STRESS, ENDOGENOUS OPIOIDS AND STEREOTYPIES IN TETHERED PIGS

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Leanne W.S. Loijens

Proefschrift

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voor Swopon

voor Elena en Raoul

Voorwoord

Bijna 12 jaar na de start van het project ligt "het boekje" dan eindelijk klaar. Was het in het begin nog de vraag *wanneer* het af zou komen, op een gegeven moment werd het de vraag *of* het af zou komen. Zelf heb ik de afgelopen jaren ook wel eens getwijfeld aan die laatste vraag. Niet omdat ik er zelf geen plezier meer in zou hebben, maar wel heb ik me regelmatig afgevraagd hoe lang ik het geduld van mijn promotor en co-promotor op de proef kon stellen...
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Loijens LWS, 2002. Stress, endogenous opioids and stereotypies in tethered pigs (Stress, endogene opioïden en stereotypieën in aangebonden varkens)

Tether housing of female pigs in narrow, individual boxes represents a chronic stressor for the animals. Pigs that are housed tethered often develop behavioural disturbances, such as stereotypies, and changes in physiological regulation. The results of the studies described in the present thesis confirm and extend previous suggestions that there is an association between stereotypies and brain opioid activity. We found a negative correlation between the intensity of stereotypy performance and opioid receptor densities in the hippocampus and the hypothalamus of pigs which had been housed tethered for two months. This correlation seemed to disappear with increasing duration of tether housing, likely as a consequence of the gradual decrease in receptor density that occurred in pigs with low levels of stereotypies. This receptor decrease might reflect glucocorticoid-induced neuronal cell loss, since we found a negative correlation between the salivary cortisol concentration and the number of neurons in the hippocampus in long-term tether housed pigs. These results accord with the idea that stereotypies represent a strategy to reduce adverse effects of chronic stress.

The chronic stress of tether housing not only leads to the development of stereotypies, it can also induce changes in physiological responsivity to further stressful stimulation. The mechanisms underlying these changes likely include alterations in endogenous opioid systems. This is indicated by our finding that antagonism of endogenous opioid activity increased the heart rate response of pigs to a stressful challenge after long-term tether housing but not after loose housing. These results provide evidence indicating that long-term tether housing leads to an increased impact of endogenous opioid systems that attenuate physiological responses to additional acute stress.

Taken together, the present thesis highlights changes in endogenous opioid activity that are induced by chronic stress and appear to prevent or reduce potentially harmful stress effects.

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Contents

Chapter 1

GENERAL INTRODUCTION

Stress

Homeostasis, the state of relative physiological stability in an organism, is a prerequisite to survive. However, physiological parameters are constantly influenced by changes in the external and internal environment. Under normal circumstances homeostasis can nevertheless be maintained by means of a set of specific behavioural and physiological routines (control). When an environmental condition threathens or disturbs homeostasis beyond the capacity of the homeostatic routine, the individual loses control and a state of stress is reached. Loss of control is a common denominator of stressful conditions (Weiss, 1968).

All environmental stimuli (positive or negative) which differ from routine induce (via neurons in the brain stem reticular core) a state of arousal in the higher parts of the central nervous system (cortex, limbic system). This reaction is subconscious and enables the individual to focus its attention on the stimulus. The arousal reaction is accompanied by sympathetic activation. Priorities in the body change and prepare it for the things to come. The decision whether to act or not and if so, in what way, is made on the basis of expectations. Incoming information is compared with what is expected (evaluation on the basis of stored information, memory) and the significance of the stimulus is estimated (appraisal). If the outcome of this appraisal process is that the stimulus threatens homeostasis, a decision with respect to the kind of biological defence has to be taken. Before going into action, the individual has to select the most appropriate response and it has to compare the benefit (reward) versus the costs (expected energy, risks, etc.). In addition, it has to set priorities between conflicting motivational states. The planning of the behavioural response (e.g. active or passive) takes place in the frontal lobe of the cortex. The amygdala plays a crucial role in the active behavioural "fight/flight" strategy, which is performed to remove or flee from the threat and thereby extinguish the arousal reaction (and thus the stress response). If it is unclear how to act (e.g. in novel or unpredictable situations, or when an

adequate response is not possible), the "conservation/withdrawal" strategy is chosen, in which the septo-hippocampal system is thought to play an essential role. Characteristic for this strategy is the maintenance of a high level of arousal. All defence mechanisms stay in the alarm state until a coping response is found. There is ample evidence that individuals differ in preference for a coping strategy, i.e. in coping style.

The response of an individual is dependent on its previous experience, and responses to one and the same or a similar (controllable) stressor decrease with repeated exposure. The individual learns to recognize the stressor and to prepare for it (anticipation). In addition, it develops refined behavioural strategies for the control of the stressor (improved fighting strategies, refined submission behaviour). Moreover, emotional changes may occur (improved self-esteem and emotional stability). The more efficient a certain challenge can be responded by a specific behavioural reaction, the less likely it is that a systemic stress response will be elicited. In the case of an uncontrollable stress situation, however, a long-lasting activation of the stress responsive systems can be observed. Since stress responses involve a redirection of both behaviour 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 or frequent stress cannot be tolerated indefinitely. The individual will experience adverse effects and this may eventually lead to (stress-related) disturbances or diseases.

Tether housing of pigs

In the present project we used an animal model of chronic stress. Prolonged tether housing of pigs was used as a paradigm of chronic restraint stress. In this housing system, pigs are restrained by a neck or breast tether and a short chain to e.g. the floor. Tether housing of pigs has been introduced in recent decades in an effort to reduce production costs by saving on both space and labour. Although these efforts may have been successful from a technological and economical point of view, the success has become increasingly overshadowed by undesirable consequences for the animals kept in these intensive systems.

Tethering can be regarded as an acute stressor for pigs. Pigs that are first tethered, fiercely resist, scream loudly and try to escape by pulling and biting the tether chain. This behavioural response is accompanied by physiological reactions

such as increased plasma levels of ACTH and cortisol, and an increased heart rate (Schouten and Wiepkema, 1991; Wiepkema and Schouten, 1992; Schouten and Rushen, 1993). Following this initial phase of resistance, the animals gradually calm down and heart rate and pituitary-adrenocortical activity normalize. However, in the long term the situation remains stressful. The animals' normal behavioural programs like foraging, exploration and social interaction as well as their defence reactions (withdrawal, fighting, behavioural inhibition) have become impracticable or ineffective, leading to a reduced controllability of the environment. As loss of control is a common characteristic of stressful situations, it can be reasoned that prolonged tether housing represents a condition of chronic stress for pigs. Long-term tether housed pigs have been found to show changes in autonomic regulatory systems (Schouten *et al.*, 1991) and pituitary-adrenocortical activity (Janssens *et al.*, 1993; 1994; 1995a,b) as well as behavioural disturbances such as stereotypies (Cronin, 1985; Wiepkema and Schouten, 1992).

Stereotypies

Definition

Numerous definitions of the term stereotypies have been formulated. The most widely used variant in ethology and animal welfare literature is the definition of Ödberg (1978) who described stereotypies as morphologically similar patterns or sequences of behavioural elements, which are characteristic per individual, are performed repetitively and have no obvious function. Examples include rocking movements in retarded children (Stone, 1964), pacing in zoo animals as bears and tigers (Meyer-Holzapfel, 1968), jumping in caged bank voles (Ödberg, 1986), tongue playing in veal calves (Wiepkema *et al.*, 1987) and bar biting and tether chewing in tether housed pigs (Cronin, 1985; Rushen, 1984; Terlouw, 1993).

Causal factors

Stereotypies can be induced in humans and animals by a variety of circumstances. They can be elicited by drugs such as amphetamine and apomorphine or may be induced by environmental factors. In addition, stereotypies may result from brain damage or psychiatric conditions (autism, schizophrenia). In this thesis, we focussed on environmentally-induced stereotypies. This class of stereotypies is mainly observed in sub-optimal environments where animals are confined and where their behaviour is restricted. It has often been suggested that animals

experience such environments as stressful and that stereotypies may arise if such conditions persist (e.g. Ödberg, 1978; Wiepkema, 1990). Some forms of stereotypies appear to be derived from attempts by the animals to escape from their adversive environment (Duncan and Wood-Gush, 1972; Cronin, 1985).
There is a growing number of examples of a relationship between stereotypies and feeding and foraging behaviour. Both inadequate nutrient intake and inadequate opportunity for foraging have been found to increase the incidence of stereotypies (Rushen, 1985; Appleby and Lawrence, 1987; Terlouw *et al.*, 1991a,b). Alternatively, stereotypies have been thought to result from restriction of locomotion. However, increasing space allowance does not always have an inhibiting effect on stereotypy performance. It has been found to reduce route tracing in canaries (Keiper, 1969) and sham chewing in pigs (Terlouw *et al.*, 1991a), but does not affect chain-chewing and excessive drinking by pigs (Terlouw *et al.*, 1991a).

Functional significance

Although stereotypies have no obvious function by definition, many authors have tried to find an explanation for their persistence. It is difficult to think of stereotypies as being purposeless considering their eliciting conditions and their significance in the time budget of animals. Stereotypies are often thought to serve a coping function, i.e. that in some way they keep animals within optimal physiological and psychological limits.
Animals subjected chronically to a constant and highly predictable environment may experience too low levels of arousal and may actively seek means of increasing arousal. In such situations, performance of stereotypies may provide sensory stimulation to compensate for the lack of appropriate exteroceptive stimulation and may thus be rewarding (Stolba *et al.*, 1983; Broom, 1983; 1987). This compensatory hypothesis does not have empirical support.
It may also be that the environment in which animals are held, elicits a state of hyper-arousal, and it has been thought that in such environments, stereotypies may enable animals to dissipate tension, frustration or anxiety engendered by the situation or to switch attention away from the aversive situation (Hutt *et al.*, 1964; Dantzer and Mormède, 1981; Rushen, 1984; Dantzer, 1986; Cronin *et al.*, 1986). Evidence supporting this de-arousal hypothesis exists. Wiepkema *et al.* (1987) found a negative relationship between tongue playing in veal calves and the

severity of abomasal lesions. Preventing stereotypy performance in pigs appeared to increase pituitary-adrenocortical activity (Dantzer and Mormède, 1981; 1983). In children, leg swinging was found to be associated with a reduction in heart rate (Soussignan and Koch, 1985). An association between stereotypies and reduced heart rate has also been reported in pigs (Schouten and Wiepkema, 1991). In pigs, stereotypies are not performed continuously but occur in bouts interrupted by bouts of non-stereotyping behaviour. The bout length of stereotypies may vary from some seconds to several minutes. A detailed analysis of the data of the latter study showed that the average heart rate during bouts of stereotypies was lower than during bouts of non-stereotyping behaviour. Heart rate decreased after the pigs had started a bout of stereotypies (Schouten and Rushen, 1993).

Individual differences in stereotypy levels

Large individual differences have been observed in level (Ödberg, 1986; Appleby and Lawrence, 1987) and type (Cronin, 1985) of stereotypies within groups of animals kept under similar conditions. These differences may be due to differences in the extent to which animals perceive and evaluate sub-optimal aspects of the environment. For example, individuals may differ in their optimal levels of arousal or in the extent to which they can predict stressors.

Individual differences in the performance of stereotypies may relate to differences in coping style. A negative correlation has been found between the acute resistance response of pigs to stress (first tethering experience) and the intensity of stereotypies shown after long-term tether housing (Schouten and Wiepkema, 1991). Benus (1988) demonstrated that mice and rats which were more aggressive towards conspecifics showed a greater tendency to develop habits and routines. Pigs which were more aggressive (in a food competition test), had a higher propensity to develop excessive drinking under restricted feeding and housing conditions (Terlouw *et al.*, 1991b).

In mice and rats it has been shown that differences between animals in their response to environmental challenges relate with individual differences in the organisation of dopamine systems (Mittleman and Valenstein, 1985; Mittleman *et al.*, 1986; Piazza *et al.*, 1989). In pigs, animals with different coping styles were found to differ in their behavioural response to the dopamine agonist apomorphine (Bolhuis *et al.*, 2000).

Neurobiological basis of stereotypies

Dopamine systems

The exact neural mechanisms underlying the performance of stereotypies are not known. Parallels have been drawn between environmentally-induced stereotypies and stereotypies induced by psychomotor stimulant drugs such as amphetamine and apomorphine. It is well established that psycho-stimulants induce stereotypic behaviour by enhancing brain dopamine (DA) release (see Robbins *et al.* (1990) for a review). Dopamine antagonists have been found to inhibit (environmentally induced) stereotypies (e.g. Kennes *et al.*, 1988). The fact that the DA system is also one of the key neurotransmitter systems involved in the central response to environmental constraints (stress) (Anisman and Zacharko, 1990; Puglisi-Allegra and Cabib, 1990; Le Moal and Simon, 1991), suggests that stress and stimulants may (partly) act upon the same neural mechanisms. An additional argument for this notion is the finding that stress and psychostimulants are interchangeable in producing behavioural sensitization (cross-sensitization). Repeated systemic injections of different types of psychostimulants lead to sensitization of the behavioural-activating effects of these drugs. Repeated exposure to mild tail pressure, inescapable footshock, food deprivation and immobilization in rats, e.g., have all been shown to increase the behavioural responses to amphetamine. Moreover, stereotypic biting induced by tail-pinch is progressively enhanced by repeated exposure either to the stressor or to amphetamine (see Piazza and Le Moal (1996) for a review). However, the finding that amphetamine- and apomorphine-induced behaviour patterns in pigs differ qualitatively from stereotypies developed under restricted feeding and housing conditions (Terlouw *et al.*, 1992) also points to differences in the underlying mechanisms.

The possible role played by DA systems in the performance of stereotypic behaviour should not be interpreted as exclusive. DA systems have complex interactions with limbic and cortical structures and increased DA release is not the only central response to stimulants or stress. It has been found, for example, that the activity of dopaminergic neurons can be stimulated by glucocorticoids (corticosterone, cortisol) (Piazza and Le Moal, 1996) and glucocorticoid secretion is a prerequisite for the development of behavioural sensitization (Rivet *et al.*, 1989; Stone, 1990). Furthermore, an increasing number of reports demonstrate the existence of interactions between dopaminergic and opioid peptide systems.

Endogenous opioid systems

Evidence for the involvement of endogenous opioids in stereotypies came from experiments in which animals were treated with opioid agonists and antagonists. Rats and mice treated with morphine or other opioid agonists show stereotyped locomotion and oral stereotypies (Robbins and Sahakian, 1981). Opioid antagonists, such as naloxone, have been found to inhibit stereotypies in pigs (Cronin *et al.*, 1985; Rushen *et al.*, 1990), horses (Dodman *et al.*, 1987) and voles (Kennes *et al.*, 1988). The degree to which naloxone reduces stereotypy performance varies between different studies. Cronin *et al.* (1985) reported a reduction of more than 50%, whereas Rushen *et al.* (1990) and Schouten and Rushen (1992) found percentages of 20-30% and 10%, respectively. The factor responsible for these differences may be the stage of development of the stereotypies. Studies in pigs (Cronin, 1985) and bank voles (Kennes *et al.*, 1988) have shown that there is a negative correlation between the time since the development of the stereotypy and the inhibiting effect of naloxone.

In stead of performing pharmacological intervention studies with opioid agonists and antagonists, Zanella *et al.* (1996) measured *post mortem* opioid receptor densities in the brain. They found a negative correlation between the intensity of (tongue-rolling and sham-chewing) stereotypies and the density of kappa opioid receptors in the frontal cortex of tether housed sows. The association appeared to be receptor type specific: mu-binding negatively correlated with tongue-rolling but not with sham-chewing and delta-binding neither related to tongue-rolling, nor to sham-chewing.

Endogenous opioid systems

Opioid peptides

In the mid seventies a variety of peptides has been described functionally similar to opiates (alkaloids obtained from the juice of the poppy *Papaver Somniferum*, such as morphine). They have therefore been termed opi*oid* (opiate-like) peptides (opioids) or endorphines (endogenous morphine). For a review on the biology of endogenous opioids, their biosynthesis, anatomy and receptor systems see Akil *et al.* (1984) and Khachaturian *et al.* (1985).

Opioids consist of three (gene) families, derived from three precursor molecules: pro-opiomelanocortin (POMC), pro-enkephalin and pro-dynorphin. All opioid peptides have the N-terminal cores for opioid activity: Tyr-Gly-Gly-Phe-Met (met-

enkephalin) or Tyr-Gly-Gly-Phe-Leu (leu-enkephalin). Removal of the N-terminal tyrosine residue yields a peptide devoid of opioid activity. Peptides derived from POMC include the opioid ß-endorphin, and the non-opioid peptides ACTH, α-MSH and γ-MSH. The POMC precursor is synthesized in both the pituitary gland as well as the brain. POMC-producing neurons in the brain can be found in two nuclei: the hypothalamic arcuate nucleus and the midbrain nucleus tractus solitarius. Especially from the arcuate nucleus, POMC-containing neurons project to a wide variety of brain areas, including limbic and brain stem nuclei.
Pro-enkephalin contains several opioid peptides: met-enkephalin, met-enkephalin-Arg^6-Phe^7-NH_2, met-enkephalin-Arg^6-Gly^7-Leu^8-NH_2 and leu-enkephalin. The third opioid precursor molecule, prodynorphin, produces three leu-enkephalin containing peptides: α/ß-neo-endorphin, dynorphin A and dynorphin B. In contrast to POMC, both pro-enkephalin- and pro-dynorphin-producing neurons are widely spread in the brain, and are part of both local circuits and larger projections. In many brain areas enkephalin-containing neurons exist alongside dynorphin-containing neurons, but whether these peptides are co-stored or co-released is not known.

Opioid receptors

Three main classes of opioid receptors have been described: mu, delta and kappa. These three classes have been shown to differ regarding distribution in the brain and pharmacological properties (Goldstein and Naidu, 1989). Following the discovery of three different classes of opioid receptors, it was speculated that each receptor class might preferentially interact with products from one precursor molecule (POMC-mu; pro-enkephalin-delta and pro-dynorphin-kappa). This was shown not to be the case. When comparing the distribution of mu-, delta- and kappa-receptors to ß-endorphin, enkephalins and dynorphins, respectively, numerous mismatches were found (Khachaturian *et al.*, 1985). Differential processing of opioid peptide precursors, together with the amounts of mu-, delta- and kappa-receptors present at the site of release might present a biological strategy for transmitting different types of opioid signals.

Functions of opioid systems

All three endogenous opioid families are clearly part of the systems that regulate the body's responses to stress. Indeed, the sites of production of endogenous opioids include the hypothalamus, the three lobes of the pituitary, and the adrenal

medulla, while the adrenal cortex is exquisitely sensitive to the products of POMC, the ACTH/ß-endorphin precursor.
Effects of opioids on a variety of physiological functions have been described. Well-known effects of opioids are inhibition of pain perception (analgesia) and induction of euphoria. Opioid systems also play a role in the regulation of respiratory, cardiovascular and gastrointestinal as well as neuroendocrine functioning. Opioid peptides have been implicated in immune function as well. In addition, opioid systems are involved in the regulation of a variety of behaviours. Opioids have been implicated in reward processes (Van Ree *et al.,* 1999). As behaviour is generally guided by avoidance of aversive stimuli and searching for rewarding stimuli, opioid systems play an important role as reinforcers of behaviour in the development of new, adaptive strategies.
For a review on the functional significance of endogenous opioid systems see De Wied and Jolles (1982), Akil *et al.* (1984) and Van Ree *et al.* (1999).

Outline of the thesis

Endogenous opioid systems are involved in a wide range of physiological processes. In addition, opioids play a role in the regulation of a variety of behaviours. Activation of endogenous opioid systems is a major response to a variety of environmental stressors. The functional consequence of this activation is generally an inhibition of behavioural and physiological stress responses (Amit and Galina, 1988; Morris *et al.*, 1990; Fontana *et al.*, 1997).
Increasing literature substantiates that chronic stress can lead to changes in responsivity to further stressful stimulation (Pike *et al.*, 1997; Bhatnagar *et al.*, 1998). The mechanisms responsible for these changes likely include alterations in endogenous opioid systems (Cuadra *et al.*, 1999). Previous research in long-term tether housed pigs has demonstrated changes in hypothalamus-pituitary-adreno-cortical (Janssens *et al.*, 1994; 1995b) and cardiovascular responsivity (Schouten *et al.*, 1991) and involvement of endogenous opioids has been suggested (Schouten and Rushen, 1993; Janssens *et al.*, 1995b). The aim of the study described in Chapter 2 was to further investigate the effects of long-term tether housing on pigs' responsivity to acute stressful stimulation (nose-sling challenge) and to study the role of endogenous opioids. In a longitudinal study, the effect of pretreatment with the opioid receptor antagonist naloxone on challenge-induced heart rate and behavioural responses was investigated, before and after long-term

tether housing.

In addition to changes in responsivity, long-term tether housed pigs often show disturbances in behaviour, such as stereotypies. There are indications that stereotypies are causally linked to endogenous opioid systems (Cronin *et al.*, 1985; 1986; Zanella *et al.*, 1996). As the intensity of stereotypy performance varies considerably between individual animals, the question arises whether high and low-stereotyping animals differ in endogenous opioid function. This question was addressed in Chapter 3 and 4. We measured opioid receptor densities in the brain of long-term tether housed pigs and investigated their possible relation to the performance of stereotypies. Three groups of animals were used which were housed tethered for 2, 5.5 and 8-9 months, respectively. In Chapter 3 receptor densities were measured in the hippocampus. In Chapter 4 the hypothalamus of the same animals was selected for receptor measurement.

The data of Chapter 3 and 4 suggested that, associated with the duration of tether housing, there was a gradual decrease in the density of opioid receptors. In Chapter 5 we tested the hypothesis that this decrease was the result of neuronal cell loss resulting from prolonged exposure to elevated levels of glucocorticoids. We investigated whether individual differences in cortisol concentrations were correlated with the number of neuronal cells and the total volume of the dentate gyrus of the hippocampus in long-term tether housed pigs.

In Chapter 3 and 4 we found a negative correlation between the density of opioid receptors in the hippocampus and the hypothalamus and the intensity of stereotypy performance. It was not clear whether the individual differences in receptor density were induced by the chronic stress of tether housing, or were already present before the animals were tethered. The latter hypothesis was further investigated in pigs that did not have a history of tether housing or stereotypy performance (Chapter 6). The animals were subjected to two acute challenge tests, and behavioural and heart rate responses were monitored. Opioid receptor densities were measured *post mortem* in the hypothalamus and the hippocampus. We tested whether there was a relationship between behavioural and heart rate measures in the two challenge tests, on the one hand, and opioid receptor densities in the hypothalamus and the hippocampus, on the other hand.

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

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Chapter 2

OPIOID ACTIVITY IN BEHAVIOURAL AND HEART RATE RESPONSES OF TETHERED PIGS TO ACUTE STRESS

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ABSTRACT

In a longitudinal experiment, effects of long-term tether housing on heart rate and behavioural responses to an acute stressor (a 15 min challenge with a nose-sling) were investigated in pigs. The animals were challenged during loose housing and again after 10-11 weeks of tether housing. To detect possible changes in endogenous opioid systems modifying these responses, the pigs were pretreated with the opioid receptor antagonist naloxone (0.5 mg/kg body weight; i.v.). In response to the nose-sling challenge the animals showed pronounced resistance behaviour and a sharp rise in heart rate. Following this initial phase of resistance the heart rate dropped to pre-challenge levels or below this line, and the pigs seemed to become sedated. Pretreatment with naloxone increased the heart rate response in animals that were long-term tether housed (n=12). No such effect was found in the control group (n=5) that was loose housed during the entire experiment. This indicates that the impact of endogenous opioid systems mitigating heart rate responses to acute stress had increased as a result of long-term tether housing. Changes in the effect of naloxone on the behavioural response were not found. Adaptive changes in opioid systems may prevent excessive physiological reactions to acute stress, and thus may serve as a coping mechanism.

INTRODUCTION

Tether housing of female pigs is still commonly used in modern pig breeding farms. When chronically subjected to this aversive housing system, pigs show behavioural and physiological disturbances, such as changes in hypothalamic-pituitary-adrenal and cardiovascular responsivity. These changes include an increased steroidogenic capacity and sensitivity to ACTH of the adrenals. This was found with various *in vivo* challenges, using either an acute experimental stressor (nose-sling), or an i.v. infusion of ACTH, or CRF and vasopressin (Janssens *et al.*, 1994; 1995a,b). In addition, an increased reactivity of the sympathetic nervous system (reflected by an increased heart rate response to feeding) was observed in long-term tether housed pigs (Schouten *et al.*, 1991).

The mechanisms responsible for these changes likely include alterations in endogenous opioid systems (Schouten and Rushen, 1993; Janssens *et al.*, 1995b). Activation of endogenous opioid systems is a major response to a variety of environmental stressors. Endogenous opioid systems are involved in a wide range of physiological functions, and also play a role in the regulation of behaviour. The

functional consequence of endogenous opioid activation is generally an inhibition of physiological and behavioural stress responses (Amit and Galina, 1988; Morris *et al.*, 1990; Fontana *et al.*, 1997).

The aim of the present study was to further investigate the role of endogenous opioids in pigs' responsivity to acute stressful stimulation and to study the effects of long-term tether housing. We used an aversive stimulus to challenge the animals, fixation with a nose-sling. This is a method commonly applied by farmers and veterinarians to perform minor procedures such as medical examinations and injections. The nose-sling procedure represents an acute stressor that activates the hypothalamic-pituitary-adrenocortical axis as well as endogenous opioid systems (Rushen and Ladewig, 1991; Rushen *et al.*, 1993; Janssens *et al.*, 1995b). In view of the role of opioid systems in the regulation of cardiovascular and behavioural responses (Holaday, 1983; Akil *et al.*, 1984; Feuerstein and Siren, 1987) we investigated the effect of pretreatment with the opioid receptor antagonist naloxone on challenge-induced responses in pigs before and after long-term tether housing. Naloxone is a non-selective opioid receptor antagonist that blocks mu-, delta- as well as kappa-opioid receptors and, thus, antagonizes the effect of opioid activity.

MATERIALS AND METHODS

Experimental design

A longitudinal experimental design was used with two groups of animals, an experimental group and a control group. During the first 11 weeks of the experiment (period I) all animals were housed loose in individual pens. At the end of this period the animals of the experimental group were moved to individual tether stalls and were housed tethered until the end of the experiment, 11 weeks later (period II). The animals of the control group were not moved. They were loose housed during the first as well as the second 11-week period.

The pigs were challenged in a 15 min nose-sling test. Tests were performed 3 and 2 weeks prior to tethering (period I) and again after 10 and 11 weeks of tether housing for the experimental group (period II). The control group was tested at time points matching those of the experimental group. Immediately before the nose-sling challenge the pigs were pretreated with an i.v. injection of either physiological saline or naloxone to investigate the involvement of endogenous opioid systems in the pigs' responses. Heart rate was measured and video recordings of the animals' behaviour were made.

Animals and housing

In the experiment 17 female nulliparous pigs (gilts) (Great Yorkshire x British Landrace, Pig Improvement Company, UK) were used. At the start of the experiment the body weight of the animals was 115 ± 10 kg (mean ± SD). For reasons discussed elsewhere (Janssens *et al.*, 1995b) all animals were surgically fitted with a permanent jugular vein catheter. Initially, all animals were housed loose in individual pens measuring 2 m x 3 m. The floor of the pens was solid concrete and covered with deep straw except for a slatted dunging area (1 m x 2 m) at the rear. After 11 weeks of loose housing the animals of the experimental group (n=12) were moved to individual tether stalls. The animals were tethered by the neck with a 50 cm long heavy gauge chain connected to the floor. The floor was solid concrete, and was covered with rubber mats where the animals stood. In the area behind the gilts a small quantity of wood shavings was placed to help keep the area dry. The animals of the control group (n=5) were housed loose during the entire experiment.

In both housing systems a 40 cm long chain was suspended above the food trough, as a substrate for stereotypies. The animals were fed 1.25 kg of a pelleted dry sow feed twice a day (at 9:00 h and 16:00 h), by hand. To prevent them from associating the presence of people with feeding, they were conditioned with a bell signal that always preceded the delivery of food. Water was available *ad libitum* through nipple drinkers. Lights were on between 7:30 h and 19:00 h and room temperature ranged from 15 to 25°C.

Nose-sling challenge

Each nose-sling challenge started at 10:15 h. A rope was tied around the upper jaw of the animals and was attached to one of the bars of the pen. The pigs were restrained in this way for 15 min. Immediately before the nose-sling was applied (at t=0 min) the animals were pretreated with an i.v. bolus injection (via the catheter) of either 0.5 mg/kg body weight naloxone (an opioid receptor antagonist) dissolved in 5 ml sterile physiological saline (0.9% NaCl; NPBI B.V., The Netherlands) or 5 ml physiological saline (control). Eight animals received a saline injection in the first nose-sling test and were injected with naloxone in the second test (1 week later); for the other nine animals the order was reversed. For the third and fourth nose-sling test (after 10 and 11 weeks of tether housing) the same procedure was applied.

Heart rate measurements

Heart rate was measured using the Polar Sport Tester™ (Polar Electro, Finland). A chest band was attached at the pig containing conductive gelled electrodes and the sensor/transmitter for heart rate measurements. On the back of the pig, fixed to the chest band, was a small plastic box (8 x 4 x 2 cm) containing the heart rate monitor. Heart rate interval capacity was set at 15 sec. Heart rate measurements started 45 min before the beginning of the nose-sling challenge and ended 165 min after the end of the challenge. Mean heart rate values were calculated for 3 time periods preceding the nose-sling challenge (period 1: -45 to -30 min; period 2: -30 to -15 min and period 3: -15 to 0 min), 3 time periods during the challenge (period 4: 0-5 min; period 5: 5-10 min and period 6: 10-15 min) and 6 time periods after the end of the challenge (period 7: 15-30 min; period 8: 30-60 min; period 9: 60-90 min; period 10: 90-120 min; period 11: 120-150 min and period 12: 150-180 min).

Behavioural observations

Each nose-sling challenge was recorded on video. Video recordings started and ended simultaneously with the heart rate measurements (at t=-45 min and t=180 min, respectively). Behavioural records were analyzed by focal animal observation. The observation time was divided in 12 time periods matching those used for the heart rate measurements. The analysis of the behaviour during the 15 min nose-sling test (periods 4, 5 and 6) included the following behavioural elements:

- resistance: pulling at the nose-sling, often screaming loudly
- standing quietly, without showing resistance, sometimes vocalizing
- lying or hanging at the nose-sling, sometimes grunting softly
  This behavioural element was called 'lying' when the animals' forelegs did and 'hanging' when they did not touch the floor.

Durations of behavioural elements were expressed as percentages of total observation time. In each of the 12 time periods the time was observed the animals were inactive (lying still, eyes closed), and the activity level (100% - %inactive) was calculated.

Statistical analysis

The heart rate data were normalized by log transformation before statistical analysis. The data were analyzed using a split-plot model (General Linear Models (GLM) procedure of SAS) (SAS, 1989). The main factors were: group (experimental or control), pig identity (nested within group), pretreatment (saline or naloxone), time (period 1 to 12) and order of pretreatment. The heart rate data measured during the first 11-week period of the experiment of both groups (loose housing conditions) and the activity level of each pig were used as covariates in the analysis. The effect of group was tested against the animal (pig identity) effect, all other factors and interactions were tested against the overall error term. The factor order of pretreatment was later erased from the analysis since it did not significantly contribute to the model. Pairwise comparisons were performed using least square mean differences at the overall 0.05 level of significance.

Since log transformation could not correct for skewedness of the behavioural data, statistical analyses were performed using non-parametric tests (SPSS, 1990). Differences in the durations of the three behavioural elements (resistance, hanging and standing) between animals of the experimental and the control group were tested using the Mann-Witney U test. Differences within groups between saline and naloxone pretreatment, as well as differences between loose and tether housing were tested by Wilcoxon matched pairs signed ranks test.

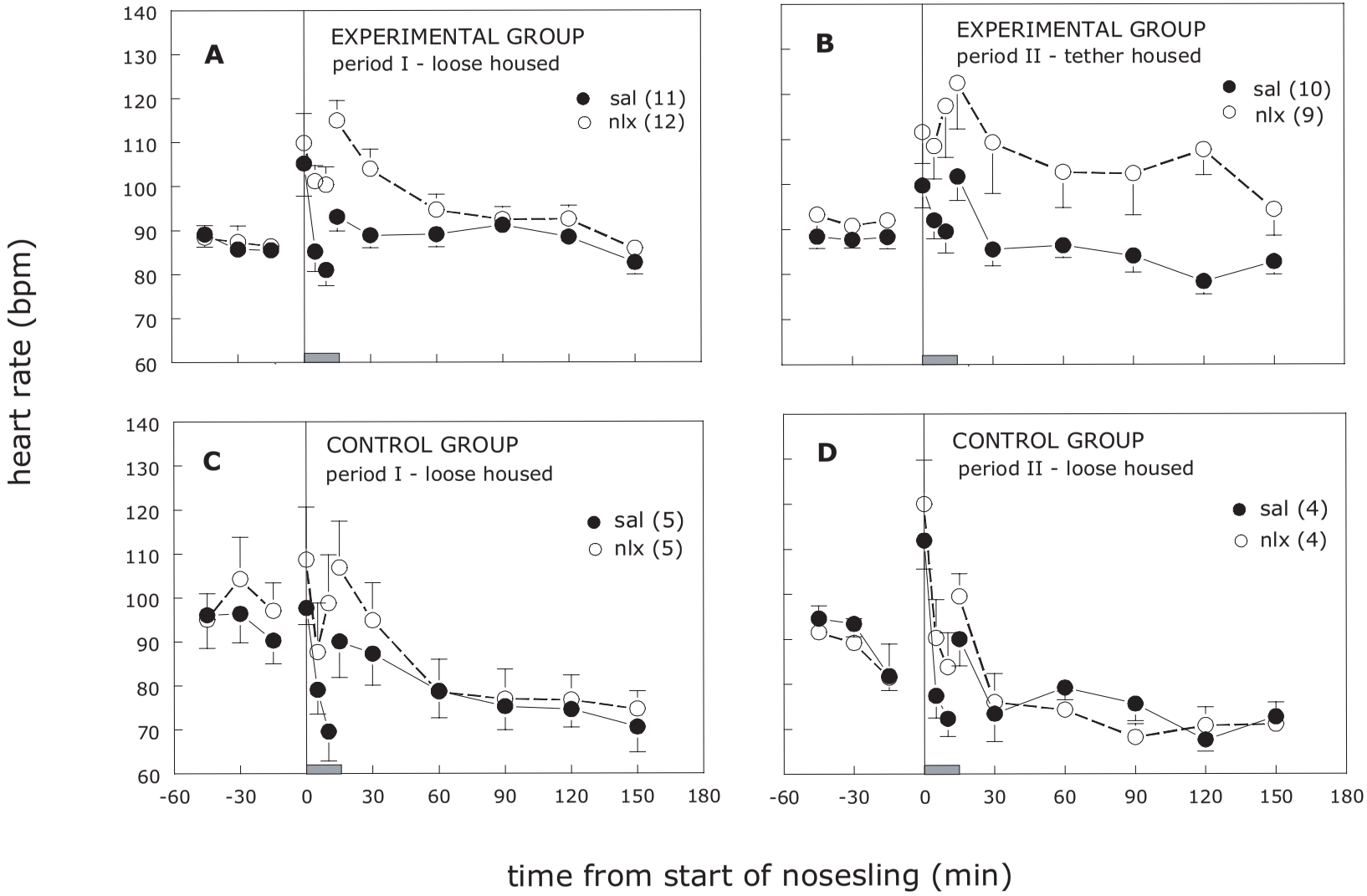
Results are expressed as mean ± SEM. The heart rate results are presented as untransformed data, with significance symbols derived from statistical analysis of the log-transformed data. The criterion for statistical significance was $p \leq 0.05$.

Due to technical problems and occasional illness of animals, a number of pigs (of both experimental and control group) could not be used for data collection at some stage during the experiment. The number of animals in each treatment is given in the Results.

RESULTS

Heart rate

The time course of the pigs' heart rate response to the nose-sling challenge is shown in Figure 1.


A
EXPERIMENTAL GROUP
period I - loose housed
sal (11)
nlx (12)
B
EXPERIMENTAL GROUP
period II - tether housed
sal (10)
nlx (9)
C
CONTROL GROUP
period I - loose housed
sal (5)
nlx (5)
D
CONTROL GROUP
period II - loose housed
sal (4)
nlx (4)
heart rate (bpm)
time from start of nosesling (min)
140
130
120
110
100
90
80
70
60
-60
-30
0
30
60
90
120
150
180

←

Figure 1. Heart rate response to a 15 min nose-sling challenge.

The experimental group was challenged while loose housed (period I; Figure A) and after 10-11 weeks of tether housing (period II; Figure B). The control group was loose housed during the entire experiment and was tested in the first (Figure C) and the second (Figure D) period of the experiment at time points matching those of the experimental group.

Immediately before the nose-sling was applied (at t=0 min) the pigs were pretreated with an i.v. injection of naloxone (0.5 mg/kg body weight) or with saline (5 ml).

The data are represented as mean ± SEM in 12 time periods (see Materials and methods) with the number of animals in each treatment in brackets. The 15 min nose-sling challenge is represented by the grey bar.

The heart rate values differed significantly over time ($F(11,234)=4.51$; $p<0.001$). The challenge induced a sharp rise in heart rate in all animals. Following this peak, heart rate dropped markedly to pre-challenge levels or even below this line. Releasing the pigs from the nose-sling induced another peak in heart rate in most animals. Thereafter, heart rate returned to baseline levels.

By using the heart rate measured during the first period of both groups (loose housing conditions) as a covariate in the analysis we corrected for group differences in this period. In the analysis we compared, thus, the experimental/ tethered group with the control/loose group in the second period.

The statistical analysis showed a significant interaction between the effects of group and pretreatment ($F(1,234)=10.36$; $p<0.01$). There was no difference in mean heart rate values between animals of the experimental/tethered group and those of the control/loose group when the animals were pretreated with saline (experimental/tethered: 89±1 bpm; control/loose: 83±2 bpm; mean±SEM; $p>0.05$). However, when pretreated with naloxone the pigs of the experimental/ tethered group showed higher mean heart rate values than those of the control/loose group (experimental/tethered: 104±2 bpm; control/loose: 86±3 bpm; $p<0.001$). The difference in heart rate between saline and naloxone pretreated animals was statistically significant only in the experimental/tethered

group (p<0.001) and not in the control/loose group (p>0.05).
The time course of the heart rate did not differ between groups and pretreatments, as there was no group*time interaction (F(11,234)=2.67), no pretreatment*time interaction (F(11,234)=0.59) and no group*pretreatment*time interaction (F(11,234)=0.39) (p>0.05 in all cases).

Table 1. Behavioural response to a 15 min nose-sling challenge.

Percentage of time (mean ± SEM) the animals of the experimental group and those of the control group spent hanging, standing and showing resistance during the 15 min nose-sling challenge, after pretreatment with saline or naloxone during the first and the second 11-week period of the experiment.

During the first period both groups were loose housed, during the second period the experimental group was tether housed, whereas the control group remained loose housed.

n = number of animals in the experimental/control group

		LOOSE		EXP.TETHERED / CONTR.LOOSE	
		SALINE (n=12/*4*)	NALOXONE (n=12/*5*)	SALINE (n=10/*4*)	NALOXONE (n=10/*4*)
Hanging	Exp.	10 ± 7[a,b]	2 ± 1[a]	0 ± 0[b]	2 ± 2
	Contr.	*4 ± 3*	*0 ± 0*	*0 ± 0*	*0 ± 0*
Standing	Exp.	84 ± 7	89 ± 4	95 ± 2	91 ± 3
	Contr.	*90 ± 3*	*92 ± 2*	*99 ± 0*	*98 ± 1*
Resistance	Exp.	6 ± 2	9 ± 2	5 ± 2	7 ± 2
	Contr.	*6 ± 1*	*8 ± 2*	*1 ± 0*	*2 ± 1*

[a] Tendency for significant difference between responses after pretreatment with saline and naloxone (p<0.10)

[b] Significant difference between responses during loose housing and tether housing (p<0.05)

Behaviour

Immediately after the nose-sling had been applied the animals started pulling the rope and screaming loudly. Gradually, the bouts of resistance behaviour occurred less frequently, the gilts became quiet and eventually some lay or hung at the nose-sling. The pigs spent 3±1% (overall mean ± SEM, n=51) of the time hanging or lying at the nose-sling and 90±1% standing quietly without showing resistance. The percentage of time showing resistance behaviour was 7±1% (Table 1).

No between group differences in behavioural response were found, neither in the first nor in the second 11-week period of the experiment. Comparisons within the experimental group of the response during loose housing with the reaction after 10-11 weeks of tether housing showed a significant decrease in lying/hanging at the nose-sling during the latter condition ($p<0.05$; after saline pretreatment). The percentage of time showing resistance and the time standing quietly without showing resistance were not affected by tether housing ($p>0.05$). There were no effects of naloxone on the behavioural response measured, although there was a trend towards less hanging during the first (loose housing) period after naloxone pretreatment as compared to saline pretreatment ($p<0.10$). Within the control group neither a naloxone effect nor differences between the first and the second period of loose housing were found.

DISCUSSION

In the present study we investigated the effect of long-term tether housing on heart rate and behavioural responses to an acute challenge with a nose-sling in pigs. In accordance with the notion that the nose-sling procedure represents an acute stressor, the animals showed resistance behaviour with loud vocalizations, and a sharp rise in heart rate. Other studies using the nose-sling reported an activation of the hypothalamic-pituitary-adrenocortical axis, resulting in increased plasma levels of ACTH and cortisol (Rushen and Ladewig, 1991; Rushen *et al.*, 1993; Janssens *et al.*, 1995b). Following this initial phase of resistance, the animals calmed down and appeared to become sedated. In some pigs this effect was so strong that they ended up lying or hanging at the nose-sling at the end of the 15 min challenge. The heart rate of the animals dropped to pre-challenge levels or even below this line in the second phase of the response. Analogous effects on behaviour and heart rate have been found after application of a "twitch" in horses (Lagerweij *et al.*, 1984).

To study the effect of long-term tether housing on the heart rate response we compared the heart rate of the two groups of animals during the second period of the experiment, when the experimental group was housed tethered and the control group was loose housed. Pretreatment with naloxone during this period increased the heart rate response of the experimental group. Naloxone, an opioid receptor antagonist, blocks endogenous opioid receptors and prevents, thus, endogenous opioids from exerting their heart rate attenuating effect. As the heart rate during the second period of the experiment was statistically corrected for group differences during the first period (when both groups were loose housed) we can make comparisons over time. Apparently, the effect of naloxone in the experimental group was higher in the second than in the first period of the experiment. This points to an increased (re)activity of endogenous opioids in these animals. In the control group that was loose housed during the entire experiment, no (changes in the) effect of naloxone were found (it should be noted, however, that the number of animals in this group was rather small (n=5)). Therefore, the increase in the impact of endogenous opioid systems observed in the experimental group cannot be attributed to factors like individual housing per se, or to the animals' increasing age, but probably resulted from tether housing. In the same animals we found evidence pointing to an increased impact of endogenous opioid systems that modulate pituitary-adrenocortical responses (Janssens *et al.*, 1995b). In another study, it has been demonstrated that the opioid-mediated inhibition of feeding-induced cardiovascular responses increased with long-term tether housing (Schouten *et al.*, 1991). Long-term tether housing apparently increases the impact of endogenous opioid systems, which mitigate the heart rate response to additional acute stimuli, regardless of the properties of the stimulus. The contention that long-term tether housing of pigs induces changes in opioid systems is also supported by our previous study, in which we found a progressive decrease in time of the density of opioid receptors in the hippocampus during long-term tether housing (Loijens *et al.*, 1999).

There was no difference in heart rate response between the two groups of animals under saline conditions. This indicates that the increased impact of endogenous opioid systems in the experimental group normalized the animals' response, thereby preventing overreaction of the cardiovascular system. Increased cardiovascular reactivity to stress is a physiological adaptive response, but in the long-term it is potentially harmful and may lead to cardiovascular disorders

(Johansson *et al.*, 1974; Kaplan *et al.*, 1983; Palatini, 1999). The increased impact of endogenous opioid systems in the experimental group may, thus, be of adaptive value. It may be a means of coping with the total loss of control induced by long-term tether housing. Pigs subjected chronically to this aversive housing system have been found to show changes in autonomic and endocrine systems comparable with those observed in human stress pathology (Schouten *et al.*, 1991; Janssens *et al.*, 1993; 1994). Long-term tether housing of pigs is, therefore, generally recognized as a condition of chronic stress, and the welfare of animals in such housing conditions is thought to be poor.
In contrast to the heart rate response, there were no (changes in the) effect of naloxone on the behavioural response to the nose-sling test. Apparently, the change in opioid (re)activity is not relevant to the behavioural response in this test. The present data illustrate that although one measure can indicate that animals have difficulties in coping with their (housing) conditions, it is essential that a variety of (stress) indicators be used if an adequate evaluation of housing systems and a justified assessment of the welfare of animals in such systems is to be obtained (Broom, 1991; Broom and Johnson, 1993).

Summarizing, our results confirm previous suggestions (Schouten *et al.*, 1991; Janssens *et al.*, 1995b; Loijens *et al.*, 1999) that the activity of the opioid system changes during long-term tether housing. The present data point to an increased impact of endogenous opioid systems modifying heart rate responses to acute stress. The adaptation of opioid systems may protect the animals from excessive physiological reactions to acute stressors and thus may be of importance as a coping mechanism.

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Chapter 3

BRAIN OPIOID RECEPTOR DENSITY RELATES TO STEREOTYPIES IN CHRONICALLY STRESSED PIGS

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ABSTRACT

Opioid receptor densities were measured in the hippocampus of chronically stressed (tethered) pigs to study the involvement of endogenous opioid systems in stereotypy performance. Three groups of animals were housed tethered for 2 (n=12), 5.5 (n=12) and 8-9 months (n=8), respectively, and the intensity of stereotypy performance was determined. Opioid receptor densities were measured post mortem using membrane binding assays with [^{3}H]naloxone as a ligand. A negative correlation was found between the density of opioid receptors and the intensity of stereotypy performance in the animals that had been housed tethered for 2 months. This correlation seemed to disappear with increasing duration of tether housing. The data further suggest that, associated with the duration of tether housing, there was a gradual decrease in the density of opioid receptors in the left hippocampal lobe of the low-stereotyping animals, but not in the right lobe, nor in the left and right lobes of the high-stereotypers. This suggests that chronic stress leads to a (asymmetrically expressed) progressive loss of opioid receptors in the hippocampus, and that stereotypies exert a mitigating effect on stress-induced changes in opioid receptor densities, supporting the hypothesis that stereotypies help the animals cope with the adverse effects of chronic stress.

INTRODUCTION

Animals in captivity may develop disturbed behaviour, often in the form of stereotypies. Stereotypies have been defined as patterns of behavioural elements that are relatively fixed in form, repetitive and without obvious function (Ödberg, 1978; Mason, 1991). Stereotypies have been described in zoo animals as bears and tigers (pacing) (Meyer-Holzapfel, 1968), and other caged animals such as voles (jumping) (Ödberg, 1986) and canaries (spot-picking and route-tracing) (Keiper, 1970). Stereotypies are also observed in farm animals as chickens (pacing) (Duncan and Wood-Gush, 1974) and veal calves (tongue playing) (Wiepkema *et al.*, 1987).

Domestic pigs often develop stereotypies when they are physically restricted for a long period of time, for instance during tether housing. Pigs that are first tethered fiercely resist, scream loudly and try to escape by pulling and biting the tether chain. They also display other responses typical for acute stress, such as increased plasma levels of ACTH, ß-endorphin and cortisol, and an increased heart rate (Cronin, 1985; Schouten and Wiepkema, 1991; Wiepkema and Schouten, 1992;

Schouten and Rushen, 1993). During long-term tether housing the aversive situation continues, and in addition to behavioural disturbances (stereotypies) the animals develop changes in endocrine and autonomic regulatory systems that are indicative of chronic stress. Chronically elevated resting plasma levels of prolactin and cortisol, flattened circadian rhythmicity of cortisol and changes in hypothalamic-pituitary-adrenal and cardiovascular responsivity have been reported (Janssens *et al.*, 1994a,b; 1995a; Schouten *et al.*, 1991).

In pigs, stereotypies include bar-biting, chewing on the tether chain, head-waving and excessive drinking (Rushen, 1984; Cronin, 1985; Appleby and Lawrence, 1987; Terlouw, 1993). Several explanations have been put forward why animals perform stereotypies (see Mason (1991) for a review). One interesting hypothesis is that stereotypies have de-arousing properties, and help the animals cope with stressful situations (Dantzer and Mormède, 1983; Wiepkema *et al.*, 1987). Indeed, evidence has been presented that the performance of stereotypies is associated with a decrease in heart rate in pigs that are aroused (Schouten and Wiepkema, 1991; Schouten and Wiegant, 1997). First indications about the underlying neurochemical mechanism of stereotypies came from experiments with the opioid receptor blocker naloxone. Naloxone can inhibit the performance of stereotypies in horses (Dodman *et al.*, 1987), bank voles (Kennes *et al.*, 1988) and pigs (Cronin *et al.*, 1985; 1986; Rushen *et al.*, 1990). This suggests that endogenous opioid peptides (endorphins) are involved. Cronin and co-workers (1985) hypothesized that in the course of their development stereotypies become linked to endorphins in the brain. As endorphins have the ability to reduce emotional distress (Lewis *et al.*, 1981), this seems an effective way of coping with stress.

Animals differ markedly in the level of stereotypies shown. This may reflect differences between individuals in sensitivity to stress, and/or differences in coping style (Mason, 1991). If stereotypies are associated with endogenous opioids in the brain, the question arises whether high and low-stereotyping animals differ in endogenous opioid function. In this study the opioid receptor density in the hippocampus of chronically stressed (tethered) pigs was determined to investigate its possible relation to the performance of stereotypies. The hippocampus was selected since (a) it plays an important role in responses to stress, particularly when possibilities for coping are limited or absent such as during chronic stress (Henry, 1986) (b) endogenous opioids and opioid receptors are abundant in the hippocampus (Akil *et al.*, 1984) (c) rats with diverging coping style, i.e.

apomorphine susceptible/unsusceptible rats differ in stress-induced hippocampal dynorphin release (Cools *et al.*, 1993), and apomorphine susceptibility in pigs too has been shown to relate to coping style (Bolhuis *et al.*, 2000) (d) neuronal cell number in the dentate gyrus of the hippocampus in long-term tether housed pigs relates to salivary cortisol concentration (Van der Beek *et al.*, 1997).

MATERIALS AND METHODS

Animals and housing

All animals used in this study were purchased from commercial breeding farms, and were allowed at least two weeks of acclimatisation in individual stalls before the start of the experiment (i.e. tethering). Three separate groups of animals were used. As the experimental set-up was such that the duration of tether housing varied between the three groups of animals, we had to synchronize the weight of the animals either at the start or at the end of the experiment. We chose the latter. The animals' weight ranged between 200 and 230 kg at the end of the experiment.

Group 1. The pigs of Group 1 were twelve crossbred primiparous sows (Dutch Landrace x Yorkshire). The animals were housed in individual tether stalls, each 65 cm wide, and were tethered by the neck with a 50 cm long heavy gauge chain connected to the floor. The floor was solid concrete and covered with rubber mats where the animals stood. In the front of each stall a food trough was placed with a nipple drinker above it. Water was available *ad libitum*. Above the food trough a 40 cm long extra chain was suspended, as a substrate for stereotypies. The animals were fed 1.25 kg of a pelleted dry sow feed at 9:00 h and 16:00 h, by hand. Immediately before each food delivery a bell was sounded to prevent the animals from associating the presence of people with feeding. Immediately after food delivery the area behind the animals was cleaned and a small quantity of wood shavings was placed there to help keep the area dry. The animals had a good view of the environment and auditory, olfactory, and visual contact was possible between the animals. Tactile contact was possible between animals in adjacent pens. Lights were on between 6:30 h and 19:00 h. Room temperature ranged from 15 to 25°C. Housing conditions were the same as has been described before (Janssens *et al.*, 1995a,b). After the animals had been housed tethered for 2 months they were slaughtered.

Group 2. Group 2 consisted of twelve crossbred sows (Great Yorkshire x Dutch Landrace (n=5) and Great Yorkshire x British Landrace (n=7) of varying parity

(1-11). The animals were kept tethered for 5.5 months under similar conditions as Group 1, and were slaughtered at the end of this period.

Group 3. Group 3 were eight crossbred gilts (British Landrace x Great Yorkshire). The animals were housed tethered under similar conditions as Group 1 and 2, for 8-9 months, and were slaughtered at the end of this period.

Behavioural observations

The behaviour of the animals was observed in the post-feeding period in which stereotypies are most pronounced.

The analysis included the following behavioural elements:

a) body posture: either standing or sitting or lying

b) activity pattern:

1) rooting: making rooting movements on the floor
2) drinker activity: any contact with the nipple drinker whether or not water was consumed
3) head in trough: eating or drinking water from the food trough or rooting, nosing or licking in the trough
4) bar biting: taking one of the bars of the pen in the mouth
5) chain manipulation: any contact with the extra chain, mostly chewing the chain
6) vacuum chewing: making chewing movements without having anything in the mouth
   or tongue playing: exposing the tongue in an unusual fashion while stretching the lower jaw horizontally
7) alert: looking around attentively, ears pricked
8) rest: lying inactive, eyes closed
9) other: all other behaviours not fitting in one of the preceding categories (including yawning, urinating, defecating, stretching, scratching)

Behavioural observations were made using a time sampling method. The behaviour was scored with a fixed interval of 1 min starting 15 min after food delivery until 45 min after delivery.

Behavioural observations of **Group 1** were made on one day after 56-68 days of tether housing. Observations were made following either the morning or the afternoon feeding session, after it had been assessed (after one month of tether

housing) that the behavioural profile of the animals was not significantly different between morning and afternoon in the 45 min post-feeding period.
One animal of Group 1 was ill at the time of observation and was not observed. It remained in the experiment, however, recovered and continued the normal procedure of the group.
For **Group 2** three morning feeding sessions at the end of the period of 5.5 months of tether housing were selected to observe the behaviour of the animals. The data of the three observation days were pooled and averaged.
The behaviour of the animals of **Group 3** was observed on one day after 6.5 months of tether housing (7 weeks before slaughtering). Observations were made both after the morning and the afternoon feeding and the values of both feeding sessions were pooled and averaged.
For each animal the relative frequency (% of total number of scores) performing each behavioural category was calculated.

Definition of stereotypies

Stereotypies have been described as morphologically similar patterns of behavioural elements, which are characteristic per individual, are performed repetitively and have no obvious function (Ödberg, 1978). Based on previous work (Cronin, 1985; Rushen *et al.*, 1990; Terlouw, 1993), and on our own observations of the animals in this study, we considered repetitive chain and bar related activities, and vacuum oral activities (vacuum chewing, tongue playing) as stereotypies. The stereotypy level of an animal was calculated as the sum of these behavioural categories.

Receptor binding assays

Membrane preparation

At the end of the period of tether housing, the animals were herd into a lorry and transported to a nearby slaughter facility. Transportation took approximately 15 min. The animals were slaughtered after electrocution immediately after arriving at the slaughterhouse. Skulls were opened, brains were collected, and the left and right lobes of the hippocampus were dissected according to Salinas-Zeballos *et al.* (1986). Only undamaged brain samples were collected. The samples were immediately frozen in isopentane (2-methyl-butane) cooled with dry ice, and stored at -70°C until use.

The samples were then defrosted, weighed and homogenized in 0.5 M Tris-HCl (pH=7.4) (10% wt/vol) using a glass homogenizer with teflon pestle (20 up and down strokes, 10,000 rpm, 4°C). The homogenates were centrifuged at 50,000 g_{av} for 20 min at 4°C, the supernatants were discarded and the pellets were resuspended in Tris-HCl (10% wt/vol). Subsequently, the samples were preincubated at 37°C for 30 min to degrade endogenous opioids present in the samples. After two wash steps with Tris-HCl, membranes were suspended (10% wt/vol) in Tris-HCl containing 0.1% (wt/vol) bovine serum albumin (BSA) (Sigma, Axel, The Netherlands). Protein concentration was determined using the method of Lowry *et al.* (1951) with bovine serum albumin as a standard.

Membrane binding assays

Binding assays were carried out with [^{3}H]-naloxone ([N-allyl-2,3-^{3}H]-naloxone, NEN, Du Pont, Den Bosch, The Netherlands) as a radioligand, in a concentration range of 0.25 nM - 15 nM (12 concentrations). Non-specific binding was assessed in parallel for each tracer concentration using excess (15 µM) non-labelled naloxone or naltrexone (Sigma). Incubations (in duplicate) were performed in a total volume of 500 µl Tris-HCl containing 0.1% BSA. Final membrane concentration was 0.5 mg protein/ml. Incubation was performed until equilibrium at 25°C for 60 min and was terminated by rapid filtration over pre-soaked Whatman GF/B filters (Brandel, St. Albans, UK). The filters were washed 3x with 5 ml saline (0.9% NaCl) (0°C), transferred to scintillation vials and incubated with 1 ml Soluene (Packard, Groningen, The Netherlands) for at least 12 hr to dissolve the radioactivity. After addition of 9 ml scintillation cocktail (Ultima Gold, Packard), filters were counted for radioactivity.

Specific binding was calculated by subtraction of non-specific binding from total binding. Specific binding ranged from 36% (highest radioligand concentration) to 77% (lowest radioligand concentration) of total binding. Filter binding was less than 10% of total binding. Displacement of radioligand binding with either non-labelled naloxone or naltrexone yielded similar values of non-specific binding. The density of naloxone binding sites (B_{max}, maximum binding capacity) and the equilibrium dissociation constant (K_D) were computed using a Scatchard plot analysis of the data.

Statistical analysis

Differences in B_{max} and K_D were tested for significance using analyses of covariance (GLM) (SAS, 1989). The model included group, parity, breed and side of the brain (left or right) as main factors, and the interactions between group and brain side and between parity and breed. Stereotypy level was included as a covariable. The factors parity and breed and their interaction were later erased from the data analyses since they did not significantly contribute to the models.

To test whether there was a relationship between the level of stereotypies and B_{max} and K_D in the left and right hippocampal lobes, Spearman rank correlations were calculated (SAS, 1989).

The criterion for statistical significance was set at $p \leq 0.05$. Results are expressed as the mean ± SEM.

RESULTS

Behavioural data

Animals showing stereotypies were found in each group. However, a high variation between individuals in the level of stereotypy performance was seen. The animals of Group 1 spend 0-77% of the observation time performing stereotypies. In Group 2 the stereotypy level ranged from 0% to 83% and in Group 3 from 10% to 85%.

Stereotypies mainly involved manipulation of the chain, 13 animals showed stereotypic chain manipulation. In Group 3 one animal showed stereotypic vacuum chewing and one had developed tongue playing as a stereotypy. In Group 1 one pig showed excessive rooting in the food trough (77% of the observation time) in a very stereotypic pattern and it was decided to include rooting as a stereotypy for this animal.

Receptor data

At the time of slaughtering some brains were damaged and could not be dissected adequately. For Group 1 there remained 9 left hippocampal lobes and 8 right hippocampal lobes. For Group 2, 11 (left) and 10 (right), respectively, and for Group 3, 8 (left) and 5 (right), respectively. A representative example of a saturation curve and Scatchard plot of naloxone binding is shown in Figure 1.

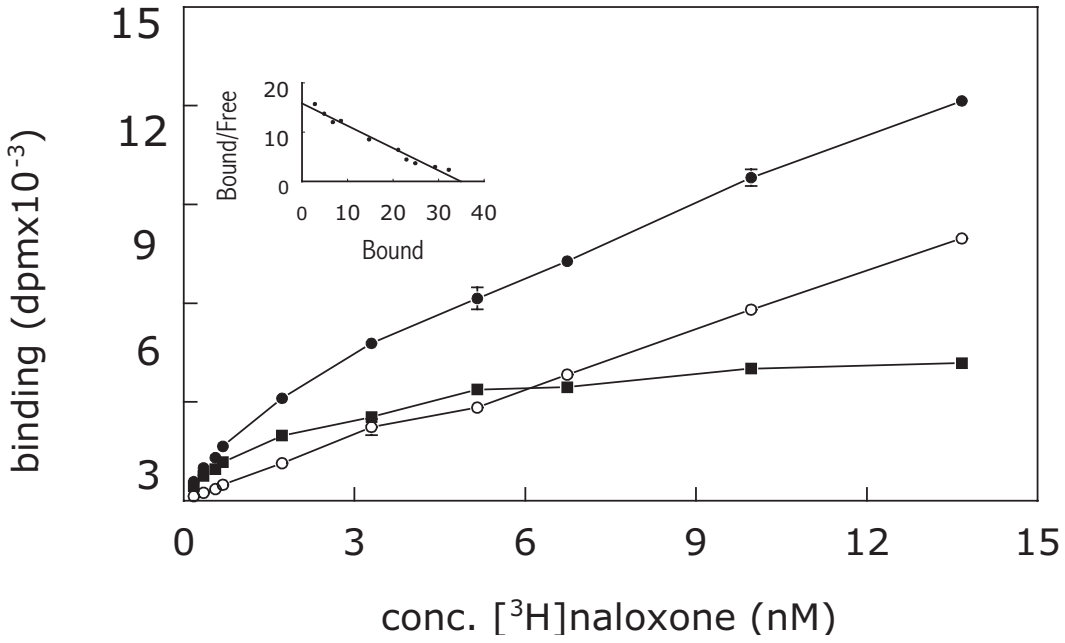

Figure 1. [^{3}H]-naloxone binding in the hippocampus (right lobe; pig # 1, Group 3): total (-●-), non-specific (-○-) and specific binding (-■-) (mean ± SEM).
Insert: Scatchard plot of the data (X-axis: [^{3}H]-naloxone bound specifically (fmol/mg protein); Y-axis: [^{3}H]-naloxone bound specifically (fmol/mg protein)/ free [^{3}H]-naloxone (nM)

The B_{max} in the hippocampus was not significantly different for the three groups of animals (F(2,43)=0.26; p=0.77). However, a highly significant difference was found between the B_{max} in the left and the right hippocampal lobe (F(1,43)= 24.22; p=0.0001), with the B_{max} in the right lobe being higher than in the left lobe. This left/right difference was found in each of the three groups, since there was no significant interaction between group and side of the brain (F(2,43)=1.20; p=0.31). The B_{max} values in Group 1 were 22±2 (n=9) (left) and 27±2 fmol/mg protein (n=8) (right), in Group 2: 20±2 (n=11) (left) and 27±1 fmol/mg protein (n=10) (right), and in Group 3: 16±2 (n=8) (left) and 29±5 fmol/mg protein (n=5) (right). The dissociation constant (K_D) in the left hippocampal lobe did not differ significantly from the K_D in the right lobe (left: K_D = 1.9 ± 0.1 nM; mean ± SEM of the three groups of animals; n=28; right: K_D = 1.8 ± 0.3 nM; n=23; p=0.70). No group differences in K_D values were found.

Correlations between stereotypy level and B_{max}/K_D

Correlations between stereotypy level and B_{max} in the left and right lobes of the hippocampus of the three groups of animals are shown in Figure 2.

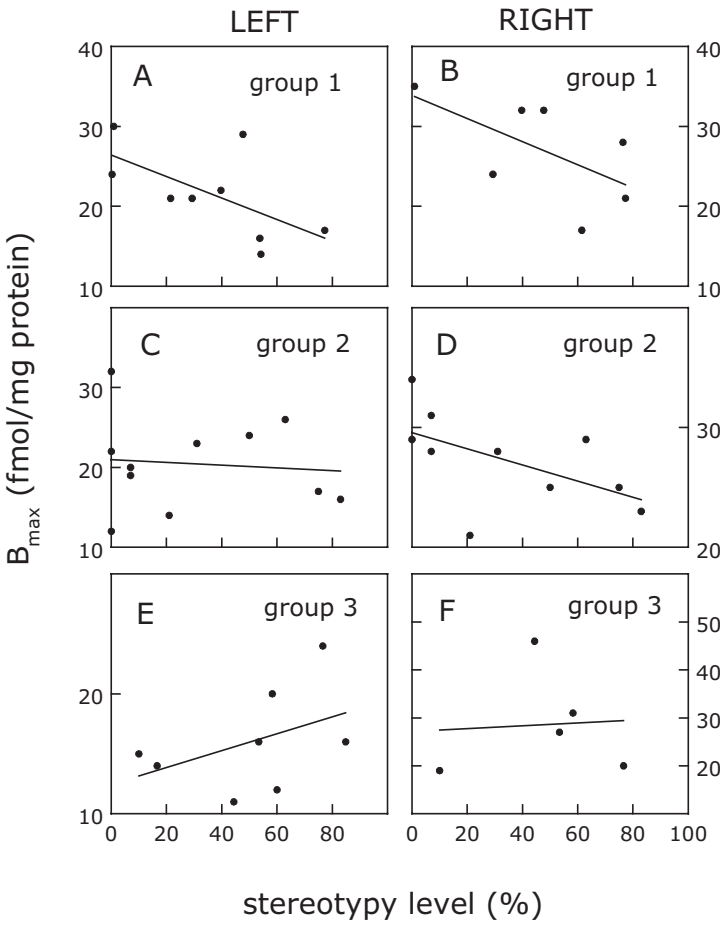

Figure 2. Relation between stereotypy level (relative frequency, % of total number of scores) and the density of naloxone binding sites (B_{max}) in the left (left panels) and right (right panels) hippocampal lobe of Group 1 (Figure 2A: left lobe and Figure 2B: right lobe), Group 2 (C and D, resp.) and Group 3 (E and F, resp.). Animals of Group 1, 2 and 3 were housed tethered for 2, 5.5 and 8-9 months, respectively.

The B_{max} correlated negatively with the stereotypy level of the animals of Group 1. The correlation was significant for the left (r=-0.73; *p*=0.04), but not for the right hippocampal lobe (r=-0.63; *p*=0.13). For Group 2, a significant negative correlation was found between the B_{max} in the right hippocampal lobe and stereotypy level (r=-0.63; *p*=0.05). For the left hippocampal lobe of Group 2 there was no correlation (r=-0.05; *p*=0.88). The B_{max} values of Group 3 did not correlate significantly with the stereotypy level of the animals (left: r=+0.48; *p*=0.23; right: r=+0.10; *p*=0.87).
The correlations between K_D and stereotypy level did not reach significance.

DISCUSSION

The present study was performed to investigate further the involvement of endogenous opioid systems in stereotypies of pigs. To that end, the density of opioid receptors in the hippocampus of individual pigs was determined using membrane binding assays with the opioid receptor antagonist naloxone. Naloxone was chosen as the ligand, since it is a non-selective opioid receptor antagonist known to inhibit stress-induced stereotypies in several species, including pigs (Cronin *et al.*, 1985; 1986; Dodman *et al.*, 1987; Kennes *et al.*, 1988; Rushen *et al.*, 1990). In view of this non-selectivity, the binding sites detected may represent μ-, κ- as well as δ-opioid receptors. The densities of naloxone binding sites (B_{max}) found are in a range comparable with data previously reported in other species, e.g. gerbils (Araki *et al.*, 1993; Kanai *et al.*, 1994) and rhesus monkeys (LaMotte *et al.*, 1978).
We found a negative correlation between the intensity of stereotypy performance and the density of opioid receptors in the hippocampus of the group that had been housed tethered for 2 months, indicating that animals displaying high levels of stereotypies have less opioid receptors in this brain region than low-stereotyping ones. A similar (negative) correlation has been found in the hypothalamus of the same animals (Loijens, Schouten, Wiegant, unpublished results). A study by Zanella *et al.* (1991) indicated that the association between stereotypies of sows (3-4 months of tether housing) and B_{max} is receptor type specific. They found that kappa-binding in the frontal cortex inversely correlated with the performance of tongue-rolling and sham-chewing stereotypies, mu-binding negatively correlated with tongue-rolling, but not with sham-chewing, and delta-binding neither related to tongue-rolling, nor to sham-chewing.

The differences in receptor density found could be the result of down-regulation in high-stereotyping animals triggered by increased release of opioids. Increased opioid release is thought to occur during stereotypies in view of the association between the performance of stress-induced stereotypies and endogenous opioid activity, as evidenced by the naloxone sensitivity of stereotypies (Cronin *et al.*, 1985; 1986; Dodman *et al.*, 1987; Kennes *et al.*, 1988; Rushen *et al.*, 1990). It is well-known, indeed, that opioid activity can affect receptor numbers in the brain, and opioid receptor up or down-regulation has been observed after prolonged treatment with opioid antagonists and agonists, respectively (Blanchard and Chang, 1988; Belcheva *et al.*, 1994).

An alternative explanation may be that the stress of tether housing was primarily responsible for increased opioid receptor density and that the high-stereotyping animals found a way to "normalize" their receptor densities which the low-stereotyping pigs did not. That low-stereotyping sows may have greater difficulties in coping with the stress of tether housing is suggested by the finding of Schouten and Wiepkema (1991) that these animals have higher mean heart rate values after feeding (when stereotypy performance is most pronounced) than high-stereotypers. Performance of stereotypies also seems to lower feeding-induced cortisol responses (Schouten *et al.*, 1996). It has been reported that acute, repeated and chronic stress can affect opioid receptor density, and both up and down-regulation have been described (Zeman *et al.*, 1988; Nabeshima *et al.*, 1985; Zanella *et al.*, 1996).

A third explanation may be that the differences in B_{max} between high and low-stereotyping animals were already present prior to tether housing and the development of stereotypies, and that they reflect differences in coping style. Types of animals using different strategies of responding to, and coping with stress have been described in pigs (Schouten and Wiepkema, 1991; Hessing *et al.*, 1994) as in other species (Von Holst, 1986; Benus *et al.*, 1987; Wiepkema *et al.*, 1987). Such types of animals may also differ in their responsivity to opioids, suggesting differences in the organization of their opioid systems (Frischknecht *et al.*, 1988; Deroche *et al.*, 1993).

When the B_{max} data of the three groups of animals were compared, striking differences were found. The negative correlation between B_{max} and stereotypies that was observed in Group 1, had partly disappeared in Group 2 and was totally absent in Group 3. It seemed that the binding sites in the left hippocampal lobe

were most sensitive in this respect. It is not likely that the factors breed or parity were involved since neither of them could explain the variance in B_{max}. Thus, duration of tether housing was the factor most likely responsible for the disappearance of the initial correlation between B_{max} and stereotypies. As no significant difference in the mean B_{max} was found between the three groups of animals, this could mean that changes in B_{max} had occurred in part of the pigs. For more detailed *post-hoc* analysis of the data, the animals were classified as high or low-stereotypers according to whether the percentage of time spent on stereotypies was above or below 50%. This yielded subgroups of animals that differed significantly in stereotyping activity (Group 1: p=0.036; Group 2: p=0.003 and Group 3: p=0.004, respectively; Mann-Whitney U test). The mean B_{max} values for these subgroups are shown in Figure 3. Statistical analysis (Kruskal-Wallis test; SAS, 1989) yielded a significant difference between the three groups of low-stereotypers in B_{max} for the left (p=0.04), but not for the right hippocampal lobe (p=0.67), nor for the left (p=0.30) and right (p=0.58) lobes of the high-stereotypers. Further analysis (Wilcoxon 2-sample test; SAS, 1989) demonstrated a significant difference in B_{max} between Group 1 and Group 3 (p=0.03), but not between Group 1 and Group 2 (p=0.14), nor between Group 2 and Group 3 (p=0.20). Indeed, these data point to gradually developing changes in receptor populations, and also to highest sensitivity of the left hippocampal lobe in this respect.

The finding that receptor density in the hippocampus decreased with the duration of tether housing in the low rather than in the high-stereotyping animals, makes it unlikely that down-regulation by stereotypy-associated, enhanced release of endogenous opioids is the underlying mechanism. In view of the putative significance of stereotypies as a coping behaviour (Dantzer and Mormède, 1983; Wiepkema *et al.*, 1987; Schouten and Wiepkema, 1991; Schouten and Wiegant, 1997), it can be reasoned that pigs performing stereotypies have lower stress levels, and are less vulnerable to detrimental effects of chronic stress, including those on neurons containing opioid receptors. There exists, indeed, literature indicating that mediators of the stress system (e.g. corticosteroids) can exert or potentiate neurotoxic effects in the brain, and that particularly hippocampal neurons are vulnerable in this respect (Tombaugh *et al.*, 1992; Watanabe *et al.*, 1992; Landfield and Eldridge, 1994; Sapolsky, 1996).

An interesting observation was the consistent difference in opioid receptor density between the left and right hippocampal lobes. In humans, cerebral lateralization, both in anatomy and function, is a well-known phenomenon (Gazzaniga, 1970; Dimond and Beaumont, 1974). However, cerebral laterality is not an exclusively human trait. Morphological, chemical and behavioural indices of brain asymmetries have been reported in a wide range of animal species, from songbirds (Arnold and Bottjer, 1985) to rats (Glick *et al.*, 1979; Diamond *et al.*, 1981; Denenberg and Yutzey, 1985) and nonhuman primates (Ettlinger and Moffett, 1964; LeMay, 1985). Recently, evidence has been provided showing that stress may influence both sides of the brain differently (Carlson *et al.*, 1988; Delrue *et al.*, 1994).

In summary, the present study demonstrates a negative correlation between the density of opioid receptors in the hippocampus and the intensity of stereotypy performance in pigs that had been housed tethered for 2 months. This correlation seemed to disappear in animals that were housed tethered for a longer period of time. The data further suggest that there is a decrease in receptor density, associated with the duration of tether housing, in low but not in high-stereotypers, possibly indicating that stereotypies may have a mitigating effect on stress-induced progressive loss of opioid receptors. This is in line with the idea that stereotypies may serve an adaptive purpose in situations of chronic stress. However, the above described contention does not exclude the possibility of pre-existing differences in opioid receptor density.

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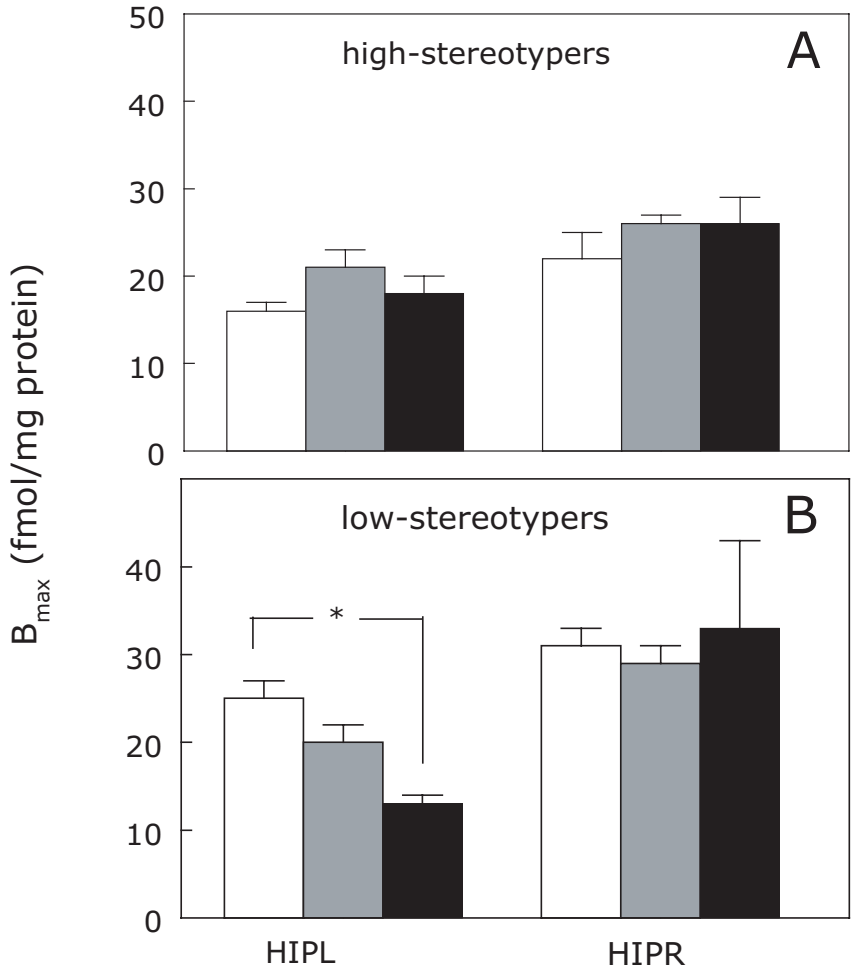

Figure 3. Density of naloxone binding sites (B_{max}) in the left and right hippocampal lobes (HIPL and HIPR) of the high-stereotypers (Figure 3A) and low-stereotypers (Figure 3B) of Group 1 (open bars), Group 2 (grey bars) and Group 3 (black bars) (mean + SEM). Animals of Group 1, 2 and 3 were housed tethered for 2, 5.5 and 8-9 months, respectively.

The number of animals per subgroup was:

high-stereotypers: HIPL: n = 3, 4 and 5 (Group 1, 2 and 3, resp.)
HIPR: n = 3, 4 and 3

low-stereotypers: HIPL: n = 6, 7 and 3
HIPR: n = 4, 6 and 2

The asterisk indicates statistical significance.

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Chapter 4

OPIOID RECEPTOR DENSITY IN THE HYPOTHALAMUS OF LONG-TERM TETHER HOUSED PIGS RELATES TO STEREOTYPIES

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ABSTRACT

Long-term tether housing of pigs has been shown to induce physiological and behavioural symptoms of chronic stress, including stereotypies. There are indications that stereotypies are causally linked to endogenous opioid systems. In the present study we investigated the involvement of endogenous opioid systems in stereotypy performance. Three groups of animals were housed tethered for 2 (Group 1), 5.5 (Group 2) and 8-9 months (Group 3), respectively, and the intensity of stereotypy performance was determined. Post mortem, densities of opioid receptors were measured in the hypothalamus using a membrane binding assay with [^{3}H]naloxone as a ligand.

A negative correlation was found between opioid receptor densities and the level of stereotypy performance in the animals of Group 1 and Group 2, but not in Group 3. Opioid receptor densities in the hypothalamus of Group 3 appeared to be lower than those of the other two groups. The results point to a relationship between stereotypies and opioid receptor densities in the hypothalamus in the 2 and 5.5 months tether housed pigs. In the animals that had been housed tethered for 8-9 months changes in receptor populations seemed to have developed, most likely as a result of long-term tether housing. The data extend our previous results in the hippocampus of the very same animals.

INTRODUCTION

Pigs in modern breeding farms are often housed in tether stalls. Literature reports indicate that this housing system represents a condition of chronic stress for pigs. Persisting disturbances in behaviour (Cronin, 1985; Wiepkema and Schouten, 1992) and in endocrine (Janssens *et al.*, 1993, 1994, 1995a,b) and cardiovascular systems (Schouten *et al.*, 1991), indicative of chronic stress, have been found in long-term tether housed pigs. In this study, we focussed on behavioural disturbances often seen in these animals, i.e. stereotypies. During stereotypy performance increased release of endogenous opioids is thought to occur in view of the association between the performance of stress-induced stereotypies and endogenous opioid activity, as evidenced by the naloxone sensitivity of stereotypies (Dodman *et al.*, 1987; Kennes *et al.*, 1988; Cronin *et al.*, 1985, 1986; Rushen *et al.*, 1990).

In a previous study we have found a negative correlation between the density of opioid receptors in the hippocampus and the intensity of stereotypy performance

in pigs that had been housed tethered for two months (Loijens *et al.*, 1999). This correlation seemed to disappear with increasing duration of tether housing. In addition, a progressive decrease over time of opioid receptor densities was observed in the pigs with low levels of stereotypies in this study. To investigate whether the relationship between stereotypies and opioid receptor density was specific for the hippocampus or whether it could also be found in other brain areas, we measured opioid receptor densities in the hypothalamus, using the very same animals. We selected the hypothalamus since (a) it plays an important role in the integration and coordination of stress responses (b) endogenous opioids and opioid receptors are abundant in the hypothalamus (Akil *et al.*, 1984) (c) in our previous research we have found evidence indicating that long-term tether housing leads to changes in endogenous opioid systems that modulate hypothalamic-pituitary-adrenocortical responses to additional acute stress (Janssens *et al.*, 1995b). These changes most probably occur at the level of the hypothalamus.

MATERIALS AND METHODS

Animals and methodology were the same as those used in our previous study (Loijens *et al.*, 1999). In short, three groups of pigs were housed tethered for 2 (Group 1; n=12), 5.5 (Group 2; n=12) and 8-9 months (Group 3; n=8), respectively. The animals were tethered with a short neck chain and were housed in individual stalls. They were fed 1.25 kg of a pelleted dry sow feed twice daily. Water was available *ad libitum*. At the end of the period of tether housing the intensity of stereotypy performance was determined using a time sampling method. The animals were observed with a fixed interval of 1 min from 15 min till 45 min after the delivery of food. The following behavioural categories were scored as stereotypies: 1) bar biting; 2) chain manipulation; 3) vacuum chewing; 4) tongue playing (previously defined by Loijens *et al.*, 1999).

At the end of the period of tether housing, the animals were slaughtered, brains were excised, and the hypothalami were dissected and stored frozen until use. The samples were then defrosted and membrane homogenates were made. Opioid receptor densities (B_{max}, maximum binding capacity) were measured using a membrane binding assay with [^{3}H]naloxone as a ligand. Membrane homogenate preparation and the membrane binding assay have been described previously by Loijens *et al.* (1999). Spearman rank correlations were calculated

between the level of stereotypies and the B_{max}. Differences in B_{max} between the three groups of animals were tested for significance using an analysis of covariance with the level of stereotypies as a covariable. Pairwise comparisons between groups were performed using least square mean differences. Data were analyzed with the SAS statistical analysis system (SAS, 1989). The criterion for statistical significance was $p \leq 0.05$. Results are expressed as the mean ± SEM.

RESULTS

Behavioural observations showed a high variation in the level of stereotypy performance between individual pigs. The range was 0-77% in Group 1, 0-83% in Group 2 and 10-85% in Group 3. Stereotypies mainly involved chain manipulation.

At the time of slaughtering some of the pigs' brains were damaged and could not be dissected adequately. For Group 1 there remained six hypothalami, for Group 2 nine, and for Group 3 three. The B_{max} of Group 1 and Group 2 showed a negative correlation with the level of stereotypy performance of the animals (r=-0.80 and r=-0.88, respectively). The correlation was significant for Group 2 (p=0.002), but not for Group 1 (p=0.10). The B_{max} of Group 3 did not correlate significantly with the stereotypy level (r=-0.50; p=0.67).

The B_{max} differed significantly between the three groups of animals ($F(2,13)$= 3.93; p=0.05). Pairwise comparisons between the groups showed a significantly lower B_{max} in Group 3 (26±4 fmol/mg protein; n=3) than in Group 2 (44±3 fmol/mg protein; n=9; p=0.01). The B_{max} in Group 1 (41±6 fmol/mg protein; n=6) tended to differ from the B_{max} in Group 3 (p=0.07), but not from the B_{max} in Group 2 (p=0.44).

DISCUSSION

In line with our observations in the hippocampus of the very same animals (Loijens *et al.*, 1999) we found a strong negative correlation between the density of opioid receptors in the hypothalamus and the level of stereotypy performance in the animals that had been housed tethered for 2 (Group 1) and 5.5 months (Group 2). The observed high individual variability in stereotypy levels agrees with other studies in pigs (Schouten and Wiepkema, 1991; Von Borell and Hurnik, 1991; Zanella *et al.*, 1996) and may relate to differences in coping style, since it has been found that the acute resistance response of pigs to stress (first

tethering experience) negatively correlates with the intensity of stereotypies shown after long-term tether housing (Schouten and Wiepkema, 1991). The present finding of a correlation between stereotypy level and opioid receptor density in the hypothalamus may, thus, suggest that individual differences in hypothalamic B_{max} relate to the coping style of the animals.

A second parallel between the present results and our previous data in the hippocampus is the lack of a correlation between opioid receptor densities and stereotypy levels in the animals that had been housed tethered for 8-9 months (Group 3). This and our observation that opioid receptor densities in the hypothalamus of Group 3 were lower than those of the other two experimental groups, may suggest that changes in receptor populations have developed and that there is a relationship with the duration of exposure to stress. A progressive loss of opioid receptors was also found in the hippocampus, but only in the low-stereotyping animals. In the present study, a classification in low and high stereotyping pigs could not be made in view of the low number of animals in Group 3 (n=3).

Loss of opioid receptors may explain why with increasing 'age' stereotypies become less sensitive to naloxone as has been found in pigs (Cronin, 1985) and voles (Kennes *et al.*, 1988). It has been hypothesized that with time stereotypies become emancipated from the original reinforcing endogenous opioid mechanism and lose their de-arousal capacities. Evidence for the notion that the effectivity of older stereotypies as a de-arousal mechanism decreases, has been found by Schouten and Rushen (1993) who observed a negative correlation between stereotypy level and postfeed heart rate in pigs after two months of tether housing, but not after 8-9 months.

In conclusion, the present data show that the relationship between stereotypies and opioid receptor density that was found in the hippocampus of 2 and 5.5 months tether housed pigs in our previous study and which seemed to disappear with increasing duration of tether housing, is not exclusive for the hippocampus, but can be found in the hypothalamus as well.

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Chapter 5

NEURON NUMBERS IN THE LEFT, BUT NOT RIGHT HIPPOCAMPAL DENTATE GYRUS CORRELATE NEGATIVELY WITH BASAL SALIVA CORTISOL LEVELS IN CHRONICALLY STRESSED PIGS

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ABSTRACT

Although the consequences of stress for the structural integrity of the rodent hippocampus have been studied extensively, relatively little is known about such effects in other species. Long-term tether housing of pigs represents a chronic stressor, as evidenced by profound behavioural changes, and autonomic and endocrine dysfunction, including hypercortisolaemia. As stress/high levels of glucocorticoids influence hippocampal viability in the rodent dentate gyrus (DG), we investigated neuron number and volume of the porcine DG after 5 months of tether housing, in relation to basal saliva cortisol levels.

Saliva was sampled in the afternoon on three consecutive days to determine free basal cortisol levels. Following perfusion fixation, systematically sampled hippocampal sections were Nissl stained and used to stereologically assess DG volume and number.

Total DG volume and neuron number were highly correlated in individual animals, both in the left ($r=0.93$; $p=0.0001$) and right DG ($r=0.92$; $p=0.0001$). Within individual animals, however, total neuron number of the left and right DG did not show a significant correlation. Basal cortisol levels were negatively correlated with volume ($r=-0.61$; $p=0.047$) and total neuron number ($r=-0.72$; $p=0.006$) of the DG in the left, but not in the right hemisphere.

These results are consistent with our previous observation of a progressive decrease in the density of opioid receptors in the hippocampus of long-term tether housed pigs, an effect that was also restricted to the left hemisphere only. Although the high correlation between basal free cortisol levels and DG number in the left hippocampus was expected to relate to altered frequencies in cell death, in a parallel study in the very same animals, no relationship was found between saliva free cortisol and the number of apoptotic cells in the DG. Hence, also reductions in e.g. ongoing neurogenesis after chronic stress may underlie these results. Alternatively, differences in DG volume could be a biological trait, determining individual feedback sensitivity of the hypothalamus-pituitary axis and hence basal free cortisol levels. These possibilities, however, await further investigation. Taken together, our results clearly demonstrate a functional lateralization in the relationship between basal cortisol secretion and volume and neuron number of the porcine DG.

INTRODUCTION

Long-term tether housing of pigs in narrow, individual boxes is still common practice in modern intensive pig breeding. This type of housing shares several features with a restraint stress paradigm. As it largely impairs normal behaviour, like e.g. exploration, it represents a serious stressor, a notion supported by the occurrence of various behavioural and physiological symptoms of chronic stress, including increased adrenocortical steroidogenic capacity and sensitivity to ACTH (Janssens *et al.*, 1994; 1995a), chronic hypercortisolaemia and a flattened diurnal rhythm of cortisol after tethering (Janssens *et al.*, 1993; 1995b). Notably, considerable variation in these symptoms exists among long-term tether housed pigs. This variation is related to individual characteristics of the animals, and is most likely associated with the animals' coping style (Schouten and Wiepkema, 1991; Wiepkema and Schouten, 1992; Hessing *et al.*, 1994).

In previous rodent studies, chronic stress or prolonged exposure to elevated concentrations of glucocorticoid (GC) hormones facilitates neuronal degeneration, particularly in the hippocampus (Sapolsky *et al.*, 1986; 1990; Sapolsky, 1996a,b; 1999) which is richly endowed with adrenocorticoid receptors in rat but also pig (Reul and De Kloet, 1985; Perreau *et al.*, 1999). In addition, GC's influence the incidence of ongoing neurogenesis and probably also apoptosis in the adult hippocampal dentate gyrus (DG) (Gould *et al.*, 1991; 1997; 1998; Gould and Tanapat, 1999). In a previous study, we have found a progressive decrease over time in the density of opioid receptors in the hippocampus of long-term tether housed pigs (Loijens *et al.*, 1999), which we expected to be the result of GC-related cell loss in the hippocampus.

We hypothesize that long-term tether housing induced changes in the activity of the hypothalamus-pituitary-adrenocortical axis that could influence volume and/or cell number in the porcine DG. In view of the individual differences in coping style of these animals, we investigate the relation between individual saliva cortisol levels and neuronal number and volume of the porcine DG.

MATERIALS AND METHODS

Animals and housing

Eleven British Landrace X Great Yorkshire gilts, 6 months of age at the start of the experiment, were housed individually in pens of 0.65 m x 3 m on a concrete floor with a grid fence in the back. The pens were separated by massive plates to

prevent visual contact between the animals. The pigs were housed tethered by a neck-chain attached to the floor by a chain of 0.5 m length. The animals were fed 1 kg of standard pelleted food two times a day (at 9:00 h and 15:30 h). Immediately before each food delivery, a bell was sounded to prevent the animals from associating the presence of people with feeding. Water was provided *ad libitum* through nipple drinkers. The temperature in the pigs' room ranged from 16°C to 25°C. Lights were on from 6:30 h - 18:30 h. The experiments were approved by the animal experimental committee of Wageningen University.

Salivary cortisol

During the 5th month of tether housing, saliva was collected for determination of resting (basal) cortisol levels on three consecutive days, 30 min before the afternoon feeding, i.e. at 15:00 h. Saliva samples were taken using cotton wool buds on which the animals were allowed to chew. The saliva was collected by centrifuging the cotton buds at 3000 rpm for 10 min at 4°C, and was stored frozen (-20°C) until radioimmunoassay. After thawing, samples were centrifuged at 3000 rpm for 10 min at 4°C, and 0.5 ml of the supernatant was extracted with 2.5 ml dichloromethane (recovery of cortisol approximately 100%). After evaporation and resuspension of the dried extract, cortisol (= free, unbound cortisol) was measured in duplicate in 25 µl samples using a radioimmunoassay (RIA), that has previously been described and validated extensively (Janssens *et al.*, 1994). The cortisol values of the three experimental days were averaged to yield one data point per animal.

Histological procedure

After 5 months of tether housing the animals were slaughtered one by one, in the morning, by perfusion fixation. The pigs were lightly anaesthetized in their home pen with 30-40 ml Stresnil i.m. (40 mg/ml azaperon) (Janssen Pharmaceutica BV, The Netherlands). Immediately following transport to the slaughter facilities, which lasted approximately 10 min, the animals were injected with 50-70 ml Hypnodil i.v. (50 mg/ml metomidate) (Janssen Pharmaceutica BV, The Netherlands) resulting in deep and complete anaesthesia. The left and right carotid arteries were then cannulated and gravity perfusion with 0.9% NaCl containing 5 ml heparin (25.000 units) (Leo Pharmaceutical Products, The Netherlands) was started. During perfusion, the animals were bled to death, and the head was separated from the

body. Following perfusion with a total volume of 5 litres of NaCl solution, the heads were perfused with 5 litres of phosphate buffered 10% formalin solution (pH=7.4). The brains were removed from the cranium and further postfixed by immersion in the same fixative for a total period of one week. After two days of postfixation, the left and right hemispheres of the brain were separated, and tissue blocks containing the hippocampal lobes were excised, and further postfixed. Blocks were then dehydrated through graded series of ethanol and xylene, and embedded in paraffin (Histowax, Klinipath, Duiven, The Netherlands). Of each tissue block, 10 µm serial sections were cut on a Reigert-Jung microtome through the complete rostro-caudal extent of the hippocampal lobes. Sections at regular intervals (left hemisphere every 1200 µm; right hemisphere every 1000 µm) were mounted on glycerin-albumin coated slides and dried at 37°C for three nights.

Morphometric analysis

For morphometric analysis, sections of the left and right hippocampus were Nissl stained using cresyl-violet, coverslipped and measured using a computer assisted imaging system (TEA Image Manager, DIFA, The Netherlands). The method for evaluation of volume and neuron number has been described previously (Van Eerdenburg *et al.*, 1992). Briefly, the volume of the dentate gyrus (DG) in each animal was estimated stereologically according to Cavalieri's principle by measuring the area of the DG in at least nine sections taken at regular intervals from rostral to caudal through the hippocampus (Sterio, 1984; Cruz-Orive, 1985a,b). For this purpose the DG was outlined as shown in Figure 1A (area in mm^2). Subsequently, the number of nucleoli was counted in a 40 x 40 µm frame, which was placed at 3 to 7 different positions in the outlined DG area (Figure 1B). All nucleoli present within the small frame were counted at different planes of focus through the section (#/mm^2). Subsequently, the total neuron number and volume of each DG was determined taking into account section thickness and intersection width. Section thickness, as estimated using differential focussing (Uylings *et al.*, 1986) did not vary between sections or between animals. Thus, no corrections were made for shrinkage and an average section thickness of 10 µm was used for calculations of total volume and neuron number of each DG.

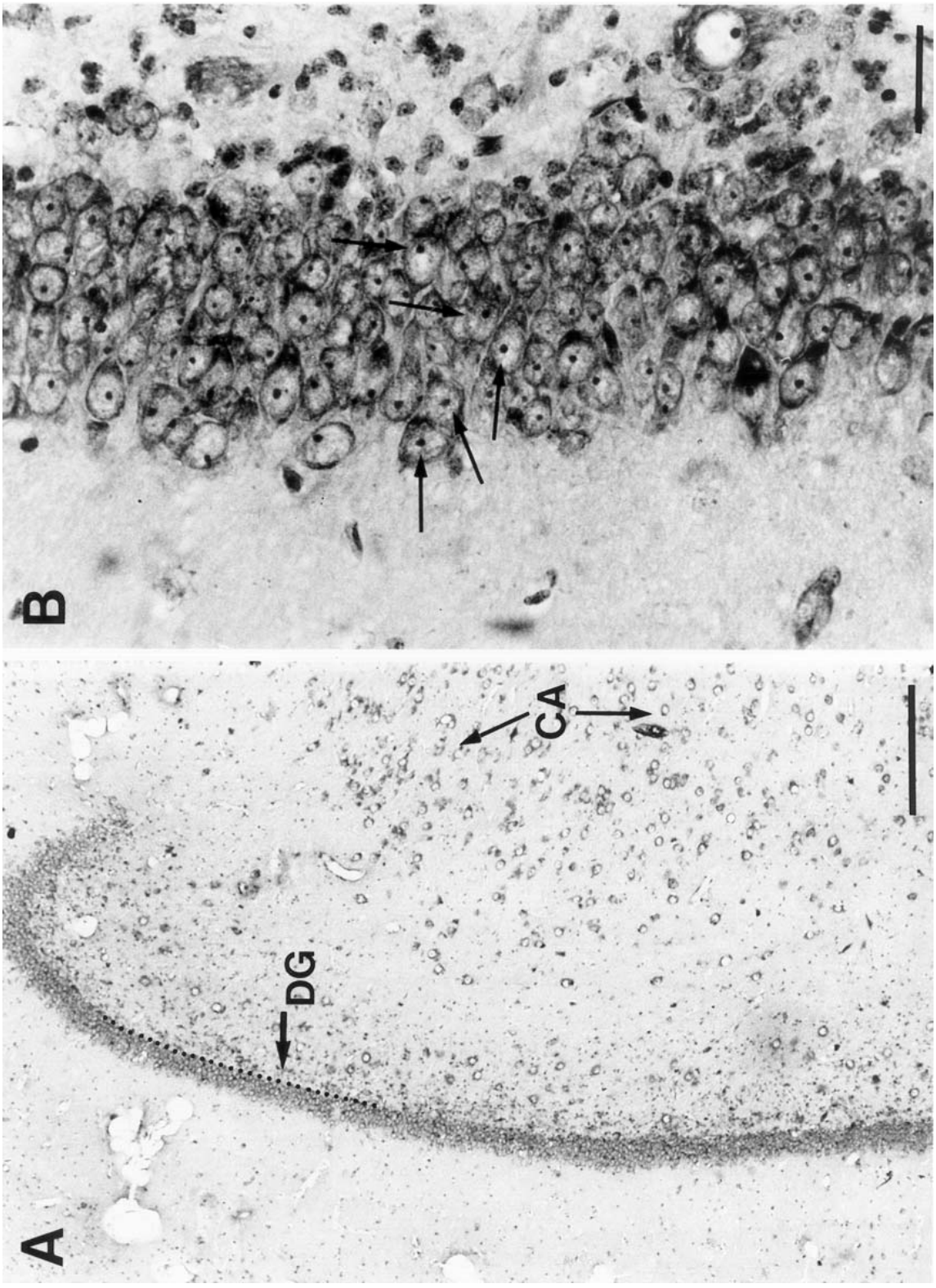
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Figure 1. Photographs of the Dentate Gyrus (DG), Nissl-stained in 10 μm paraffin sections of the pig hippocampus. The DG was outlined as indicated by the dotted line in panel A at a low (10x) magnification (bar=200μm). The number of nucleoli was counted in small 40 x 40 μm frames placed in different parts of the DG at a higher (40x) magnification (panel B, bar=20 μm) as a measurement of cell density. Arrows in panel B point to single nucleoli in individual neurons of the DG. CA: caudate putamen. See text for further details.

Statistical analysis

Data were analyzed using the SAS statistical analysis system (SAS, 1989). Pearson's correlation coefficients were calculated between cortisol levels and morphometric data. Wilcoxon matched pairs signed-rank test was used to assess differences in total volume and neuron number between the left and right DG. Results were expressed as the mean ± SEM, and a value of $p<0.05$ was considered to be significant.

RESULTS

The number of sections in which the DG was present in our animals ranged from 9 to 13 in the left and from 9 to 14 in the right hemisphere. An example of the exact localization and size of the porcine DG in the left hippocampal lobe is drawn in Figure 2.

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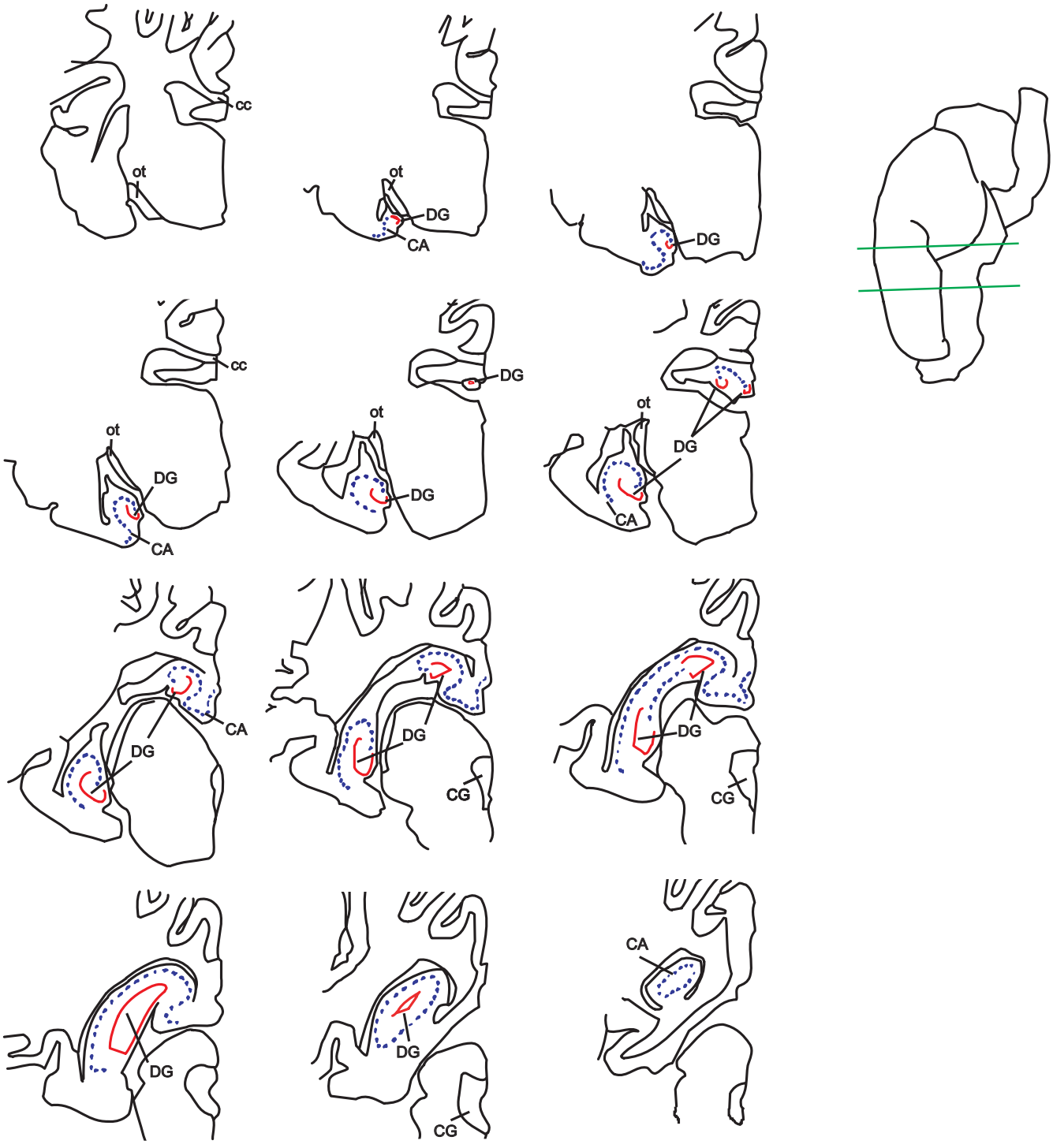
Figure 2. Camera Lucida drawings of the hippocampus in the left hemisphere of a representative animal (#I-07).

Sections were taken at regular intervals through the complete rostral to caudal extent of the hippocampus (order of drawings: left to right, top to bottom). In each figure, the dentate gyrus (DG) is indicated by a solid line, the caudate putamen (CA) is indicated by a dotted line; CG: central grey; ot: optic tract; cc: corpus calosum.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In each of the sections, the total area (mm^3) was measured and plotted as shown in Figure 3A, with the area under the curve representing the total volume (mm^3) of the DG in that particular animal.

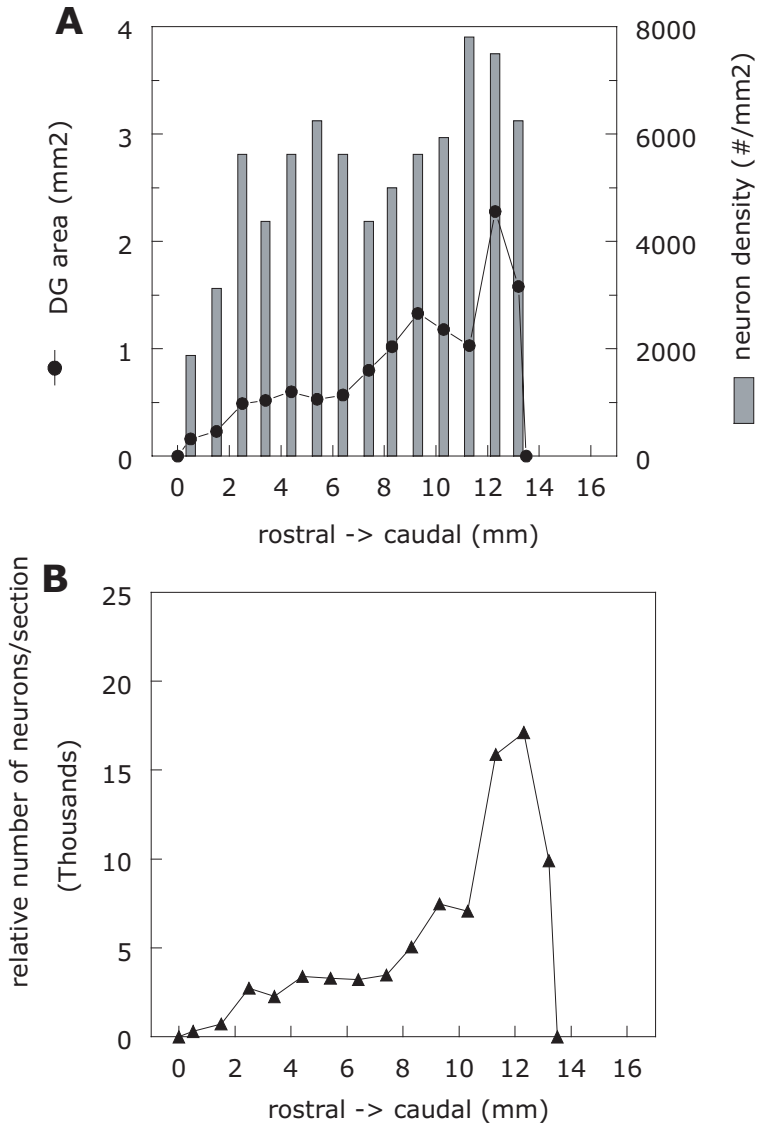

Figure 3. Measurement of the area (panel A) and the cell density (panels A+B) in sections taken at regular intervals through the complete rostral to caudal extent of the dentate gyrus in the right hippocampal lobe of a representative animal (#N-09).

The neuron density of the DG in each section (#/mm^2) as measured using nucleoli counts, is indicated by the grey bars in Figure 3A. The density did not show much variation within a section, but neuron density was slightly lower in sections of the rostral part of the DG compared to sections in the medial and caudal part of the DG. The number of neurons in the DG was calculated for each section by multiplying the area (in mm^2) and the neuron density. These data were plotted as in Figure 3B, the area under the curve representing the total number of neurons in the DG of that particular animal.

Both total neuron number and volume of the DG in the left and right lobe showed a considerable variation between animals (see Figure 4). Yet, the total neuron number was significantly correlated with volume, both in the left (r=0.93; p=0.0001) and in the right (r=0.92; p=0.0001) DG. The total neuron number was not significantly different between the left (73747 ± 4001 thousand) (mean ± SEM) and the right DG (74368 ± 4823 thousand) (p>0.05). Also, the volume of the left DG (12.7 ± 0.6 mm^3) was comparable to that found in the right DG (12.4 ± 0.6 mm^3). Within individual animals, there was no significant correlation (p>0.05) between left and right volume or neuron number (total cell number: r=0.271; p=0.210; total volume: r=0.382; p=0.246, see Figure 4).

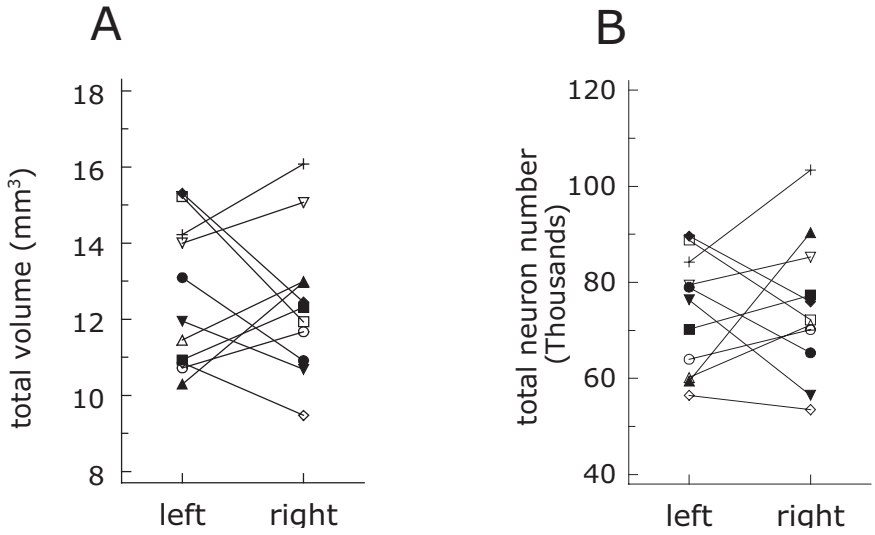

Figure 4. Relation between total volume (panel A) and total neuron number (panel B) of the DG in the left and right hemisphere in each individual animal.

Basal free cortisol in saliva was determined in undisturbed animals. The cortisol concentration varied only slightly within, but differed considerably between animals. Average basal cortisol concentrations were negatively correlated with total neuron number (r=-0.72; p=0.006) and volume (r=-0.61; p=0.047) of the left hippocampal lobe. No correlation was found between basal cortisol and these morphometric parameters in the DG of the right hemisphere (p>0.05) (Figure 5).

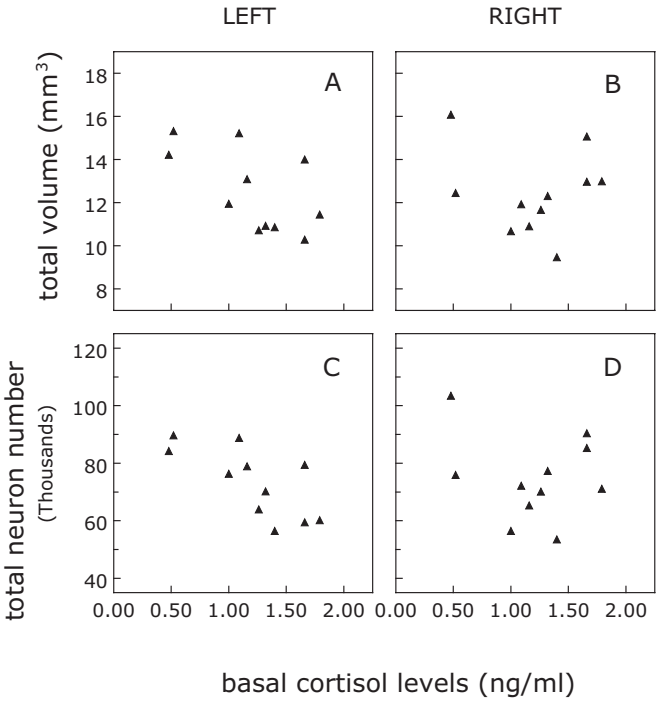

Figure 5. Correlation of basal (prefeeding) salivary cortisol levels with total volume (panels A,B) and neuron number (panel C,D) in the left (A,C) and right (B,D) dentate gyrus of the hippocampus. Correlation coefficients and p values are indicated in each panel.

DISCUSSION

In this study we investigated possible consequences of chronic stress on the porcine dentate gyrus (DG). Although the total volume and neuron number of the DG of the hippocampus varied considerably among individual, tether housed

pigs, the left, but not the right hemisphere, displayed a highly significant, negative correlation with the basal salivary cortisol concentration. Evidently, the total volume of the DG depends on both the number of cells and their volume. In view of the extremely high positive correlation found between neuron number and volume (i.e. left: r=0.93; right: r=0.92; $p<0.0001$), however, the differences between individual pigs in total volume of the DG are most likely a direct result of those in neuron number.

Assuming that the negative correlation between the concentration of cortisol in saliva and number of neurons in the DG is indeed causal, this correlation could be explained by glucocorticoid (GC)-induced changes in neuronal turnover and loss. These may have occurred during the chronic stress period starting from tether housing onwards. This explanation is consistent with the already somewhat older concept that chronic stress or prolonged GC exposure can induce extensive neuronal damage in rodent brain, mainly in the hippocampal CA subregions (Sapolsky, 1990; 1996b). Recent studies in rats, however, failed to find indications for such a substantial loss after stress or different steroid treatments (Sousa *et al.*, 1998) but, interestingly, did show changes in the DG (Sousa *et al.*, 1999). Furthermore, stress can inhibit ongoing cell birth in the adult DG (Gould *et al*., 1991; 1997; 1998; Cameron and Gould, 1994), which could over time, contribute significantly to overall reductions in DG neuron number and volume as well.

Our previous research has demonstrated a marked individual variability in the symptoms of chronic stress, including chronic hypercortisolaemia (Janssens *et al*., 1993; 1995b). This variability is likely related to differences between animals in stress responsivity (Hessing *et al*., 1994). The cortisol concentration in saliva in the present study is indicative for the unbound, biologically active fraction of circulating GCs, which represents approximately 5% of the total concentration of bound and unbound cortisol in plasma. It can be envisaged that the differences between individuals in this parameter are proportionally related to a similar variation in feedback. Subsequently, this may influence the number of neurons in an animal's hippocampus. In addition, we have previously shown that long-term tether housing of pigs leads to a progressive decrease in the density of opioid receptors in the hippocampus (Loijens *et al*., 1999), an effect that was also lateralized and observed primarily in the left hippocampus. This indicates that

structural changes in the porcine hippocampus can indeed be induced by tether housing.

The fact that the presently observed negative correlation between saliva cortisol and DG neuron number was only found in the left, but not in the right hemisphere, suggests that the left DG in pigs may be more sensitive to the effect of GCs on neuronal turnover and death. Similarly, in studies in rats (Carlson *et al.*, 1988) and mice (Delrue *et al.*, 1994; Tabibnia *et al.*, 1999) steroid hormones were indeed shown to influence both sides of the brain in a different way. Furthermore, a recent study in humans showed a significant correlation between the severity of depression (a condition associated with chronic HPA activation and altered basal cortisol levels) and hippocampal volume as measured by MRI in the left lobe only (Vakili *et al*., 2000).

At this point in time, we can only speculate as to the exact mechanism(s) underlying the putative GC-induced (lateralized) change in neuronal turnover during tether housing, be it increased death and/or decreased birth of neuronal cells. Interestingly, in a separate study using brain sections of the very same pigs (Lucassen, Van der Plas, Van der Beek, Schouten, Van Eerdenburg, De Kloet and Wiegant, unpublished results) ongoing apoptosis was clearly evident in the hippocampus, and most prominent in the DG. However, in none of the hippocampal subregions a correlation was found between saliva cortisol level and number of apoptotic cells. It should be kept in mind, however, that apoptosis is a rapid process, and that even massive, simultaneous apoptotic cell death requires observations made within a narrow time window in order to be detected (Perry *et al.*, 1998a,b). In addition, the intriguing possibility that GC-related effects on adult neurogenesis may have influenced neuronal numbers in the DG during the period of tether housing, requires further investigation. In a different cohort of animals, we have recently identified numerous cells in the hilus and DG that were positive for the proliferation marker Ki-67, suggesting that also in the adult porcine hippocampus, cell birth and proliferation continues to occur (Kallivretaki *et al.*, 2001).

Although the exposure to chronic stress in our study may have influenced lateralized effects in the DG, no absolute differences in cell number between the left and right DG were found. This observation is consistent with several recent morphometrical studies reporting that chronic stress and/or high exogenous GCs have no effect on hippocampal cell numbers in rats, monkeys and tree-shrews

(Bodnoff *et al*., 1995; Leverenz *et al*., 1999; Souza *et al*., 1998; 1999; Vollman-Honsdorf *et al*., 1997; Lucassen *et al.*, 2001a,b). These studies, however, focussed primarily on maintenance of hippocampal cell numbers in young and aged animals submitted to chronic unpredictable stress, or changes induced by exogenous GC application or by different types of stress. Moreover, they did not address the issue of lateralization of stress or GC effects on the DG.

An alternative, more speculative explanation for the negative correlation between DG neuronal number and salivary free cortisol concentrations, could be that the number of neurons in the DG is a biological trait of pigs that governs basal secretion of cortisol. Indeed, individual differences in basal plasma cortisol levels have been found in piglets at an age as early as 3 weeks (Hessing *et al.*, 1994), but whether or not those differences correlate with DG neuronal numbers remains to be investigated. Interestingly, several studies in rats suggested that the extent of the damaging and disorganizing effects of GC exposure are more dramatic when present in neonatal and young, rather than in adult animals (Souza *et al.*, 1998; 1999; Tanapat *et al.*, 1998; Uno *et al.*, 1994). The latter may be relevant for our present animal cohort as well, as they were tethered from 6 months of age, which is relatively young for this species. However, using several markers for pathological changes, no indications for overt pathological alterations could be found in our parallel study of the same animals (Lucassen, Van der Plas, Van der Beek, Schouten, Van Eerdenburg, De KLoet and Wiegant, unpublished results). Furthermore, the anatomical distribution pattern of apoptotic cells is more consistent with ongoing (developmental) cell death rather than with one caused by GC overexposure.

In conclusion, the presently found highly significant, negative correlation between salivary cortisol concentrations and the number of neurons in the left hippocampal DG of tether housed pigs accords with the notion that, under stressful conditions, GCs can have detrimental effects on brain neurons. In addition, the data indicate a profound functional lateralization in the relationship between the DG and basal cortisol secretion in this species.

Whether or not the present results point to a causal relationship between cortisol concentration and neuronal number of the DG, and, if so, whether these differences between individuals are ‘nature’ (i.e. ontogenic and present from birth) or ‘nurture’ (i.e. acquired during life due to ‘neurotoxic’ actions of GCs) requires further study.

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Chapter 6

BRAIN OPIOID RECEPTOR DENSITY REFLECTS BEHAVIOURAL AND HEART RATE RESPONSES IN PIGS

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ABSTRACT

Results from our previous research indicate that long-term tether housed pigs with high or low levels of stereotypies show differences in the density of endogenous opioid receptors in the hippocampus and the hypothalamus. It was not clear whether the differences in opioid receptor density were induced by the chronic stress of tether housing or stereotypy performance, or were already present before the animals were tethered and reflected differences in coping style. The latter possibility was tested in the present experiment. We used a group of eighteen non-stereotyping pigs that had no experience with tether housing and investigated whether the animals differed in the density of endogenous opioid receptors in the brain and if so, whether these differences were related to the animals' reactions to acute challenges. The pigs were subjected to two tests: an open field test and a tethering test. Behavioural reactions as well as heart rate responses were measured. Opioid receptor densities were determined post mortem in the hippocampus and the hypothalamus using a membrane binding assay with [^{3}H]naloxone as a ligand. Animals differed widely in their responses to the two tests. A relationship was found between behavioural and heart rate responses and densities of naloxone binding sites in the hippocampus and the hypothalamus. The data suggest that endogenous opioid systems in the brain contribute to differences in stress responding between individual pigs.

INTRODUCTION

In reaction to stressful situations animals show coordinated behavioural and physiological responses that are aimed at coping with the stressor. However, animals of the same species may value the same situation differently, and therefore, the same stressor does not necessarily result in identical responses in all individuals. Several authors have reported different modes of coping with stressful situations, each with its own physiological concomitants (Corson and Corson, 1976; Henry and Stephens, 1977; Von Holst, 1986; Bohus *et al.*, 1987; Benus *et al.*, 1990; Sapolsky, 1990; Verbeek *et al.*, 1994). Interestingly, individuals may differ in 'coping style', i.e. in preference for a coping strategy. Coping styles are likely determined by genetic constitution as well as by early experience (Kagan and Levi, 1974; Van Oortmerssen *et al.*, 1985; Bohus *et al.*, 1987; Suomi, 1987). Important, in this respect, is the way in which an individual estimates the situation and its possibilities to cope (Smelik, 1987).

Evidence that the behavioural and physiological responses of individual animals to a stressor are consistent over time has also been found in farm animals, including pigs (Hessing *et al.*, 1994; Spoolder *et al.*, 1996; Ruis *et al.*, 1997; 2000; Erhard and Mendl, 1999), although it is not without debate (e.g. Jensen *et al.*, 1995a,b). Animals with distinct coping styles may differ in vulnerability to the development of behavioural disturbances, like stereotypies (Wiepkema *et al.*, 1987; Schouten and Wiepkema, 1991).

Stereotypies have been associated with endogenous opioid activity (Cronin *et al.*, 1985; Kennes *et al.*, 1988; Zanella *et al.*, 1996), although other authors have questioned the evidence (Dantzer, 1991; Rushen, 1993). If there is a causal relation between stereotypies and endogenous opioid release, one would expect that animals with high or low levels of stereotypies would differ in the organization of their endogenous opioid systems. In a recent study in tether housed pigs we have found a negative correlation between the intensity of stereotypy performance and the density of naloxone binding sites in the hippocampus (Loijens *et al.*, 1999) and the hypothalamus (Loijens, Schouten, Wiegant, unpublished results). It was not clear whether the differences in opioid receptor density were induced by the chronic stress of tether housing or stereotypy performance, or were already present before the animals were tethered and reflected differences in coping style. The latter hypothesis was further investigated in pigs that did not have a history of tether housing or stereotypy performance. To detect differences in coping style, the animals were subjected to two acute challenge tests, an open field test and a tethering test and behavioural and heart rate responses were analyzed. Subsequently, they were slaughtered, and opioid receptor densities were measured in the hypothalamus and the hippocampus. Regression analysis was carried out to test whether there was a relationship between behavioural and heart rate measures in the two tests, on the one hand, and opioid receptor densities in the hypothalamus and hippocampus, on the other hand.

MATERIALS AND METHODS

Animals and housing

In this study 18 nulliparous female pigs (Large White x British Landrace, Pig Improvement Company, United Kingdom) were used. The animals were 7 months old and weighed 105 ± 5 kg (mean ± SD) at the start of the experiment. They were housed pairwise in stalls measuring 2.2 m x 2.8 m. The stalls had a solid

concrete floor at the front, covered with wood shavings, and a slatted dunging area at the back. A food trough, which was divided in two parts, was placed at the front side of each stall. At the backside two nipple drinkers were situated. The animals were fed standard pelleted food by hand, twice a day, at 8:00 h and 16:00 h (2.5 kg/day). Water was available *ad libitum*. Lights were on between 6:00 h and 21:00 h. Room temperature ranged from 15 to 20°C.
The animals had all shown two or more oestrous cycles at the beginning of the experiment. Oestrous detection was carried out daily (at 8:30 h) with a vasectomized boar, and by inspection of external oestrous signs. On the days the animals were in oestrus, no tests were done.

Experimental design

The experiment started one month after the animals had arrived at the experimental accommodation, in order to give them sufficient time to acclimatize and get used to handling and the procedure of heart rate measurement. The experiment consisted of two challenge tests that were conducted in the same order for all animals with an interval of at least one day. Pigs were tested individually. Test 1 was an open field test, test 2 a tethering test. During both tests video recordings of the animals' behaviour were made, and the heart rate was recorded. Vocalizations were recorded on the audio track of the videotape. After the two tests had been completed for all pigs, the animals were slaughtered. The interval between test 2 (tethering test) and the day of slaughtering was 35 ± 17 days (mean ± SD) (range 9-63 days). Brains were collected and the hypothalamus and hippocampus were dissected. Membrane preparations were used in a radioligand binding assay to assess the density of opioid receptors in each brain sample.

Open field test

Procedure

The open field was part of a corridor outside the pigs' room measuring 4.3 m x 2.3 m, divided in 12 equal squares (1.08 m x 0.77 m, 4 rows of 3 squares). It was surrounded by concrete walls at two sides, doors at one end and two sliding doors (used as entrance and exit) at the other end. The open field test always started at 14:30 h. Before the animal's introduction into the open field, the pig was led to another pen, similar to its home pen (the heart rate pen). Here the animal was equipped with a Polar Sport Tester™ to measure the heart rate, and it was left on

its own for 15 min. All pigs had experienced this procedure three to four times. After the 15 min rest the pig was led to the open field. 5 Min after the pig's introduction into the open field, a metal bucket was lowered from the ceiling, which, after its noisy contact with the concrete floor, was fixed above the centre squares of the open field at such a height that the pig could touch it but not bite in it. After another 10 min in the open field the pig was led back to its home pen. At the end of each open field test, the floor of the open field was scrubbed.

Behavioural observations

Video recordings of the pigs' behaviour were analyzed by focal animal observation. Behavioural observations included:

1) rooting: making rooting movements at the floor or sniffing: sniffing at the floor, walls or doors
2) locomotion: walking, keeping the head up
3) alert: standing motionless while looking around attentively, ears pricked
4) bucket:
    a) looking at the bucket: looking in the direction of the bucket while keeping distance
    b) sniffing close to the bucket, without touching
    c) touching the bucket
5) pushing against the sliding doors
6) urinate
7) defecate
8) other: any activity other than those mentioned above

Observations included duration and frequency of the behavioural elements described above, and were made separately for the first 5 min of the open field test (before the introduction of the bucket) and the last 10 min (after the introduction of the bucket). Durations of behavioural elements were expressed as percentages of observation time.

The number of line crossings in the open field were counted and the time the pig spent in the centre or the border squares of the open field, respectively, was measured. The number of vocalizations was scored.

The latency to

1) first contact with the bucket

2) first push against the sliding doors
3) first vocalization
   a) after the start of the open field test and
   b) after the introduction of the bucket
4) first entrance in one of the centre squares of the open field
   a) after the start of the open field test and
   b) after the introduction of the bucket, was determined.

The time was measured 1) it took to get the pig out of its home pen, 2) the pig walked from the heart rate pen to the open field and 3) back from the open field to the heart rate pen. The animals were gently pushed if not willing to move voluntarily.
The number of defecations was counted and at the end of the test, faeces were collected and weighed.

Heart rate measurements

Heart rate was measured using the Polar Sport Tester™ (Polar Electro, Finland). A chest band was attached to the pig containing conductive gelled electrodes and the sensor/transmitter for heart rate measurements. On the back of the pig, fixed to the chest band, was a small plastic box (8 x 4 x 2 cm) containing the heart rate monitor. Heart rate interval capacity was set at 5 sec. Mean heart rate values were calculated for 1) the last 5 min of the 15 min rest period in the heart rate pen, before the pig's introduction into the open field 2) the first 5 min of the open field test and 3) the last 10 min of the open field test.

Tethering test

Procedure

The tethering test always started at 9:30 h. The procedure was similar to that of the open field test. However, after the 15 min rest period in the heart rate pen, the test pig was now led to the tethering pen. The tethering pen (0.85 m x 2.80 m) was adjacent to the heart rate pen. The pig received a small quantity of food and it was tethered while it was eating. The animals were tethered by a neck chain attached to the floor by a 50 cm long heavy gauge chain. The tethering test lasted for two hours. During the entire test, video recordings of the pig's behaviour were made, and heart rate was measured. At the end of the tethering period the animal

was released, the heart rate equipment was taken off and the pig was led back to its home pen.

Behavioural observations

Video recordings of the pigs' behaviour were analyzed by continuous observation of the activity pattern:

1) pulling at the tether chain
2) chain manipulation: other behaviour directed at the tether chain, mostly chewing or rooting at the chain
3) rooting: making rooting movements on the floor
4) bar biting: biting on the bars or other parts of the pen
5) inactive: lying down, showing no overt activity
6) other: all other activities not fitting in categories 1 to 5 (mainly standing quietly between bouts of chain pulling)

Observations included duration and frequency of the behavioural elements described above. Durations of behavioural elements were expressed as percentages of observation time. The number of vocalizations was counted.

Heart rate measurements

Heart rate was measured using the Polar Sport Tester™. Heart rate interval capacity was set at 5 sec. Mean heart rate values were calculated for the last 5 min of the 15 min rest period in the heart rate pen (before the pig's transport to the tethering pen), and for 7 periods during the tethering test: 0-5 min after the start of tethering (0-5 min); 5-10 min; 10-15 min; 15-30 min; 30-60 min; 60-90 min and 90-120 min.

Receptor binding assays

At the end of the experiment the animals were transported to a slaughterhouse and were slaughtered after electrocution. Skulls were opened, brains were excised, and the hypothalamus and left and right lobes of the hippocampus were dissected on ice according to Salinas-Zeballos *et al.* (1986). The time between electrocution and freezing of brain samples was approximately 30 min. Only undamaged brain samples were collected. Samples were immediately frozen in isopentane (2-methyl-butane) cooled with dry ice, and stored at -70°C until use. Only the hypothalami

and right hippocampal lobes were used for receptor binding assays.
Membrane suspensions of the brain samples were made and the density (B_{max}, maximal binding capacity) and the equilibrium dissociation constant (K_D) of opioid binding sites were measured using a membrane binding assay with [^{3}H]-naloxone as a ligand, as described previously (Loijens *et al.*, 1999).

Statistical analysis

Differences between the 0-5 min period and the 5-15 min period of the open field test in the relative durations of behavioural elements were tested using Wilcoxon matched pairs signed-rank test. Differences in mean heart rate values in the three time periods of the open field test were analyzed by normal analysis of variance (General Linear Models (GLM) procedure). The three time periods were treated as three levels of a repeated measure factor. Pairwise comparisons between mean heart rate values in the pre-test period and those in subsequent time periods were performed using F-tests. The heart rate data of the tethering test were analyzed using the same method.
Wilcoxon matched pairs signed-rank tests were used to test for differences in B_{max} and K_D between the brain regions studied. Spearman rank correlations were calculated between the B_{max} values in the hypothalamus and those in the hippocampus.
The data were further analyzed using stepwise regression to test whether there was a relationship between the behavioural parameters, and heart rate measures described above, on the one hand, and the B_{max} in the hypothalamus and hippocampus on the other hand. As it was an exploratory analysis, we included the complete ethogram in the analysis. Two additional variables were included in the regression analysis: 1) the time between behavioural testing and slaughter sample collection and 2) the day in the oestrous cycle. The significance level for entry into the regression model, and the level for staying in the model were p=0.15 (SAS, 1989). Regression analysis was performed for the 0-5 min and 5-15 min period of the open field test and for the first 60 min (see Results page 94) of the tethering test.
All statistical analyses were performed using the SAS statistical package (SAS, 1989). The criterion for statistical significance was set at $p \leq 0.05$. Results are expressed as the mean ± SEM.

RESULTS

Open field test

A high variation between individual animals was seen in their reaction to the open field test. Large individual differences were found in the pigs' behavioural reaction as well as in their heart rate response to the test. Due to technical problems video recordings of the behaviour of one animal in the 0-5 min period and one animal in the 5-15 min period of the test were not complete (yielding n=17 for both parts of the test).

Behaviour

It took 20 ± 9 sec (mean ± SEM; range 2-133 sec) to get the animals out of their home pen. The pigs walked in 68 ± 7 sec (range 38-128 sec) from the heart rate pen to the open field and in 71 ± 6 sec (range 40-109 sec) back from the open field to the heart rate pen.

The behavioural response of the pigs to the open field test is shown in Table 1.

During the first 5 min in the open field, the pigs spent most of the time exploring their new environment by sniffing and rooting at the floor, walls and doors. The relative duration of sniffing/rooting was significantly less in the last 10 min of the test ($p<0.01$). During the latter period the animals were more alert ($p<0.05$). Significant differences between the two periods in the time spent in other behavioural elements were not found.

Faeces were collected at the end of the test and weighed 179 ± 50 grams (range 0-640 grams). The number of defecations was 0.3 ± 0.7 (mean ± SD) (range 0-2) before and 0.9 ± 0.9 (range 0-2) after the introduction of the bucket.

Heart rate

Transport and exposure to the open field increased the heart rate of the animals (Figure 1). In both periods during the pigs' stay in the open field mean heart rate values were significantly higher than the pre-test level (0-5 min period: $p<0.01$; 5-15 min period: $p<0.05$). The heart rate during the first 5 min of the open field test was not significantly different from that in the last 10 min ($p>0.05$).

Table 1. Behavioural response of pigs (n=17) to an open field test.
Behavioural parameters are expressed as mean ± SEM (range), before and after the introduction of the bucket (0-5 min and 5-15 min period, respectively).
The asterisks indicate significant differences between the two time periods.

Behavioural element	0-5 min	5-15 min
Time sniffing/rooting	66 ± 4% (21-90%)	36 ± 5% (3-77%)**
Time alert	20 ± 4% (0-66%)	32 ± 5% (5-93%)*
Time walking	4 ± 1% (0-18%)	4 ± 1% (0-22%)
Time pushing doors	2 ± 1% (0-14%)	5 ± 2% (0-22%)
Time other behaviour	8 ± 1% (0-16%)	14 ± 2% (1-24%)
Latency to push doors (sec)	231 ± 20 (9-300)	
Number of vocalizations	24 ± 5 (0-76)	91 ± 18 (1-324)
Latency to first vocalization (sec)	91 ± 19 (4-300)	21 ± 4 (3-56)
Number of line crossings	41 ± 5 (14-100)	55 ± 8 (5-115)
Time in centre squares	16 ± 2% (4-34%)	23 ± 5% (2-90%)
Latency to enter centre (sec)	23 ± 6 (1-111)	39 ± 13 (1-182)
Time bucket		9 ± 1% (1-21%)
Latency to touch bucket (sec)		200 ± 49 (12-600)

* = $p \leq 0.05$; ** = $p \leq 0.01$

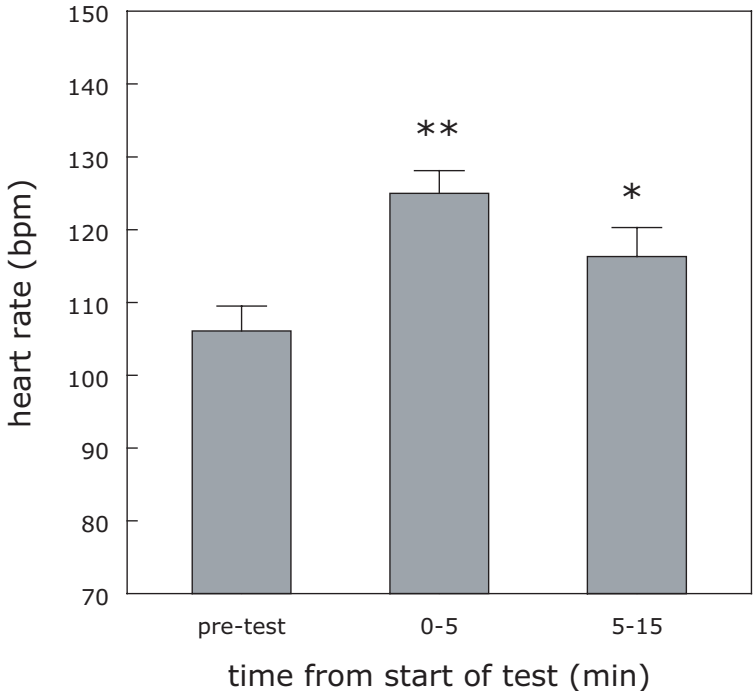

Figure 1. Heart rate response of pigs (n=17) to an open field test.

Heart rate (beats per minute: bpm) (mean + SEM) during the last 5 min of the 15 min rest period in the heart rate pen (before the start of the open field test), during the first 5 min of the open field test, and during the last 10 min of the test. The asterisks indicate significant differences from pre-test value:

* = $p \leq 0.05$; ** = $p \leq 0.01$

Tethering test

Analogous to the open field test, the tethering test led to high individual variability in the pigs' reaction. Large differences were found between individual animals in their behavioural reaction as well as in their heart response to the test. Two animals had to be released from the tether stall (15 and 30 min after the start of tethering, respectively), because they were in danger of severely wounding themselves. Data from these animals were excluded from any further analysis.

Behaviour

As soon as the animals became aware of their tethered condition, they started pulling at the tether chain and screaming. The intensity of this breakout behaviour differed greatly between individual pigs. After a period of fierce resistance, the breakout behaviour gradually became less intense and eventually most animals lied down. The pigs were mainly inactive when lying, with only occasional outbursts of breakout behaviour. At t=30 min (30 min after the start of tethering) 14 out of the 16 animals were still active. At t=60 min this number was 6, and at t=90 min and t=120 min 3 animals had not yet lied down. In view of these numbers, it was decided not to include the last 60 min of the test in the statistical analysis.
The behavioural pattern of the pigs in the first 60 min of the tethering test is shown in Table 2.

Table 2. Behavioural response of pigs (n=16) to tethering.
Behavioural parameters are expressed as mean ± SEM (range) during the first 60 min of the tethering test.

Behavioural element	mean ± SEM (range)
Time pulling at tether chain	13 ± 2% (3-34%)
Time manipulating chain	17 ± 3% (0-47%)
Time rooting floor	4 ± 1% (0-10%)
Time biting bars	7 ± 2% (0-26%)
Time inactive	4 ± 1% (0-17%)
Time other behaviour (mainly standing quietly between bouts of chain pulling)	55 ± 4% (35-85%)
Number of vocalizations	558 ± 86 (62-1169)

Heart rate

Tethering led to a sharp, time dependent rise in heart rate (Figure 2). The highest heart rate values were found in the first 5 min after the start of tethering. Thereafter, the heart rate decreased gradually but remained significantly higher than the pre-test level until 30 min after the start of tethering. In the 90-120 min period after the start of tethering the heart rate dropped below the pre-test level.

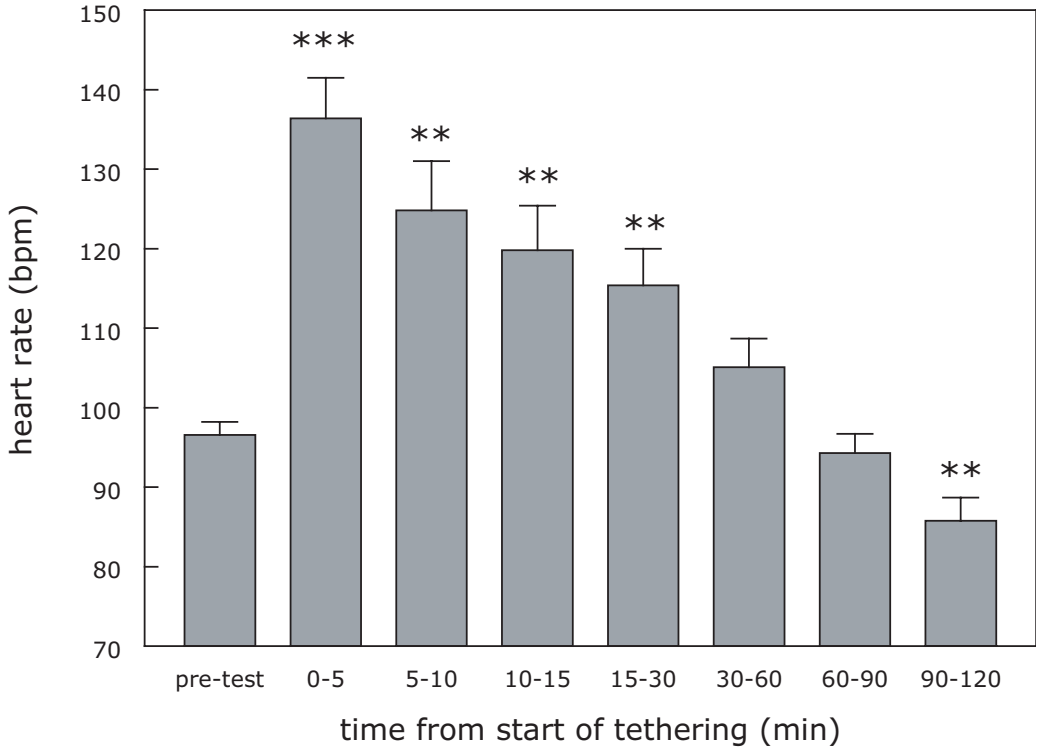

Figure 2. Heart rate response of pigs (n=16) to tethering.

Heart rate (beats per min: bpm) (mean + SEM) during the last 5 min of the 15 min rest period in the heart rate pen (before the start of the tethering test), and during seven time periods of the tethering test.

The asterisks indicate significant differences from pre-test value:

** = $p \leq 0.01$; *** = $p \leq 0.001$

Receptor data

At the time of slaughtering some brains were damaged and could not be dissected adequately. There remained 16 hypothalami and 17 right hippocampal lobes.

The dissociation constant (K_D) in the hypothalamus was 1.4±0.1 nM (mean±SEM) and was significantly lower than the K_D in the (right) hippocampal lobe (2.2±0.2 nM) ($p<0.05$). The density of naloxone binding sites in the hypothalamus was 31.5 ± 2.2 fmol/mg protein. This value was significantly higher than the B_{max} in the hippocampus (19.6 ± 0.5 fmol/mg protein) ($p<0.0005$). The B_{max} values in the two brain regions did not correlate (r=+0.16; $p=0.56$).

Regression analysis

Regression analysis was used to test whether there was a relationship between the behavioural parameters and heart rate values that were measured in the two tests, on the one hand, and the B_{max} in the hippocampus and the hypothalamus on the other hand. Analyses were carried out for the 0-5 min and 5-15 min period of the open field test and for the first 60 min of the tethering test.

Open field test: 0-5 min

Regression analysis with the B_{max} in the hippocampus as the dependent variable yielded a model with one independent variable, the latency to enter the centre squares of the open field after the start of the test ($F(1,11)=4.06$; $p=0.07$; $R^2=0.27$; estimated regression coefficient=+0.04). When the B_{max} in the hypothalamus was tested as the dependent variable, five independent variables were included in the model (yielding R^2 of the model=0.86; $F(5,7)=8.61$; $p=0.007$): (1) the time to get the animals out of their home pen ($F(1,7)=21.43$; $p=0.002$; (partial) $R^2=0.43$; estimated regression coefficient=+0.22) (2) the latency to the first vocalization after the start of the test ($F(1,7)=19.06$; $p=0.003$; $R^2=0.14$; estimated regression coefficient=+0.12) (3) the time the animals spent in the centre squares of the open field ($F(1,7)=13.31$; $p=0.008$; $R^2=0.14$; estimated regression coefficient=-0.23) (4) the latency to enter the centre squares of the open field after the start of the test ($F(1,7)=4.95$; $p=0.062$; $R^2=0.10$; estimated regression coefficient=-0.14) and (5) the time the animals spent alert ($F(1,7)=2.88$; $p=0.134$; $R^2=0.06$; estimated regression coefficient=-0.09).

Open field test: 5-15 min

Regression analysis with the B_{max} in the hippocampus as the dependent variable resulted in a model with two independent variables (yielding R^2 of the model=0.32; $F(2,12)=2.79$; $p=0.101$): (1) the time the animals spent looking at the bucket ($F(1,12)= 4.31$; $p=0.060$; $R^2=0.16$; estimated regression coefficient=-0.07) and (2) the latency to enter the centre squares of the open field after the introduction of the bucket ($F(1,12)=2.84$; $p=0.118$; $R^2=0.16$; estimated regression coefficient= +0.02). When the B_{max} in the hypothalamus was tested as the dependent variable, three independent variables were included in the model (yielding R^2 of the model=0.62; $F(3,11)=6.02$; $p=0.011$): (1) the time the animals spent alert ($F(1,11)=12.94$; $p=0.004$; $R^2=0.37$; estimated regression coefficient=+0.06) (2) the latency to cross a line after the introduction of the bucket ($F(1,11)=3.33$; $p=0.095$; $R^2=0.15$; estimated regression coefficient=-0.11) and (3) the latency to sniff close to the bucket ($F(1,11)=2.97$; $p=0.113$; $R^2=0.10$; estimated regression coefficient=-0.03).

Tethering test

Regression analysis with the parameters of the tethering test as the independent variables and the B_{max} in the hippocampus as the dependent variable led to a model with two variables (yielding R^2 of the model=0.69; $F(2,8)=8.76$; $p=0.010$): (1) the heart rate in the period 15-30 min after the start of tethering ($F(1,8)=14.41$; $p=0.005$; $R^2=0.58$; estimated regression coefficient=+0.14) and (2) the time the animals spent manipulating the chain ($F(1,8)= 2.81$; $p=0.132$; $R^2=0.11$; estimated regression coefficient=+0.002). When the B_{max} in the hypothalamus was tested as the dependent variable a model with three independent variables came out (yielding R^2 of the model=0.66; $F(3,7)=4.61$; $p=0.044$): (1) the heart rate in the 10-15 min period after the start of tethering ($F(1,7)=11.70$; $p=0.011$; $R^2=0.26$; estimated regression coefficient=-0.49) (2) the heart rate in the heart rate pen before the start of tethering ($F(1,7)=7.87$; $p=0.026$; $R^2=0.18$; estimated regression coefficient=+1.03) and (3) the number of vocalisations ($F(1,7)=4.57$; $p=0.070$; $R^2=0.22$; estimated regression coefficient=-0.02).

DISCUSSION

In the present study we measured opioid receptor densities in the hippocampus and the hypothalamus of pigs without a history of tether housing or stereotypy

performance. Opioid receptor densities appeared to be associated with behavioural and heart rate responses measured in the open field and the tethering challenge test. The variables (1) time between behavioural testing and slaughter sample collection and (2) day in the oestrous cycle could not explain any of the variance in opioid receptor densities. The data may therefore suggest that the differences in B_{max} reflect different coping strategies. Differences in the density of opioid receptor sites imply differences in brain opioid function. As endogenous opioids are part of the systems that regulate the body's responses to stress (Akil *et al.*, 1984), it can be hypothesized (and our results support this hypothesis) that animals differing in brain opioid function show differences in the processing of (stress) stimuli in the brain and, thus, in their response to stressful situations (i.e. challenge tests).

Evidence exists indicating that animals that adopt different response patterns (coping strategies) when stressed, differ in the organization of their endogenous opioid systems. Deroche and co-workers (1993) observed individual differences between rats in their vulnerability to the psychomotor and addictive effects of morphine. These differences were found to be predicted by the animals' reactivity to a novel environment. Studies in apomorphine susceptible and unsusceptible rats have shown that (a) the differences in apomorphine susceptibility reflect differences in coping style and (b) these types of animals differ in stress-induced hippocampal dynorphin release (Cools *et al.*, 1993). Apomorphine susceptibility in pigs has also been found to relate to coping style (Bolhuis *et al.*, 2000).

The binding sites detected in the present study may represent mu, delta as well as kappa opioid receptors, since we used naloxone as the ligand in the receptor binding assays. Naloxone is a non-selective opioid receptor antagonist with a preference of about 10-fold for mu over kappa, and a still greater preference for mu over delta opioid receptors, as demonstrated in guinea pig whole brain preparation (Goldstein and Naidu, 1989). Naloxone was used since (a) it has been proven an effective antagonist of stereotypies *in vivo* (Cronin *et al.*, 1985; Kennes *et al.*, 1988; Rushen *et al.*, 1990) and (b) individual differences in the intensity of stereotypy performance in tether housed pigs (which are likely related with the coping style of the animals) were found to be associated with the density of naloxone binding sites in the hippocampus (Loijens *et al.*, 1999) and the hypothalamus (Loijens, Schouten, Wiegant, unpublished results).

Two tests were used to challenge the animals. The tethering test was the animals' first experience with tethering. In general, pigs that are first tethered fiercely resist,

vocalize and try to escape by pulling and biting the tether chain. They also display other responses typical for acute stress, such as increased plasma levels of ACTH and cortisol, and an increased heart rate (Schouten and Wiepkema, 1991; Wiepkema and Schouten, 1992; Schouten and Rushen, 1993). The open field test was used as a 'novel environment' test. The two tests can be regarded as challenge tests since the animals are brought in a situation, which they cannot predict or control. Reduced predictability and/or controllability are generally recognized as a common denominator of stressful conditions (Weiss, 1968; 1970; Wiepkema, 1990). An interesting observation in the regression analysis of the open field test was that only behavioural parameters were included in the model, while the analysis of the tethering test resulted in a model with behavioural as well as heart rate measures. This may relate to the fact that the open field test is a milder stressor than the tethering test, leading to a more moderate increase in heart rate and less variability between individual animals.

Regression analysis of the open field parameters showed higher values for R^2 of the model when the B_{max} in the hypothalamus was analyzed as compared to the B_{max} in the hippocampus. This may suggest that opioid systems in the hypothalamus play a more important role in the animals' responses to the open field test than those in the hippocampus. The R^2 values for the tethering test were both high suggesting an equal involvement of hippocampal and hypothalamic opioid systems. This seems to be in line with the hypothesis of Henry (1976) that the hippocampus plays an important role under conditions where the animals have lost control over the situation.

An open field test similar to the one we have used in our study (including a novel object) has been applied in other studies with pigs as well. Hessing and colleagues (1994) tested piglets in an open field, which had been classified as either aggressive/resistant (A/R) or non-aggressive/non-resistant (NA/NR), based on the outcome of the animals' behaviour in social confrontation tests and backtests. The authors demonstrated differences between these two groups in the number of vocalizations, in the latency to touch the novel object and the contact time with the novel object. Regression analysis in the present study showed a significant relationship between B_{max} values in the hypothalamus and the hippocampus, on the one hand, and behavioural parameters of the open field test, on the other hand. However, neither the animals with high nor those with low B_{max} values in the hypothalamus or the hippocampus seemed to correspond with the A/R and NA/NR

animals of Hessing and co-workers. The animals with high hypothalamic B_{max} values resembled the A/R piglets of Hessing *et al.* in that they had a short latency to inspect the bucket, but they did not (like the A/R animals) have less contact with the bucket and they did not vocalize less than their group mates in the open field test (although they had a longer latency to the first vocalization and they did vocalize less in the tethering test). The only difference between the animals with high and low B_{max} values in the hippocampus which corresponds with the differences between the A/R and NA/NR piglets of Hessing *et al.*, was the time they were interested in the bucket. However, the two studies differed in experimental design. In contrast to our experimental set-up, Hessing and his co-workers preselected their animals and only tested piglets that were either A/R or NA/NR but no 'doubtful' animals. The starting populations of the two studies were, therefore, different.

Lawrence *et al.* (1991) tested pigs in open field, which were selected on the basis of their response in a series of handling tests. The authors demonstrated differences between high and low responders (animals that were difficult and easy to handle, respectively) in their interest in a novel object. The high responders appeared to pay more attention to the novel object. In the present study we observed differences between pigs with high and low hippocampal B_{max} values in the time the animals spent looking at the bucket, but we have no evidence that these animals also differed with respect to ease of handling. The animals did not, like those of Lawrence *et al.*, show differences in the latency to leave their home pen. However, as the starting populations of the two studies were, again, different comparison is difficult.

In conclusion, our data reveal individual differences in the density of opioid receptors in the hypothalamus and the hippocampus in pigs which appear to be related to behavioural and heart rate responses of the animals in stressful situations. The results suggest that endogenous opioid systems in the brain contribute to differences in responding to stress between individual pigs.

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Chapter 7

SUMMARY AND GENERAL DISCUSSION

Summary

Stress, of both physical and emotional origin, can have profound effects on various physiological functions as well as on behaviour. Relatively little is known, however, with respect to the effects of chronic stress and the mechanisms that are involved. One of the few animal models available for this kind of research was used in this thesis: prolonged restraint of pigs by a neck-tether was used as a chronic stress paradigm. In our previous research with long-term tether housed pigs, changes in autonomic cardiac regulation (Schouten *et al.*, 1991) and pituitary-adrenocortical activity (Janssens *et al.*, 1993; 1994; 1995a,b) as well as behavioural disturbances such as stereotypies (Cronin, 1985; Wiepkema and Schouten, 1992) have been found. The present thesis was mainly focussed on stereotypies and their possible link with endogenous opioid systems. An association between stereotypies and endogenous opioid activity has been proposed on the basis of findings that treatment with the opioid receptor antagonist naloxone reduces the time spent stereotyping (Cronin *et al.*, 1985; Kennes *et al.*, 1988; Rushen *et al.*, 1990).

Chronic stress may not only lead to the development of stereotypies, it can also induce changes in responsivity to further stressful stimulation (Pike *et al.*, 1997; Bhatnagar *et al.*, 1998). The mechanisms underlying these changes likely include alterations in endogenous opioid systems (Cuadra *et al.*, 1999). Changes in (cardiovascular and hypothalamus-pituitary-adrenocortical) responsivity have also been found in long-term tether housed pigs (Janssens *et al.*, 1994; 1995a; Schouten *et al.*, 1991). The aim of the study described in Chapter 2 was to further investigate the role of endogenous opioids in pigs' responsivity to acute stressful stimulation (15 min fixation with a nose-sling) and to study the effects of long-term tether housing. Our results provide evidence indicating that long-term tether housing (10-11 weeks) leads to an increased impact of endogenous

opioid systems mitigating heart rate responses to additional acute stress.
In Chapter 3 and 4 we further investigated the relationship between endogenous opioid systems and stereotypies. To this aim we measured opioid receptor densities in the brain of long-term tether housed pigs with varying degrees of stereotypies. Three groups of animals were used which were housed tethered for 2, 5.5 and 8-9 months, respectively. A negative correlation was found between the intensity of stereotypy performance and opioid receptor densities in the hippocampus (Chapter 3) and the hypothalamus (Chapter 4) of pigs which had been housed tethered for two months. This correlation seemed to disappear with increasing duration of tether housing. In addition, we observed a decrease in opioid receptor density, both in the hippocampus and in the hypothalamus, which was found to be associated with the duration of tether housing.
In Chapter 5 we tested the hypothesis that the decrease in opioid receptor densities that was observed in Chapter 3 and 4 could be the result of glucocorticoid-related neuronal cell loss. We investigated the relationship between basal cortisol concentrations and the number of neuronal cells and the total volume of the hippocampal dentate gyrus in long-term tether housed pigs. The negative correlations found, accord with such a relationship.
In Chapter 3 and 4 we reported individual differences in brain opioid receptor densities in long-term tether housed pigs, which appeared to be related with the level of stereotypy performance of the animals. We hypothesized that these differences might not be the result of long-term tether housing or stereotypy performance. In Chapter 6 we tested the hypothesis that individual differences in opioid receptor density were present in animals without a history of tether housing or stereotypy performance and reflected differences in coping style. Two acute tests were used to challenge the animals. A relation was found between behavioural and heart rate responses in the two challenge tests and opioid receptor densities in the hippocampus and the hypothalamus.

Adaptation to long-term tether housing

Changes in endogenous opioid systems

In response to stressful stimulation autonomic, neuroendocrine and behavioural systems can be activated in order to eliminate the stressor or withdraw from it. If coping is successful, the stress response is terminated and homeostasis is

re-established. However, if the situation cannot be dealt with successfully, a state of chronic stress is reached.

When tethered for the first time, pigs show behavioural and physiological reactions that are characteristic for acute stress. The animals try to break out by pulling and biting at their tether chain while vocalizing loudly. Heart rate increases as well as plasma levels of ACTH and cortisol (Schouten and Wiepkema, 1991; Janssens *et al.*, 1995b). After this initial resistance the animals slowly calm down and heart rate and ACTH and cortisol levels gradually return to prestress levels. The time course of this decline varies. Whereas heart rate normalizes within hours, hypersecretion of cortisol is maintained for at least three complete oestrous cycles after tethering (approximately 9 weeks) (Janssens *et al.*, 1995b). The normalization of the heart rate, ACTH and cortisol levels suggests that adaptive changes occur during the chronic stress of long-term tether housing. However, this does not imply normalization of cardiovascular and hypothalamo-pituitary-adrenocortical function. Long-term tether housed pigs have been found to show changes in cardiovascular and hypothalamo-pituitary-adrenocortical responsivity (Schouten *et al.*, 1991; Janssens *et al.*, 1994; 1995b).

The mechanisms responsible for these changes likely include alterations in endogenous opioid systems. It has been demonstrated that the opioid-mediated inhibition of feeding-induced cardiovascular responses increases with long-term tether housing (Schouten and Rushen, 1993). In addition, evidence has been found indicating that the impact of endogenous opioid systems modulating pituitary-adrenocortical responses to acute stress (nose-sling) had increased after 10-11 weeks of tether housing (Janssens *et al.*, 1995b). The results of the study described in Chapter 2 (in which the very same animals were used) point to an increased impact of endogenous opioid systems mitigating heart rate responses to additional acute stress. It may be speculated that the gradual reduction of the hypercortisolaemia (described previously) also results from an increase in the impact of endogenous opioid systems.

The increased impact of endogenous opioid activity may prevent over(re)activity of the cardiovascular and hypothalamo-pituitary-adrenocortical systems. Activation of these systems is a physiological adaptive response, but in the long-term it can be harmful and may ultimately lead to pathophysiological states (Kaplan *et al.*, 1983; Gold *et al.*, 1986a,b; Palatini, 1999). The increased impact

of endogenous opioid systems may, thus, be of adaptive value.
The effectiveness of these changes in endogenous opioid systems may, however, be of transient nature. In Chapter 3 and 4 we found a progressive decrease in brain opioid receptor density in the hippocampus (in the low-stereotypers, not in the animals with high levels of stereotypies) and the hypothalamus with increasing duration of tether housing. We hypothesize that this decrease is a result of neuronal cell loss resulting from prolonged exposure to elevated levels of glucocorticoids. The inverse correlation between basal cortisol concentrations and the number of neuronal cells and total volume of the dentate gyrus that we found in chapter 5 supports this hypothesis.

Development of stereotypies

In addition to changes in pituitary-adrenocortical activity and cardiovascular systems, long-term tether housed pigs may develop persisting disturbances in behaviour, often in the form of stereotypies. Studies in horses (Dodman *et al.,* 1987), bank voles (Kennes *et al.,* 1988) chickens (Savory *et al.*, 1992) and pigs (Cronin *et al.*, 1985; 1986; Rushen *et al.*, 1990) have demonstrated that stereotypies can be blocked by opioid receptor antagonists, such as naloxone. In view of these studies, it has been hypothesized that there is a link between stereotypies and endogenous opioid release and that stereotypies are repeated over and over again because of the reinforcing and addictive properties of opioids (Cronin *et al.*, 1985; 1986; Van Ree and De Wied, 1983). As opioids have calming effects as well (Lewis *et al.*, 1981), performance of stereotypies may be a coping mechanism for animals housed under stressful conditions. By blocking the effects of endogenous opioids, naloxone, in this interpretation, makes stereotypies purposeless, and, as a result, their performance decreases. It has been reported, however, that naloxone injection caused human subjects to feel dizziness, nausea and headache (Cohen *et al.*, 1983). Hence the naloxone effect on stereotypy performance could be due to a side-effect (Broom and Johnson, 1993). It has also been argued that, if stereotypies are rewarding, pretreatment with naloxone should result in a temporary increase in the behaviour before its disappearance (Dantzer, 1991).
An alternative to using opioid antagonists is to look for direct evidence of endogenous opioid activity in animals showing stereotypies. Rushen *et al.* (1990) found evidence of an opioid-based reduction in sensitivity to pain in pigs

following feeding (using a tail-flick test). Contrary to their expectations, however, animals showing high levels of stereotypies were more responsive to the painful stimulus than low-stereotypers. Gilham *et al.* (1994) measured plasma beta-endorphin levels in horses with a stereotypic disorder (cribbing) and in non-cribbing controls. Beta-endorphin levels in the cribbers appeared to be half those of the controls. Schouten and Rushen (1992) have demonstrated that the effect of naloxone on stereotypic chain playing was larger in animals with low levels of stereotypies than in high-stereotypers.
There was, thus, a clear need to extend the research on the relation between stereotypies and endogenous opioid systems. The observed negative correlations between the intensity of stereotypy performance and opioid receptor density in the hippocampus (Chapter 3) and the hypothalamus (Chapter 4) that were found in the present thesis accord with the idea that a relationship between stereotypies and endogenous opioid activity exists. A similar correlation has been found in the frontal cortex (Zanella *et al.*, 1996).

It has often been suggested that stereotypies may help animals cope with adverse conditions, such as long-term tether housing, whether or not by means of activation of endogenous opioid systems. The 'coping' hypothesis of stereotypies is supported by evidence suggesting that animals with high levels of stereotypies have fewer physiological signs of stress. Wiepkema *et al.* (1987) found a negative relationship between tongue-playing in veal calves and the severity of abomasal lesions. Leg-swinging in children was found to be associated with a reduction in heart rate (Soussignan and Koch, 1985). An association between stereotypies and reduced heart rate has also been found in pigs (Schouten and Wiepkema, 1991). Heart rate appeared to be lower during bouts of stereotypies than during bouts of non-stereotyping behaviour (Schouten and Rushen, 1993). This has also been found in calves (Seo *et al.*, 1998). Stereotypies may also lead to changes in the activity of the hypothalamo-pituitary-adrenocortical axis. Kostal *et al.* (1992), e.g., have found lower concentrations of corticosteroids in poultry showing a spot-pecking stereotypy.
There are, however, examples in which stereotypies do not seem to be associated with a reduction in physiological stress symptoms. Biting and licking stereotypies of veal calves, e.g., have no influence on abomasal lesions (Wiepkema *et al.*, 1987), and Schouten *et al.* (1991) and Terlouw *et al.* (1991)

did not find an increase in heart rate or plasma cortisol levels in pigs prevented from displaying stereotypies. However, as the term stereotypies applies to a group of heterogeneous behaviour patterns, properties of one form of stereotypies are not necessarily those of another (Mason, 1991). In addition, the contradictions in the data may be explained by differences in experimental set-up of the studies. In the studies of Schouten *et al.* and Terlouw *et al.*, e.g., chain removal was accompanied by an increase in alternative stereotypies as well as in drinking behaviour. The authors speculate that performance of these behaviours caused a reduction in heart rate and cortisol levels.

A factor, which may explain part of the conflicting results, could be the stage of development of the stereotypy. The effectivity of stereotypies as a de-arousal mechanism seems to decrease with the 'age' of the stereotypy. Schouten and Rushen (1993) have found a negative correlation between stereotypy level and postfeed heart rate in pigs after two months of tether housing, but not after 8-9 months. As the naloxone sensitivity of older stereotypies decreases as well (Cronin, 1985; Kennes *et al.*, 1988), it has been hypothesized that with time stereotypies become emancipated from the original reinforcing endogenous opioid mechanisms and lose their de-arousal capacities. At first sight, the results of Chapter 3 and 4 of the present thesis in which we found that the correlation between stereotypies and brain opioid receptor density disappeared with increasing duration of tether housing, seem to support this hypothesis. However, the loss of correlation should particularly be attributed to a decrease in opioid receptor density in the animals with low levels of stereotypies.

Welfare

The results of the studies described in the present thesis provide evidence indicating that long-term tether housing of pigs leads to changes in endogenous opioid systems and development of stereotypies. As has been discussed earlier, these adaptive changes may help the animals to cope with the adverse conditions of tether housing. However, coping in the sense that the pigs can free themselves from the physical restraint, is not possible. It can be speculated that the animals perceive chronic loss of control (chronic stress) in this situation, and that their welfare is, thus, reduced. Indeed, persisting disturbances in endocrine and cardiovascular systems, indicative of chronic stress, have been found after prolonged tether housing. Tether housed pigs develop hypercortisolaemia and an

increased steroidogenic capacity and sensitivity to ACTH of the adrenals (Janssens *et al.*, 1993; 1994; 1995a,b). In addition, overreaction of the sympathetic nervous system has been found after prolonged tether housing (Schouten *et al.*, 1991). Chronically increased (re)activity of the stress system can have profound physiological consequences and may ultimately lead to stress-related disturbances or diseases. If health problems arise the animals clearly have lost their struggle to withstand the environmental demands, and their welfare is compromised.

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SAMENVATTING

Inleiding

Stress, zowel fysieke als psychologische, kan grote invloed hebben op verschillende fysiologische processen en op gedrag. Er is echter nog maar weinig bekend over de effecten van chronische stress en de mechanismen die hieraan ten grondslag liggen. In het onderzoek dat in dit proefschrift wordt beschreven, werd gebruik gemaakt van een diermodel voor chronische stress: het aangebonden varken. In dit uit de intensieve veehouderij afkomstige huisvestingssysteem worden varkens vastgebonden (aangebonden) met behulp van een nekbeugel die via een korte ketting verankerd is aan de vloer. Wanneer varkens voor het eerst worden aangebonden verzetten ze zich heftig en proberen ze los te breken door te trekken aan de aanbindketting. Ontsnappen is echter niet mogelijk. Tijdens aangebonden huisvesting worden de dieren sterk belemmerd in het uitvoeren van hun natuurlijke gedragsrepertoire en hun mogelijkheden tot contact met soortgenoten zijn zeer beperkt. Verder is er sprake van een uiterst stimulus-arme omgeving. Langdurige huisvesting onder deze condities gaat dus gepaard met stimulus-deprivatie en ernstig chronisch controle-verlies en voldoet daarmee aan de kenmerken van een chronische stressor. Onderzoek aan langdurig aangebonden gehuisveste varkens heeft aangetoond dat bij deze dieren hormonale en cardiovasculaire stoornissen voorkomen en afwijkingen in het gedrag, bijvoorbeeld in de vorm van stereotypieën. Stereotypieën zijn vormen van gestoord gedrag die karakteristiek zijn voor het individu en worden gekenmerkt door hun relatief constante vorm in de tijd, eindeloos lijkende herhaling en hun ogenschijnlijke zinloosheid in de gegeven context. Het bekendste voorbeeld van stereotypieën is waarschijnlijk het “ijsberen” van tijgers en beren in dierentuinen.

Het in dit proefschrift beschreven onderzoek richtte zich met name op stereotypieën en hun mogelijke relatie met endogene opioïd systemen. In de literatuur wordt een verband tussen stereotypieën en endogene opioïd activiteit gesuggereerd. Stereotypieën (bij o.a. aangebonden varkens) blijken te kunnen worden geantagoneerd door behandeling van de dieren met naloxon, een opioïd

receptor-antagonist. Dit leidde tot de hypothese dat stereotypieën eindeloos worden herhaald vanwege de belonende en verslavende eigenschappen van opioïden. Aangezien opioïden ook kalmerende effecten hebben, zouden stereotypieën een copingsmechanisme kunnen vormen voor dieren die onder stressvolle omstandigheden zijn gehuisvest. Naloxon zou stereotypieën "nutteloos" maken door het antagoneren van de effecten van endogene opioïden met als resultaat dat dieren geen of minder stereotypieën uitvoeren.

Overzicht van de in dit proefschrift beschreven studies

Chronische stress kan niet alleen de ontwikkeling van stereotypieën induceren, het kan ook leiden tot langdurige veranderingen in responsiviteit op additionele stressors. Herhaalde of langdurige blootstelling aan een oncontroleerbare stressor heeft in het algemeen een toename van de gedragsfysiologische stress-respons tot gevolg. Ook bij aangebonden varkens zijn veranderingen in responsiviteit gevonden. Het ligt voor de hand dat dergelijke veranderingen berusten op veranderingen in hersenfuncties. Er zijn aanwijzingen dat endogene opioïd systemen betrokken zijn bij veranderingen in responsiviteit. In de studie beschreven in *hoofdstuk 2* werd de rol van endogene opioïd systemen bij veranderingen in responsiviteit nader onderzocht. Het doel van het experiment was het effect van langdurig aangebonden huisvesting op de responsiviteit (hartslag, gedrag) van varkens op een acute stressor te onderzoeken en om de betekenis van endogene opioïden bij veranderingen in responsiviteit na te gaan. Hiertoe werden varkens (experimentele groep) zowel vóór aanbinden als na langdurig (10-11 weken) aangebonden huisvesting blootgesteld aan een "nose-sling" challenge. Hierbij werden de dieren gedurende 15 minuten strak vastgezet aan de stangen van hun hok met behulp van een touw dat aan een lus om de bovenkaak werd gelegd. De dieren verzetten zich hier heftig tegen en hun hartslag steeg sterk. Om veranderingen in endogene opioïd systemen te onderzoeken werden de dieren voorbehandeld met naloxon. Dit leidde tot een toename van de hartslagrespons. Naloxon, een opioïd receptor-antagonist, blokkeert endogene opioïd receptoren en voorkomt op deze manier dat endogene opioïden hun hartslagverlagend effect kunnen uitoefenen. Het effect van naloxon bleek groter te zijn in de aangebonden situatie dan wanneer de dieren vrijlopend waren (vóór aanbinden).

Controle-dieren die gedurende het hele experiment vrijlopend gehuisvest waren, lieten geen verandering in het effect van naloxon zien. Het is dus waarschijnlijk dat de toename in de impact van endogene opioïd systemen die in de experimentele groep werd waargenomen het gevolg was van aangebonden huisvesting. De resultaten van een parallelle studie aan dezelfde dieren wezen erop dat ook de impact van endogene opioïd systemen die voor een demping van de hypothalamus-hypofyse-bijnierschors (HHB)respons zorgen, was toegenomen na 10-11 weken aangebonden huisvesting.
De toegenomen impact van endogene opioïden kan over(re)activiteit van het cardiovasculaire en het HHB-systeem voorkomen. Activatie van deze systemen is een fysiologisch adaptieve respons, maar te sterke en/of langdurige activatie kan schadelijk zijn en kan uiteindelijk leiden tot stoornissen en (verhoogde kwetsbaarheid voor) ziekten. De toegenomen impact van endogene opioïd systemen heeft dus waarschijnlijk een adaptieve waarde.
Er werd geen (verandering in het) effect van naloxon op de gedragsrespons gevonden. Blijkbaar zijn de veranderingen in opioïd (re)activiteit niet relevant voor de gedragsrespons op de nose-sling-challenge.

In *hoofdstuk 3 en 4* werd de relatie tussen endogene opioïd systemen en stereotypieën onderzocht. Hiertoe werden opioïd receptordichtheden gemeten in de hersenen (hippocampus, hypothalamus) van varkens na langdurig aangebonden huisvesting. Er werden drie groepen dieren onderzocht die respectievelijk 2, 5.5 en 8-9 maanden aangebonden waren en die in meer of mindere mate stereotypieën hadden ontwikkeld. In de eerste groep (2 maanden aangebonden) werd een negatieve correlatie gevonden tussen enerzijds het stereotypie-niveau van de dieren en anderzijds de opioïd receptordichtheid in de hippocampus (hoofdstuk 3) en de hypothalamus (hoofdstuk 4). De correlaties leken te verdwijnen in langer aangebonden dieren. Verder werd er een afname in opioïd receptordichtheid gevonden, zowel in de hippocampus als in de hypothalamus, die geassocieerd was met de duur van aanbinden.

In de studie beschreven in *hoofdstuk 5* werd de hypothese getest dat de in hoofdstuk 3 en 4 gevonden afname in opioïd receptordichtheid het gevolg was glucocorticoid-gerelateerd neuronaal celverlies. In de literatuur wordt een verband gesuggereerd tussen excessieve of chronisch verhoogde glucocorticoid

(cortisol, corticosteron)-niveaus en celdood. De hersenen en met name hersencellen met veel glucocorticoid-receptoren, zoals bijvoorbeeld in de hippocampus, lijken bij uitstek gevoelig te zijn voor de katabole invloed van (langdurig) hoge glucocorticoid-concentraties. In hoofdstuk 5 werd de relatie onderzocht tussen het basale cortisol-niveau en het volume en celaantal van de hippocampale dentate gyrus bij varkens na langdurig aangebonden huisvesting. De negatieve correlaties die gevonden werden, duiden op een dergelijke relatie.

In hoofdstuk 3 en 4 werden individuele verschillen gevonden tussen langdurig aangebonden gehuisveste varkens in opioïd receptordichtheid in de hersenen die gerelateerd leken te zijn met het stereotypie-niveau van de dieren. Mogelijk zijn deze verschillen niet het gevolg van langdurig aangebonden huisvesting of van het uitvoeren van stereotypieën, maar waren ze al aanwezig vóór aanbinden en was er een relatie met de 'coping'stijl van de dieren. Dit werd onderzocht in *hoofdstuk 6*. Er werd gebruik gemaakt van varkens die geen aanbind-verleden hadden en die geen stereotypieën vertoonden. De dieren werden blootgesteld aan twee acute challenge testen (open-field-test, aanbindtest) en hartslag- en gedragsresponsen werden gemeten. Er werd een relatie gevonden tussen enerzijds opioïd receptordichtheden in de hippocampus en de hypothalamus die na de slacht werden gemeten en anderzijds hartslag- en gedragsresponsen op de twee challenge-testen. De resultaten wijzen erop dat verschillen in endogene opioïd systemen in de hersenen bijdragen tot verschillen in stress reactiviteit tussen individuele varkens.

Conclusie

Langdurig aangebonden huisvesting van varkens kan leiden tot een toegenomen activiteit van endogene opioïden, waardoor extreme, en daardoor mogelijk schadelijke responsen van o.a. het cardiovasculaire systeem op stressoren worden tegengegaan (hoofdstuk 2). Waarschijnlijk is dit voor de dieren een manier om te 'copen' met de aversieve situatie waarin ze zich bevinden. De effectiviteit van deze veranderingen in endogene opioïd systemen is mogelijk slechts van tijdelijke aard aangezien de opioïd receptordichtheid in de hersenen (hippocampus, hypothalamus en mogelijk ook in andere hersengebieden) progressief afneemt met de duur van aanbinden (hoofdstuk 3 en 4). Deze afname zou het resultaat kunnen zijn van neuronaal celverlies dat optreedt als

gevolg van langdurige blootstelling aan verhoogde glucocorticoïd-niveaus. De resultaten van de studie beschreven in hoofdstuk 5 waarin een negatieve correlatie werd gevonden tussen het basale cortisol-niveau en het aantal neuronale cellen en totaal volume van de dentate gyrus ondersteunen de hypothese van glucocorticoïd-geïnduceerde neurotoxiciteit.

Ook het ontwikkelen van stereotypieën is waarschijnlijk een manier om te copen met stressvolle condities, zoals langdurig aangebonden huisvesting. Het mechanisme dat hieraan ten grondslag ligt is mogelijk een activatie van endogene opioïden. De resultaten van de experimenten beschreven in hoofdstuk 3 en 4 wijzen op een verband tussen stereotypieën en endogene opioïd activiteit in dieren die relatief kort aangebonden staan. Deze relatie lijkt te verdwijnen in langer aangebonden dieren, wat mogelijk een verklaring is voor het feit dat de effectiviteit van stereotypieën als een de-arousal mechanisme afneemt met de 'leeftijd' van de stereotypie.

De mogelijkheden van aangebonden varkens om te copen met de stressvolle situatie waarin ze zich bevinden lijken dus af te nemen naarmate de situatie langer aanhoudt. Uit studies met langdurig aangebonden gehuisveste varkens blijkt dat deze dieren stoornissen vertonen in endocriene en cardiovasculaire systemen. Zo zijn bij aangebonden varkens verhoogde basale plasma cortisol-spiegels gevonden (hypercortisolaemie) en een toegenomen steroïdogene capaciteit en gevoeligheid van de bijnieren voor ACTH. Bovendien neemt tijdens aangebonden huisvesting de reactiviteit van de sympatische tak van het autonome zenuwstelsel toe. Deze toegenomen (re)activiteit van het stress systeem kan vergaande fysiologische gevolgen hebben en kan uiteindelijk leiden tot stress-gerelateerde stoornissen of ziektes. Het is dan ook aannemelijk dat aangebonden huisvesting bijdraagt tot de hoge frequentie van gezondheids-problemen bij aangebonden varkens in de praktijk van de veehouderij.

PUBLICATIONS & ABSTRACTS

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CURRICULUM VITAE

Leonie Wilma Simone (Leanne) Loijens werd geboren op 28 maart 1965 te Heerlen. Ze groeide op in Nuth, doorliep de H.A.V.O. aan het Broekland College te Hoensbroek en vervolgens het Atheneum B aan het St. Janscollege, eveneens in Hoensbroek. In september 1984 begon zij met haar studie biologie aan de toenmalige Landbouwhogeschool te Wageningen. Tijdens de doctoraalfase deed zij de volgende afstudeervakken: ethologie, dierfysiologie, gezondheids- en ziekteleer en celbiologie/immunologie en een stage celbiologie/immunologie bij het Instituut voor Dierhouderij en Diergezondheid, ID/DLO te Lelystad. Ze studeerde af in augustus 1990. Per 1 november van datzelfde jaar trad zij in dienst van de Nederlandse organisatie voor Wetenschappelijk Onderzoek (NWO) en werkte gedurende vier jaar als onderzoeker in opleiding bij de leerstoel Ethologie en de leerstoelgroep Fysiologie van Mens en Dier van Wageningen Universiteit aan het onderzoek dat leidde tot dit proefschrift.

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