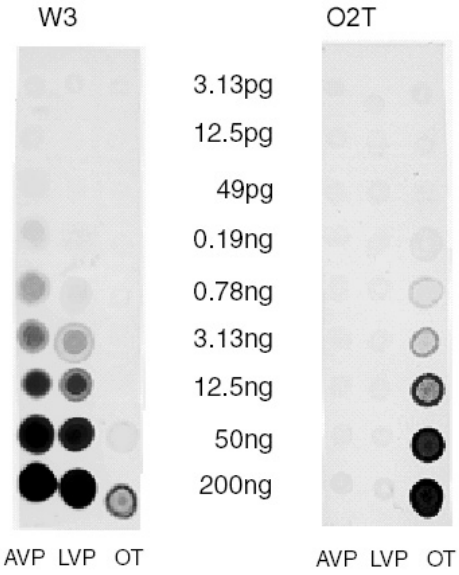


Figure 1. Dot blot membranes immunocytochemically stained with either vasopressin (W3; left) or OT (O2T right) antibody. Each membrane consisted of three rows with synthetic AVP, LVP and OT in the indicated amount.

Pre-incubation of the rabbit antisera with protein A was necessary to abolish non-specific staining of neurons in the PVN. This observation was consistent with previous studies (Van der Beek et al., 1992), where it was also shown that the non-specific staining usually observed with rabbit antisera in magnocellular neurons of the PVN can be effectively prevented by masking the Fc portion of IgGs with protein A. Pilot experiments showed that protein A in a final dilution of 1:100 was effective in this respect for both W3 and O2T.

Immunostaining of the PVN

In the animals used in this study, expression of VP, CRH and OT peptides was found throughout the PVN. The pattern of staining from the 3rd ventricle to lateral, however, differed between the peptides and was restricted to specific areas in the PVN (Figure 2a). At each cross-sectional level, the PVN was divided into four regions based on the distribution of CRH, VP and OT immunocytochemical staining (Figure 2a and 2b). The periventricular (pv) region, defined as the region

		Spot								
		1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*
Ab	*peptide*	200ng	50ng	12.5ng	3.13ng	0.78ng	0.19ng	49pg	12.5pg	3.13pg
W3	AVP	100	95	64	35	16	7	3	1	2
	LVP	100	84	51	19	6	5	4	5	3
	OT	25	6	5	5	5	6	7	5	6
O2T	AVP	10	6	6	7	7	7	7	7	7
	LVP	7	7	7	7	7	7	7	7	7
	OT	79	60	23	7	4	2	2	3	2

Table 1. Dot blot membranes staining of W3 and O2T expressed in % of staining darkness compared to the staining of homologous antigen (AVP for W3 and OT for O2T). W3 stained well with LVP either in high or low concentrations. W3 also showed to cross-reaction with OT, mainly in high concentrations. Antibody O2T showed clear staining with OT and hardly any cross-reaction with AVP or LVP.

between the 3rd ventricle and a line drawn 0.05 μm lateral from the 3rd ventricle, contained virtually no immunoreactivity for each of the three peptides. The antero-medial region (am, bordering the pv and further lateral) was defined as the region in which OT containing neurons were most abundant, with a gradient from dorsal to ventral, and in which hardly any CRH or VP containing neurons were present. The medial region (me) was defined as the region in which CRH and OT containing neurons were abundant, also with a gradient from dorsal to ventral. In the medial region also VP containing neurons were present, mainly in the dorsal area. Finally, the lateral region (la) of the PVN, often referred to as the "wing" of the PVN, contained VP neurons and only a few OT containing neurons, which were mainly located dorso-lateral. These clearly distinguishable regions of the PVN were present in all of the 15 evaluated levels that together covered the total length of the porcine PVN (1800 μm). The distribution of CRH, VP and OT containing neurons within each level of the porcine hypothalamus is presented in schematic drawings of one representative animal (Figure 3).

Neurons expressing CRH, VP and OT were identified by the presence of dark cytoplasmatic staining and a clear nucleus in the cell. Counting of neurons containing, CRH, VP and OT in the distinguishable regions of the PVN was performed manually in the collected images with an interspace of 90 μm throughout the PVN. The average numbers of, CRH, VP and OT containing

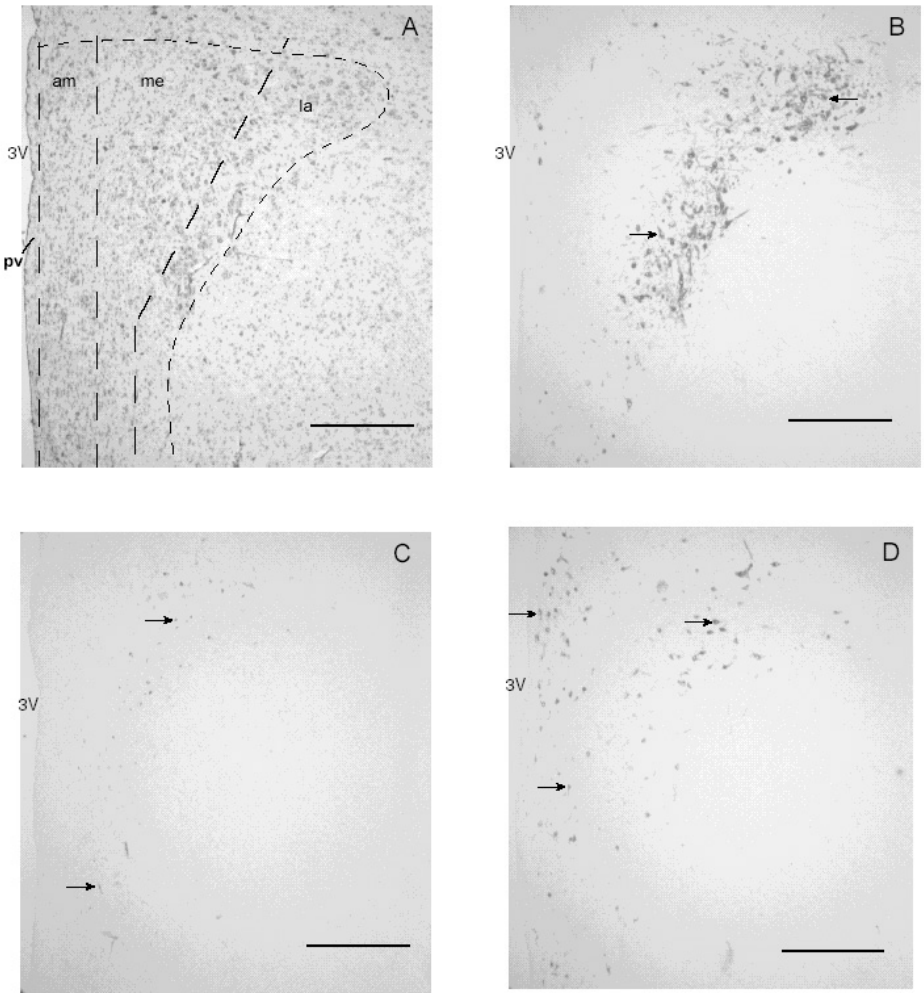


Figure 2a. Nissl staining with schematic representation of the different regions in level 7 of the porcine PVN, based on the distribution of VP, CRH and OT, (A) and distribution of VP (B), CRH (C) and OT (D) of level 7 of a representative animal. Arrows point out individual cells. pv: periventricular region, am: anteromedial region, me: medial region, la: lateral region, 3V third ventricle. Bar = 500 micrometer.

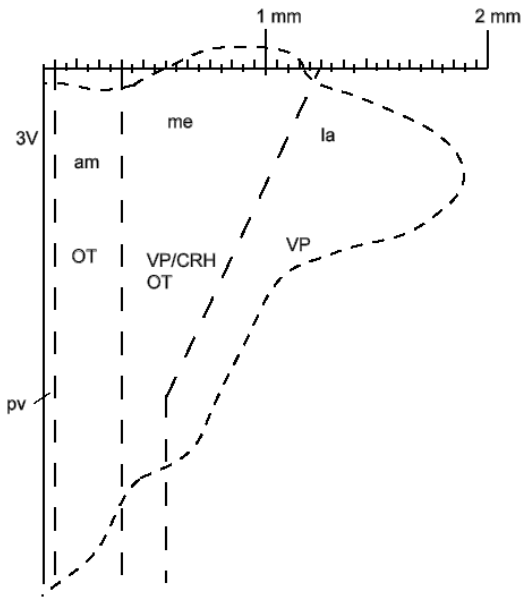
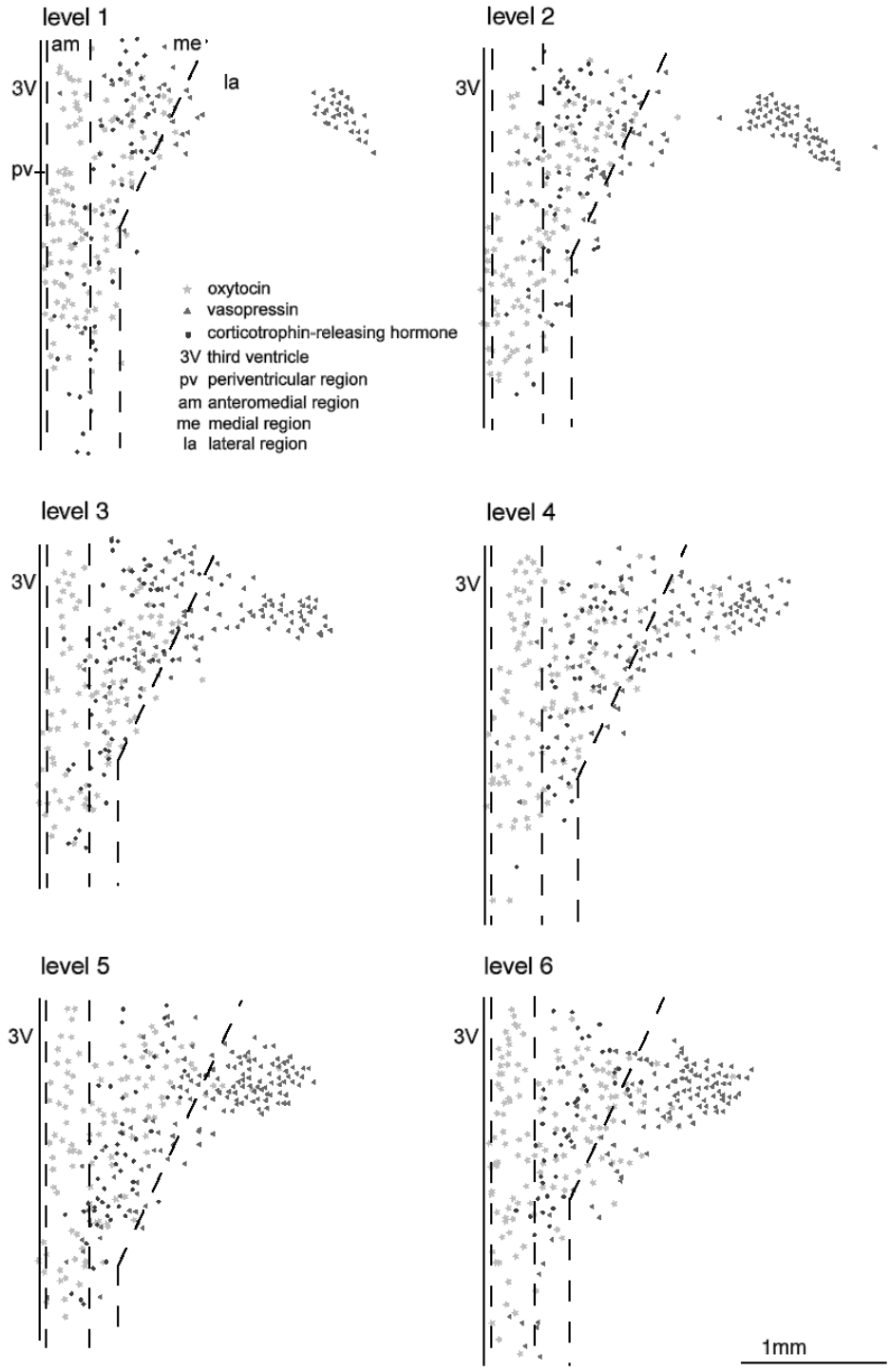


Figure 2b. Schematic representation of the different regions in a level of the porcine PVN, based on the distribution of VP, OT and CRH. pv: periventricular region, am: anteromedial region, me: medial region, la: lateral region, 3V third ventricle.

neurons are presented in figure 4 for each level and region of the PVN of all 5 animals. Despite the heterogeneity of the group (breed and sex) we found great similarity in staining, distribution and number of the different neuron-types as indicated by the small variance in group data. The number of OT containing neurons in the anteromedial region (am) of the PVN were particularly high in the rostral and midrostrocaudal (level 5-9) part and gradually decreased towards the caudal part of the PVN. Most CRH containing neurons in the medial region (me) of the PVN were found in the midrostocaudal part of the PVN (levels 5-9). In the lateral region (la) of the PVN, most VP containing neurons were present in the midrostocaudal part of the PVN, whereas in the medial region most VP containing neurons were present in the rostral part of the PVN.

Figure 3. Distribution of vasopressin (VP), oxytocin (OT) and corticotropin-releasing hormone (CRH) throughout the PVN of the pig. Immunoreactive cell bodies are represented with symbols. These schematic drawings are derived from adjacent 6 μm thick sections; the distance between sections is 90 μm. The total PVN was approximately 1800 μm in length leading to a total of 15 represented levels from rostral to caudal. **Next pages**.


level 1
am
me
la
3V
pv
level 2
3V
oxytocin
vasopressin
corticotrophin-releasing hormone
3V third ventricle
pv periventricular region
am anteromedial region
me medial region
la lateral region
level 3
3V
level 4
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level 5
3V
level 6
3V
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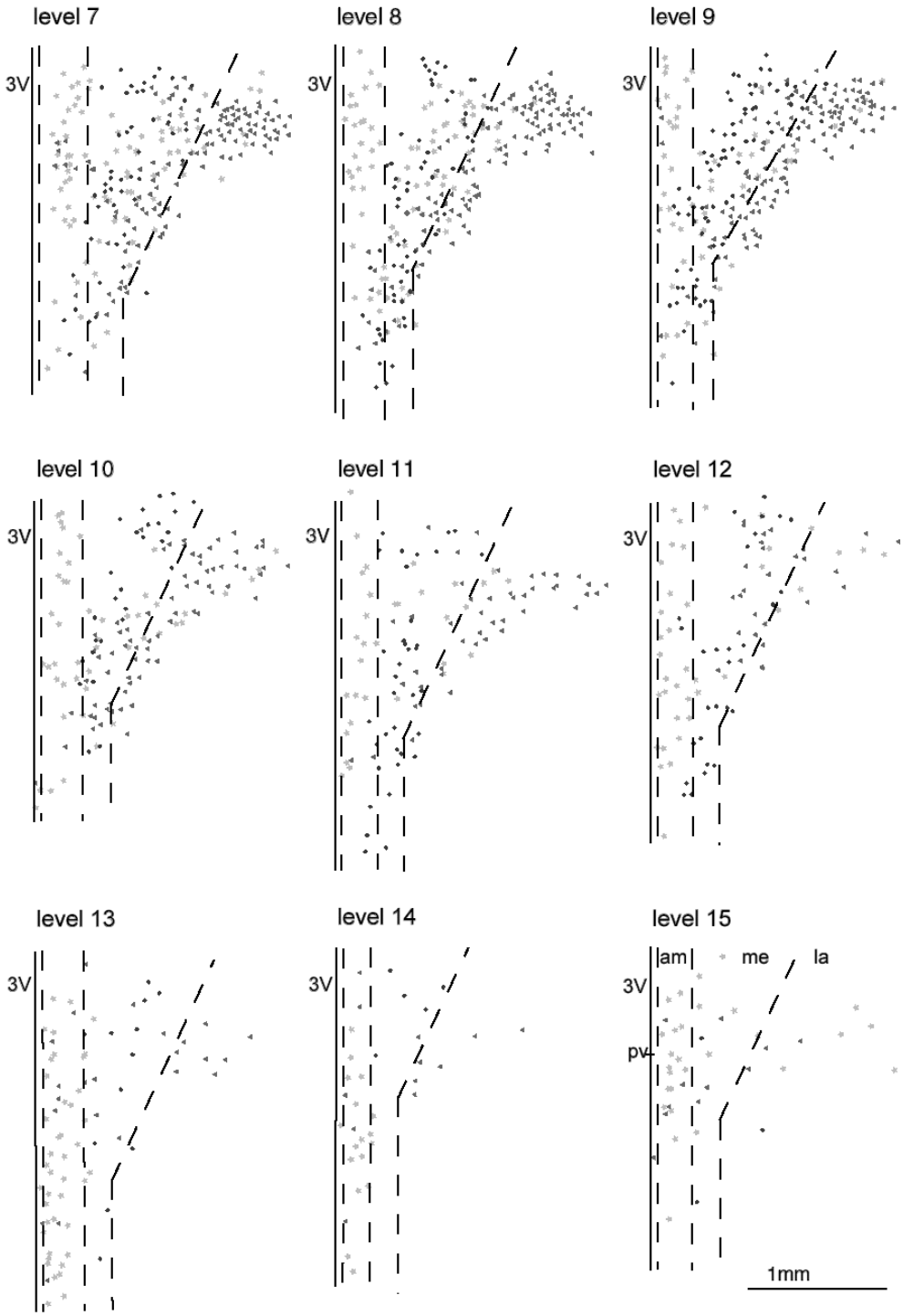
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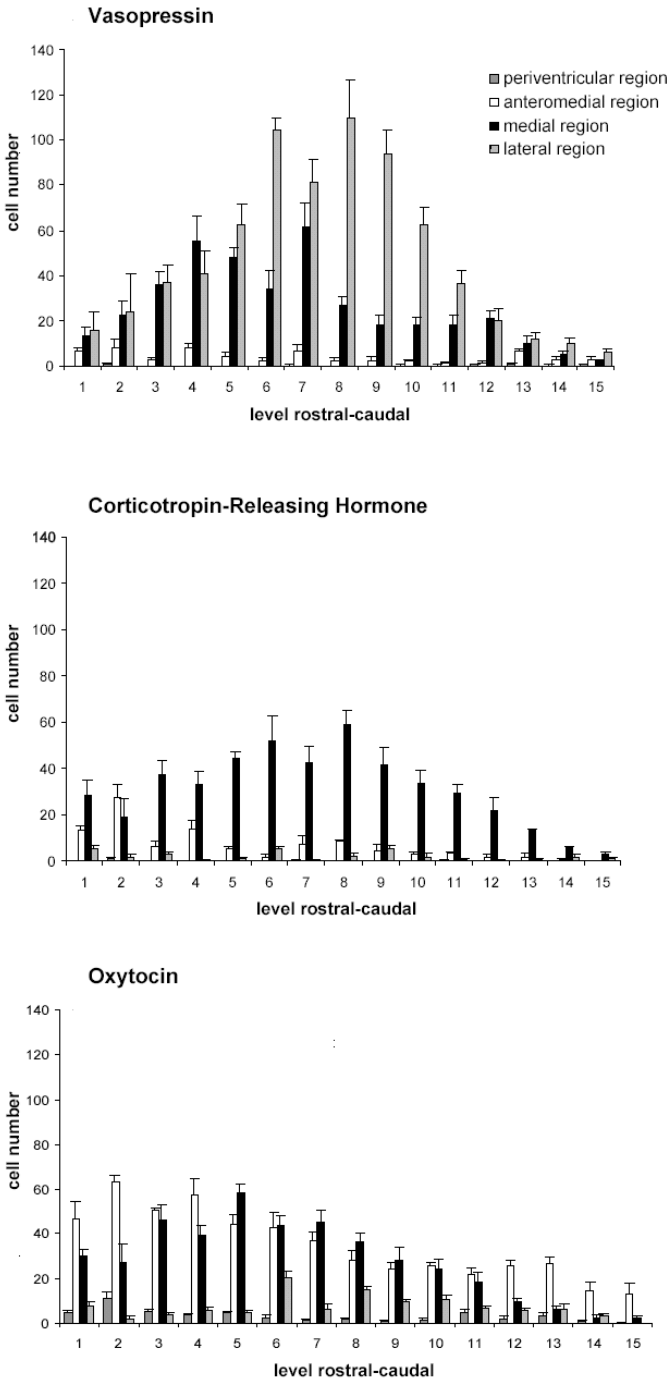

Figure 4. The number of neurons containing VP, CRH or OT in each level of the PVN of the pig throughout the total PVN. Bars represent mean + SEM.

Discussion

The present study describes the distribution of CRH, VP and OT neurons in the pig PVN by means of immunocytochemistry. It was shown that CRH containing neurons are most abundant in the medial region of the PVN throughout the PVN. The distribution of both VP and OT containing neurons was not restricted to a particular region of the PVN although the medial and lateral regions contained most VP neurons, whereas OT was most abundant in the antero- and medial region of the PVN throughout the PVN.

The presently proposed subdivision of the pig PVN in four regions is based on the distribution of CRH, VP and OT containing neurons (with the main focus on CRH containing neurons). This subdivision differs from that proposed by Seeger (1987) who distinguished three regions in the PVN of the pig based on cell size and localization; i.e. the pars verticularis, pars horizontalis, and pars parvocellularis. In contrast, in the PVN of the rat, eight regions have been recognized (Swanson and Kuyper, 1980; Swanson and Swachenko, 1983; Swanson et al., 1986; Hallbeck, 1999a), and in the human PVN a total of five different regions can be distinguished (Koutcherov et al., 2000). These regions are based upon cell size, neuro-peptide or transmitter content and in- and output projections. For the pig PVN, a previous study on the localization of immunoreactive VP and OT (Van Eerdenburg et al., 1992; Vellucci and Parrott, 1997) only focused on Seegers subdivision of the pig PVN. In the present study, the distribution of VP and OT containing neurons was in accordance with the studies mentioned above, however, a distinction between the pars verticularis and pars horizontalis could not be made. In contrast, two distinct regions in the pars parvocellularis were clearly present, mainly because of the localization of CRH found in the present study. In addition, in the present study we found that each peptide showed a distinct laterally oriented anatomical distribution leading to our proposed subdivision of the PVN into four lateral regions, which was consistent between the animals in our study, as is illustrated in figure 4 by the small variance between animals.

To our knowledge this is the first time the localization and distribution of CRH peptide was studied using immunocytochemistry in the pig PVN. The present findings are distinctly different from previous observations on CRH mRNA expression in the pig PVN using ISH (Vellucci and Parrott, 1998). In that study, neurons expressing CRH mRNA were found in young challenged pigs in the region (pars parvocellularis) bounded medially by the 3rd ventricle and laterally by the magnocellular area. In contrast, we found the distribution of CRH peptide in the pig PVN to be restricted to the medial region of the PVN and not bordering the 3rd ventricle directly. The difference in methods used to identify the CRH containing neurons might account for this discrepancy between our work and that of Velucci

and Parrott (1998). It is well established that in situ hybridization is a more sensitive technique than immunocytochemistry for the detection of peptidergic cell bodies (Hallbeck 1999a; Simmons et al., 1989; Hermanson et al., 1995), particularly when long cRNA probes are used. However, Vellucci and Parrott used a short oligonucleotide probe complementary to the rat CRH gene, which may have hybridized less specifically with porcine CRH then expected. Also, in situ hybridization with a porcine cRNA probe performed in our lab showed a distribution of CRH mRNA containing neurons similar to the distribution presently found for CRH peptide (Chapter 5), thus supporting a relatively restricted localization of CRH in the medial region in the porcine PVN. In addition, the distribution of CRH peptide found in the present study in the pig PVN is comparable to that reported for the rat, sheep, hamster, goat and hedgehog (Papadopoulos et al., 1985; Delville et al., 1992; Aguilera, 1998; Ceccatelli et al., 1989; Kikusui et al., 1997) making it more likely to be indeed restricted to the medial region of the PVN.

The subdivision of the rat PVN in magno- and parvocellular areas is well established (Sawchenko et al., 1984; Ceccatelli et al., 1989). In this species, CRH is mainly localized in the parvocellular region of the PVN, whereas VP is mainly localized in the magnocellular region. In contrast, OT is localized in the magnocellular as well as in the parvocellular region. In the present study, however, we did not measure the size of individual neurons to distinguish magno- from parvocellular neurons, although CRH and OT containing neurons in the antero-medial and medial regions of the PVN seemed to be smaller in diameter compared to the VP containing neurons in the lateral region. Therefore, we suggest that the medial region, that contains most CRH neurons, as well as the part bordering the 3rd ventricle (antero-medial region), that contains mainly OT neurons, are parvocellular. Furthermore, we suggest that the lateral region of the PVN, containing VP and OT, is likely to be a magnocellular region.

Given our present results, the distribution of the three peptides in the pig PVN seems to be quite similar to that found in other species. Indeed, OT neurons are generally found to be located near the 3rd ventricle and in the medial region of the nucleus (Swaab et al., 1975; Meister, 1990; Arima and Aguilera, 2000). CRH neurons in the pig PVN were scattered throughout the medial region of the PVN together with mainly OT neurons but also VP neurons were observed. This is also observed in sheep (Papadopoulos et al., 1985) and to a less extend also in rat (Sawchenko et al., 1984; Arima and Aguilera, 2000), but not in human (Koutcherov et al., 2000). VP neurons were mainly found in the most lateral regions of the pig PVN, which has also been described in the rat and human (Whitnall, 1988; Harding et al., 1995; Hallbeck et al., 1999a,b). Taken together, we

can conclude that the pig has a quite similar anatomical localization of OT, VP and CRH compared to other species. Although at present we do not have insight in the input and output projections of these neurons in specific sub-regions of the PVN, this may suggest that the hypothalamic CRH neuro-secretory system in the pig is regulated in a similar fashion as has been described in the rat.

It is known that CRH neurons centered in the parvocellular division of the rat PVN are capable of producing and secreting VP under normal, but in particular under stress situations (Sawchenko et al., 1984; Whitnall and Gainer, 1988; Kovacs and Sawchenko, 1996; Ma et al., 1999a). This VP, co-released with CRH, potentiates the actions of CRH to stimulate ACTH secretion from the anterior pituitary (Gibbs, 1986; Gillies et al., 1982; Whitnall, 1993; Aguilera, 1994, 1998; Ma et al., 1999a,b). If the same mechanism holds true for the pig, it could well be that part of the CRH containing neurons in medial region of the PVN are able to secrete VP too. Some of the immunoreactive VP found in the medial region of the PVN could therefore well be co-localized with CRH in the present study. With the presently used techniques, however, this was difficult to determine. Assuming that the regulation of the hypothalamic CRH neuro-secretory system is under the same organization as has been proposed for the rat, we expect that possible co-localization of CRH and VP will be primarily found in the medial region of the PVN, since CRH containing neurons are most numerous in this region.

In conclusion, the distribution patterns of VP, OT and CRH containing neurons were consistent between animals using the present delineation of the PVN subregions based on immunocytochemical staining. We can also conclude that the pig has a quite similar anatomical localization of VP, OT and CRH compared to other species. Whether the hypothalamic CRH/VP neuro-secretory system in the pig is regulated in a similar fashion as has been described in the rat remains to be elucidated.

Acknowledgements

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Chapter 3

Sex differences in the expression of vasopressin and corticotropin-releasing hormone in the paraventricular nucleus of pre-pubertal pigs

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Abstract

In rodents, stress is known to induce expression of vasopressin (VP) in paraventricular corticitropin-releasing hormone (CRH) neurons, with the degree of VP expression relating to duration and intensity of the stress. The response of the individual may differ depending on coping strategy as has been shown for several species including the pig. In addition, the activity of the hypothalamus-pituitary-adrenocortical (HPA) axis appears to be sexual dimorphic. In the present study, we investigated the effects of standard barren versus slightly enriched housing during rearing in group housed male and female piglets. The coping strategy of the individual animal was assessed early in life using the backtest. At 26 weeks of age, just before the onset of puberty, the expression of CRH and VP peptide in the paraventricular nucleus (PVN) of high and low responding pigs was determined by means of immunocytochemistry.

Neither housing condition nor coping strategy affected the amount of CRH or VP peptide in the PVN. Sex, however, affected both VP and CRH peptide content in the PVN. Male pigs, standard castrated on day 3 after birth, showed higher VP and CRH peptide content than female pigs possibly as a result of actions of gonadal steroid hormones during early development.

Barren housing conditions are thought to be stressful to the animals as evidenced by behavioral, autonomic and endocrine changes. Based on our results we suggest that the minimal enrichment provided by the straw bedding does not have enough impact for group housed animals to result in changes in the activity of the HPA axis at the hypothalamic level.

Introduction

Stressful life events and chronic stress conditions can increase behavioral, autonomic and endocrine responsiveness to subsequent challenges (sensitization), thereby increasing the vulnerability of individuals to stress-related diseases. Rearing and housing conditions, and interventions routinely used in pig husbandry are often stressful by themselves, and therefore potentially sensitizing (Schouten and Wiepkema, 1991; Janssens et al., 1995; Geverink et al., 1998).

The hypothalamus-pituitary-adrenocortical axis (HPA axis) plays a key-role in adaptation to stress (Whitnall, 1993, review). Corticotrophin releasing hormone (CRH), secreted by parvocellular neurons in the paraventricular nucleus (PVN) of the hypothalamus, is the major regulator of the secretion of Adrenocorticotropic Hormone (ACTH) from the pituitary that drives adrenal glucocorticoid release, namely cortisol in the pig. Under acute stress conditions, the activity of the CRH neurons is up-regulated and vasopressin (VP) co-expression is induced in these parvocellular neurons (Kovacs and Sawchenko 1996; Ma et al.,

1997). VP is only a weak ACTH secretagogue on its own, but acts synergistically with CRH to release ACTH, and is thought to play an important role in sustaining pituitary responsiveness during repeated or chronic stress (Antoni, 1993; Gillies et al., 1982; Aguilera, 1994, 1998). It has been shown in rats that repeated restraint desensitizes the transcriptional response of CRH. In contrast, the VP response pattern to this daily restraint shows an increase (Aguilera, 1994, 1998). Rats adapted to a chronic homotypical stress, however, show an enhanced transcriptional response of both CRH and VP in the parvocellular PVN to a novel stressor. The up-regulation of VP under restraint stress suggests that VP synthesis in the PVN plays an important role in activating the HPA axis under conditions of chronic stress. At the same time it could maintain the ability of the animal to respond to a novel stressor during the adaptation to homotypic stress (Ma et al., 1999). These arguments suggest that the functional state of CRH and VP systems may be used as a neurobiological index of cumulative stress.

It has been demonstrated that pigs housed in a standard barren environment have a blunted circadian rhythm in salivary cortisol compared to pigs housed in an enriched environment (De Jong et al., 1998, 2000). This may also indicate decreased welfare, since a blunted rhythm in cortisol has been reported in situations of chronic stress in several species including pigs and rodents (Janssens et al., 1995; Makino et al., 1995), and during depression in humans (Deuschle et al., 1997; Yehuda et al., 1996). This suggests that a barren housing condition may be considered a chronic stressor affecting the regulation of the HPA axis. In addition, it is known that during early postnatal development in the pig, environmental manipulation may result in permanent changes in HPA axis function. Separating boars from their mother for one hour for the first 14 days of their life already changes the HPA axis function permanently, as reflected in increased release of ACTH at slaughter (Weaver et al., 2000a). Based on findings in rats (Tilders et al., 1993) we hypothesize that, in pigs too, the degree of expression of VP and CRH, and especially the co-localization of these peptides in the PVN will be an indicator of altered HPA axis activity.

In the pig, as in rodents, the existence of individual stress coping strategies has been reported (Schouten and Wiepkema, 1991; Mendl et al., 1992; Hessing et al., 1993). An indication of the coping strategy can be obtained early in life by measuring the degree of resistance displayed in the so-called backtest (Hessing et al., 1993). In this test, a piglet is restrained on its back for 1 min and the reaction is scored by counting the number of escape attempts. The piglets are tested twice with a one-week interval. Piglets are classified as “high-resisting” (HR) if they show more than 2 escape attempts in each test. Piglets that show 2 or less escape attempts in each test are classified as “ low-resisting” (LR). There is also a group of

piglets (20%) that are not consistent over both test and classified as switchers or intermediates.

The classification of pigs in this test as HR or LR is to a certain extent predictive for the way pigs will response to stressful events later in life. It has been shown that HR and LR animals differ in a variety of features such as the behavioral response to apomorphine (Bolhuis et al., 2000), basal cortisol concentration, aggression, and HPA axis reactivity (Hessing et al., 1994; Ruis et al., 2000; Geverink et al., 2002a,b). In addition, some behavioral, pathological and immunological characteristics of pigs were reported to depend on the interaction between backtest qualification and housing conditions (Schrama et al., 1997; Bolhuis et al., 2003). These results indicate that the two behavioral strategies are highly correlated with different physiological and neuroendocrine responses, and that the backtest is a valuable tool for determination of these coping strategies of piglets in early life.

Taken together, the regulation of CRH and VP at the level of the hypothalamus may differ between pigs depending on the individual coping strategy (HR and LR). Since LR pigs seem to be more responsive to changes in the environment (Bolhuis et al., submitted), we hypothesize that housing under standard barren conditions may be reflected by higher CRH and VP peptide expression in the PVN compared to enriched housing, especially in LR pigs.

Standard housing conditions in pig husbandry involve group housing of castrated males and female piglets until slaughter. Yet, it is well established that both in animals, as well as humans, the activity of the HPA axis is sexually dimorphic (Rhodes and Rubin, 1999, review). Sexually dimorphic AVP nuclei have been found in the lateral septum, medial nucleus, hypothalamus, and bed nucleus of the stria terminalis (De Vries et al., 1984; De Vries and Boyle, 1998). In addition, a sexual dimorphism in the supraoptic nucleus (SON) as well as the vasopressin and oxytocin containing nucleus (VON) in the pig hypothalamus was demonstrated at the end of puberty (Van Eerdenburg and Swaab, 1994). Studies on sexual dimorphism of the CRH system are scarse, and report contradictory results. Therefore, the objective of the present study was to investigate the effect of housing conditions, coping strategy and sex on the expression of CRH and VP in the PVN of group housed piglets by means of immunocytochemistry.

Materials and Methods

Animals and Housing

Brains were obtained from groups of pigs reared under contrasting housing conditions derived from field practice; i.e. in standard/barren versus slightly enriched pens (Bolhuis et al., unpublished). Each pen ($7m^2$) had a solid area (70%) and a dunning area. Enriched pens contained additional straw bedding, but were otherwise similar to barren pens. Barrows and gilts of the crossbred [Large White x (Duroc x English Landrace)] x Pietrain were used for this study. The pigs were born from 6 multiparous sows either in a barren or enriched environment at the experimental farm in Wageningen. Pigs stayed in the same environment until the end of the experiment. At 10 and 17 days of age they were individually subjected to the backtest (Hessing et al., 1993). Boars were standard castrated on day 3 after birth. Piglets were weaned at 4 weeks of age and litters were culled to 10 piglets per group. Nests were not stratified for sexes and coping strategy resulting in a variation between the litters used in the experiment. Pigs were fed commercial feed ad-lib and had free access to water from a nipple drinker.

The backtest was performed on postnatal days 10 and 17 (Hessing et al., 1993, see table 1). Unfortunately, none of the female piglets born and housed under enriched environment were characterized as LR. Therefore there is no group of LR female pigs housed under enriched conditions included in the present study.

At the age of 26 weeks, the pigs were transported from Wageningen to the slaughter facilities in Lelystad using a commercial livestock lorry. Two groups were transported per day, i.e. one group raised in the enriched environment and one group in the barren environment. At 06.00 hr, the pigs were moved out of their pen into the transport box. The pigs were transported in the transport box to the lorry and loaded. The groups were penned separately on the same deck of the lorry. At arrival at the slaughter facilities, each group was separately unloaded and kept unmixed. At ± 11.00 hr the first group was driven to the stunning pen and pigs were slaughtered according to normal commercial practices after manual electrical stunning.

Slaughter, collection and processing of brain tissue

After the animals were killed, brains were removed from the skull and immersed in freshly prepared 10 % paraformaldehyde (Merck) (pH 7.2) in 0.1 M phosphate buffered solution for 14 days at 4°C. The post mortem time was very constant; time from electrocution until the moment that brains were put into fixative was 30-45 min. After 14 days, the hypothalamus was dissected and immersed in the same

Sex	Coping strategy	Housing condition	N
Male	LR	Barren	4
Male	LR	Enriched	5
Male	HR	Barren	6
Male	HR	Enriched	6
Female	LR	Barren	4
Female	HR	Barren	6
Female	HR	Enriched	6

Table 1. Number of male and female piglets classified as HR or LR in each housing condition.

fixation solution for another week, dehydrated through series of graded ethanol, embedded in paraffin (Histowax©, (melting point 56-58°C), Klinipath B.V., Duiven, Holland) and stored at room temperature until sectioning. Serial 6 μm sections were cut, and selected sections taken at regular intervals (90μm) were mounted on gelatin coated slides and stored at room temperature. For staining, sections were deparaffinized with xylene (Merck), and sections were hydrated with graded series of ethanol and washed in Tris-buffered saline (TBS) for 5 minutes. Every 15th section (interval of 90μm) was stained with cresyl violet (0.5% cresyl violet (BDH) in acetic buffer, pH 3.8) in order to localize the PVN-region in the hypothalamus before selecting section for immunocytochemical staining.

Immunocytochemistry

Immunocytochemical staining for CRH and VP was performed on two series of adjacent sections taken at 90 μm intervals throughout the region in which the PVN was present yielding approximately 21 sections per peptide staining per animal.

The monoclonal rat anti-CRH (PFU 83, IgG2a subclass) used, was directed to the C-terminal part (amino acids 38-39) of rat/human CRH (generous gift from Prof. dr. F.J.H. Tilders, Amsterdam, for more details see: Van Oers et al., 1989). After deparaffination and hydration of the slides, endogenous peroxidase activity was blocked with 0.5% H_2O_2 in methanol for 10 minutes, followed by washing in TBS for 5 min. Sections remained in TBS until incubation. Incubations with PFU 83, diluted 1:80.000, were performed overnight in staining buffer (0.05 M Tris (Sigma), 0.5 M NaCl (Sigma), pH 7.4, containing 0.5% Triton-X-100 (BDH), and 0.25% gelatin (Merck)) at room temperature in moist boxes with a lid to prevent evaporation. The following day, the sections were washed in TBS (3 × 10 min) and

incubated for 90 minutes at room temperature with a biotinylated goat-anti-rat IgG (H+L) serum (Vector) diluted 1:300 in staining buffer. Thereafter, sections were rinsed in TBS (3 × 10 min) and incubated for 2 hours with rabbit ABC-complex (Vector), diluted 1: 600 in staining buffer. The sections were subsequently washed in TBS. Staining was visualized (20 min, room temperature) with filtered DAB-substrate prepared by dissolving 30 mg diaminobenzidine (DAB, 10 mg tablets (Sigma)) in 60 ml TBS with 1% imidazole (Merck) added for intensification of the end product and activated by adding 0.03 % H_2O_2 (Sigma). Finally, the sections were washed, dehydrated in graded series of ethanol and coverslipped using DEPEX (DBH).

Antiserum raised in rabbit against arginin-vasopressin "W3" (RMI, Utrecht, final dilution 1:6000) was used for immunostaining of lysine-vasopressin (Chapter 2). Sections were incubated with the primary antibody, diluted in staining buffer, and kept overnight at room temperature (26°C) as described above. Subsequently, sections were incubated for 90 min at room temperature with biotinylated goat-anti-rabbit IgG serum (Vector) diluted 1:500 in staining buffer, thoroughly washed with TBS, and subsequently incubated with rabbit- ABC complex diluted 1:1000 in staining buffer for 2 hours at room temperature. Finally, the sections were incubated (20 min room temperature) in filtered DAB-substrate and coverslipped as described above.

Pre-absorption with oxytocin

A previous study (Chapter 2) indicated that oxytocin (OT) specific antibodies were present in the W3 antiserum. Therefore, W3 antiserum (final dilution 1:6000) was routinely pre-absorbed with OT (Sigma, final concentration 2.08 ng/ml) to eliminate OT-staining and frozen until further use. Briefly, diluted peptide solution was pipetted in 24 aliquots of 10 µl onto gelatin-coated nitrocellulose sheets and the press-blotting procedure was performed according to procedures described previously by Van der Beek et al. (1992) and Karman et al. (Chapter 2). Nitrocellulose sheets of 22.4 cm^2 were used for absorption of 2.5 ml antiserum diluted for immunocytochemistry. The antiserum was absorbed twice with one peptide-coated nitrocellulose sheets in small sealed bags to allow optimal contact between antigen and antibody. The first absorption was performed 3-4 hrs and the second absorption with a fresh peptide-coated sheet for 1 hr, both at room temperature.

Pre-incubation with protein A

To prevent nonspecific, Fc-mediated staining of rabbit Ab's that is normally seen in the porcine hypothalamus, protein A (Sigma) was added to the diluted rabbit antiserum (VP) according to procedures described previously by Van der Beek (1992). The antibody-prot A mix was subsequently used for immunocytochemical staining performed as described in the section "immunocytochemistry". The concentration of Protein A used was determined in a pilot experiment (dilutions 1:50, 1:100, 1:400). The 1:100 dilution yielded optimal results and was therefore used routinely for further immuno-staining with the (preabsorbed) rabbit antiserum (W3).

For technical reasons, immunocytochemical staining was performed in 4 separate runs for each peptide. To allow comparison of the staining between runs, sections of two animals were included in each run and used as controls.

Image Analysis

Images of the PVN at the right side of the 3rd ventricle (standardized to 15 images per pig brain) were captured using a microscope (2.5 x 0.63 magnification) with a digital black-and-white CCD camera (Sony, XC-77CE) coupled to a PC. The fifteen consecutive sections (90 μm apart) containing the PVN were selected using several criteria, including the end of the PVN and the location of the VON. The size of the captured images was 530 x 795 mm (horizontal x vertical).

To be able to compare CRH and VP data of multiple staining runs, each section was captured and analyzed with standardized procedures. This method was checked for reproducibility by comparing the results from the material of the two animals stained in all staining runs. The comparison showed no difference in the area measurements of CRH and VP positive nuclei. Thereafter, images were analyzed using an image analysis program (Scion Image Beta, version 4.02; Scion Corporation, Maryland, USA) using gray level threshold discrimination (Bethea et al., 1992). Briefly, a background correction was made by capturing each image of the PVN at the same gray level (i.e. the average gray level of the entire image). After capturing all images, the mean background was calculated for each staining procedure by measuring the gray level in an area that did not contain CRH and VP -positive cells in a representative selection of the images (10-40%). Using these background measurements the gray level threshold was determined: the mean maximal gray level in background + 3 x mean S.D. of the background was used as the threshold, which theoretically results in a $P < 0.001$ to include false positive nuclei in the measurement procedure. Using this threshold, the image was converted in a binary image in which gray levels above threshold were colored red (i.e. the positive staining) and particles smaller than a minimal number of pixels (n

= 3) were excluded by hand. Artifacts that were colored red were also excluded. Finally, the area covered by the CRH and VP containing cells was counted automatically. Using this method, only dark stained CRH and VP cells were measured, which corresponds to neurons with a high concentration/density of CRH and VP. Neurons with a low concentration of cellular CRH or VP from which the staining intensity did not reach threshold levels were not included in our area measurement result. According to a previous study performed in our lab (Chapter 2) co-localization of VP and CRH was most likely to occur in the medial region of the PVN of the pig, therefore from each of the 15 successive sections only the medial region of the PVN was examined. We found no significant differences in total area of CRH and VP-positive cells between the left and right side of the brain (Van der Beek and Karman, unpublished data) allowing us to measure only one side of the PVN.

Since we used automated measurements of the area positively stained for CRH and VP as a read out parameter for peptide expression in this study, we first compared cell counts of peptide positive cells with the area above threshold level in a random sample of 90 sections originating from 6 animals. We found a significant positive correlation between cell counts and area for both peptides, i.e. $r=0.79$, $p<0.01$ for CRH and $r= 0.89$, $p<0.01$ for VP, thus validating the use of automated measurements of stained area as a read out parameter.

Statistical analysis

Distribution of CRH or AVP peptide content was compared between the groups by means of GLM repeated measurement with factors coping strategy, housing condition and sex, and post-hoc analysis using the Bonferroni test of significance. Total area of CRH or AVP peptide content (area under the curve) was compared between the groups by means of univariate analysis of variance and the Bonferroni test of significance was used for post-hoc analysis, Statistical analysis was performed with the SPSS software system, version 11.0. Because of the lack of effect of coping strategy on CRH and VP content in the present study, LR and HR pigs housed under the same conditions were considered as one group and reexamined for possible effects of housing condition. A $p< 0.05$ was considered significant.

Results

Immunocytochemistry

Immunocytochemistry was performed to investigate possible effects of housing condition during rearing, coping strategy and sex on VP and CRH peptide expression in the PVN of pigs. Immunocytochemistry resulted in a clear cytoplasmic staining of VP and CRH-ergic neurons in the PVN.

VP peptide expression in PVN

In general, expression of VP was predominantly seen in neurons located in the lateral and medial region of the PVN, which is in accordance with a previous study from our lab on the localization of VP, CRH and OT in the PVN of the pig (Chapter 2; Figure 1A and 2). Since co-localization of VP with CRH, and thus involvement in the regulation of the HPA axis, was most likely to be found in the medial region of the PVN, only data of VP expression in the medial region are represented in the figures. However, inclusion of the expression of VP in the lateral region did not alter our reported results below. Coping strategy did neither affect the total amount of peptide expressed (i.e. area under the curve, Figure 4) ($F(0.384)=3.84$) nor the distribution ($F(0.730)=3.84$) (data not shown) of VP throughout the medial region of the PVN. Also, housing condition did not affect total VP expression ($F(0.109)=3.84$) or the distribution throughout the PVN ($F(0.089)=3.84$) (Figure 3 and 4) ($p> 0.05$ in all cases). In contrast, sex significantly affected the total VP staining area measured ($F(11.303)=3.84$; $p<0.01$)

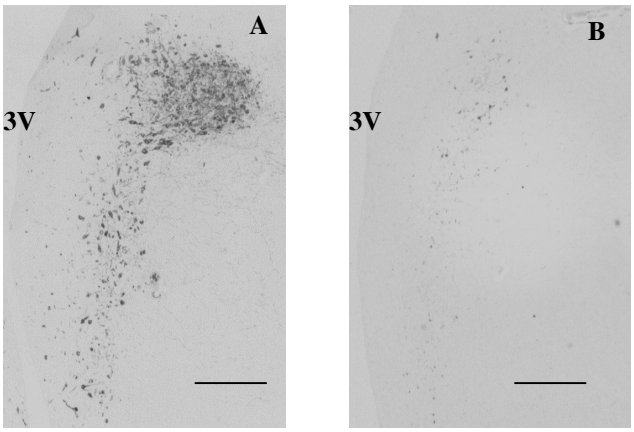

Figure 1. Distribution of VP (A) and CRH (B) of level 7 of a representative animal. 3V, third ventricle. Bar = 500 micrometer.

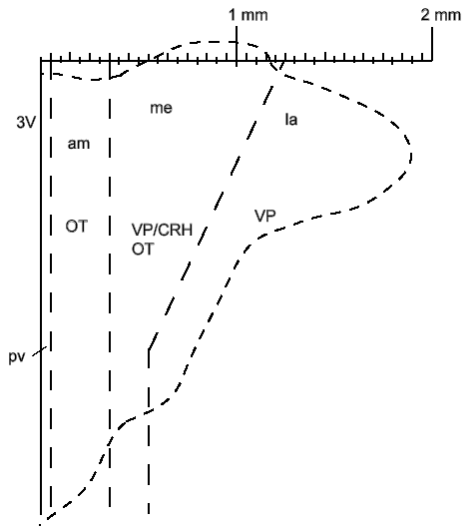

Figure 2. Schematic representation of the different regions of the porcine PVN, based on the distribution of VP, OT and CRH in our previous studies. pv, periventricular region; am, anteromedial region; me, medial region; la, lateral region; 3V, third ventricle.

(Figure 4), but not the distribution pattern of VP in the PVN ($F(0.760)=3.84$; $p>0.05$) (Figure 3). Post hoc analysis revealed that the overall expression of VP peptide was significantly higher in male pigs compared to female pigs ($F(11.303)=3.84$; $p<0.01$) (Figure 4).

CRH peptide expression in PVN

Expression of CRH was mainly seen in neurons in the medial region of the PVN of the pigs, which is in accordance with previous results from our lab (Chapter 2; Figure 1B and 2). Coping strategy did not affect the distribution (data not shown) ($F (4.430) = 3.84$) or total amount (Figure 6) ($F(0.008) =3.84$) of CRH peptide expressed in the medial region PVN. No differences were observed in the distribution pattern ($F (2.018) = 11.1$) or amount ($F(0.584) = 3.84$) of CRH throughout the PVN between the different housing-conditions (Figure 5 and 6) ($p>0.05$ in all cases). Barren housed males seemed to have higher CRH levels, but this was not significant ($F (1.706) = 3.84$; $p>0.05$). Again, sex significantly affected the total amount of CRH peptide, but not the distribution ($F (2.516) = 3.84$; $p>0.05$) of CRH in the PVN (Figure 5 and 6). The overall expression of CRH peptide was significantly higher in male pigs compared to female pigs ($F(12.481$; $p<0.01$) (Figure 6).

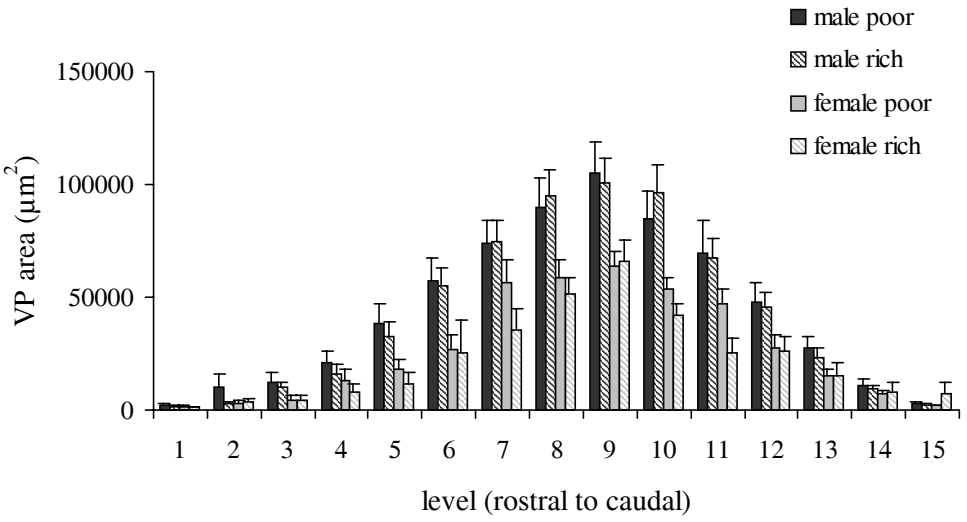

Figure 3. Distribution of VP from rostral to caudal throughout the PVN in male and female pigs reared under different housing conditions (barren = poor, enriched = rich). No effect of housing condition was observed in the amount or distribution of VP peptide expression. Sex affected the quantity but not the general distribution pattern of VP in the PVN. Bars represent mean + SEM.

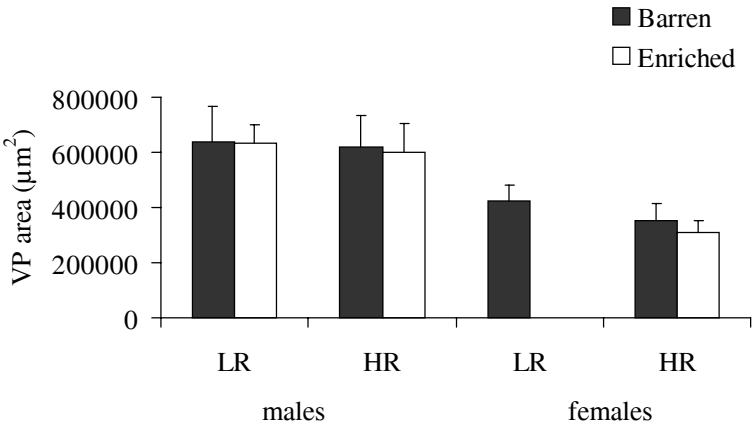

Figure 4. Area under the curve of VP, expressed in the medial part of the PVN (level 3 – 13) of male and female pigs reared under different housing conditions (barren = poor, enriched = rich), characterized for coping strategy (HR or LR). No effects of coping strategy or housing condition were observed on the quantity of VP peptide. Sex significantly affected the quantity of VP in the PVN, with a higher VP content in the PVN of males compared to females. ($p < 0.01$). Bars represent mean + SEM, n= 4-8.

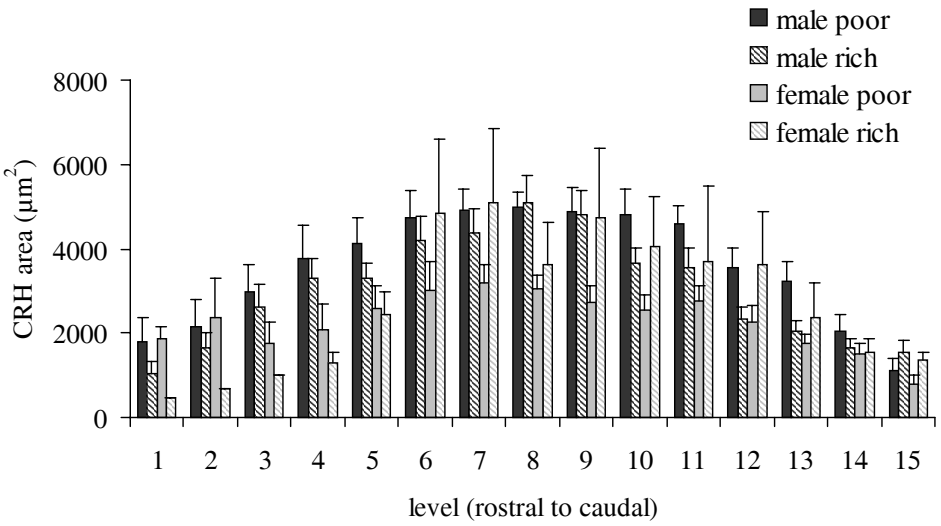

Figure 5. Distribution of CRH from rostral to caudal throughout the PVN of male and female pigs reared under different housing conditions (barren = poor, enriched = rich). No effect of housing condition was observed on the quantity or distribution of CRH peptide expression. Sex significantly affected the quantity but not the distribution of CRH in the PVN. Bars represent mean + SEM

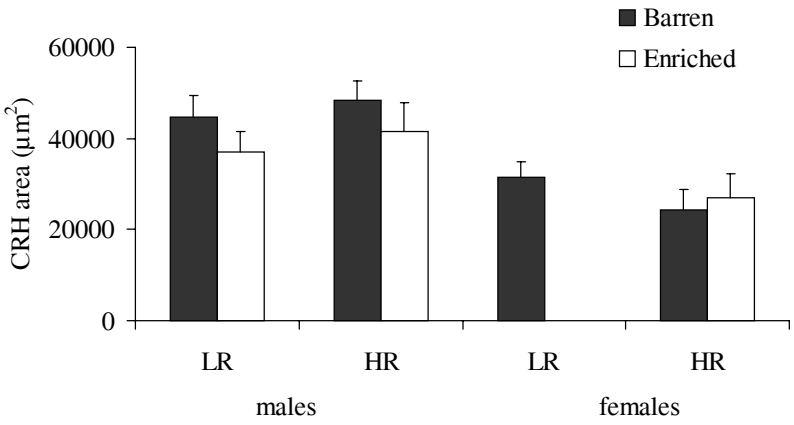

Figure 6. Area under the curve of CRH expression in the medial part of the PVN (level 3 – 13) of male and female pigs reared under different housing conditions (barren = poor, enriched = rich), characterized for coping strategy (HR, LR). No effects of coping strategy or housing condition were observed on the total area immunopositive for CRH peptide Sex affected the quantity of CRH in the PVN, males showing higher CRH content in the PVN than females. ($p < 0.01$). Bars represent mean + SEM, n= 4-8.

Discussion

In the present study, we found no effects of enrichment or coping strategy on VP or CRH peptide expression in the PVN of group housed pigs. However, sex affected both VP and CRH peptide content in the PVN, with castrated male pigs having higher VP and CRH peptide content in the PVN than female pigs. These sex differences may be due to modulation of the HPA axis by gonadal steroids in the pig early during development.

It has been shown that pigs contrasting for their response in the backtest early in life (HR and LR pigs) also differ in behavioral and physiological responses to stressful events later in life. For instance, HR and LR pigs differ in the level of aggression and reaction to a novel object (Hessing et al., 1993, 1994), as well as in cardiovascular and immune responses to challenges at later age (Hessing et al., 1995; Schrama et al., 1997; Bolhuis et al., 2003). Bolhuis et al. (2000) also showed that HR and LR pigs vary in the behavioral response to the dopamine agonist apomorphine, suggesting that HR and LR pigs may vary in dopamine signaling in the brain (Cools et al., 1990; Piazza et al., 1991). The same animals as used in the present study have been tested for behavioral, physiological and immune responses, which are presented and discussed in another paper (Bolhuis et al., unpublished). It was shown that these responses were highly correlated with the backtest response suggesting that HR/LR indeed reflect different coping strategies. In contrast, in the present study no effect of coping strategy was found on the expression of VP and CRH in the hypothalamus. This suggests that CRH and VP signaling may be similar between coping strategies under these (group) housing conditions that do not differ to a great extent, i.e. straw bedding added to the barren environment. A second explanation may be that the circumstances in which these pigs were reared and housed, which are very different from standard procedures in pig husbandry, did not lead to a divergent development of the activity of the CRH and VP system in these animals. Apart from the space restriction, it is common practice in modern pig husbandry to wean piglets at 3 or 4 weeks of age, in order to increase the number of litters one sow can have during her (short) reproductive life. Also, standard (stressful) pig husbandry procedures include castration of the male piglets, teeth clipping, ear tattooing, and tail docking, all carried out at 3 days of age. On top of that piglets may be mixed into new groups several times during their life to create fattening groups that are made up of individuals who are similar in breed, size and age. This mixing results in fighting of the pigs in order to settle social hierarchy positions. This would imply that the differentiation in housing conditions without concurrent social stress induced by stratification, mixing of nests and transport, might have been too small to induce differences in CRH and VP expression between coping strategies. Further research has indeed established

that LR and HR pigs housed under more severe stress conditions (i.e. individual housing) do show differences in CRH and VP signaling in the PVN (Chapter 4,5). Because of the lack of difference in CRH and VP content in the present study, LR and HR pigs housed under the same conditions were considered as one group and reexamined for the effect of housing condition.

In the present study, housing condition did not significantly affect CRH or VP peptide expression in the PVN (or cortisol responses, Bolhuis et al., unpublished). Yet, CRH levels were consistently, but not significantly, higher in poor males compared to rich males, not in females. This lack of an effect of housing conditions is in contrast to findings of other investigators who found effects of similar comparisons of different housing conditions, albeit on different HPA parameters. For example, De Jong and colleagues (1998), demonstrated that pigs housed in a barren environment were affected by the housing condition, as reflected in a blunted circadian rhythm in salivary cortisol compared to pigs housed under enriched environments. In addition, pigs kept under enriched environment show greater exploratory behavior of novel environments during the husbandry than pigs under barren conditions (De Jong et al., 1998). In the rat, environmental enrichment primarily accelerated habituation to novelty and improved spatial learning and memory. However it does not significantly alter basal and response levels of plasma ACTH and corticosterone to an acute stressor compared to barren environment (Pham et al., 1999; Larsson et al., 2002). Yet, environmental enrichment has also been found to have profound and long-lasting neural and physiological consequences in the rat. Higher hippocampal expression of 5-HT1A receptor (Rasmuson et al., 1998), glucocorticoid receptor (Mohammed et al., 1993) and changes in the expression of a large number of genes (Rampon et al., 2000) are only a few of changes that occur.

Housing under barren conditions can lead to a chronic stress situation, and it has been shown in the rat that exposure to chronic stress alters the HPA axis responses to subsequent stressors (Aguilera et al., 1994, 1998) as well as basal HPA axis activity. It has been suggested that in the context of chronic stress a shift from CRH- to VP-mediated modulation of the pituitary-adrenal function may occur in order to adapt to homotypic stress while at the same time maintaining the ability to respond to novel stressors (Ma et al., 1998, 1999). The lack of an effect on CRH and VP expression in the present study suggests that the housing conditions for these parameters, in contrast to other behavioral and endocrine parameters, were not contrasting enough. This may be the result of a stress dampening effect provided by social support in group housing conditions as proposed by Pearce and colleagues (1989). It was shown that pigs housed in dense groups displayed moderate to low levels of fear of humans compared to small and less dense groups.

Therefore, in the present study, even though the pigs were housed under barren conditions the size of groups might have protected the pigs from the stress-induced situation to a certain extend. This contention is supported by data of an experiment performed with hippocampus material of these very same animals, which showed that there is an effect of housing condition on the dentate gyrus. The hippocampus of barren housed pigs showed more apoptosis compared to neurogenesis, and the amount of GR was decreased (Kallivretaki et al., 2001, 2003). Since the hippocampus is closely involved in the regulation of the activity of CRH and VP in the PVN, effects of stress might first become evident in the hippocampus, and may only become evident in the hypothalamus during more severe stress. The trend, albeit not significant, for consistently higher CRH levels in barren housed male pigs compared to enriched housing might be the first sign of it. Another point that has to be taken into account is that the pigs were transported to the slaughter facilities for collection of brain material, which in fact represents a novel stressor for the animals. Since it is known that rats adapted to a chronic homotypical stress show an enhanced transcriptional response of CRH and VP in the parvocellular PVN to a novel stressor, the transport might have influenced the changed state of the CRH neuron, masking the effect of the housing conditions.

Our finding that VP peptide content is higher in (castrated) male compared to female pigs is in accordance with other studies in which sexually dimorphic VP nuclei were found in the lateral septum, medial nucleus, hypothalamus, and bed nucleus of the stria terminalis (De Vries et al., 1984; De Vries and Boyle, 1998). In these studies, male mammals showed greater cell number and fiber density than female mammals. Gonadal steroids are believed to organize the sexual dimorphism during development and maintain this situation in adulthood. In addition, Ishunina and Swaab (1999) described a sex difference in the size of VP neurons in the human PVN, where males had larger cell size compared to females. This was suggested to be the results of inhibitory actions of estrogens in adulthood on the activity of VP neurons in the human SON and PVN. The results of the present study indicate that also in pig, sex differences exist in VP neurons. However, whether this is due to greater cell number or larger cell size could not be determined due to technical limitations and needs further research. In addition, even though we have to take into account that the male pigs were castrated at a very young age, the neonatal testosterone surge, thought to be important for sexual differentiation of the hypothalamus, occurs in pig immediately after birth (Ford et al., 1990; Meusy- Desolle, 1975). Therefore the sexual dimorphism found in the VP system of the PVN in the pig is most likely organized by gonadal steroids during development and does not require the presence of gonadal hormones during life like was also found in the rat (De Vries et al., 1985; Viau et al., 1999).

Interestingly, however, Van Eerdenburg and Swaab (1994) concluded that sexual differentiation in pig occurs much later compared to that seen in other mammalian species. Sexual dimorphism in the SON and VON in the pig was not evident at the end of puberty, but only occurred between puberty and adulthood, in which neuron numbers increased in females. These findings, however, do not have to conflict with our present observations, since the sexual dimorphism of the SON and the VON seem to be driven by ovarian steroids, whereas the sexual dimorphism of the VP-containing systems is more likely to be testosterone driven (De Vries and Boyle, 1998).

Studies on sex differences of the CRH system are scarce, some report lower CRH gene expression in the PVN in females compared to males (Patchev et al., 1995; Givalois et al., 1997) while others report the opposite, i.e. males having lower CRH expression compared to females (Dunčko et al., 2001). Other studies failed to confirm possible gender differences in CRH mRNA levels in the PVN (Almeidi et al., 1997). The estrous cycle may be a possible explanation for the variance found between different studies. It has been suggested that ovarian steroids modulate the activity of the HPA axis. Estrogen may stimulate the HPA axis by a direct effect on CRH expression. It has been demonstrated that estradiol treatment increased CRH mRNA levels in the PVN of OVX female rats, rhesus monkeys and sheep (Rivier, 1999; Roy et al., 1999; Wood et al., 2001). In addition, testosterone has been suggested to be required for the inhibitory effects of cortisol on stress-related HPA function (Viau et al., 1999; Rhodes and Rubin, 1999). Since the male pigs in the present study had virtually no testosterone, inhibition of cortisol on CRH synthesis might have been reduced, resulting in higher CRH levels in our males compared to females. Moreover, the female pigs used in the present study were pubertal and might not have been fully cycling yet. Therefore they were not regularly exposed to estradiol, the ovarian steroid, which has been demonstrated to increase CRH mRNA in OVX female rats and rhesus monkeys (Rivier, 1999; Roy et al., 1999). The relatively low levels of circulating estrogen could also explain why female pigs showed lower CRH levels in the PVN compared to male pigs, but only when we assume that these female pigs were not fully cycling and that estrogen stimulates the HPA axis in pig as it does in other species (Rivier, 1999; Dunčko et al., 2001; Wood et al., 2001).

In conclusion, the present study shows that coping strategy and housing condition did not significantly affect the HPA axis at the level of CRH and VP peptide expression. Yet, behavioral and immunological responses in these animals were affected by barren housing condition. In addition, the hippocampi of these same animals under barren housing conditions showed more apoptosis and less neurogenesis. Since the hippocampus is the main input drive for the activity of

hypothalamic CRH and VP systems in the PVN, these changes may only be the beginning of a dysfunctional HPA axis and eventually lead to a change in the degree of CRH and VP expression in the PVN. Sex affected both CRH and VP peptide content in the PVN of the pig, males showing higher levels of both CRH and VP immunoreactivity in the PVN. It is known that testosterone can inhibit the HPA axis while estrogen may enhance its function. To which extend gonadal steroids are involved in these sexual differences found in the present study requires further research.

Acknowledgements

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Chapter 4

Individual housing affects vasopressin but not corticotropin-releasing hormone peptide expression in the paraventricular nucleus in pigs depending on coping strategy

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Abstract

Differences in coping strategy present at birth as well as housing conditions may influence autonomic and endocrine stress responses. In rats, corticotropin-releasing hormone (CRH) and vasopressin (VP) produced in the parvocellular system of the paraventricular nucleus (PVN) both play an important role in stress responses. There is evidence that the activity of these systems is altered in stress-induced sensitization. This suggests that the functional state of CRH and VP systems in the pig PVN could be used as a neurobiological index of stress vulnerability, an important aspect of animal welfare. We investigated effects of coping strategy and individual, confined housing (i.e. chronic stress) on the expression of VP and CRH peptide in the PVN in young adult female pigs (gilts).

To determine the coping strategy, animals were subjected to the backtest on days 10 en 17 after birth. High-resisting (HR) and low-resisting (LR) piglets were housed in mixed groups until 7 months of age. Thereafter, half of the animals were housed individually (IND), while the other half remained group-housed (GRO). At 14 months of age, gilts were sacrificed, brains collected and processed for immunocytochemistry to determine VP and CRH peptide content in the PVN (area in μm^2) using computer assisted image analysis (SCION).

GRO-housed gilts had significantly higher basal cortisol concentrations than IND-housed gilts. Housing or backtest did not affect the initial cortisol response to an acute stressor (nose sling). In IND HR gilts, however, the cortisol concentration took longer to return to basal levels compared to LR gilts, indicating a protracted stress response. Our immunocytochemical data showed a clear effect of coping strategy in IND, but not in GRO housed gilts; IND housed HR gilts showed significantly higher VP levels compared to IND housed LR gilts ($p<0.05$). Also IND housed HR gilts showed slightly higher VP levels compared to GRO housed HR gilts, albeit not significantly ($p=0.076$). Neither housing condition nor coping strategy affected the CRH peptide content in the porcine PVN.

This study shows, that HR and LR gilts react to chronic stress, induced by individual housing, with different neuro-adaptations: HR gilts up-regulate VP in neurons of the medial PVN, whereas LR gilts do not. We suggest that the higher levels of VP peptide in the medial region of the PVN play a role in potentiating the actions of CRH to stimulate ACTH secretion from the anterior pituitary. The observed differences between HR and LR gilts in PVN-VP levels may be the consequence of differences in glucocorticoid feedback or glucocorticoid receptor expression, which requires further investigation. In addition, the adaptations in the HPA axis that we found induced by the individual housing condition in pigs resemble those reported for human psychopathologies, and may thus be interpreted as a (pre) pathological sign. In conclusion, the results from the present study

clearly show that the mode of adaptation of pigs to chronic stress may differ between individuals depending on the coping strategy, and suggest that individual housing is not an optimal housing condition for pigs.

Introduction

In current pig husbandry individual, confined housing of breeding sows in stalls is still common practice. Pigs are social animals. Individual housing deprives the animals of contacts with conspecifics thereby causing chronic stress (Janssens et al., 1995; Ruis et al., 2001a,b). Chronic stress can lead to altered behavioral, autonomic and endocrine responsiveness to subsequent challenges (sensitization), increase the vulnerability of the animal to stress-related diseases, and compromise its welfare.

It is well established that the hypothalamus-pituitary-adrenocortical axis (HPA axis) plays a central role in stress responses (Whitnall, 1993, review). Corticotrophin-releasing hormone (CRH) secreted by parvocellular neurons in the paraventricular nucleus (PVN) of the hypothalamus, regulates the secretion of adrenocorticotropin releasing hormone (ACTH) from the pituitary (Whitnall, 1993, review). Under stress conditions, in rats, CRH is up-regulated in the parvocellular neurons and additional vasopressin (VP) expression in these neurons is switched on (Kovacs and Sawchenko 1996; Ma et al., 1997). VP is only a weak ACTH secretagogue on its own, but acts synergistically with CRH (Antoni, 1993; Gillies, 1982; Aguilera, 1994, 1998). In rats, it has been shown that repeated exposure to restraint stress desensitizes the CRH, but not the VP transcription response in the PVN to subsequent restraint. In contrast, the VP response to daily restraint shows an increase (Aguilera, 1994, 1998). This up-regulation of VP suggests that VP in the PVN plays an important role in activating the HPA axis under conditions of chronic stress and may be required to maintain its ability to respond to novel stressors during adaptation to homotypic stress (Ma et al., 1999). This suggests that the functional state of the CRH and VP systems in the PVN may be used as a neurobiological index of cumulative stress and stress vulnerability, an important aspect of animal welfare.

In the pig, like in many other species (Koolhaas et al., 1999, review), individual behavioral and physiological stress response patterns or coping strategies have been observed. An indication for the coping strategy of individual pigs can be obtained early in life by measuring the degree of resistance in the so-called “backtest” at day 10 and 17 of age. In this test a pig is restrained on its back and the number of escape attempts is determined. The classification of pigs in this test as “high-resisting” (HR, making more than 2 escape attempts in each test) or “low-resisting” (LR, making less than 2 escape attempts in each test) is to a certain

extent predictive for the way pigs will respond to stressful events later in life. Indeed, it has been shown that HR and LR animals differ in a variety of features such as the behavioral response to apomorphine (Bolhuis et al., 2000), basal cortisol concentration, aggression and hypothalamic-pituitary-adrenocortical (HPA) reactivity (Hessing et al., 1994; Ruis et al., 2000; Geverink et al., 2002a,b). In addition, some behavioral, pathological and immune characteristics of pigs were reported to depend on the interaction between backtest typification and housing conditions (Schrama et al., 1997; Bolhuis et al., 2003).

Most studies on individual coping strategies in pigs have used relatively young animals, i.e. piglets and fattening pigs. However, with respect to welfare of adult pigs, for instance breeding sows, it is of interest to know whether the backtest response in early life relates to individual physiological or behavioral characteristics when pigs grow older. Recently, Geverink et al. (2003) found that differences between adult HR and LR breeding pigs in basal behavioral and physiological variables are particularly expressed under conditions of chronic stress such as individual housing. In addition, it has been established that HR and LR animals differ in their response to an acute stressor. During fixation with a nose sling, young adult female HR pigs (gilts) vocalized more, showed a lower heart rate response, and a more protracted cortisol response than LR pigs (Geverink, 2002b). Thus, coping strategies as assessed in the backtest early in life may indeed be predictive of stress responsivity in adulthood.

Based on the above data we hypothesized differences in the regulation of CRH and VP at the level of the hypothalamus between adult pigs characterized as HR and LR in the backtest in early life. To test this, we investigated the effects of individual confined housing (i.e. chronic stress) on the expression of CRH and VP peptide in the PVN in young adult female pigs (gilts) displaying different coping strategies (HR or LR) by means of immunocytochemistry. Assuming that the regulation of the hypothalamic CRH neuro-secretory system of the pig resembles that of the rat, we expected changes in the CRH and VP system to occur primarily in the medial region of the PVN, since CRH containing neurons are most numerous in this region (Chapter 2). Therefore, in the present study, expression of CRH and VP peptides was measured in the medial region throughout the PVN.

Materials and Methods

Animals

Pig brains were obtained from a large experiment, which included contrasting housing conditions derived from field practice, that is individual vs. group housing of gilts. (Geverink, 2002a).

Subjects and housing have been described in detail previously (Geverink et al., 2002a). Briefly, in two identical successive experimental runs, a total of 48 nulliparous crossbred gilts (Pietrain × ((Large White Duroc) × British Landrace)) kept at the experimental farm of the Wageningen University were studied. They were selected out of a large pool of gilts bred at a commercial farm (Straathof, The Netherlands), based on their response in the backtest at 10 and 17 days of age. Briefly, each piglet was gently removed from its pen and put on its back on a farrowing mat positioned on a table. It was restrained in this supine position for 60 sec by placing one hand over the throat and the other loosely on the hind legs. A piglet making more than two escape attempts in each test was classified as an HR whereas when less than two escape in each test were made it was classified as an LR. For each experimental run 12 LR and 12 HR gilts were selected out of the pool of tested animals. They were weaned at 4 weeks of age and brought to the experimental farm at the Wageningen University, at 10 weeks of age. They were housed in groups of 12 (6 HR and 6 LR) until 20 weeks of age when groups were split resulting in four groups of six (3 HR and 3 LR). At 7 months of age, animals from half of the groups were housed in individual stalls. Thus in each experimental run, eventually 12 gilts remained group-housed (GRO) and 12 gilts were individually housed (IND) for the remainder of the experiment.

Group housing pens (3.80 × 3.15 m) had 65% solid floor and 35% slatted floor. A metal food trough (3.80 m long) was fitted at the front of the pen. The animals had access to a nipple drinker in the dunging area and a chain was attached above the trough. Individual pens were 0.60 m wide and 2.20 m long. They were made of partitions with vertical bars and placed in one row. HR and LR gilts were housed alternately. Each stall had a concrete floor (1.30 × 0.60 m) in the front and slatted floor (0.90 × 0.60 m) at the rear end. A metal trough was fitted at the front, with a nipple drinker in the middle. Above the food trough, a chain was attached that was easily accessible to the pig. For all rooms, an automatically controlled heating and ventilation system was used, set to maintain room temperature at 18°C. Furthermore, individual stalls had floor heating to compensate for heat loss through the concrete floor. In addition to natural light, artificial lights were on from 07.00 until 19.00 hr. Twice a day, at 08.30 hr and 15.00 hr, the animals were fed a commercial pelleted diet according to the Dutch standard for breeding pigs. All

pigs were accustomed to the experimenters and to the procedures of saliva sampling to avoid unwanted stress reactions.

Synchronization of estrus in gilts

A synthetic progesterone (altrenogest, Regumate, Intervet, Boxmeer, The Netherlands) was used to synchronize the estrus cycle in the gilts used in the present study (Kraeling et al., 1981, Stevenson and Davis, 1982) in order to reduce the influence of estrogen on the activity of the HPA axis (Rivier, 1999; Dunčko et al., 2001; Wood et al., 2001). Gilts were fed 20 mg of altrenogest per day, sprayed on their feed, for 18 consecutive days, regardless of the stage of the estrus cycle (Stevenson and Davis, 1982). Gilts were sacrificed 10 days after ending the treatment, which is known to be a large enough time interval for every gilt to return to estrus and reach the luteal phase of the cycle (Stevenson and Davis, 1982).

Nose sling procedure

Fixation with a nose sling was used to challenge the gilts. This technique of temporally restraining pigs is commonly used in veterinary practice and represents an acute stressor that activates the HPA axis. The nose sling test was performed at ten months of age and the procedure and the results of the whole group have been described in detail previously (Geverink et al. 2002b). Briefly, a familiar experimenter led a gilt individually to the test room, which was unfamiliar to the animal. Then, the experimenter tied a rope around the upper jaw of the gilt, attached the rope to a bar, and kept the gilt restrained in this way for 5 minutes. Thereafter, the gilt was brought back to her home cage. The test room was thoroughly cleaned after each gilt. All six gilts out of the same room (either a group or stall housing room) were tested on the same day, between 12.00 and 12.45 hr, and HR and LR gilts were tested alternately. Group and stall housed gilts were tested alternately on successive days.

Saliva collection and cortisol analysis

Saliva samples were collected at several time points before and after the nose sling test in the home pen of the animal. Start of the nose sling for each pig was defined as t= 0 min. Sampling times were –90, -30, -5, +15, +45 and +90 min. After the t = -5 min sample, animals were moved to the test-room, subsequently tested as described above and returned to their home pen before the t = +15 min was taken. Samples were collected by allowing the pig to chew on two cotton buds (Hartmann, Nijmegen, The Netherlands) until they were thoroughly moisturized. The cotton buds were placed in test tubes and centrifuged for 5 min at 2800 rpm to collect the saliva, which was stored at – 20°C until assay. Cortisol concentration in

saliva was measured using a commercial RIA kit (Coat-a-Count, Diagnostic Products, Apeldoorn, The Netherlands) modified for pig salivary cortisol (Ruis et al., 1997). Behavioral and hormone analysis were performed by N.A. Geverink and previously reported in detail (Geverink et al., 2002a).

Tissue collection and preparation

The healthy, intact animals were sacrificed at 14 months of age. Approximately half an hour before the canulation, animals were sedated with stressnil in their home pen, and transported to the slaughter facility in a transport box. Animals were anaesthetised with ketamine and brains were perfused via the ascending aorta with 5 liters of saline, followed by 5 liters of freshly prepared 6 % phosphate buffered formaldehyde (Merck, 37 % formalin, pH 7.4) as described previously (van der Beek et al, 1992; 2003). Brains were removed from the skull, postfixed in the 6% formalin solution for 2 days at 4°C. Thereafter the hypothalamus was dissected and immersed in the same fixative for another 2 days, dehydrated through series of graded ethanol, embedded in paraffin (Histowax©, (melting point 56-58°C), Klinipath B.V., Duiven, Holland) and stored at room temperature until sectioning. Serial 6 μm sections were cut, and selected sections taken at regular intervals (90 μm), were mounted on gelatin-coated slides and stored at room temperature. For staining, sections were deparafinized with xylene (Merck), and sections were hydrated with graded series of ethanol and washed in Tris-buffered saline (TBS) for 5 minutes. Every 15th section was stained with cresyl violet (0.5% cresyl violet (BDH) in acetic buffer pH 3.8) in order to localize the PVN in the hypothalamus.

Immunocytochemistry

Immunocytochemical staining for CRH and VP was performed on two consecutive series of sections taken at 90 μm intervals throughout the region in which the PVN was present. This amounted to approximately 15 sections per peptide staining in each animal, since the pig PVN is approximately 1800 μm in length (Chapter 2).

The monoclonal rat anti-CRH (PFU 83, IgG2a subclass) that was used, was directed to the C-terminal part (amino acids 38-39) of rat/human CRH (generous gift from Prof. dr. F.J.H Tilders, Amsterdam, for more details see: Van Oers et al., 1989). After deparafination and hydration of the slides, endogenous peroxidase activity was blocked with 0.5% H_2O_2 in methanol for ten minutes, followed by washing in TBS for 5 min. Sections remained in TBS until incubation. Incubations with PFU 83, diluted 1:80.000 in staining buffer (0.05 M Tris (Sigma), 0.5 M NaCl

(Sigma), pH 7.4, containing 0.5%Triton-X-100 (BDH), and 0.25% gelatine (Merck)) were performed at room temperature overnight in moist boxes with a lid to prevent evaporation. The following day, the sections were washed in TBS (3 × 10 min) and incubated for 90 minutes at room temperature with a biotinylated goat-anti-rat IgG (H+L) serum (Vector) diluted 1:300 in staining buffer. Thereafter, sections were rinsed in TBS (3 × 10 min) and incubated for 2 hours with rabbit ABC-complex (Vector), diluted 1: 600 in staining buffer. The sections were subsequently washed in TBS. Staining was visualized (20 min room temperature) with filtered DAB-substrate prepared by dissolving 30 mg diaminobenzidine (DAB, 10 mg tablets (Sigma)) in 60 ml TBS with 1% imidazole (Merck) added for intensification of the end product and activated by adding 0.03 % H_2O_2 (Sigma). Finally, the sections were washed, dehydrated in graded series of ethanol and coverslipped using DEPEX (DBH).

Rabbit antiserum "W3" (RMI, Utrecht, final dilution 1:6000) was used for immunostaining of VP. Sections were incubated with the primary antibody, diluted in staining buffer, and kept at room temperature (26°C) overnight as described above. Subsequently the sections were incubated with biotinylated goat-anti-rabbit IgG serum (Vector), diluted 1:500 in staining buffer (90 min at room temperature), followed by rabbit- ABC complex diluted 1:1000 in staining buffer for 2 hours at room temperature. In between incubations sections were thoroughly washed with TBS. The sections were subsequently incubated (20 min room temperature) in filtered DAB-substrate as described above and coverslipped.

Pre-absorption with oxytocin

A previous study (Chapter 2) indicated that antibodies cross-reacting with oxytocin (OT) were present in the W3 antiserum. Therefore, W3 antiserum (final dilution 1:6000) was routinely pre-absorbed with OT (final concentration 2.08 ng/ml) to eliminate OT-staining, and frozen until further use. Briefly, diluted peptide solution was pipetted in 24 aliquots of 10 µl onto gelatin-coated nitrocellulose sheets and the press-blotting procedure was performed as described previously (Chapter 2; van der Beek et al., 1992). Nitrocellulose sheets of 22.4 cm^2 were used for absorption of 2.5 ml antiserum diluted for immunocytochemistry. The antiserum was absorbed twice with peptide-coated nitrocellulose sheets in small sealed bags at room temperature to allow optimal contact between antigen and antibody. The first absorption was performed 3-4 hrs and the second absorption, with a fresh peptide-coated sheet, for 1 hr.

Pre-incubation with protein A

Protein A (Sigma) was added to the diluted rabbit antiserum, (VP) to prevent nonspecific, Fc-mediated staining of rabbit Ab's normally present in the porcine hypothalamus, according to procedures described previously by Van der Beek (1992). The antibody-prot A mix was subsequently used for immunocytochemical staining performed as described in the section "immunocytochemistry". The concentration of Protein A used was determined in a pilot experiment (dilutions 1:50, 1:100, 1:400). The 1:100 dilution yielded optimal results and was therefore used routinely for further immunostaining with the (preabsorbed) rabbit antisera (W3).

Image analysis

Images of the PVN at the right side of the 3rd ventricle (standardized to 15 images per pig brain) were captured using a microscope (2.5 x 0.63 magnification) with a digital black-and-white CCD camera (Sony, XC-77CE) coupled to a PC. Animals that did not show staining in some of the levels examined or missed levels due to damage of the section were excluded from further analysis The fifteen consecutive sections (90 mm apart) containing the PVN were selected using several criteria, including the clearly distinguishable end of the PVN and the location of the VON (van Eerdenburg et al, 1992). The size of the captured images was 530 x 795 mm (horizontal x vertical). By capturing images immediately adjacent to the 3rd ventricle at the indicated magnification, we captured the entire PVN in one image.

Thereafter, images were analyzed using an image analysis program (Scion Image Beta, version 4.02; Scion Corporation, Maryland, USA) using gray level threshold discrimination as previously described by Bethea et al. (1992). To be able to compare CRH and VP data of multiple stainings, each section was captured and analyzed with standardized procedures. This method was checked for reproducibility by comparing sections of two animals that were included in all staining runs. These resulted in a comparable area of CRH and VP positive cells in each animal used in all runs. A background correction was made by capturing each image of the PVN at the same gray level (i.e. the average gray level of the entire image). After capturing all the images, the mean background was calculated for each staining procedure by measuring the gray level in selected part of the image that did not contain CRH and VP -positive structures in a representative selection of the images (10-40%). Using these background measurements the gray level threshold was determined: i.e. the mean maximal gray level in background + 3 x mean S.D. of the background was used as the threshold, which theoretically results in a $P < 0.001$ to include false positive cells in the counting procedure. Using this

threshold, the image was converted in a binary image in which gray levels above threshold were colored red (i.e. the positive staining) and particles smaller than a minimal number of pixels (n = 3) were excluded. Artifacts that were colored red were excluded by hand. Finally, the area of CRH and VP positive staining was measured automatically. Using this method, only dark stained CRH and VP cells were measured, which corresponds to neurons with a high concentration/density of CRH and VP. Neurons with a lower concentration of cytoplasmatic CRH or VP from which the staining intensity did not reach threshold levels were not included in our area measurement result. According to a previous study performed in our lab (Chapter 2), co-localization of VP and CRH was most likely to occur in the medial region of the PVN of the pig. Therefore, from the 15 successive sections only the medial region of the PVN was examined. Using this approach, we found no significant differences in total area of CRH and VP-positive cells between the left and right side of the brain (van der Beek and Karman, unpublished data) thus allowing us to measure only one side of the PVN.

Statistical analysis

Statistical analysis was performed with the SPSS software system, version 11.0 for Windows. The effect of housing and backtest type on cortisol concentrations before and after the nose sling was studied by means of General Linear Model (GLM) repeated measurement and univariate analysis of variance. Distribution of CRH or AVP peptide content was compared between the groups by means of GLM repeated measurement. Total area of CRH or AVP peptide content was compared between the groups by means of univariate analysis of variance and the Bonferroni test of significance was used for post-hoc analysis. A $p < 0.05$ was considered significant.

Results

Cortisol before and after the nose sling test

The data shown are adapted from Geverink et al. (2002b) after reanalysis including only the data from the 48 animals used in the present immunocytochemical study. In general, the changes in cortisol for the selected animals were comparable to that reported for the whole group. Group-housed (GRO) gilts showed significantly ($F(5.355)=3.84$; $p<0.05$) higher cortisol concentrations than individual-housed (IND) gilts at t= -90 min (see Table 1). There was a significant increase after the nose sling stressor ($p< 0.01$) in all groups, and cortisol responses 15 minutes after the nose sling stressor were comparable between housing-conditions and coping strategies ($F(1.367)=7.81$; $p>0.05$). At t=+45 min cortisol levels for IND HR gilts were still significantly elevated compared to basal levels measured at -90 min ($p<0.05$), whereas the other groups showed levels comparable to basal. At t=+90 min the cortisol levels in each group were back to basal levels. At t=+90 min GRO gilts again showed significant ($F(6.029)=3.84$; $p<0.05$) higher cortisol concentrations than IND gilts.

VP peptide expression in the PVN

Immunocytochemistry resulted in a clear cytoplasmic staining of VP and CRH-containing neurons in the PVN. In general, expression of VP (i.e. measured as area of VP positive neurons and area under the curve) was observed mainly in neurons located in the lateral and medial region of the PVN of the pigs (see Figure 1A), which is comparable to that reported by our lab in a previous study in which

	t= -90	t = -30	t = -5	t = +15	t = +45	t = +90
GRO HR	1.17 ± 0.25	0.97 ± 0.39	1.29 ± 0.36	2.02 ± 0.24	1.06 ± 0.27	1.35 ± 0.34
GRO LR	1.10 ± 0.34	0.90 ± 0.19	0.94 ± 0.16	2.40 ± 0.36	1.35 ± 0.27	1.56 ± 0.52
IND HR	0.65 ± 0.10	0.68 ± 0.11	0.47 ± 0.05	2.23 ± 0.20	1.06 ± 0.21	0.46 ± 0.14
IND LR	0.63 ± 0.10	0.68 ± 0.11	0.84 ± 0.24	1.91 ±0.27	0.76 ± 0.14	0.66 ± 0.12

Table 1. Mean salivary cortisol concentrations before and after a 5-min fixation with a nose sling in group (GRO) and individual (IND) housed HR and LR gilts. Basal cortisol levels (t= -90 min) were lower in IND gilts compared to GRO gilts ($p<0.05$). However, cortisol responses 15 minutes after the nose sling stressor were comparable between groups. At t=+45 cortisol was back to basal levels, except for IND HR gilts ($p< 0.05$), and at t=+90 cortisol levels were lower in IND gilts compared to GRO gilts ($p<0.05$). Data represent mean + SEM; N=4-11.

we investigated the localization of VP, CRH and OT in the PVN of the pig in detail (Chapter 2; Figure 2). We found a clear interaction ($F(6.031)=3.84$; $p=0.021$) between housing condition and coping strategy on the amount of VP peptide in the medial region of the PVN; IND HR gilts showed a higher expression of VP compared to IND LR gilts ($p < 0.05$) (Figure 3A). Also, IND housed HR gilts showed higher VP levels compared to GRO housed HR gilts, albeit not significantly ($p=0.076$). Finally, IND HR gilts tended ($F(2.348)=7.81$; $p= 0.09$) to have a different distribution of VP peptide compared to IND LR gilts mainly due to higher VP levels in the midrostrocaudal and caudal parts of the PVN (Figure 4).

CRH peptide expression in the PVN

Expression of CRH (i.e. measured as area of VP positive neurons and area under the curve) was mainly seen in neurons in the medial region of the PVN of the pigs (see Figure 1B), which is also comparable to that reported by our lab in a previous study in which we investigated the localization of VP, CRH and OT in the PVN of the pig in detail (Chapter 2; Figure 2). Neither coping strategy nor housing conditions significantly affected the quantity ($F(0.003)=3.84$; $F(1.697)=3.84$, respectively) (Figure 3B) or distribution ($F(2.725)=3.84$; $F(0.404)=3.84$, respectively) (Figure 5) of CRH peptide throughout the PVN. ($p> 0.05$ in all cases).

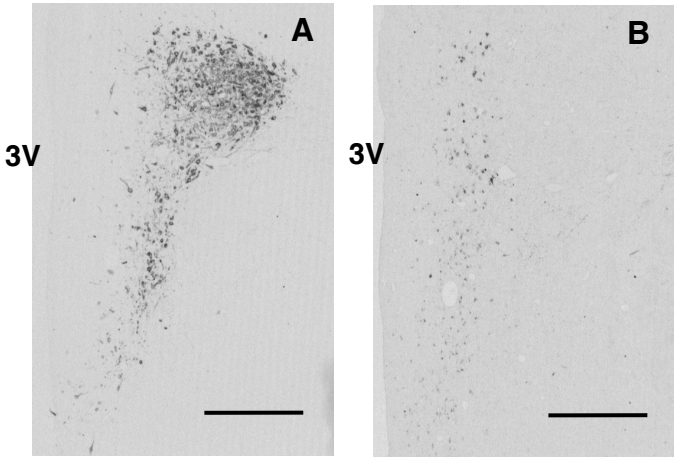

Figure 1. Distribution of VP (A) and CRH (B) of level 6 of a representative animal. 3V, third ventricle. Bar = 500 micrometer.

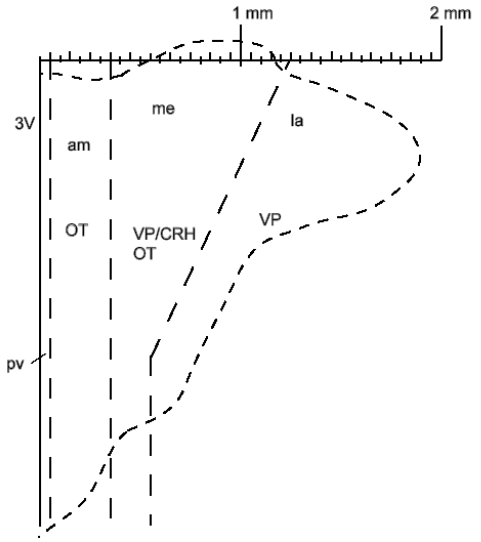

Figure 2. Schematic representation of the different regions in a level of the porcine PVN, based on the distribution of VP,OT and CRH. pv, periventricular region; am, anteromedial region; me, medial region; la, lateral region; 3V, third ventricle.

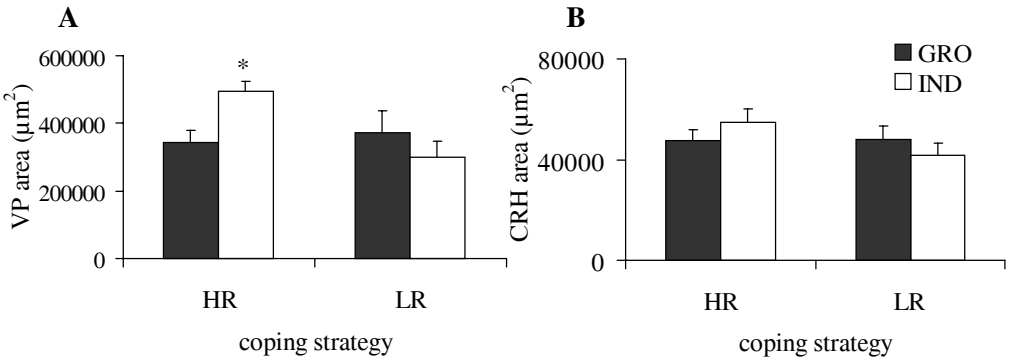

Figure 3. Area under the curve of VP (**A**) and CRH (**B**) peptide expression in the medial region of level 3–13 of the PVN of one-year-old female pigs with different coping strategies (HR or LR) after group- (GRO) or individual (IND) housing. These data show an interaction between housing condition and coping strategy for VP content in the PVN. ($p < 0.05$). Housing condition nor coping strategy affected the CRH content in the PVN of these gilts. Bars represent mean + SEM; n = 5-8.

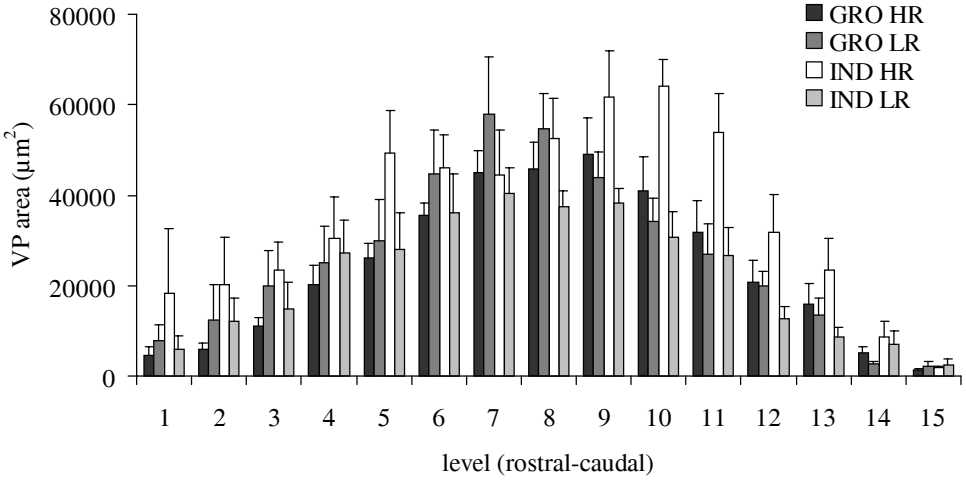

Figure 4. Distribution pattern of VP peptide expression in the medial region of level 1–15 of the PVN of one-year-old female pigs with different coping strategies (HR or LR) after group- (GRO) or individual (IND) housing. These data show that IND HR gilts tended to have a different distribution pattern compared to IND LR gilts (p= 0.09).
Bars represent mean + SEM; N = 5-8.

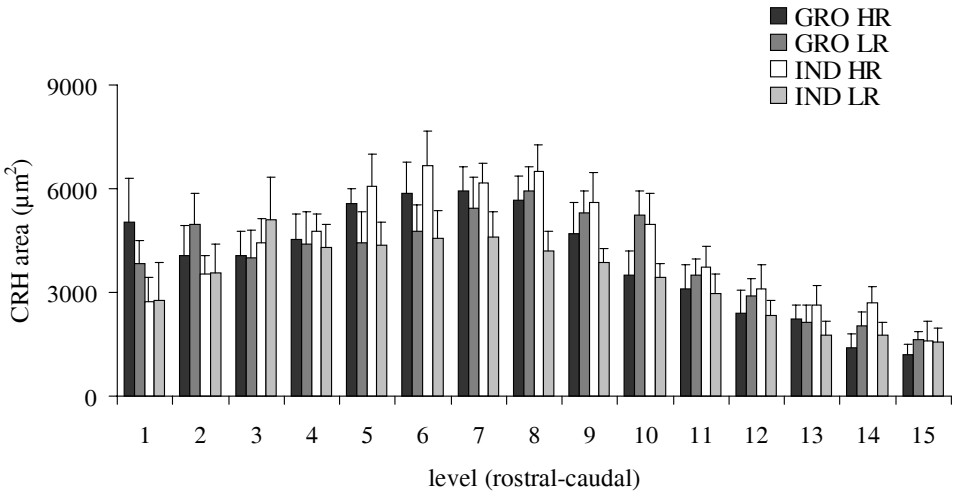

Figure 5. Distribution pattern of CRH peptide expression in the medial region of level 1–15 of the PVN of one-year-old female pigs wtih different coping strategies (HR or LR) after group- (GRO) and individual (IND) housing . Housing condition nor coping strategy affected the CRH distribution in the PVN of these gilts.
Bars represent mean + SEM. N= 5-8.

Discussion

The present study was designed to investigate the effect of different housing conditions and coping strategies on the amount of CRH and VP peptide in the PVN of pigs. We found a clear interaction between the backtest qualification and housing condition for VP, but not for CRH peptide expression. Thus, the VP response induced by individual housing appears to depend on the coping strategy of the animal, with HR animals showing an increase in VP peptide levels and LR animals not. It has been suggested that the functional state of the CRH and VP systems in the PVN may be used as neurobiological index of cumulative stress and stress vulnerability. Consequently, the results suggest that HR gilts may be be more susceptible to stress sensitisation than LR gilts.

The increased VP expression in IND HR gilts resembles a response that is seen in rat after repeated immobilization or daily restraint. In these rats daily repeated immobilization did not affect CRH peptide stores but caused a progressive increase in VP stores and in the number of CRH nerve endings containing VP in the external zone of the median eminence the major projection site of PVN CRH neurons (De Goeij et al., 1991). Similarly it has been shown that parvocellular VP mRNA levels rise after repeated immobilization (Bartanusz et al., 1993), suggesting a change in neuronal activity. The higher levels of VP found in the present study may therefore be due to changes in neural activity as well. In rodents, VP acts synergistically with CRH and is thought to play an important role in sustaining pituitary responsiveness to a novel stressor during chronic stress. Another mechanism by which VP facilitates ACTH regulation in the rat is by decreasing the sensitivity of glucocorticoid feedback at the level of the pituitary (Aguilera, 1994,1998). In the pig, VP may regulate pituitary corticotroph function in the same way. The fact that the cortisol response to an acute stressor was not different between GRO- and IND housed animals may support this assumption.

In IND HR gilts, VP peptide levels appeared to be higher in the medial region throughout the PVN compared to the other groups, but highest levels of VP peptide were found in the midrostrocaudal and caudal (posterior) part of the pigs PVN. In rats, neurons of the posterior magnocellular subdivision contain VP, which project to the posterior pituitary where VP is released in the bloodstream (Swanson and Kuypers, 1980). On the other hand, neurons of the medial parvocellular subdivision that contain CRH, VP (Swanson et al., 1983) and other releasing hormones (Brownstein et al., 1982) send axons to the median eminence, where they regulate the hormonal output from the anterior pituitary (Antoni et al., 1983). At present we do not have insight in the input and output projections of these neurons in specific sub-regions of the pig PVN. Assuming that the projection

of the hypothalamic CRH and VP neuro-secretory system is under the same organization as has been proposed for the rat, we suggest that the higher levels of VP peptide in the midrostrocaudal and caudal part of the medial region of the PVN indeed play a role in potentiating the actions of CRH to stimulate ACTH secretion from the anterior pituitary (Gibbs, 1986; Gillies et al., 1982; Whitnall, 1993; Aguilera, 1994, 1998; Ma et al., 1999a,b). Indeed, the finding that HR pigs react to the stress of individual housing with an increase in VP levels, and a protracted cortisol response support this suggestion.

The adaptations found in the HPA axis of IND HR gilts (increased levels of VP together with a prolonged cortisol response to stress and altered basal cortisol levels) are reminiscent of those found in patients with major depression (Purba et al., 1996; van Londen et al., 1997). Holsboer (1995) postulated that the mechanism underlying these findings in depression includes weakened glucocorticoid feedback function in the hippocampus and the hypothalamus, leading to enhanced synthesis and release of CRH and VP. Irrespective whether similar mechanisms underlie our present findings, the adaptations found in HR pigs may be interpreted as (pre-) pathological signs, indicating that individual housing is detrimental for pigs welfare.

In contrast, the LR gilts did not show any change in VP peptide levels under IND housing. Yet, the lower basal cortisol concentration observed 90 minutes before and 90 minutes after application of the nosesling in IND gilts have been suggested to be induced by chronic stress (De Jong et al, 1998). There is evidence from human studies that hypocortisolism occurs in some conditions of ongoing stress (Heim et al., 2000; Mason et al., 2002). Hippocampal mineralocorticoid receptors (MRs) are important in terms of the control of inhibitory tone over the HPA axis, whereas during stress the levels of glucocorticoids are under the negative feedback control of glucocorticoid receptor (GR) in the hippocampus. It has been shown that aged rats generally have reduced MR (and GR) expression and an increased basal HPA activity and prolonged stress-induced ACTH release. In contrast, tricyclic antidepressants increase expression of hippocampal MRs and decrease basal and stress-induced HPA activity (de Kloet et al., 1998). In addition, since GR activation also suppresses the hippocampal output, resulting in an inhibition of PVN neurons (de Kloet et al., 1998; 2000), changes in the GR receptors could also have resulted in the observed changes in cortisol under individual housing. Irrespective whether similar mechanisms underlie our present findings, the decrease in basal cortisol in IND LR pigs could also be interpreted as (pre) pathologic.

In conclusion, this study shows that HR and LR gilts react to chronic stress, induced by individual housing, with different neuro-adaptations: HR gilts

up-regulate VP in neurons of the medial PVN, whereas LR gilts do not. Given its association with altered cortisol output under basal and stress-stimulated conditions, this up-regulation may serve similar functions in the regulation of the HPA axis as are known for VP co-expressed with CRH in neurons of the PVN in rodents and other species. These results support the hypothesis based on behavioural studies in these very same animals that effects of coping strategy only become evident under extreme housing conditions, such as individual housing. In addition, the alterations in the HPA axis that we found induced by individual housing in pigs resemble those reported for human psychopathologies. Our present data therefore underscore the notion that individual housing is detrimental for the welfare of pigs.

Acknowledgements

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Chapter 5

Corticotropin-releasing hormone mRNA expression in the paraventricular nucleus in pigs is affected by individual housing depending on coping strategy

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Abstract

The hypothalamus-pituitary-adrenal (HPA) system of female pigs (gilts) with different coping strategies shows diverging stress-responses, particularly under conditions of chronic stress. High-resisting (HR), but not low-resisting (LR) pigs, show an increase in vasopressin (VP), but not corticotropin-releasing hormone (CRH) levels in the paraventricular nucleus (PVN) under individual housing (i.e. chronic stress) compared to group housing. VP is thought to act synergistically with CRH and may play an important role in sustaining pituitary responsiveness during chronic stress. Moreover, individual housed gilts showed decreased basal cortisol levels, independent of coping strategy, suggesting that changes at the level of the PVN may have occurred also in LR pigs. Therefore, the objective of the present study was to investigate the effects of individual confined housing (i.e. chronic stress) on the expression of CRH mRNA in the PVN of young adult female pigs (gilts) displaying different coping strategies (HR or LR) using a digoxigen (DIG)- labeled probe for in situ hybridization.

HR and LR piglets were group housed until 7 months of age. Thereafter, half of the animals were housed individually (IND), while the other half remained group-housed (GRO). At 14 months of age, gilts were sacrificed, brains collected and processed for in situ hybridization to determine CRH mRNA content in the PVN (area in μm^2) using computer assisted image analysis (SCION).

LR, but not HR, gilts showed a significant increase in CRH mRNA expression in the PVN under individual compared to group housing. These results extend our previous observations in these very same animals that showed a differential effect of individual housing on VP peptide expression in the PVN of HR and LR pigs. Thus, LR pigs react to chronic housing stress with an up-regulation of CRH, while HR pigs react with up-regulation of VP in the PVN. These results indicate fundamental differences between HR and LR pigs in adaptational changes induced by chronic stress in the HPA axis and it likely explains previously found differences between HR and LR pigs in HPA responses. Since the adaptations in the CRH/VP system found in HR and LR pigs show similarities with those observed in human stress-related psychopathologies, they likely indicate that individual housing may exceed the adaptive capacity of pigs, and seriously compromises the welfare of the animals.

Introduction

It is generally acknowledged that behavioral, physiological and endocrinological responses to stress show considerable variance between individuals of the same species. There is evidence that the individual mode of responding to stress or 'coping strategy' is, at least in part, dependent on biological traits. In the pig, like in many other species (Koolhaas et al., 1999, review), the existence of individual coping strategies has been established (Hessing et al., 1993). An indication for the coping strategy of pigs can be obtained in the first weeks of life by measuring the degree of resistance behavior in the so-called "backtest". In this test, a piglet is restrained on its back and its reaction is scored by counting the number of escape attempts. Indeed the behavioural response in this test is, to a certain extent, predictive for the pig's responses to a variety of challenges and stressful events later in life. It has been shown, for instance, that pigs selected for high-resistance (HR) or low-resistance behavior (LR) in the backtest markedly differ in behavioral and hypothalamus-pituitary-adrenal (HPA) axis responses to novelty stress (Hessing et al., 1993, 1994), basal cortisol levels, aggressive behavior, stereotyped behavioural responses to apomorphine, and responses to immunological challenges (Hessing et al., 1995; Bolhuis et al., 2000; Ruis et al., 2001a,b; Geverink et al., 2002a,b, 2003). Apart from their acute reactions to acute stressful stimuli, HR and LR pigs also differ in their long-term adaptations to chronic stress. Pigs are social animals, and individual housing in stalls is thought to represent a chronic stressor. Indeed, it induces adaptations that are generally recognized as typical of chronic stress (Ruis et al., 2001a,b; Geverink et al., 2002b, 2003). Recently, we have found that adaptations induced by individual housing also depend on the coping strategy of the animals, as indicated by interaction effects of housing and backtest type on behaviour and immune function (Schrama et al., 1997; Bolhuis et al., 2003).

In a previous study, we found an interaction effect of coping strategy and housing condition on vasopressin (VP) peptide expression in the paraventricular nucleus (PVN) of these animals. Individually housed HR, but not LR gilts, showed an increase in VP levels in the PVN compared to group housed gilts, while the expression of corticotropin-releasing hormone (CRH) peptide was not significantly affected. In rats, exposure to chronic intermittent stress leads to up-regulation of VP in parvocellular CRH neurons in the PVN. Since VP acts synergistically with CRH on the anterior pituitary as an ACTH secretagogue, it is thought that up-regulation of VP is an adaptive response that secures HPA responsiveness during chronic stress and plays an important role in sustaining pituitary responsiveness during chronic stress. Yet, apart from an adaptive VP response, rats adapted to a chronic homotypical stress, also show an enhanced transcriptional response of

CRH in the parvocellular PVN without increased peptide levels (Aubry et al., 1999). Moreover, even though individual housed LR gilts did not show an up-regulation of VP, they did show decreased basal cortisol levels, suggesting that changes at the level of the PVN may have occurred. Therefore, the objective of the present study was to investigate the effects of individual confined housing (i.e. chronic stress) on the expression of CRH mRNA in the PVN of young adult female pigs (gilts) displaying different coping strategies (HR or LR) using a digoxigen (DIG)- labeled probe for in situ hybridization.

Materials and Methods

Animals

Pig brains were obtained from a large experiment, which included contrasting housing conditions derived from field practice, that is individual vs. group housing of gilts. (Geverink, 2002a).

Subjects and housing have been described in detail previously (Geverink et al., 2002a). Briefly, in two identical successive experimental runs, a total of 48 nulliparous crossbred gilts (Pietrain × ((Large White Duroc) × British Landrace)) kept at the experimental farm of the Wageningen University were studied. They were selected out of a large pool of gilts bred at a commercial farm (Straathof, The Netherlands), based on their response in the backtest at 10 and 17 days of age. Briefly, each a piglet was gently removed from its pen and put on its back on a farrowing mat positioned on a table. It was restrained in this supine position for 60 sec by placing one hand over the throat and the other loosely on the hind legs. A piglet making more than two escape attempts in each test was classified as an HR whereas when less than two escape in each test were made it was classified as an LR. For each experimental run 12 LR and 12 HR gilts were selected out of the pool of tested animals. They were weaned at 4 weeks of age and brought to the experimental farm at the Wageningen University, at 10 weeks of age. They were housed in groups of 12 (6 HR and 6 LR) until 20 weeks of age when groups were split resulting in four groups of six (3 HR and 3 LR). At 7 months of age, animals from half of the groups were housed in individual stalls. Thus in each experimental run, eventually 12 gilts remained group-housed (GRO) and 12 gilts were individually housed (IND) for the remainder of the experiment.

Group housing pens (3.80 × 3.15 m) had 65% solid floor and 35% slatted floor. A metal food trough (3.80 m long) was fitted at the front of the pen. The animals had access to a nipple drinker in the dunging area and a chain was attached above the trough. Individual pens were 0.60 m wide and 2.20 m long. They were made of partitions with vertical bars and placed in one row. HR and LR gilts were

housed alternately. Each stall had a concrete floor (1.30 × 0.60 m) in the front and slatted floor (0.90 × 0.60 m) at the rear end. A metal trough was fitted at the front, with a nipple drinker in the middle. Above the food trough, a chain was attached that was easily accessible to the pig. For all rooms, an automatically controlled heating and ventilation system was used, set to maintain room temperature at 18°C. Furthermore, individual stalls had floor heating to compensate for heat loss through the concrete floor. In addition to natural light, artificial lights were on from 07.00 until 19.00 hr. Twice a day, at 08.30 hr and 15.00 hr, the animals were fed a commercial pelleted diet according to the Dutch standard for breeding pigs. All pigs were accustomed to the experimenters and to the procedures of saliva sampling to avoid unwanted stress reactions.

Synchronization of estrus in gilts

A synthetic progesterone (altrenogest, Regumate, Intervet, Boxmeer, The Netherlands) was used to synchronize the estrus cycle in the gilts used in the present study (Kraeling et al., 1981, Stevenson and Davis, 1982) in order to reduce the influence of estrogen on the activity of the HPA axis (Rivier, 1999; Dunčko et al., 2001; Wood et al., 2001). Gilts were fed 20 mg of altrenogest per day, sprayed on their feed for 18 consecutive days, regardless of the stage of the estrus cycle (Stevenson and Davis, 1982). Gilts were sacrificed 10 days after ending the treatment, which is known to be a large enough time interval for every gilt to return to estrus and reach the luteal phase of the cycle (Stevenson and Davis, 1982).

Tissue collection and preparation

The healthy, intact animals were sacrificed at 14 months of age. Approximately half an hour before the canulation, animals were sedated with stressnil in their home pen, and transported to the slaughter facility in a transport box. Animals were anaesthetised with ketamine and brains were perfused via the ascending aorta with 5 liters of saline, followed by 5 liters of freshly prepared 6 % phosphate buffered formaldehyde (Merck, 37 % formalin, pH 7.4) as described previously (van der Beek et al, 1992; 2003). Brains were removed from the skull, postfixed in the 6% formalin solution for 2 days at 4°C. Thereafter the hypothalamus was dissected and immersed in the same fixative for another 2 days, dehydrated through series of graded ethanol, embedded in paraffin (Histowax©, (melting point 56-58°C), Klinipath B.V., Duiven, Holland) and stored at room temperature until sectioning. Serial 6 μm sections were cut, and selected sections taken at regular intervals (90μm), were mounted on gelatin-coated slides and stored at room temperature. For staining, sections were deparafinized with xylene

(Merck), and sections were hydrated with graded series of ethanol and washed in Tris-buffered saline (TBS) for 5 minutes. Every 15th section was stained with cresyl violet (0.5% cresyl violet (BDH) in acetic buffer pH 3.8) in order to localize the PVN in the hypothalamus.

In situ hybridization

Every 30th section of the PVN (180 μm interval) was mounted on superfrost slides and stored at room temperature until further use. For staining, sections were deparafinized with xylene (Merck), and sections were hydrated with graded series of ethanol and washed in PBS for 5 minutes. Slides were placed into 10 mg/ml proteinase K dissolved in PBS at 37°C for 10 minutes and fixed in freshly prepared chilled 4 % paraformaldehyde in PBS for 5 minutes. The slides were washed with PBS three times for 2 minutes and transferred to 0.25% acetic anhydride in 0.1M triethanolamine (pH 8.0) for 10 minutes. This was followed by washing twice in PBS for 2 minutes.

Pig CRH cRNA probes were derived from 3'-noncoding cDNA fragments which were cloned in MLM573 (kindly provided by Prof. dr. P. Burbach). The pig CRH fragment is a 650 basepair (bp) EcoRI-Not I fragment. DIG-labeled antisense transcripts were generated on linearized plasmids using T3 RNA polymerase (Promega, Leiden, the Netherlands). As a control for non-specific binding, sense RNA probes were made using T7 polymerase (Promega).

Prehybridization, hybridization and washing of tissue sections

Prehybridization was performed at room temperature with 300 μl hybridization buffer (50% formamide, 5× SSC, 5× Denhardts, 250 μg/ml bakers yeast and 500 μg/ml sonificated salmon sperm DNA) per slide covered with parafilm for 2 hrs. The hybridization mixture was prepared by adding 400 ng DIG-cRNA per ml hybridization buffer, then heated for 5 min at 80°C to degrade the probe and chilled on ice. The prehybridization solution was gently removed and 150 μl of hybridization mixture per slide was spread over the sections using parafilm. The sections were covered with NESCO-film (Omnilabo) and special care was taken to avoid air bubbles. The hybridization was performed overnight at 72°C in milliQ-humidified chambers. The chambers were sealed off to prevent evaporation. Slides were washed by placing them vertically in a rack immersed in 2 × SSC at 72°C to remove the NESCO-film. Washing was performed in 0.2 × SSC at 72°C for 2 hours after which the sections were air dried for 30 seconds to adjust to room temperature before they were placed in 0.2 × SSC at room temperature for 5 min.

Slides were washed with into ISH buffer 1 (0.1M TrisHCL, 150 mM NaCL, pH 7.4) for 5 min, and then placed horizontally and incubated with ISH buffer 1 containing 10% Heat Inactivated Foetal Calf Serum (FCS) for 1 hour at room temperature. Thereafter slides were moved to a cold room and after removal of ISH buffer 1, slides were incubated over night with anti-DIG AP antibody diluted 1:5000 in buffer 1 containing 1 % FCS at 4°C.

On day three, slides were washed in ISH buffer 1 at room temperature (3 × 5 min) followed by a wash step for 5 min in ISH buffer 2 (0.1 M TrisHCL, 50mM MgCL2 and 0.1 M NaCL, pH 9.5). To visualize the reaction product, slides were placed horizontally again and incubated with 1ml freshly prepared staining solution (200 µl (2%) NBT-BCIP stock solution (Roche) and 1 ml Levamisol (2.4 mg / ml, Sigma) into 8.8 ml ISH buffer 2) for 4 hours. The color reaction was performed in the dark and stopped by transferring the slides into a rack containing T10E5 (10mM TrisHCL, 5mM EDTA, pH 8.0). Sections were mounted in a water-based mounting medium (Kaiser's gelatin), as organic solvent-based ones (DEPEX) were found to result in loss of the colored precipitate.

Image Analysis

Of each animal 3 images of the PVN at the right side of the 3rd ventricle were captured using a microscope (2.5 x 0.63 magnification) with a digital black-and-white CCD camera (Sony, XC-77CE) coupled to a PC. The three consecutive sections (180 µm apart) containing the PVN were selected using the end of the PVN and the location of the VON, and were in accordance with level 5, 7 and 9 of the peptide map reported in a previous study from our lab (Chapter 2). Some animals that did not show any staining in the levels examined or missed these levels due to tissue damage or technical problems were excluded from further analysis completely.

Thereafter, images were analyzed using an image analysis program (Scion Image Beta, version 4.02; Scion Corporation, Maryland, USA) using gray level threshold discrimination as previously described by Bethea et al. (1992). To be able to compare CRH mRNA data of multiple stainings, each section was captured and analyzed with standardized procedures. This method was checked for reproducibility by comparing one animal of which sections were included in all staining runs. CRH mRNA staining (area) in this animal indeed proved to be similar across staining runs. A background correction was made by capturing each image of the PVN at the same gray level (i.e. the average gray level of the entire image). After capturing all the images, the mean background was calculated for each staining procedure by measuring the gray level in an area that did not contain

CRH mRNA-positive cells in a representative selection of the images (10-40%). Using these background measurements the gray level threshold was determined: the mean maximal gray level in background + 3 x mean S.D. of the background was used as the threshold, which theoretically results in a $P < 0.001$ to include false positive cells in the counting procedure. Using this threshold, the image was converted in a binary image in which gray levels above threshold were colored red (i.e. the positive staining). Artifacts that were colored red were excluded by hand. Finally, the total area of CRH mRNA positive cells was determined automatically; only particles between a minimum and maximum number of pixels (3 -1000) were included in the measurement, which included all the CRH mRNA positive neurons since these were all smaller than 1000 pixels (Karman, unpublished observation). Using this method, only dark stained cells were measured, which correspond to neurons with a high concentration/density of CRH mRNA. Neurons with a low concentration of nuclear CRH mRNA from which the staining intensity did not reach threshold levels were not included in our area measurement. According to a previous study performed in our lab (Chapter 2) co-localization of VP and CRH most likely occurred in the medial region of the PVN of the pig. Therefore, only the medial region of the PVN was examined in the 3 sections analyzed. We found no significant differences in total area of CRH mRNA-positive cells between the left and right side of the brain (Karman, unpublished observation) allowing us to measure only one side of the PVN. Thereafter areas under the curve, which was defined as the sum of the measured CRH mRNA areas in the three sections, were compared between the groups.

Statistical analysis

Statistical analysis was performed with the SPSS software system, version 11.0. Total area of CRH mRNA content was compared between the groups by means of General linear model (GLM) univariate with factors coping strategy, housing condition, and one-way ANOVA. The Bonferroni test of signifcance was used for post-hoc analysis post-hoc analysis. Correlations between CRH mRNA and peptide were analyzed with a two-tailed Pearson correlation. For this correlation, area measurements from sections, immuncytochemically stained for CRH peptide in a previous study at the corresponding levels evaluated for CRHmRNA were used (Chapter 4). The used CRH peptide sections preceded the one selected for in situ hybridization. A $p<0.05$ was considered significant.

Results

In situ hybridization

In the present study the effect of chronic stress (i.e. of individual housing) on CRH mRNA expression in pigs with different coping strategies was investigated, using a digoxigen- labeled cRNA probe. In situ hybridization resulted in clear cytoplasmatic staining of CRH-ergic neurons located in the medial region of the PVN as described for CRH peptide in a previous study from our lab (Chapter 2 and 4).

CRH mRNA expression in the PVN

Expression of CRH mRNA was mainly seen in neurons in the medial region of the PVN of the pigs (Figure 1), which is in accordance with data from our previous study in which CRH peptide localization was determined in these same animals (Chapter 4). Sense cRNA probes showed no detectable hybridization signal.

In the present study, CRH mRNA expression in each animal was examined in 3 consecutive sections in accordance with level 5, 7 and 9 of the PVN (Chapter 2) and areas under the curve, which were defined as the sum of the measured CRH mRNA areas in the three sections, were compared between the groups.

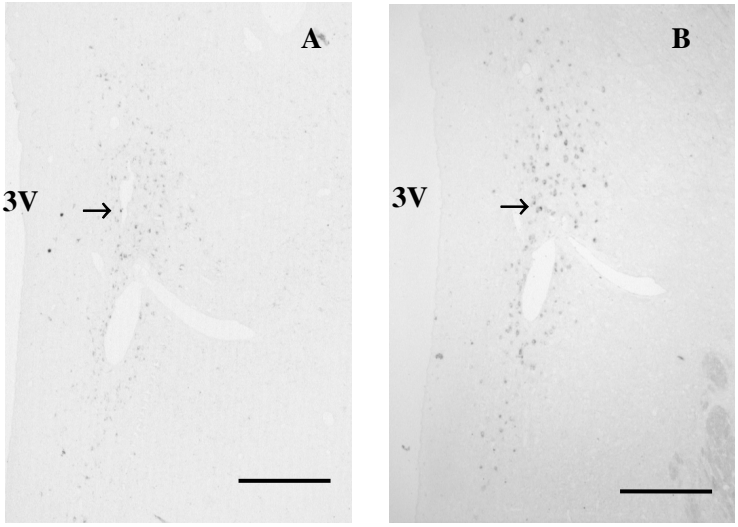

Figure 1. Distribution of neurons containing CRH peptide (**A**), and CRH mRNA (**B**) in level 5 of a representative animal. 3V, third ventricle. Arrowheads indicate the localization of neurons positive for CRH peptide or mRNA. Bar = 500 micrometer.

Our data showed a clear interaction of housing condition × coping strategy on the total area positive for CRH mRNA in the medial region of the PVN ($F(4.862)=3.84$; $p< 0.05$); individually housed LR gilts showed a significantly higher expression of CRH mRNA in the medial region of the PVN compared to all other groups (Figure 2A). In Figure 2B the total area positive for CRH peptide in the medial region of the PVN of the same levels measured in adjacent sections is shown. No differences between the groups were observed.

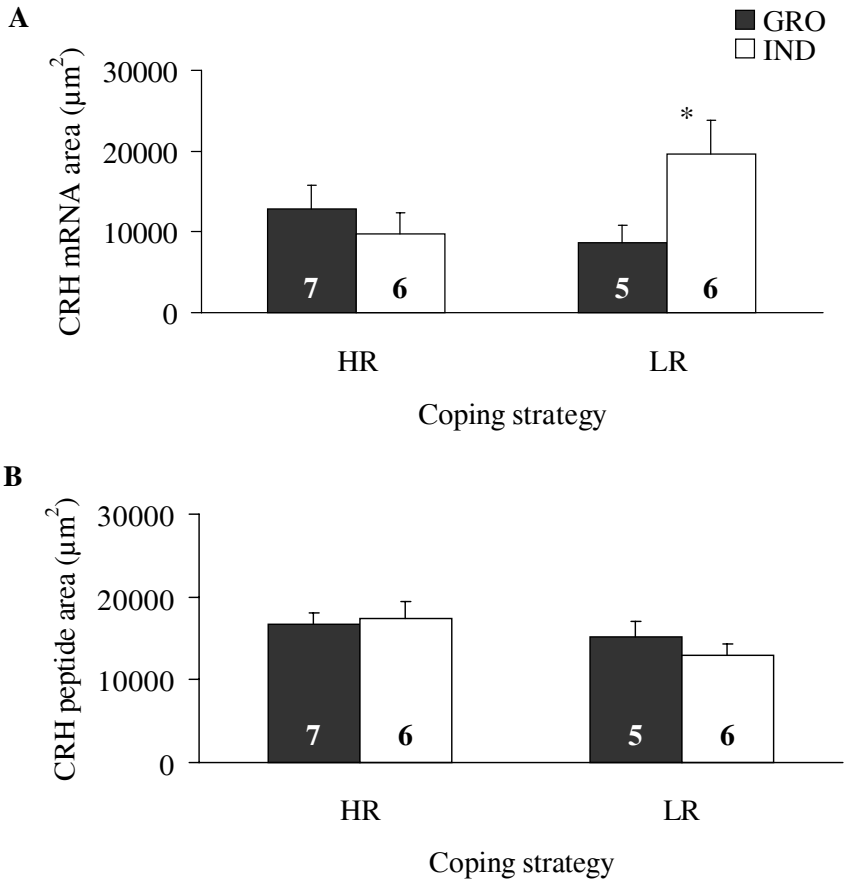

Figure 2. Area under the curve of CRH mRNA (**A**) and CRH peptide (**B**) in adjacent sections measured in the medial region of the 3 levels of the PVN (level 5,7,and 9) in 14-month-old female pigs displaying different coping strategies (HR or LR). For the last 5 months they were either housed in groups (GRO) or individual (IND). A significant interaction of coping strategy × housing condition was found in CRH mRNA expression ($p<0.05$). CRH peptide levels were comparable between groups.
Bars represent mean + SEM.

We also investigated whether a correlation existed between the CRH peptide and mRNA content in the selected sections of the PVN in these animals. Indeed we found a significant positive correlation between peptide content and mRNA content in both IND HR and LR gilts (r= 0.58, p= 0.02; r=0.68, p=0.005 resp.). In GRO HR gilts the positive correlation just escaped significance (r=0.56, p=0.06) (Figure 3).

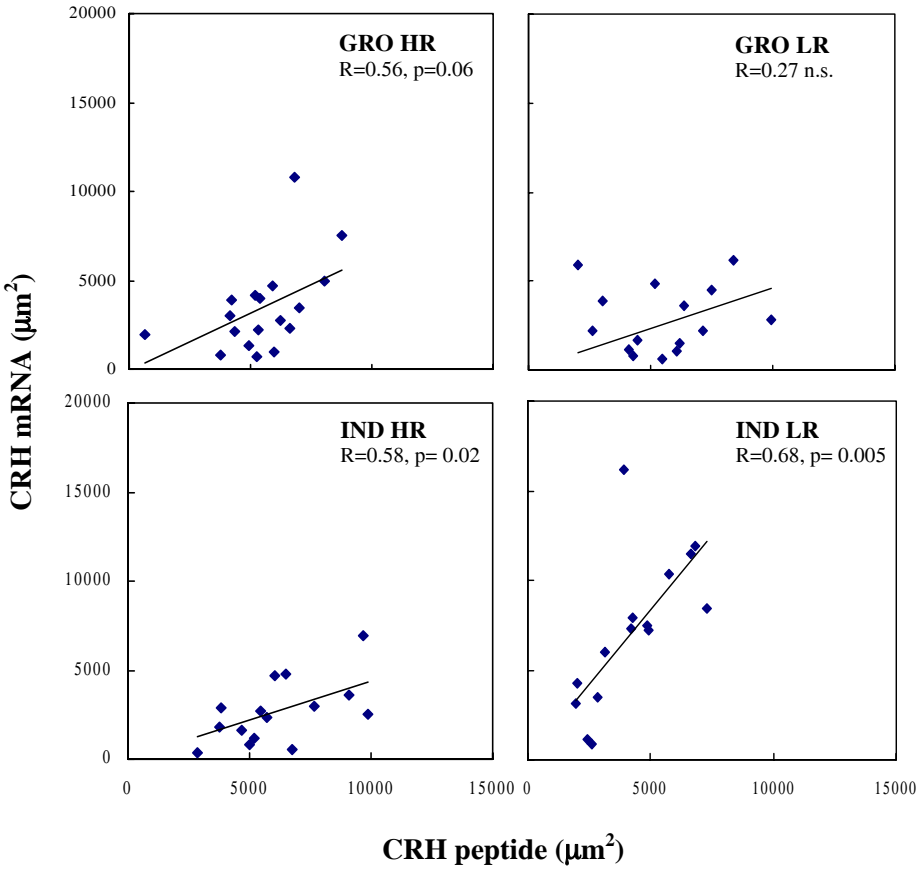

Figure 3. Correlation of CRH peptide area with CRH mRNA area in the 3 levels of the PVN in of female pigs housed under different housing conditions (group (GRO) and Individual (IND)) and displaying different coping strategies (HR or LR).

Discussion

In the present study we investigated CRH mRNA in the PVN of pigs by means of in situ hybridization using a digoxigen (DIG)- labeled cRNA probe. Indeed we found CRH mRNA positive cells in the medial region of the PVN. This localization corresponded with that described for CRH peptide in our previous study that was performed on adjacent sections of the PVN of the very same animals (this Chapter and Chapter 4). We found a clear interaction between the backtest qualification and housing condition for CRH mRNA expression. Thus, the CRH mRNA response induced by individual housing appears to depend on the coping strategy of the animal, with LR, but not HR animals showing an increase in CRH mRNA levels. In addition, we found positive correlations between CRH mRNA and peptide after individual housing, but not in group housed HR and LR gilts, suggesting an altered HPA axis activity in individual housing. These results extend our previous observations on the expression of VP in the PVN of these very same animals. Thus, LR pigs react to chronic housing stress with an up-regulation of CRH, while HR pigs react with up-regulation of VP in the PVN. It has been suggested that the functional state of the CRH and VP systems in the PVN may be used as neurobiological index of cumulative stress and stress vulnerability. Consequently, the results underscore the notion that individual housing is detrimental for the welfare of pigs, independent of coping strategy

To our knowledge this is the first time that changes in CRH mRNA levels following experimental manipulation in the pig were studied using in situ hybridization with a DIG-labeled cRNA probe. The present findings on the distribution of CRH mRNA in the pig PVN are distinctly different from previous observations made by others using in situ hybridization (Vellucci and Parrott, 1998). In that study, neurons expressing CRH mRNA were found in young restraint challenged pigs in the region of the PVN bounded medially by the 3rd ventricle and laterally by the magnocellular area (pars parvocellularis). In contrast, we found the distribution of CRH mRNA in the pig PVN to be restricted to the medial region of the PVN and not bordering the 3rd ventricle directly, and resembling that of CRH peptide. The difference in methods used to identify the CRH neurons might account for this discrepancy. It is well established that in situ hybridization is a sensitive technique for the detection of peptidergic cell bodies (Hallbeck 1999a; Simmons et al., 1989; Hermanson et al., 1995), particularly when long cRNA probes are used. However, Vellucci and Parrott (1998) used a short oligonucleotide probe complementary to the rat CRH gene, which may have hybridized less specifically with porcine CRH then expected and may have hybridized to other peptides, resulting in a less restricted distribution of neurons thought to be positive for CRH mRNA. In situ hybridization with a longer porcine

DIG-labeled cRNA probe, as used in the present study, apparently allows detection of CRH mRNA containing neurons with a higher sensitivity, yielding a distribution of CRH mRNA that was similar to that found for CRH peptide in the PVN of the pig (this Chapter and Chapter 4). In addition, the distribution of CRH mRNA found in the present study for the pig PVN is comparable to that reported for the rat, sheep, hamster, goat and hedgehog (Papadopoulos et al., 1985; Delville et al., 1992; Aguilera, 1998; Ceccatelli et al., 1989, Kikusui et al., 1997) making it more likely, indeed, that CRH neurons are restricted to the medial region of the PVN in the pig too.

In the present study, LR gilts responded to the chronic stress of individual housing with higher levels of CRH mRNA, while CRH mRNA levels in HR gilts were unaffected by housing condition. In contrast, CRH peptide levels in the PVN of these very same animals were comparable between the experimental groups. The unchanged CRH peptide content may suggest accelerated transport of CRH peptide to the median eminence. It cannot be excluded that the acute stress of transport before slaughter contributed to the increase of CRH mRNA. Indeed, it has been shown that mRNA changes in response to stress can actually be detected within minutes (Herman, 1995). It should be noted, however, that the increase in CRH mRNA occurred only in LR and not in HR pigs, although the latter were subjected to exactly the same procedure of transport and slaughter. Yet, there are also other possibilities that may have led to the findings at CRH mRNA and peptide level in the present study. For example, CRH mRNA is increased due to enhanced transcription and less breakdown, whereas the translation of CRH peptide is not enhanced. Alternatively, the translation of CRH peptide is increased, yet, simultaneous changes in, breakdown, maturation, transport and/or release, however, may have resulted in the unchanged peptide levels. Interestingly, we found a positive correlation between CRH mRNA and peptide in individually housed gilts, and also in group-housed HR gilts, albeit not significantly. This correlation suggests that the measured CRH mRNA has indeed been translated into peptide, indicating activation of the CRH system in these animals by their housing condition. Therefore, the correlation between CRH mRNA and peptide might be a general indication of an adapted HPA axis, suggesting that individual housing induced changes independent of coping strategy.

The observed increase in CRH mRNA in IND LR animals was not associated with an increase in the cortisol response to an acute stressor (nose sling) compared to GRO LR animals, basal cortisol levels, however, were lower in IND LR compared to GRO LR gilts (Chapter 4). This could be due to a desensitization of the CRH receptors in the pituitary in these animals (Aguilera, 1998). In addition, the adaptation found in the HPA axis of IND LR gilts (increased CRH mRNA) is

reminiscent of those found in patients with major depression (increased CRH in CSF, Nemeroff, 1984), suggesting that the increased CRH mRNA in IND LR pigs may be interpreted as a (pre) pathological sign. Depression is most often associated with an increased cortisol level, however, there are also studies in which cortisol levels in depressed patient do not change, probably because of the mild to moderate character of the depression (Heim et al., 2000; Mason et al., 2002). Holsboer (1995) postulated that the mechanism underlying these findings in depression includes weakened glucocorticoid feedback function in the hippocampus and the hypothalamus, leading to enhanced synthesis and release of CRH and VP. Irrespective whether similar mechanisms underlie our present findings, the adaptation found in LR pigs may be interpreted as (pre-) pathological signs, indicating that individual housing is detrimental for pigs welfare.

These results extend our previous observations on the expression of VP in the PVN of these very same animals. In that study, the opposite was found for VP peptide levels in the PVN. HR gilts responded to individual housing with an increase in VP levels while LR gilts did not show any change (Chapter 4). VP acts synergistically with CRH as an ACTH secretagogue, and plays an important role in sustaining the pituitary responsiveness during chronic stress when the response to CRH desensitizes (Antoni, 1993; Scaccianoce et al., 1991; Dallman, 1992; Aguilera, 1994; 1998; Aguilera and Rabadan-Diehl, 2000). It could be that the up-regulation of VP seen in IND HR gilts serves to secure reactivity of the HPA axis to acute stress during a chronic stress situation, as is also seen in rats (De Goeij et al., 1992b; Makino et al., 1995; Ma and Lightman, 1998) Yet, the cortisol response to a nose sling procedure in IND HR animals took longer to return to basal levels compared to IND LR animals (Chapter 4). The adaptations found in the HPA axis of IND HR gilts (increased levels of VP together with a prolonged cortisol response to stress and altered basal cortisol) are reminiscent of those found in patients with major depression (Purba et al, 1996; van Londen et al., 1997) and it was suggested that these adaptations found in HR pigs may also be interpreted as (pre-) pathological signs, indicating that individual housing is detrimental for pigs welfare.

In two rat lines selected for divergent sensitivity for apomorphine-induced gnawing, the apomorphine susceptible (apo-sus) and apomorphine unsusceptible (apo-unsus) rats, it has been established that differences in coping strategy are associated with the stress-response pattern of the HPA axis (Cools et al., 1990). Apo-sus rats are marked by a higher level of CRH gene expression, which resulted in higher ACTH but lower total corticosterone plasma levels under basal conditions compared to apomorphine-unsusceptible (apo-unsus) rats (Rots et al., 1995). When stressed, the c-fos activity in CRH neurons in apo-sus rats was reduced whereas the

ACTH release and corticosterone release was increased and prolonged compared to apo-unsus rats (Mulders et al., 1995). It was suggested that the prolonged elevated activity of the HPA axis of apo-sus rats might be the result of a weaker negative feedback of glucocorticoids on the ACTH release at the level of the pituitary (Rots et al., 1995). In addition, it has been observed that in adult apo-sus rats hippocampal MR levels are increased under basal conditions compared to apo-unsus rats, suggesting a potential difference in glucocorticoid function between apo-sus and apo-unsus rats (Rots et al., 1996).

Apomorphine, used to select for gnawing score in the rat, induces stereotyped behaviour in several other species as well, including the pig. The behavioural effects of different doses of apomorphine have been studied systematically in pigs (Terlouw et al., 1992), and these, like rats, also vary in their response to the drug (Fry et al., 1981; Terlouw et al., 1992). It has now been established that the response to apomorphine in pigs is linked to the individual coping strategy as characterized by the response in the backtest (Bolhuis et al., 2000). HR pigs have a higher susceptibility for apomorphine-induced stereotypies than LR pigs, as indicated by higher levels of stereotyped snout contact with the floor following apomorphine treatment. The lack of an increase in CRH mRNA in the PVN of HR gilts under conditions of chronic stress is similar to the hypothalamic stress response observed in apo-sus rats. Also, apo-sus and HR gilts show similarities in their cortisol response to stress (a prolonged stress induced increase in cortisol levels) suggesting that HR gilts are comparable with apo-sus rats. This would support the notion that also in HR and LR pigs, differences could be found in the glucocorticoid negative glucocorticoid feedback system and MR/GR expression.

In conclusion, this study shows that one-year-old HR and LR gilts differ in their CRH mRNA level in the PVN under conditions of chronic stress caused by individual housing. These results extend our previous observations on the expression of VP in the PVN of these very same animals. Thus, LR pigs react to chronic housing stress with an up-regulation of CRH, while HR pigs react with up-regulation of VP in the PVN. This divergence of adaptations in the CRH/VP system of the PVN indicates a fundamental difference in the make up in the brain circuitry that governs HPA function. It likely explains previously found differences between HR and LR pigs in HPA responses, and given the pivotal role of the HPA axis and its (neuro) hormones in behavioral and physiological adaptation in general, it may also be pertinent to other responses that have been found to differ between HR and LR pigs. Since the adaptations in the CRH/VP system found in HR and LR pigs show similarities with those observed in human stress-related

psychopathologies, they likely indicate that individual housing may exceed the adaptive capacity of pigs, and seriously compromises the welfare of the animals.

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Chapter 6

Is vasopressin co-localized in corticotropin-releasing hormone containing neurons of the porcine paraventricular nucleus under stress?

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Abstract

In the rat and other species, stress is known to induce an increase in the expression of vasopressin (VP) in parvocellular corticotropin-releasing hormone (CRH) neurons of the paraventricular nucleus of the hypothalamus (PVN). In previous studies in pigs we found that individual housing, a chronic stress situation for pigs, resulted in increased levels of VP peptide and CRH mRNA in the medial PVN, a region thought to represent the 'parvocellular region' of the porcine PVN. It remains to be established, however, whether or not the observed increase in VP represents co-expression of VP in CRH neurons.

In the present study, we investigated whether co-localization of CRH and VP in the medial region of the pig PVN occurs and whether it is a stress-induced phenomenon. To that end, we used an immunocytochemical approach with double staining for CRH and VP, and compared staining in the PVN of individually housed (IND) and group housed (GRO) pigs.

The results revealed co-localization of CRH and VP in neurons in the pig PVN. The number of neurons expressing CRH alone and the number of neurons co-localizing CRH and VP were increased in IND compared with GRO pigs, although this difference was not statistically significant. The percentage of CRH positive neurons co-localizing VP was comparable between GRO and IND housed gilts. Neurons with intense staining for both CRH and VP, however, were found only in IND and not in GRO gilts. These results indicate that, like in other species, prolonged exposure to stress may lead to up-regulation of VP gene expression in CRH neurons in the PVN of the pig.

Introduction

Under stress conditions in the rat, co-expression of VP has been found in a subset of CRH neurons (Whitnall et al., 1988). VP is only a weak ACTH secretagogue on its own, but acts synergistically with CRH and is thought to play an important role in sustaining pituitary responsiveness during chronic stress (Antoni, 1993; Gillies, 1982; Aguilera, 1994, 1998). Moreover, co-localization of VP in CRH containing neurons has been observed following adrenalectomy in the rat (Whitnall, 1988), after chronic intermittent stress and chronic psychological stress (De Goeij et al., 1992a,b). In humans co-expression of VP and CRH is found in subjects who suffered from stress-related psychopathology, indicating that VP is up regulated in the parvocellular PVN (Raadsheer et al., 1994; Purba et al., 1996). Thus, VP co-localization in CRH neurons is likely to be a sign of hyperactivity of these neurons, and may be used as a neurobiological parameter for cumulative stress.

In a previous study in which we investigated the distribution of CRH and VP containing neurons in the pig PVN we found an overlap between the localization of CRH and VP immunoreactive neurons in the medial region of the PVN (Chapter 2). Furthermore, the studies described in chapter 4 and 5, showed that individual housing, which is a chronic stress situation for pigs, resulted in up-regulation of VP or CRH depending on the coping strategy of the animals. Pigs classified as high responders (HR) in the backtest reacted with an increase in VP peptide, whereas low responding (LR) pigs showed an increase in CRH mRNA following individual housing. Yet, in these studies we could not determine whether the change in VP observed in HR pigs indeed reflects an increase in co-expression of the peptide in CRH neurons. In the present study we sought to answer this question.

Therefore the aims of this study were to investigate 1) if co-localization of VP in CRH containing neurons occurs in the PVN of the pig, and if so, 2) whether this is a stress-induced phenomenon as has been reported for the rat.

To this end we used immunocytochemical double staining for VP and CRH on paraffin sections containing the midrostrocaudal PVN, in which we investigated the medial region of the PVN, the region where CRH neurons are most abundant and thought to represent the parvocellular equivalent in the pig (Chapter 2). Selected sections from group housed and individual housed HR pigs were compared to establish the effect of housing stress on co-localization.

Materials and Methods

Animals

Pig brains were obtained from a large experiment, which included contrasting housing conditions derived from field practice, i.e. individual housing vs. housing in groups. (Geverink, 2002a). The same brains were also used in chapter 4 and 5.

Subjects and housing conditions have been described in detail previously (Geverink et al., 2002a). Briefly, in two identical successive experimental runs, a total of 48 nulliparous crossbred gilts (Pietrain × ((Large White Duroc) × British Landrace)) kept at the experimental farm of the Wageningen University were studied. These animals were selected out of a large pool of gilts bred at a commercial farm (Straathof, The Netherlands), based on their response in the backtest at 10 and 17 days of age. Briefly, each piglet was restrained on its back for 1 min and its reaction is scored by counting the number of escape attempts. A piglet making more than two escape attempts in each test was classified as an high resister (HR) whereas when less than two escape attempts in each test were made it was classified as an low resister (LR). They were housed in groups of 12 (6 HR

and 6 LR) until 20 weeks of age when groups were split resulting in four groups of six (3 HR and 3 LR). At 7 months of age, half of the animals were housed individual. Thus in each experimental run, eventually 12 gilts remained group-housed and 12 gilts were individually housed (For more details see Geverink et al., 2002a). For the present study only HR animals have been used.

Group housing pens (3.80 × 3.15 m) had 65% solid floor and 35% slatted floor. Individual pens were 0.60 m wide and 2.20 m long. They were made of partitions with vertical bars and placed in one row. HR and LR gilts were housed alternately. Each pen had a concrete floor (1.30 × 0.60 m) in the front and slatted floor (0.90 × 0.60 m) at the rear end. For all rooms, an automatically controlled heating and ventilation system was used, set to maintain room temperature at 18°C. Furthermore, individual pens had floor heating to compensate for heat loss through the concrete floor. In addition to natural light, artificial lights were on from 07.00 until 19.00 hr. Twice a day, at 08.30 h and 15.00 h, the animals were fed a commercial pelleted diet according to the Dutch standard for breeding pigs (For more detail on housing conditions see Geverink et al., 2002a).

Tissue preparation

The healthy, intact animals were sacrificed at 14 months of age. Approximately half an hour before the canulation, animals were sedated with stressnil in their home pen, and transported to the slaughter facility in a transport box. Animals were anaesthetised with ketamine and brains were perfused via the ascending aorta with 5 liters of saline, followed by 5 liters of freshly prepared 6 % phosphate buffered formalin (Sigma, 37 % formalin). Brains were removed from the skull, postfixed in the 6% formalin solution for 2 days at 4°C. Thereafter the hypothalamus was dissected and immersed for another 2 days, dehydrated through series of graded ethanol, embedded in paraffin (Histowax©, (melting point 56-58°C), Klinipath B.V., Duiven, Holland) and stored at room temperature until sectioning. Serial 6 µm sections were cut, and selected sections taken at regular intervals, were mounted on gelatin-coated slides and stored at room temperature. For staining, sections were deparafinized with xylene (Merck), and hydrated with graded series of ethanol, followed by washing in Tris-buffered saline (TBS) for 5 minutes. Every 15th (90 µm interval) section was stained with cresyl violet (0.5% cresyl violet (BDH) in acetic buffer pH 3.8) in order to localize the PVN before selecting sections of the hypothalamus for immunocytochemical staining.

Co-localization of VP and CRH

Immunocytochemical double staining for CRH and VP was performed on a fourth series of sections taken at 180 μm intervals throughout the region in which the PVN was present in order to demonstrate co-localization of CRH and VP. The staining was a modification of the immunocytochemical double staining method of Claassen et al. (1986) and Raadsheer et al. (1995), resulting in blue CRH cells, red VP cells and purple CRH and VP containing neurons. CRH was stained blue by alkaline phosphatase (AP), and VP was stained red using AEC chromogen coupled to the avidin-biotin complex.

The monoclonal rat anti-CRH (PFU 83, IgG2a subclass) was used that was directed to the C-terminal part (amino acids 38-39) of rat/human CRH (generous gift from Prof. dr. F.J.H. Tilders, Amsterdam, for more details see: Van Oers et al., 1989), Rabbit antiserum "W3" (RMI, Utrecht) was used for immunostaining of VP. Protein A (Sigma, dilution 1:100) was added to the diluted antiserum mixture to prevent nonspecific, Fc-mediated staining of rabbit Ab's normally present in the porcine hypothalamus, according to procedures described previously by Van der Beek et al. (1992) and Karman et al. (Chapter 2). After deparaffination and hydration, slides were placed in plastic containers containing 200 ml 0.01M sodium citrate buffer, which had been preheated to 100°C in a microwave oven (700 watt). Containers with slides were placed in the microwave oven and fluid was kept boiling for 10 min, this in order to retrieve masked antigens in the tissue. After heating, the containers were removed from the oven and allowed to cool down for 15 min (Lucassen et al., 1995). Slides were rinsed in cold TBS and rehydrated to alcohol 100%. Thereafter endogenous peroxidase activity was blocked with 0.5% H_2O_2 in methanol for 20 minutes, dehydrated and washed in TBS for 5 min. Sections remained in TBS until incubation. Incubations with combined PFU 83 (final concentration 1:80.000) and W3 (final concentration1:4000) together with prot A (final concentration 1:100) in staining buffer (0.05 M Tris (Sigma), 0.5 M NaCl (Sigma), pH 7.4, containing 0.5%Triton-X-100 (BDH), and 0.25% gelatine (Merck)), was performed at room temperature (26°C) overnight in moist boxes with a lid to prevent evaporation.

The following day, the sections were washed in TBS (3 × 5 min) and incubated for 90 minutes at room temperature (26°C) with a biotinylated goat-anti-rabbit IgG (H+L) serum (Vector) diluted 1:300 in staining buffer. Thereafter, sections were rinsed in TBS (3 × 5 min) and incubated for 2 hours with both AP-labeled goat-anti-rat IgG (H+L) serum (K.P.L., Maryland, USA) together with a rabbit ABC-complex (Vector), diluted 1: 50 and 1: 600, respectively in staining buffer. The sections were subsequently washed in TBS. The AP activity was

demonstrated by incubating exactly 15 min at 37°C with filtered suspension prepared by dissolving 1 mg Fast Bleu BB base (Sigma) into 50 ml 2 N HCL and 50 ml 4% NaNO which was added to 8 ml Tris-HCL (pH8.5) containing 1 mg Naphtol-AS-AX phosphate (Sigma), 50 ml N,N-dimethylformamide and 5 mg levamisole (Sigma). The sections were subsequently washed, in TBS (3 × 5 min) and incubated (10 min, RT) in filtered AEC-substrate prepared by dissolving 4 mg of 3-amino-9ethylcarbazole (Sigma) in 250 ml N,N-dimethylformamide, which was added to 9.75 ml 50mM Na-acetate (pH 5.0) and activated by 50 ml 30% H_2O_2. Finally the sections were washed and coverslipped with Kaisers Glycerin-gelatin.

Analysis

In 3 immunocytochemically double stained sections of the PVN taken at 180 μm intervals, corresponding to level 5,7 and 9 as reported previously (Chapter 2), the number of CRH and both CRH and VP containing cells (blue, blue/red, purple) were counted manually using a Zeiss microscope with 40 x objective and 12.5 x ocular lenses.

Statistical analysis

The total number of CRH cells, and the number of cells containing both CRH and VP were normally distributed and analyzed by a two tailed t-test.

Results

CRH or VP peptide expression in PVN

Expression of CRH was mainly seen in neurons in the medial region of the PVN of the pigs, which is in accordance with a previous study in which CRH immunocytochemistry was performed on these very same animals (Chapter 4). The majority of neurons that only stained for VP in all subjects were present in the lateral region of PVN, which is also in accordance to the study mentioned previously, in which also VP immunocytochemistry was performed. In addition, neurons that only stained for VP were also found in the medial region.

Co-localization of VP and CRH

Neurons in the PVN reacting with anti-VP alone, anti-CRH alone, or with anti-CRH and anti-VP were stained red, blue or purple respectively (Figure 1). The reaction products were distributed over the entire cytoplasm of the stained cell. The red VP neurons were brightly stained, but the majority of blue CRH neurons were faintly stained, as were the double-labeled purple VP and CRH neurons. In

Animal	Condition	Very faint blue cells	Faint Blue cells	Blue cells	Blue cells with very faint red	Blue cells with faint red	Purple cells
72	GRO	+++	++	-	++	+	-
76	GRO	++	-	-	+	-	-
108	GRO	++	-	-	+	-	-
84	GRO	++	-	-	++	-	-
74	IND	++	++	-	++	-	±
78	IND	+++	++	-	++	++	++
87	IND	+++	-	++	±	-	++
106	IND	+++	±	-	±	-	±
110	IND	++	-	-	±	-	-

Table 1. Immunocytochemical double staining for CRH and VP.
Cell profiles in 3 sections.

- none	+ 6-9	+++ 50-99
± 1-5	++ 10-49	++++ ≥100

Very faint, faint, blue cells only contained CRH. Bleu cells with very faint red, faint and purple cells contained both CRH and VP.
Notice that only individual housed gilts had purple cells (p=0.04).

addition, we observed faintly blue stained CRH neurons that also contained faintly red "vesicle like structures" (blue + red, Figure 1). These neurons were also considered to show co-localization of VP and CRH (see Table 1).

The majority of CRH neurons in all subjects showed very faint blue staining (only CRH). Neuronal co-localization of CRH and VP in the PVN was found in every animal (Table 1). Most co-localization was seen in neurons stained faintly blue with very faint red "vesicle like structures" inside. Only IND housed gilts showed purple stained neurons co-localizing CRH and VP, and the number of these cells differed significantly between IND and GRO housed animals (p=0.04). Both the total number of CRH neurons (including blue, blue + red as well as purple neurons, see Figure 2), and the total number of neurons co-localizing CRH and VP (including both the blue + red as well as purple neurons, see Figure 2) were higher in IND than in GRO housed gilts, albeit not significantly. The percentage of CRH cells co-localizing VP (including both the blue + red as well as purple neurons, see Figure 2) amounted 21% and 18 % respectively for GRO and IND gilts, and did not differ significantly between housing conditions.

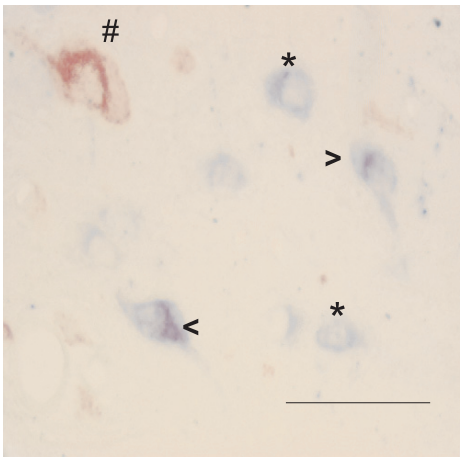

Figure 1. Representative picture of immunocytochemical double staining on a PVN section (level 7). Photo taken in medial region of PVN. CRH cells stained blue (*), VP cells stained red (#) and CRH and VP containing cells stained purple (>). Bar = 50 micrometer.

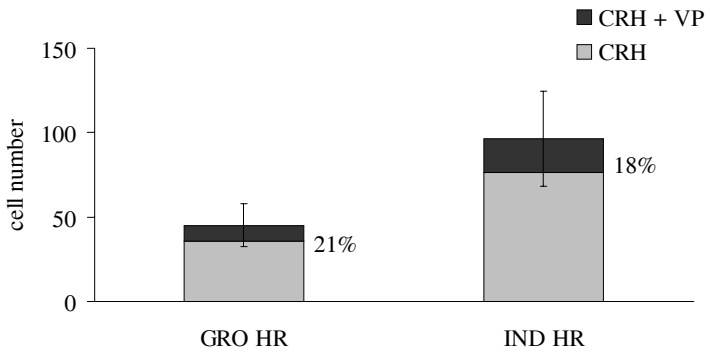

Figure 2. Mean number of CRH cells (very faint, faint and blue cells) and CRH cells co-localizing VP (blue cells with very faint and faint red as well as purple cells) in level 5, 7 and 9 of the PVN. The increase in CRH cells as well as CRH cells co-localizing VP in individual housed (IND) versus group housed (GRO) gilts was not statistically significant. The percentage of CRH cells co-localizing VP did not differ between the groups. n=4-5. Bars represents mean ± SEM.

Discussion

In the present study we showed, for the first time, the presence of neurons that co-localize CRH and VP in the PVN of the pig. We also found a significant increase in the number of neurons showing intense double staining for CRH and VP (purple cells) in gilts subjected to the chronic stress of individual housing as compared with GRO animals. In addition, the total number of CRH neurons and neurons containing both CRH and VP was also increased in IND compared to GRO housed gilts, albeit not significantly. Together, these results indicate that CRH neurons in the PVN of the pig can up-regulate the expression of VP in response to stress, as has been reported for other species.

In the rat, colchicine administration or adrenalectomy is required to visualize CRH peptide and co-localization of VP and CRH with immunocytochemical techniques (Sawchenko et al., 1984, Whitnall 1988, 1990). Our present data show that in pigs, like in humans (Raadsheer et al., 1995), such treatment is not needed to visualize the CRH and co-localization of CRH and VP in neurons in the medial region of the PVN. We found immunocytochemically stained CRH neurons in all pigs investigated, and the localization and distribution of these CRH neurons were in accordance with our previous findings obtained with a more sensitive immunocytochemical staining and detection method; the ABC - DAB-method in these very same animals (Chapter 4). The CRH neurons visualized in the present study appeared to be less intensely stained than the ones observed in our previous study, and this may indeed be due to the less sensitive AP detection method used (Ohtani et al., 1987; Raadsheer et al., 1995). Yet, we found neurons stained for both VP and CRH in all pigs. Although the double staining observed was faint in most cases, i.e. blue CRH neurons with faint red VP-containing vesicle like structures inside, IND gilts showed a considerable number of neurons with purple staining, clearly evidencing neuronal co-localization of CRH and VP in the pig PVN. The fact that VP co-localization in CRH neurons was observed as vesicle like structures, may suggest that CRH and VP in the parvocellular PVN of pigs are not packed and thus not secreted in the same vesicles, which may indicate that CRH and VP secretion in the pig might be under different regulation.

In the rat there are two types of parvocellular CRH neurons in the PVN: VP-containing and VP-deficient neurons (Whitnall, 1988). The VP containing subset of CRH neurons is selectively activated by stress (De Goeij et al., 1992a,b). Also in humans it has been demonstrated that stress can induce VP expression in CRH neurons. Purba et al. (1996) showed an increase in VP expressing neurons in the parvocellular part of the PVN, however, no subsets of CRH neurons selectively activated by stress were reported.

Thus the question arises if in the pig also two types of CRH neurons exist. In the rat, the two major subpopulations can not only be discriminated based on the expression of VP, but also based on topographic distribution within the PVN (Whitnall and Gainer, 1988). Such a distribution difference between CRH and CRH with VP neurons could not be detected in the pig PVN. The neurons containing both CRH with VP appeared to be scattered from dorsal to ventral in the medial region of the PVN in each level observed. Thus, it appears that the parvocellular CRH/VP neurons in the pig are not distributed in clusters at all. This finding suggests that functionally distinct subpopulations of CRH neurons may not exist in the pig PVN, although the absence of clustered distribution evidently does not exclude the existence of such subpopulations.

Individual housing increased the number of CRH neurons and CRH with VP neurons albeit not significantly. Yet, IND housed gilts showed more intense double staining (purple neurons) compared to GRO gilts, which did not show any purple neurons whatsoever. Since individual housing is considered a stressful situation, the more intensive double staining could be interpreted as a sign of hyperactivity of these CRH neurons in the IND gilts. From this we can conclude that CRH neurons react in the same way as seen in rat. The CRH containing neurons in the pig are also capable to co-express VP under conditions of chronic stress. Moreover, the number of CRH neurons was increased under individual housing conditions, albeit not significantly, probably due to the low number of animals included in the study. This may suggest some activation of the CRH system after all, even though CRH mRNA expression was not higher in IND than in GRO housed HR gilts (Chapter 5). Yet, changes in mRNA are not necessarily associated with changes in peptide content and vice versa, probably due to simultaneous changes in synthesis, breakdown, maturation, transport and/or release.

In a previous study (Chapter 4) we observed that IND HR gilts always had higher CRH peptide content compared to GRO HR gilts, but this was not significantly different. The fact that we observe a clearer increase in CRH peptide in IND HR gilts in the present study may be due to differences in sensitivity of the techniques used in these studies. The AP detection method used in the present study was less sensitive, and likely detects only neurons with high CRH content.

Summarizing, we found a clear co-localization of VP and CRH in the pig PVN. In addition, the number of CRH and CRH/VP neurons were increased in IND versus GRO pigs, albeit not significantly, and only IND housed gilts showed neurons with very intense double staining for CRH and VP. Based on these data we conclude that, as has been shown in other species, CRH neurons in the PVN of the pig that likely regulate the secretion of ACTH in the pituitary can co-express VP,

and that the expression of VP in these neurons is up-regulated during chronic stress. VP expression in the medial PVN may therefore be used as a neurobiological indication of cumulative stress.

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Chapter 7

Summary and general discussion

Introduction

In response to stressors, the central nervous system of mammalian species evokes physiological responses that ultimately result in the activation of the hypothalamic- pituitary-adrenal (HPA) axis. The responses of this neuro-endocrine regulatory system are presumed to have crucial adaptive and homeostatic value during periods of stress and are critical for the survival in individuals. Yet, there is a large body of evidence that previously experienced stress can alter the responsivity of animals and humans to subsequent challenges and stressors (sensitization) (Bruijnzeel et al., 2001, Jansen et al., 2003), and increase their vulnerability to stress and stress related pathology (Munck et al., 1984).

CRH and VP signaling in the brain play a pivotal role in behavioral, autonomic and endocrine stress-responses, with CRH being more potent than VP in stimulating ACTH release from the pituitary (Gillies et al., 1982; Scaccianoce et al., 1991). It has become evident that VP may be important to maintain corticotroph responsiveness during chronic stress (Antoni, 1993; Gillies, 1982; Scaccianoce et al., 1991; Dallman, 1992; Aguilera, 1994, 1998; Aguilera and Rabadan-Diehl, 2000). In rodents, stress is known to induce the expression of VP in paraventricular CRH neurons (Kovacs and Sawchenko 1996; Ma et al., 1997; Schmidt et al., 1997), with the degree of VP expression relating to the duration and intensity of stress (Schmidt et al., 1997; Ma and Lightman, 1998). In addition, it has now become evident that not only chronic or frequent stress, but even a single stressful event can alter the CRH and VP signaling in the brain, which may result in stress-related diseases in the long-run (Van Dijken et al, 1992, 1993; Tilders et al., 1993; Aubry et al., 1999; Bruijnzeel et al., 2001). This suggests that the functional state of CRH and VP systems may be used as a neurobiological index of cumulative stress.

In previous research with standard barren and minimal enriched conditions, barren housed pigs showed changes in cortisol regulation as well as behavioral disturbances (De Jong et al., 1998, 2000). In addition, it was shown recently that, compared to socially housed pigs, isolated gilts generally develop a higher state of fearfulness, and become more responsive to environmental changes (Ruis et al., 2001a,b). Relatively little is known, however, about the neuro-endocrine mechanism involved in stress responses in the domestic pig. The knowledge about this economically important species is highly relevant for the assessment of stress and welfare of domestic mammals under current husbandry regimes.

Susceptibility for stress varies between individuals, given the considerable variation in behavioral and endocrine responsiveness to (or in anticipation of) relevant environmental changes that threaten homeostasis (stress). This variability has been shown to exist in many species, including the pig (Bohus et al., 1987,

Benus et al., 1991, Hessing et al., 1993). Research has documented the existence of basically two different ways of coping with stressful situations. 1. Active coping, resembling the fight/flight response, and 2. Passive coping, resembling the withdrawal response (Henry and Stephens, 1977). The interesting notion is that each individual appears to have a preference for one or the other coping strategy, determined by their genetic background and early life experience (Van Oormerssen et al., 1985; Bohus et al., 1987; Benus et al., 1991). Several studies have shown that the two behavioral strategies are highly correlated with different physiological and neuro-endocrine mechanisms (Von Holst, 1986; Bohus et al., 1987; Sapolsky, 1990; Schouten and Wiepkema, 1991). In the pig, an indication for the behavioral strategy can be obtained in the first two weeks after birth (Hessing et al., 1993). In a so-called backtest piglets can be classified as "high-resisting" (HR) or "low-resisting" (LR) by the number of escape attempts they display. This classification based on a behavioral stress response of the young piglet has been shown to be predictive of aspects of the mode of responding to stress (or stress coping strategy) later in life, and therefore should be taken into account when investigating the effect of stress on pigs.

Summary of findings

The main purpose of this thesis was to study to which extent individual coping strategies and rearing and housing conditions determine the susceptibility of an animal for stress sensitization. To this end we studied the activity of the CRH and VP systems in the hypothalamus of pigs with different coping strategies, reared and/or housed under conditions normally found in current pig husbandry (i.e. barren vs. enriched pens during rearing, and group vs. individual housing).

In order to study the activity of both the CRH and VP system in the PVN of pigs we used immunocytochemistry procedures to measure the amount of CRH and VP peptide expressing neurons, and in situ hybridization to measure the amount of CRH mRNA expression. Since the degree of co-localization of VP in CRH neurons in particular seems to be related to the duration and intensity of stress in the rat, we also performed an experiment to gather more information about possible co-localization of VP in CRH neurons in the pig PVN.

With respect to patterns of peptide expression in hypothalamic regions regulating hypothalamic-pituitary-adrenal axis activity, only few studies are available that describe the CRH and VP cell populations in the pig (Watkins and Choy, 1977; Weindl et al., 1984; Van Eerdenburg et al., 1992; Vellucci and Parrott, 1997, 1998), albeit not in great detail. Therefore, the aim of the experiment described in chapter 2 was to investigate these cell populations in more detail in order to be able to investigate possible changes in CRH and VP following

experimental manipulation in the pig performed in the following experiments. Our results provided us with a detailed map of the anatomical location of CRH, VP and OT throughout the porcine PVN. The general distribution pattern appeared to be comparable to that described for other species. If the same mechanism regulating ACTH secretion in the rat holds true for the pig, we would assume that part of the CRH containing neurons in medial region of the PVN are able to secrete VP as well. Although we used alternating sections in our analysis we believe that some of the immunoreactive VP found in the medial region of the PVN could be co-localized with CRH. Further analysis was consistently performed on the medial region of the PVN, since CRH neurons were most numerous in this region.

In chapter 3 we investigated the effect of barren vs. enriched group housing condition from birth onwards during rearing, individual coping strategy and sex on the expression of CRH and VP peptide by means of immunocytochemistry. This study showed that neither housing condition nor coping strategy significantly affected CRH and VP peptide expression. Sex, however, affected both the amount of VP and CRH peptide in the PVN, with male pigs showing higher VP and CRH peptide levels than female pigs. The sexual difference is likely to be the result of specific actions of gonadal steroid hormones during early development, since the males in this study were castrated on day 3 after birth. Also, the female pigs were still pubertal and not exposed to cyclic levels of estrogens. The lack of effect on CRH and VP expression in the present study suggests that the housing conditions tested, although they clearly affected some behavioral and endocrine parameters (Bolhuis et al., submitted), were not contrasting enough with respects to stress load to result in changes in the activity of the HPA axis at the hypothalamic level. This may be the result of a stress reducing effect of social support that may occur under group housing conditions.

In chapter 4 and 5 we investigated the effect of coping strategy and individual, confined housing (i.e. chronic stress) on the expression of VP and CRH peptide (Chapter 4) and the expression of CRH mRNA (Chapter 5) in the PVN in young adult virgin female pigs (gilts). Our data showed a clear effect of coping strategy in individual (IND), but not in group (GRO) housed gilts. IND housed HR gilts showed significantly higher VP levels than IND housed LR gilts. Neither housing condition nor coping strategy affected CRH peptide expression. The transcriptional data, however, revealed that LR, but not HR, gilts showed significantly higher levels of CRH mRNA expression in the PVN under IND housing condition. GRO housed animals showed comparable levels to IND HR gilts irrespective of coping strategy. Interestingly, the CRH mRNA expression showed a significant correlation with CRH peptide content area in the PVN of IND gilts only. This correlation may suggest that the measured CRH mRNA has been

translated into peptide, indicating activation of the CRH system in these animals by their housing condition. The correlation between CRH mRNA and peptide might be a general indication of an altered HPA axis, suggesting that individual housing is not an optimal housing condition for pigs, independent of coping strategy.

These data clearly show that the VP and CRH systems in the PVN are differentially affected by housing condition depending on coping strategy. Also, changes in mRNA are not always reflected by similar changes in peptide content and vice versa, probably due to simultaneous changes in synthesis, breakdown, maturation, transport and/or release. These results indicate that HR and LR gilts adapt to a chronic stress situation in a different way. HR gilts respond with an increased VP level, whereas LR pigs respond with an increase in CRH mRNA levels. The observed differences between HR and LR may be caused by differences in glucocorticoid feedback or glucocorticoid receptor expression but the exact underlying mechanism waits to be elucidated. In addition the adaptations observed in HR and LR pigs show similarities with those observed in human psychopathologies, and could be interpreted as (pre) pathologic. This suggests that individual housing is not an optimal for pigs, and may likely compromise welfare.

We assumed that the regulation of the hypothalamic CRH and VP neuro-secretory system in the pig is similar to that in the rat, and that the observed increase in VP peptide expression in individually housed HR gilts (Chapter 4) in fact represents co-localization with CRH. In chapter 6 we finally tackled this assumption, i.e. we studied the possible co-localization of VP in CRH neurons in the pig PVN using an immunocytochemical double staining. In addition we investigated if the number of CRH neurons and co-localization of VP in CRH neurons in the PVN of HR pigs in particular was affected by housing conditions. We found that, indeed, co-localization of CRH and VP occurs in the pig PVN. Individual housing increased both single labeled CRH as well as double labeled CRH with VP containing neurons, albeit not significantly. The percentage of double-labeled CRH with VP neurons, however, was comparable between GRO and IND housed gilts. Yet, only IND housed gilts showed neurons with very intense double labeling, which may be interpreted as a sign of hyperactivity of these CRH neurons. This confirms that the CRH and VP neuro-secretory system in the pig resembles that of the rat, and that the increase in VP observed in chapter 4 may indeed be interpreted as co-localization of VP in CRH neurons. These data therefore indicate that IND HR gilts adapt to chronic stress with up-regulation of VP in CRH containing neurons.

Measuring the activity of the CRH and VP in the PVN

The levels of CRH and VP peptide measured as immuno-positive area (μm^2) were used to determine the activity state of the hypophysiotropic CRH system, and may therefore serve as a neurobiological index of cumulative stress. The CRH mRNA levels in the PVN, again determined as immuno-positive area (μm^2), were used as a tool for assessment of CRH gene expression and activity of the CRH system at the gene transcription level. In the rat, it is known that colchicine administration, which blocks fast axonal transport, or adrenalectomy is required to visualize CRH neurons and co-localization of VP and CRH (Sawchenko et al., 1984, Whitnall and Gainer 1988; Whitnall, 1990). In the pig, however, like in humans (Raadsheer et al., 1994, 1995), such treatment was not needed to visualize the neurons containing CRH (and VP) in the medial region of the PVN.

The degree of VP expression in CRH neurons seems to relate to the duration and intensity of stress or activation of the neurons. In the present study, VP levels were measured in a single immunocytochemical staining of the PVN in the region where it was known that CRH neurons were present (medial region), in all of the experiments, except in one case. This was mainly due to the technical difficulties we encountered to perform a quantifiable double immunocytochemical staining for CRH and VP in the same section. Based on a study in humans, co-localization may only be found after a long period of HPA axis functioning, as evidenced by the fact that co-localization of CRH and VP was only found in subjects older than 40 years of age (Raadsheer et al., 1995). We assumed that automated measurements of the VP expression in successive sections in the medial region of the PVN would also give us valuable information about the activity of the VP system involved in the regulation of the HPA axis, assuming anatomical and functional similarity of the hypothalamic CRH and VP neuro-secretory system in the pig and the rat (Gibbs, 1986; Gillies et al., 1993; Whitnall, 1993; Aguilera, 1994, 1998; Ma et al., 1999a,b). The fact that we found a clear co-localization of VP and CRH in PVN neurons in our HR gilts and also up-regulation of VP co-expression in these neurons following stress (Chapter 6), likely indicates such similarity. Double labeling was relatively scarce, but the very intense double staining observed (purple) particularly in PVN neurons of IND HR pigs may be interpreted as a sign of hyperactivity of these CRH neurons. Yet, functionally distinct subpopulations of CRH neurons, as has been reported in rat (Whitnall and Gainer, 1988), do not seem to exist in the pig PVN, as the double labeled CRH -VP neurons appeared to be scattered from dorsal to ventral in the medial region, and in each rostro-caudal level. Whether co-localization of CRH and VP as found in gilts

at 1 year is an age-dependent phenomenon in the pig too, as has been reported in human (Raadsheer et al., 1995) can not be concluded although we were previously unable to detect co-localization of CRH and VP in the younger animals (26 weeks) discussed in chapters 2 and 3. Moreover, we have to emphasize that we only investigated co-localization in HR gilts, since VP levels in these animals were highest in chapter 4. In patients suffering from depression an increased activity of CRH neurons has been observed, as evidenced by a higher number of CRH-expressing neurons as well as a higher number of neurons co-expressing VP and CRH in the PVN (Raadsheer et al., 1994). Since the adaptation to chronic stress observed in both HR and LR gilts show similarities with those observed in these human patients with stress-related psychopathology, it would have been of interest if we had investigated co-localization in LR gilts as well.

Effect of sex

It is well established that there are sex differences in the HPA axis, and these differences occur at virtually all levels of the axis. There are sex-related differences in the levels of type I and II glucocorticoid receptors (MR and GR respectively) in the hippocampus and hypothalamus (Turner and Weaver, 1985; Carey et al., 1995; MacLusky et al., 1996); the expression of CRH and VP (Viau and Meaney, 1996); the secretion of ACTH from the anterior pituitary (reviewed by Rhodes and Rubin, 1999) and the circadian patterns of cortisol release (Kitay, 1961; Gaskin and Kitay, 1970). It is thought that the differences observed between males and females reflect differential effects of the sex steroids on the HPA axis, as the activity of many proteins which regulate the axis are directly regulated by the steroids, and sex steroid receptors are present at many levels of the axis (Bethea et al., 1996; Madigou et al., 1996). It is known that testosterone can inhibit the HPA axis (Viau, 1999; Rhodes and Rubin, 1999) while estrogen may enhance its function (Rivier, 1999; Roy et al., 1999; Wood et al., 2001). It is therefore not surprising that also in the pig, sex differences in the regulation of the HPA axis were reflected by a sexual dimorphism in CRH and VP levels in the PVN (Chapter 3). In addition, even though we have to take into account that the male pigs were castrated at a very young age, the neonatal testosterone surge, thought to be important for sexual differentiation of the hypothalamus, occurs in pig immediately after birth (Ford et al., 1990; Meusy- Desolle, 1975). The sexual dimorphism found in CRH and VP system of the PVN in the pig is most likely organized by gonadal steroids during development and does not require the presence of gonadal hormones during life like was also found in the rat (De Vries et al., 1985; Viau et al., 1999). Based on these results we can conclude that in the pig sex differences are present in the HPA axis at the level of the PVN, which may in turn influence

other levels of the HPA axis. To which extend gonadal steroids are involved in these sex differences requires further research. Yet, these sex differences should be taken into account in the planning and design of experiments, particularly in the field of stress research, since they may profoundly influence the experimental outcome.

Adaptation to an enriched or barren environment

In rodents, animals from an enriched environment show less behavioral stress in novel environments as evidenced by less manipulative behavior. Also, they perform better in learning tasks compared to animals housed under barren conditions (De Jong et al., 1998, 2000). It has been hypothesized that their better cognitive performance was due to a more adaptive HPA axis response, since they show less behavioral stress in novel environments (Uphouse, 1980; Mohammed et al., 1990, 1993). However no significant differences in basal corticosterone levels between enriched and barren housed rodents have been reported (Pham et al., 1999), nor in corticosterone levels following stress (Larsson et al., 2002). Yet, it was recently reported that enriched housed animals showed a higher expression of glucocorticoid receptors in the hippocampus compared to barren housed animals (Mohammed et al., 1993; Olsson et al., 1994). In addition, it was shown that environmental enrichment during the early postnatal period (handling) attenuated the reactivity of stress-related neuronal circuitries in the adult rat brain, while prolonged maternal separation have the opposite effect (Liu et al., 2000). As adults, animals that had been handled during the first weeks of life showed reduced plasma ACTH response to stress and reduced hypothalamic CRH mRNA levels under basal conditions compared to controls (Meaney et al., 1991; Plotsky and Meaney, 1993). In adult animals, stress-induced corticosterone concentration was lower and declined faster back to baseline levels after stress if animals were handled at postnatal age (Liu et al., 1997). The observed increase in expression of hippocampal GR in adult rats (Meaney and Aitken, 1985; Liu et al., 1997) is suggested to induce the enhanced glucocorticoid feedback efficacy observed in these animals (Meaney et al., 1989). These differences in glucocorticoid feedback sensitivity are associated with reduced resting state levels of mRNA encoding for CRH and VP in PVN neurons and in the median eminence levels of CRH and VP immunoreactivity (Meaney et al., 1991; Plotsky and Meaney, 1993). High levels of GR would induce a proper glucocorticoid negative feedback mechanism, whereas low levels of GR reduce the glucocorticoid negative feedback. This suggests that the IND housed gilts in the present thesis might have had a reduced GR level in the hippocampus, resulting in impaired negative feedback and the higher levels of CRH and VP.

Pigs, when exposed to a mildly enriched environment indeed show less manipulative social behavior, whereas barren housed pigs develop abnormal agonistic behavior (Schouten, 1986; Beattie, 1995, 1996). The basal cortisol concentration, during the light period, however, is higher in enriched compared to barren housed pigs. Yet, there are indications that barren housed pigs have an impaired long-term spatial memory compared to enriched housed pigs (De Jong et al., 2000), and it was suggested that rearing conditions in pigs may affect brain morphology. These data suggest that pigs show similar behavior as rats in particular tests.

The behavioral, autonomic and endocrine stress responses to some procedures and interventions routinely used in pig husbandry practice are well documented. Adaptations to specific stress paradigms at the level of CRH and VP levels in the PVN of the pig, however, have not been reported in the literature yet. Since CRH and VP signaling in the brain plays a pivotal role in behavioral, autonomic and endocrine stress-responses, it was hypothesized that altered activity of either one or both peptide systems could be responsible for the changes observed in cortisol levels during the day and/or the behavioral response to novelty. In chapter 3, however, this hypothesis was not confirmed, since we found no changes in the amount of CRH and VP peptide in the PVN, but note that mRNA levels were not examined. The lack of an effect on CRH and VP expression suggests that the housing conditions may not have been contrasting enough to result in an effect at the level of the hypothalamus. This may be due to stress reducing effect provided by the social contact between conspecifics in group housing as proposed by Pearce and colleagues (1989). The hippocampus of these very same barren housed pigs however, showed more apoptosis and less neurogenesis, and GR numbers were decreased (Kallivretaki et al., 2001). These changes are suggestive for reduced glucocorticoid feedback in barren housed animals, as has been suggested for the rat (Fernández-Teruel et al., 2002). Since the hippocampus is rich in corticosteroid receptors and closely involved in the regulation of CRH and VP in the PVN, effects of a barren environment might first become evident in the hippocampus before affecting the CRH and VP levels. The trend, although not significant, for higher CRH levels in barren housed male pigs might be the first sign of this sequence of events. The GR data indicated that HR and LR pigs adapt to mild stress in a similar way.

Even though some behavioral, pathological and immune characteristics of pigs were reported to depend on the interaction between backtest qualification and housing conditions (Schrama et al., 1997; Bolhuis, 2003), no effects were found of housing condition on CRH and VP expression in the PVN of pigs of either coping strategy, suggesting that the housing conditions used in the present study may not

have been contrasting enough to result in diversion of this neurobiological index at the hypothalamic level.

Individual housing

Ruis et al., (2001a,b) have already emphasized, that the pig is a socially living animal, which requires social contact with conspecifics. Although the regulations for individual housing of pigs are getting tougher (amended EC Council Directive 91/630/EEC, 1991), individual housing of sows is still common practice in pig husbandry, even though it may likely lead to chronic stress. Ruis et al. (2001a,b), recently showed that, compared to socially housed pigs, isolated gilts generally develop a higher state of fearfulness, and become more responsive to environmental changes. Moreover, there is evidence to indicate that HR and LR gilts differ in their ways to adapt to a social isolation challenge (Ruis et al., 2001b). LR gilts appeared to recover more quickly from social isolation than HR gilts. Especially for the HR gilts, the isolation challenge seemed to have long lasting effects. This is in accordance with results Geverink and colleagues reported for the HR and LR breeding pigs used in the experiments described in chapter 4, 5 and 6 of this thesis. They found that differences between adult HR and LR breeding pigs in basal behavioral and physiological variables were particularly expressed under conditions of chronic stress such as individual housing (Geverink et al., 2003). In addition, it has recently been established once more that HR and LR animals differ in their response to an acute stressor independent of housing condition. During fixation with a nose sling, HR young adult female pigs (gilts) vocalized more and showed a lower heart rate. Also, it took longer for their cortisol response to return to preceding 'basal' concentrations (Geverink et al., 2002b). These observations suggest, that restraint is likely a more stressful experience for HR than for LR gilts. In accordance with these results, we showed in chapter 4 and 5 that marked differences between the two coping strategies in VP peptide and CRH mRNA levels also became evident under "extreme housing" conditions. These results indicate that HR and LR gilts differ fundamentally in their way to adapt to individual housing at the level of the hypothalamus. They showed that LR gilts adapt to a chronic stress situation by up-regulation of the CRH system, whereas HR gilts appear to adapt to chronic stress situations by an activation of the VP system in the PVN and, presumably, a desensitization of the CRH system as has been reported for rats (Ma et al., 1999). The activation of VP is supported by the fact that we found an intense double staining for CRH and VP in neurons in IND HR gilts, which might be a sign of hyperactivity of these CRH neurons in favor of VP. These data suggest a shift of the hypothalamic CRH/VP signal in favor of VP

following prolonged psychosocial stress, a pattern similar to that found in immobilization stress studies (Whitnall, 1989).

In general our results provide additional evidence to show that individual housing is a stressful experience for both HR and LR animals, as evidenced by the increase of VP peptide and CRH mRNA respectively. They confirm and extend the notion that individual housing induces chronic stress, which may eventually lead to stress related diseases.

Coping strategy and the susceptibility to stress-related diseases

A little over a decade ago, the group of Cools et al. (1990) selected two rat lines representing the two extremes of susceptibility for the effects of the dopamine agonist apomorphine that are naturally present in a normal Wistar population, in order to investigate factors involved in individual biobehavioural differences. Selection was accomplished using the apomorphine-induced gnawing response as a criterion, resulting in an apomorphine-susceptible (apo-sus) line consisting of rats with high gnawing score, and apomorphine-unsusceptible (apo-unsus) line displaying an extremely low gnawing score. The apomorphine susceptibility in the apo-sus rats is linked to an enhanced responsiveness specifically of the nigrostriatal and tuberoinfundibular dopamine pathways (Rots et al., 1996).

It has been established that other differences between the two selected rat lines from the Cools lab can be observed in the response to an open field test situation. In this test apo-sus animals show more locomotor activity than apo-unsus rats (Rots et al., 1995, 1996). Apart from this behavioural difference the lines also differ in their HPA axis response pattern. Apo-sus rats display a higher and prolonged stress induced increase in ACTH and free corticosterone release. In addition, the ACTH-response to exogenous CRH administration is more pronounced in apo-sus than in apo-unsus rats (Van Eekelen et al., 1992). Apo-sus rats are marked by a higher level of CRH gene expression which results in higher ACTH but lower total corticosterone plasma levels under basal conditions compared to apo-unsus rats (Rots et al., 1995). Yet, when stressed, the c-fos activity in CRH neurons in apo-sus rats is reduced whereas the ACTH release is increased compared with apo-unsus rats (Mulders et al., 1995), which is surprising in view of the stimulatory effect of CRH on ACTH release. It was suggested that the prolonged elevated activity of the HPA axis of apo-sus rats might be the result of a weaker negative feedback of glucocorticoids on the ACTH release at the level of the pituitary (Rots et al., 1995). Likewise there may be a differential regulation of synthesis and release of CRH in apo-sus and apo-unsus rats. In addition, it has been observed that in adult apo-sus rats hippocampal MR levels are increased under basal conditions compared to apo-unsus rats, suggesting a potential

difference in glucocorticoid function between apo-sus and apo-unsus rats (Rots et al., 1996).

Apart from the differences in the nervous and endocrine systems, apo-sus and apo-unsus rats also differ in their immunological system. There is evidence that the HPA axis interacts with the immune system very closely (Munck and Guyre, 1990). Glucocorticoids both induce an inhibition as well as an activation on certain immunological responses. It has been shown that glucocorticoids inhibit the response of TH1- lymphocytes, but stimulate the differentiation and/or activation of TH2 cells. This indicates that glucocorticoid shift the balance towards dominance of the TH2 system, which can result in altered susceptibility to certain diseases. It was shown that apo-sus rats have a relative dominance of the TH2 system (Kavelaars et al., 1997), which makes them more susceptible to peridontitis than apo-unsus rats (Breivik et al., 1996, 2000). Moreover it has been found that the recovery from gastric ulceration produced by restraint-in-water-stress is significantly slower in apo-sus than apo-unsus rats (Degen et al., 2003). This finding can be explained by the fact that the stress-induced increase in corticosterone, which slows the recovery from ulcerations (Carpani-de-Kaski et al., 1995), is both less and shorter-lasting in apo-unsus rats than in apo-sus rats (Rots et al., 1995). These data suggest that genetic selection for coping strategy predict stressor susceptibility and vulnerability for diseases.

Recently, also in mice differences in coping strategy were found to be associated with differential regulation of the HPA system under basal, acute and chronic psychosocial stress conditions. LAL (Long Attack Latency, low to non-aggressive) mice showed lower basal ACTH and corticosterone levels under basal conditions compared to short attack latency, high aggressive (SAL) mice. GR and MR mRNA in the hippocampus and CRH mRNA in the hypothalamus were not different between the two lines under basal condtions. During an acute stressor, LAL mice showed a higher and prolonged stress-induced increase in plasma corticosterone compared to SAL mice, while the absolute ACTH response did not differ (Veenema et al., 2002, 2003), LAL mice also showed an increase in hippocampal MR mRNA. In addition, CRH mRNA expression 24 hrs after an acute stressor was significantly higher in LAL mice compared to SAL mice (Veenema et al., 2002). The observation of a prolonged acute stress-induced increase in corticosterone levels in LAL mice reveals an impaired termination of the stress response. Yet, after a chronic psychological stressor (defeat stress) no difference in CRH mRNA was found between LAL and SAL mice (Veenema et al., 2003), yet CRH mRNA in defeated SAL mice was higher compared to controls (no stress). Moreover, chronic stress (defeat stress) induced increased ACTH levels in LAL compared to SAL mice and also elevated corticosterone levels and lower

hippocampal MR: GR ratio compared to LAL controls (no stress). A reduced capacity of especially hippocampal MRs has been hypothesized to be involved in the HPA system dysregulation found in depression. In view of the profound differences in behavior and stress reactivity, these mouse lines were suggested to present an interesting model for studying the mechanism underlying individual variation in susceptibility to stress-related psychopathology. Moreover, based on the above data is has been suggested that LAL mice appear to be more susceptible to psychosocial stress than SAL mice (Veenema et al., 2003).

These findings support the idea that genetic selection for coping strategy predicts stressor susceptibility and that in this case particularly LAL mice are more susceptible to a psychosocial stressor. The apparent discrepancy between the findings that in rat the more active and in mice the more passive coping strategy seems to be more susceptible to stress-related diseases may not be as contrasting as it appears at first sight. Apo-sus rats and LAL mice both show similarities in their endocrinological response (an enhanced and prolonged stress induced increase in corticosterone levels), which in both species has been suggested to be due to a reduced containment of the stress response system by corticosterone. Yet, the underlying mechanism appears to be different between the two models. Both models, however, show that selecting on coping strategy predicts the susceptibility to stress-related diseases.

It has been shown that HR and LR pigs differ in a variety of features such as basal cortisol concentration, aggression and HPA reactivity (Hessing et al., 1994; Ruis et al., 2000; Geverink et al., 2002a; this thesis). Apomorphine, used to select for gnawing score in the rat, induces stereotyped behavior in several other species as well, including the pig. The behavioral effects of different doses of apomorphine have been studied systematically in pigs (Terlouw et al., 1992) and pigs, like rats also vary in their response to the drug (Fry et al., 1981; Terlouw et al., 1992). It has now been established that the response to apomorphine in pigs is linked to the individual coping strategy characterized by the backtest (Bolhuis et al., 2000). It has been suggested that the higher levels of snout contact with the floor observed in apomorphine treated HR pigs, as compared to LR pigs, reflect higher susceptibility to the drug in this type of animal.

It has been reported that the HR gilts used in the present project showed lower cortisol levels during the light period compared to LR gilts at 6 months of age under group-housing conditions (Geverink et al., 2003), which was not observed at 11 months of age. Yet after individual housing for 5 months cortisol levels in HR gilts were lower compared to LR gilts at the age of 11 months. After an ACTH-challenge at 5 months of age no differences were found in cortisol levels between group housed HR and LR gilts (Geverink et al., 2003). During an acute

(nose sling) stressor at 11 months of age, however, HR gilts showed a prolonged acute stress-induced increase in cortisol compared to LR gilts, which was not dependent on housing condition (Geverink et al., 2002b). In addition HR gilts vocalized more during the acute stressor, indicating that it was a more stressful situation for them, compared to LR gilts (Geverink et al., 2002b).

HR and LR pigs also differ in their immunological system. At 10 weeks of age, LR pigs had a higher keyhole limpet haemocyanin (KLH) induced antibody titer (Bolhuis et al., in prep.) compared to HR pigs, indicating that LR pigs may have a relative dominance of the TH2 system. This finding may be explained by the higher level of cortisol in LR pigs, which is thought to enhance antibody synthesis (Hessing et al., 1995; Kavelaars et al., 1997). Yet, at 12 months of age no effect of coping strategy on the humoral or cellular immune response in the gilts was found. This finding may be explained by the fact that the stress-induced increase in cortisol is longer lasting in 12-month-old HR gilts, shifting the balance between the TH1 and TH2 system towards that seen in LR gilts, which in the long-run could lead in HR animals to an increase in their susceptibility to certain stress-related diseases, such as auto-immune diseases, like rheumatoid arthritis (Kavelaars et al., 1997), which are dependent on a TH1 response.

In the present study we showed that hypothalamic CRH and VP peptide levels are not significantly different between HR and LR pigs when housed under conditions of mild stress (Chapter 3). When chronically stressed, however, VP peptide levels were increased in HR gilts (Chapter 4), whereas CRH mRNA levels were increased in LR gilts (Chapter 5). In addition, co-localization of VP in CRH neurons appeared to be higher in individually housed HR gilts compared to group housed HR gilts (Chapter 6). Both types of pigs appear to show similar effects in adaptation to chronic stress compared to certain rat and mouse lines selected for diverging coping strategies. Whereas HR gilts show similarities in their cortisol response to apo-sus rats, LR gilts show similarities in higher CRH mRNA levels with LAL mice.

The observed prolonged stress induced increase in cortisol level in HR gilts might be the result of a weaker negative feedback of glucocorticoids on the ACTH release at the level of the pituitary, as reported for apo-sus rats and LAL mice. This impaired negative feedback that develops under conditions of chronic stress may be explained by elevated release of VP from hypophysiotropic neurons in the PVN. It is known that VP can reduce the sensitivity of glucocorticoid feedback through mechanisms that involve interaction of transcription factors induced by the VP and the glucocorticoid receptor (Webster and Cidlowski, 1999), thereby facilitating and maintaining ACTH secretion in spite of high circulating levels of glucocorticoid during stress. Likewise, the hippocampal MR:GR ratio may be different between

HR and LR gilts resulting in a potential difference in glucocorticoid function, which may account for the observed increase in CRHmRNA in LR gilts.

The observed increase in VP levels in HR gilts is similar to that found in patients suffering from depression. Purba et al. (1996) reported an increase in the number of vasopressin-expresssing neurons in the parvocellular part of the PVN of depressives. This confirms the view of Holsboer et al. (1995, 1996) that the increase in VP in depressives explains the observed increase in cortisol in those patients as a result of disturbed negative feedback at the level of the pitutiary. Yet, the observed increase in CRH mRNA in LR is also in accordance to findings in depression. Nemeroff et al. (1984) reported elevated CRH in the CSF of patients suffering from depression. These data could suggest that also in depressive patients subtypes exist that adapt differentially to stress, as we have found for HR and LR pigs. Evidence for the existence of subcategories of depressive patients that differ in symptomatology and plasma VP has indeed been reported (van Londen et al., 1997, 1998a,b; de Winter et al., 2003). On the other hand it cannot be excluded that the increase in CRH in CSF, the non-supression in the dexamethasone supression test and the increase in VP in the PVN go together and result in the increased HPA activity found in 40-90% of patients with major depression (Holsboer et al., 1996). These data indicate that selection for coping strategy in the pig presents an interesting model for studying mechanisms underlying individual variation in susceptibility to stress-related psychopathology.

This study shows, that HR and LR gilts react to chronic stress, induced by individual housing, with different neuro-adaptations: LR pigs react to chronic housing stress with an up-regulation of CRH, while HR pigs react with up-regulation of VP in the PVN. This divergence of adaptations in the CRH/VP system of the PVN indicates a fundamental difference in the make up of the brain circuitry that governs HPA function. It possibly explains previously reported differences between HR and LR pigs in HPA responses, and given the pivotal role of the HPA axis and its (neuro) hormones in behavioral and physiological adaptation in general, it may also be pertinent to other responses that have been found to differ between HR and LR pigs. As the adaptations in the CRH/VP system found in HR and LR pigs show similarities with those observed in human stress-related psychopathologies, and in animal models where the occurrence is associated with increased vulnerability for disease, they probably indicate that individual housing may exceed the adaptive capacity of pigs and seriously compromise the welfare of the animals.

Implications of the studies

The experiments described in this thesis provide evidence that adult pigs selected for diverging coping strategies at an early age differ in the way they adapt to individual housing at the level of the PVN. The diverging differences found in CRH and VP levels in the PVN support the differences in basal behavioral and physiological variables between these adult HR and LR pigs that were found expressed under conditions of chronic stress. Moreover, the changes in activity of CRH and VP observed are similar to those observed in humans suffering from depression, suggesting that these changes may be interpreted as (pre) pathological signs in the pig. The present data therefore support the notion that individual housing is detrimental for the welfare of pigs, independent of coping strategy. Selection of coping strategy to maintain the common practice of individual housing of breeding pigs will therefore not benefit the welfare of the animals. To improve pig welfare and minimize the risk of stress-related diseases, the focus should be on optimizing the pigs' housing conditions.

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Samenvatting

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Opgroei- en huisvestingcondities van het varken kunnen de gedragsmatige autonome en (neuro)endocriene stress responsen beïnvloeden. Het effect van stress is sterk afhankelijk van de individuele coping-(adaptatie) strategie van het dier. De coping strategie van een varken kan vlak na de geboorte vastgesteld worden met behulp van een backtest. In deze test wordt een big op zijn rug gelegd en gedurende één minuut in deze houding vastgehouden. Doet een big veel pogingen om te ontsnappen, dan heeft het een relatief agressief karakter en een actieve manier van omgaan met stress (High-Resisting, HR). Weinig weerstand in de rugtest geeft aan dat het dier voornamelijk rustig en een passieve manier van omgaan met stress heeft (Low-Resisting, LR).

Ook is aangetoond dat eerdere ervaringen met stress, zowel eenmalige als chronische, kunnen leiden tot veranderingen in de respons op volgende stressoren (stress-sensitisatie). Deze veranderde gevoeligheid komt tot uiting in veranderingen in neuro-biologische karakteristieken, welke de regulatie van gedragsmatige, autonome en (neuro)endocriene responsen sturen. Corticotropin-releasing hormoon (CRH) en vasopressine (VP), welke gemaakt worden in de hersenen, spelen een belangrijke rol bij de regulatie van deze responsen. CRH is een neuropeptide dat in de hypothalamus wordt geproduceerd door neuronen in de nucleus paraventricularis (PVN). CRH stimuleert de afgifte van adrenocorticorticotropic hormone (ACTH) uit de hypofyse wat vervolgens de afgifte van bijnierschors hormonen, cortisol in het varken, stuurt. CRH speelt een essentiële rol in de respons van de hypothalamus-hypofyse-bijnier (HPA)-as op stress. Onder normale condities wordt CRH tonisch afgegeven, waardoor er altijd lage basaal niveaus van cortisol in het lichaam aanwezig zijn. Lage concentraties cortisol zijn noodzakelijk voor het goed functioneren van vrijwel alle weefsels/cellen. Zij beïnvloeden celgroei, celdeling en celmetabolisme en zorgen voor de beschikbaarheid van energie.

Het effect van CRH op de afgifte van ACTH wordt gepotentieerd door VP, eveneens gemaakt door neuronen in de PVN. Er wordt verondersteld dat de CRH neuronen van de PVN hyperactief worden tijdens stress condities en zowel CRH en VP gaan produceren. Door vervolgens de hypofyse en de bijnierschors te activeren, heeft dit een chronische verhoging in basale cortisol spiegels tot gevolg, wat leidt tot ongewenste katabole effecten. Het voorgaande suggereert dat de functionele staat van de neuropeptiden VP en CRH gebruikt kan worden als neurobiologische index voor stressgevoeligheid.

De huidige opgroei- en huisvestingcondities van varkens zijn stressvoller dan vroeger en kunnen er toe leiden dat de stressgevoeligheid van de dieren, wellicht afhankelijk van hun copingstrategie, toeneemt. Dit kan uiteindelijk ook

leiden tot een veranderde gevoeligheid voor (stressgerelateerde) ziekten. Hoofdstuk 1 geeft een overzicht van de huidige condities waaronder varkens in de intensieve veehouderij worden gehouden en de backtest karakterisering van varkens in relatie tot verschillende stress responsen. Daarnaast wordt de signalering van CRH en VP en hun regulatie op de HPA-as onder normale condities en onder stress condities in andere diersoorten dan het varken in detail besproken.

In dit promotie-onderzoek zijn histochemische (immunocytochemie en in situ hybridisatie) technieken gebruikt om het effect van huisvestingcondities op CRH en VP systeem in de PVN van varkens met verschillende copingstrategieën te bestuderen. Deze neurobiologische data geven essentiële informatie over de gevolgen van stress bij varkens op lange termijn gehuisvest onder verschillende condities. Deze kennis kan gebruikt worden om het welzijn van het varken als productiedier in de toekomst te verbeteren.

Overzicht van de in dit proefschrift beschreven studies

In tegenstelling tot de rat is er in het varken weinig bekend over de CRH en VP signalering in de nucleus paraventricularis (PVN) van de hypothalamus en de effecten van stress. Hoewel er een paar studies bekend zijn waarin de distributie van CRH en VP in de PVN van het varken worden beschreven, is dit helaas niet heel gedetailleerd. Het lag daarom voor de hand om alvorens naar het effect van huisvestingconditie en copingstrategie op de activiteit van het CRH en VP systeem te kijken, eerst in detail de locatie en distributie van CRH en VP producerende cellen in de PVN van het varken in kaart te brengen. Deze studie staat beschreven in hoofdstuk 2, waarin 5 hypothalami van varkens van 6 maanden oud, werden gebruikt om de CRH, VP en oxytocine (OT) neuronen in kaart te brengen. Op basis van de strikte locatie van de cellen positief voor elke van de drie neuropeptiden, was het mogelijk om de PVN in te delen in 4 (lateraal georiënteerde) zones. Direct liggend aan het derde ventrikel was er een smalle periventriculaire regio waar vrijwel geen immuno-reactieve cellen gevonden werden. Daar direct naast lag een gebied (anteromediale regio) dat vooral gedomineerd werd door OT positieve cellen. Uiterst lateraal van het 3e ventrikel werden vooral cellen gevonden die VP bevatten, de laterale regio. Tussen deze 2 laatste gebieden lag een gebied waarin zowel CRH als VP cellen gevonden werden, de mediale regio. De distributie van deze 3 peptiden ten opzichte van het ventrikel was door de hele (1800 μm van rostraal naar caudaal) PVN grofweg hetzelfde en leek veel op wat ook in de rat is waargenomen. Aangenomen werd dat ook de organisatie van de CRH neuro-secretie systeem bij varkens vergelijkbaar is met die in de rat. Op basis van deze aanname wordt verwacht dat mogelijk effecten op CRH en VP

signalering/activiteit en wellicht co-lokalisatie van deze 2 peptiden vooral in de mediale regio van de PVN te vinden zullen zijn.

Met deze kennis kon in het onderzoek het effect van arme (groeps)-huisvesting (beton) v.s. rijke (beton met stro) (groeps)-huisvesting in varkens met verschillende copingstrategieën bestuderen (hoofdstuk 3). Het is bekend dat varkens gehuisvest in een arme omgeving, agressiever zijn en meer moeite hebben met het leren van een gedragstaak. Ook is ontdekt dat arm gehuisveste dieren een lager basaal cortisol niveau in het bloed hebben dan rijk gehuisveste dieren. Dit zou kunnen duiden op een veranderd HPA systeem en wellicht een veranderde signalering van CRH en VP. Helaas vonden wij in dit experiment geen effect van huisvesting op de hoeveelheid CRH en VP neuropeptide in de mediale regio van de PVN. Ook de individuele copingstrategie bleek geen effect te hebben op de hoeveelheid CRH en VP signalering in de mediale regio. Dit zou er op kunnen wijzen dat de arme huisvesting conditie niet stressvol genoeg is om de CRH en VP signalering te beïnvloeden, of de verrijkte huisvesting niet verrijkt genoeg. Wel kwam in het onderzoek een duidelijk sekse verschil in de hoeveelheid CRH en VP naar voren. Mannetjes hadden meer CRH en VP peptide in de PVN dan vrouwtjes.

In hoofdstuk 4 en 5 werd het effect van individuele huisvesting op de activiteit van CRH en VP in varkens met verschillende copingstrategieën bestudeerd. In hoofdstuk 4 werd de hoeveelheid CRH en VP peptide bestudeerd, terwijl in hoofdstuk 5 de hoeveelheid CRH mRNA in deze dieren werd bestudeerd. In deze studies werden één jaar oude vrouwelijke varkens (gelten) die groepsgehuisvest waren vergeleken met één jaar oude gelten die de laatste 5 maanden individueel hadden gestaan. In deze studie bleek duidelijk dat individuele huisvesting, afhankelijk van de copingstrategie, de regulatie van de HPA-as had beïnvloed. De hoeveelheid VP peptide bleek in HR, maar niet in LR gelten verhoogd te zijn. Deze verhoging in VP peptide zou nodig kunnen zijn om het stimulerende effect van CRH op de hypofysaire afgifte van ACTH te potentieren tijdens de voortdurende chronische stress van de individuele huisvesting. De hoeveelheid CRH peptide was in alle groepen vergelijkbaar, maar de hoeveelheid CRH mRNA bleek in individueel gehuisveste LR gelten verhoogd te zijn ten opzichte van de andere groepen. Dit suggereert dat LR gelten met een verhoogde activiteit van het CRH systeem op de individuele huisvesting reageren. HR gelten lijken niet in staat tot een verhoogde activiteit van het CRH systeem tijdens chronische stress en vertonen daarom waarschijnlijk een verhoogd VP peptide niveau, zoals dat ook in de rat is waargenomen tijdens chronische stress. Deze resultaten wijzen erop dat varkens, gekarakteriseerd met verschillende copingstrategieën, verschillen in de adaptatie op individuele huisvesting. De verschillen in CRH en VP signalering tussen de twee copingstrategieën komen

overeen met de gedragsmatige en fysiologische responsen die in deze dieren zijn gevonden al 3 maanden na individuele huisvesting. De gevonden verschillen in CRH en VP signalering tussen de twee copingstrategieën, lijken bovendien veel op veranderingen die optreden in de PVN van patiënten die aan depressiviteit lijden. Hieruit zou gesuggereerd kunnen worden dat de veranderingen die optreden in het varken na individuele huisvesting als (pre)pathologisch geïnterpreteerd kunnen worden. Samengevat suggereren deze resultaten dat in individueel gehuisveste HR gelten een verschuiving in het hypothalame CRH/VP signaal optreedt ten voordele van VP, zoals dit ook gevonden wordt in immobilisatie studies in de rat. Dit impliceert dat individuele huisvesting een stressvolle conditie voor HR gelten is. Echter, de verandering in CRH mRNA juist in de LR gelten suggereert dat deze een verhoogde activiteit van het CRH systeem hebben onder individuele huisvesting. Deze vorm van huisvesten zorgt voor aanpassing van de HPA-as regulatie ongeacht de copingstategie en is voor beide type varkens dus geen optimale huisvesting conditie.

Het is bekend dat onder stress condities in de rat, co-lokalisatie van VP in CRH neuronen optreedt. Ook bij de mens treedt co-lokalisatie van VP in CRH neuronen op, zoals bij patiënten die lijden aan stress- geïnduceerde depressie. In de voorgaande hoofdstukken werd ervan uitgegaan dat de regulatie van het CRH en VP systeem in het varken vergelijkbaar is aan dat van de rat en de mens. In hoofdstuk 6 wilden we definitief vaststellen of de verandering in VP niveaus zoals die gerapporteerd zijn in hoofdstuk 4, daadwerkelijk co-lokalisatie van VP in CRH neuronen representeert. Het eerste doel was daarom vast te stellen of co-lokalisatie van VP in CRH neuronen in de PVN van het varken optreedt. Vervolgens werd onderzocht of individuele huisvesting (chronische stress) het aantal CRH en CRH-VP neuronen in varkens beïnvloedde en dus stress afhankelijk zou zijn. Er werd gebruik gemaakt van de HR dieren uit hoofdstuk 4 en 5 en een immunologische dubbelkleuring waarmee CRH en VP in dezelfde cel werden aangetoond. In deze studie werd gevonden dat het varken wel degelijk co-lokalisatie van VP in CRH neuronen vertoonde en dat varkens onder individuele huisvesting meer CRH en CRH-VP neuronen hadden, maar dit verschil was niet significant. Een interessante observatie in deze studie was dat individueel gehuisveste HR dieren een intensievere dubbelkleuring vertoonden. Dit duidt wellicht op een verhoogde activiteit van deze CRH neuronen en hiermee samenhangend een teken van stress.

Conclusie en aanbevelingen

Met de neurobiologische data die in dit proefschrift worden beschreven, hoop ik meer informatie over de lange termijn gevolgen van stress bij varkens gehuisvest onder verschillende condities en met verschillende copingstrategieën te genereren, en deze kennis te gebruiken om het welzijn van het varken te bevorderen.

De experimenten die in dit proefschrift staan beschreven, tonen aan dat verschillen in CRH en VP signalering in de PVN van HR en LR varkens vooral tot uitdrukking komen onder extreme condities zoals individuele huisvesting. Dit komt overeen met gedragsresultaten in deze dieren waarin gevonden werd dat verschillen tussen HR en LR dieren vooral tot uitdrukking kwamen onder individuele huisvesting. Bovendien lijken de veranderingen die optreden in CRH en VP expressie in het varken erg veel op veranderingen die optreden bij patiënten die lijden aan depressiviteit. Gebaseerd op de resultaten in dit proefschrift, kan geconcludeerd worden dat huisvestingscondities waarschijnlijk de stressgevoeligheid in zowel HR als LR varkens bepalen. De adaptatie van de regulatoire neuropeptiden voor de HPA-as zoals gevonden in dit proefschrift, suggereren dat individuele huisvesting verstrekkende gevolgen heeft voor de weerbaarheid van de dieren. Alhoewel de aanpassingen verschillen tussen dieren met een verschillende copingstrategie, valt voor beide aan te bevelen huisvestingscondities te kiezen waar stress tot een minimum beperkt is. Het blijkt in de praktijk dus niet mogelijk varkens te selecteren die de “juiste” copingstrategie hebben om goed te kunnen adapteren aan individuele huisvesting. Dit houdt praktisch in dat huisvestingscondities voor varkens zorgvuldig gekozen moeten worden om het risico voor stress- gerelateerde ziekten te minimaliseren.

List of publications

List of publications

Karman, A.G., Bolhuis, JE., Jousma, E., Wiegant, V.M., Van der Beek, E.M. Sex differences in the expression of vasopressin and Corticotropin-releasing hormone in the paraventricular nucleus of pre-pubertal pigs. *In preparation*

Karman, A.G., Van der Beek, E.M., Swarts, J.J.M., Kastelijn, J., Wiegant, V.M. The distribution of vasopressin, corticotropin-releasing hormone and oxytocin containing neurons in the paraventricular nucleus of the porcine hypothalamus. *Submitted*

Karman, A.G., van Kesteren-Buiting, A.T.D., Geverink, N.A., Van Eerdenburg, F.J.C.M., Wiegant, V.M., Van der Beek, E.M. Individual housing affects vasopressin (VP) but not corticotropin-releasing hormone (CRH) peptide expression in the paraventricular nucleus (PVN) in pigs depending on coping strategy. *In preparation*

Karman, A.G., van Kesteren-Buiting, A.T.D., Loos, M., Wiegant, V.M., Van der Beek, E.M. Corticotropin-releasing hormone mRNA expression in the paraventricular nucleus in pigs is affected by individual housing depending on coping strategy. *In preparation*

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Abstracts

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Go raibh maith agat!

Ariëlla Gezina Karman werd geboren op 26 december 1975 te Naarden en groeide op in Huizen. Na het behalen van haar VWO diploma aan het Christelijk College "Stad en Lande" te Huizen in 1994, is ze begonnen aan de studie Medische Biologie (afstudeerrichting Neurobiologie) aan de Vrije Universiteit in Amsterdam. Tijdens de docteraalfase heeft zij twee onderzoeks stages verricht, waarvan zij de eerste uitvoerde in Amsterdam op de afdeling Anatomie en Embryologie van de Vrije Universiteit. Haar tweede onderzoeks stage verrichtte zij in Cambridge bij de afdeling experimentele psychologie van Cambridge University. In februari 1999 behaalde zij haar docteraal examen.
Van maart 1999 tot juni 2003 was Ariëlla werkzaam als Onderzoeker in Opleiding (OIO) op een NWO-gesubsidieerd project, uitgevoerd op de leerstoelgroep Fysiologie van Mens en Dier van de Wageningen Universiteit. Dit proefschrift is daarvan het resultaat.

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Cover: A.G. Karman
Front: photo of a brain of a 6-month-old gilt
Back: photo of immunocytochemical double staining on a PVN section. CRH cells stained blue, VP cells stained red and CRH and VP containing cells stained purple.

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