NNO 201,2559

STELLINGEN

1. Verandering van huisvestingscondities kan de timing van de pre-ovulatiore Luteïniserend Hormoon (LH) piek bij het varken beïnvloeden. (Dit proefschrift)

2. Endogene Opioïde Peptiden (EOP) zijn betrokken bij de regulatie van de timing van de pre-ovulatoire LH piek. (Dit proefschrift)

3. Bij studies met naltrexon dient men er rekening mee te houden dat de gevoeligheid van varkens voor deze opiaat-receptor antagonist een aanzienlijk individuele variatie vertoont. (Dit proefschrift)

4. Bij het plaatsen van een intracerebroventriculaire canule in dieren die een voorhoofdsholte ontwikkelen kan men zich het beste oriënteren op het neurocranium in plaats van op de externe schedelbeenderen. (Dit proefschrift)

5. Met transrectale echografie kan naast het moment van ovulatie (Soede *et al.*, 1992) ook de folliculaire ontwikkeling nauwkeurig worden bepaald, waarmee deze methode een belangrijke bijdrage kan leveren aan onderzoek naar factoren die invloed hebben op deze processen bij varkens. (**Soede NM, Noordhuizen JPTM, and Kemp B** 1992 The duration of ovulation in pigs, studied by transrectal ultrasonography, is not retelated to early embryonic diversity *Theriogenology* **38** 653-666)

6. The dissociation of peptides with the modified and, more generally, subobtimal anchor residue side chains, may explain the presence of empty Major Histocompatibility Complex (MHC) class I molecules and free MHC class I heavy chains at the cell surface. (Neefjes et al., 1993 European Journal of Immunology 23(4) 840-845)

7. Een perfecte beheersing van taal is nog geen garantie voor een goede communicatie

8. Het eerste axioma van Watzlawick "Men kan niet niet beïnvloeden", geeft aan dat bij proefdierkundig onderzoek het onmogelijk is de proefdieren naief te houden.

9. Bij de interpretatie van onderzoek is de waarde van "het gemiddelde" weliswaar belangrijk doch relatief nietszeggend zonder de waarde van de afzonderlijke samenstellende componenten te weten.

10. Het inkoppen van een voorzet is vaak moeilijker dan op het eerste oog lijkt en hangt niet alleen af van de inkopper maar ook van de voorzetter.

11. Het bezuinigen op onderwijs en gezondheidszorg getuigt van een evengrote intelligentie als het afzagen van de tak waar men op zit. In beide gevallen hangt de afloop af van de ondergrond waarop men denkt terecht te komen.

12. Het geven van kleuren aan een kabinet wekt ten onrechte de indruk dat politiek kinderspel is.

13. Men wordt groot door klein te blijven.

14. Het begeleiden van studenten is als bakken in Croma®: Je moet er even bij blijven door het beste resultaat.

15. De ironie van het leven: De belangrijkste levenslessen worden gedoceerd door hen die zijn overleden.

16. Met de invoering van het basisonderwijs is de negatieve spiraal van de kwaliteit van het totale onderwijsstelsel ingezet.

Stellingen bij het proefschrift: Neuro-Endocrine Regulation of LH Secretion in Cyclic Pigs van John Dierx, 15 januari 1999

Neuro-Endocrine Regulation of LH Secretion in Cyclic Pigs

John Dierx

Promotores: Dr. V.M. Wiegant
Hoogleraar in de Psychoneuroendocrinologie

Dr. D. van der Heide
Hoogleraar in de algemene fysiologie van mens en dier

Neuro-Endocrine Regulation of LH Secretion in Cyclic Pigs

John Dierx

Proefschrift

ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van de landbouwuniversiteit Wageningen
dr. C.M. Karssen
in het openbaar te verdedigen
op vrijdag 15 januari 1999
des namiddags te half twee in de Aula.

Afbeelding op de omslag: Astrologische klok te Praag

Met dank aan Erwin Hellegering
en Kobien van der Luit voor adviezen
voor de lay-out

CIP-DATA KONINKLIJKE BIBLIOTHEEK, DEN HAAG

Dierx, John

Neuro-Endocrine Regulation of LH Secretion in Cyclic Pigs
John Dierx
Thesis Agricultural University Wageningen -With ref.- With summary in Dutch
ISBN 90-5485-957-1
Subject headings: reproductive hormones/ endogenous opioid peptides/ female pig

Contents

Chapter I	General introduction	9
Chapter II	Change in housing conditions alters the timing of the preovulatory LH surge in sows	21
Chapter III	Naltrexone dose dependently increases luteinizing hormone and prolactin during the luteal phase in gilts: Individual variation in response	33
Chapter IV	Pulsatile release of luteinizing hormone during the follicular phase in gilts: Possible role of endogenous opioid peptides	45
Chapter V	Effect of oral treatment with naltrexone of cyclic gilts during the follicular phase of the oestrous cycle on the GnRH stimulated, hypophysial LH release *in vitro*: A pilot study	59
Chapter VI	A new technique for implantation of a chronic cannula in the lateral brain ventricle of postpubertal, freely moving pigs	69
Chapter VII	Repeated intracerebroventricular treatment with naltrexone during the follicular phase advances the termination of the preovulatory LH surge in cyclic, freely moving gilts	85
Chapter VIII	Summary and General discussion	97
	References	103
	Publications & Abstracts	121
	Samenvatting	123
	Dankwoord	129
	Curriculum vitae	131

Chapter I

General introduction

GENERAL INTRODUCTION

Sexual reproduction is one of the most conserved features during evolution and it is one of the most important activities for the survival of animal species. When organisms of a certain species don't reproduce, this species will soon be extinct. In general, reproduction appears to be as normal and essential as eating and both processes are postponed when survival of the organism is threatened e.g. by predators or catastrophes of nature. In reproduction in vertebrates it usually takes two to tango, meaning that the eventual production of offspring is the result of a complicated interplay between two organisms of different sexes. Since in the present thesis attention will be focused on neuro-endocrine processes in reproduction in the female gender, this introduction will be restricted to this side of the interplay.

The Reproductive Axis

The ability to reproduce depends on the presence of the organs that constitute the reproductive axis or hypothalamo-pituitary-gonadal (HPG) axis. The neurones containing the decapeptide gonadotropin releasing hormone (GnRH), also known as luteinizing hormone releasing hormone (LHRH), are dispersed throughout the ventral forebrain with a majority located in the medial preoptic area (MPOA) and mediobasal hypothalamus (MBH) in a variety of species (Halász *et al.*, 1989; Kraeling and Barb, 1990; Levine *et al.*, 1991; Herbison *et al.*, 1993; Jarry *et al.*, 1995). Most of these neurones project to the median eminence (ME), where GnRH is secreted directly in the pituitary portal circulation (Lehman and Karsch, 1993). Subsequently, GnRH selectively stimulates the gonadotrophic cells in the anterior pituitary gland to secrete and synthesise the gonadotropins luteinizing hormone (LH) and follicle stimulating hormone (FSH)(Clayton and Catt, 1981). Both FSH and LH are glycoproteins, that share the same α unit but have a different ß subunit accounting for the biologic difference in hormonal action (Franz, 1988). These gonadotropins affect the gonads by promoting cell proliferation, synthesis and secretion of gonadal steroids (oestrogens, progesterone and testosterone), gametogenesis and eventually trigger ovulation. The gonadal steroids, in turn, exert a feedback at the level of the pituitary and hypothalamus, direct or indirect on the gonadotrophic cells and GnRH neurones respectively (Figure 1).

Oestrus cycle

The hormonal profiles show periodic changes that are repeated over and over again during reproductive life. These menstrual (in humans and non-human primates) or oestrous cycles (other mammals), refer to the shedding of uterine endometrium and the accompanying bleeding or to the cyclic occurrence of increased activity (oestrum is Greek for "gadfly") shown by the female during the period of increased receptivity to the sexual advances of the

male (Fink, 1988). These reproductive cycles can be devided in a luteal and follicular phase (Figure 2). Roughly, the phase of the cycle from ovulation of the oocyte(s) until menstrual bleeding or breakdown of the corpus luteum (remainder of ovarian follicle(s)) is termed the luteal or secretory phase. The phase from cessation of menstrual bleeding or breakdown of corpus luteum ending with the ovulation of the matured oocyte(s), is termed the follicular or proliferative phase (Franz, 1988).

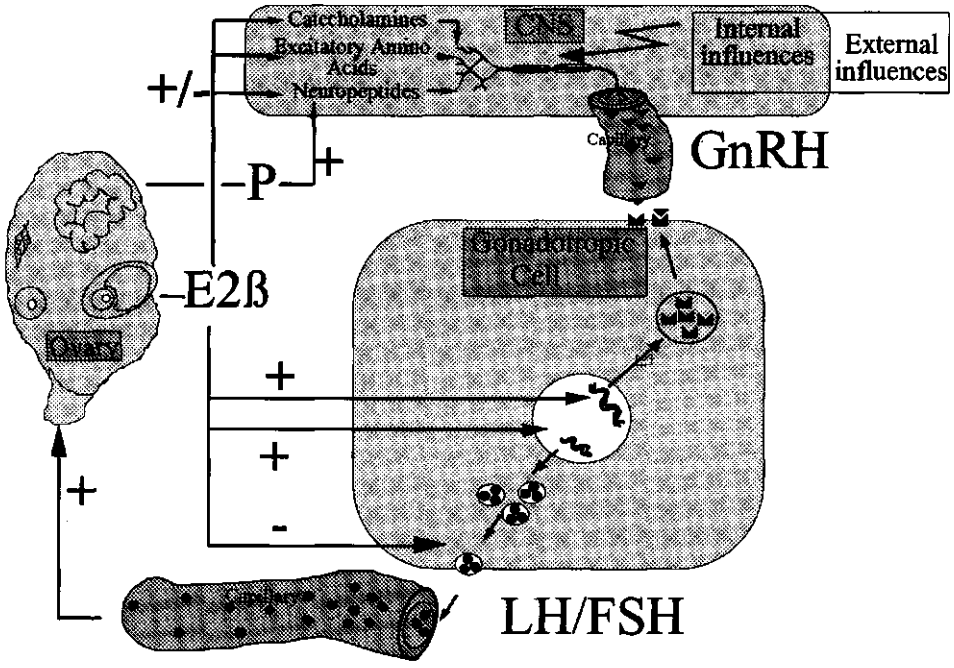

***Figure 1**: Schematic representation of the reproductive axis in the female. GnRH (▼) is secreted from the hypothalamus into the portal blood vessels and stimulates the secretion of the gonadotropic hormones (LH and FSH; ●) from the pituitary. LH and FSH increase synthesis and secretion of ovarian steroids (E2ß and P), which, together with several internal and external influences, exert a direct or indirect "positive" and negative feedback on the secretion of GnRH, GnRH-receptors (▬) and/or gonadotropins.*

Pulsatility of GnRH and Gonadotrophin Release

It is now well established that GnRH is released in rhythmic secretory bursts and that this pulsatile pattern of secretion functions as the primary neuro-endocrine determinant of pituitary pulsatile LH secretion (Yen *et al.*, 1972; Carmel et al, 1976; Levine *et al.*, 1982). Temporal association between GnRH and gonadotropin pulses has been confirmed in a variety of species (Levine *et al.*, 1985; Pau *et al.*, 1986; Karsch *et al.*, 1987; Urbanski *et al.*, 1988), though less compelling for FSH than for LH. The physiological mechanism of the pulsatile pattern of gonadotropin secretion was discovered by Yen *et al.* and his group (1972).

They have reported data indicating that the pulsatile release of endogenous GnRH must be a consequence of a synchronous discharge of GnRH containing neurones effected by some neural signal generator or oscillator, referred to as the "GnRH pulse generator" (Yen *et al.*, 1972). Furthermore, the physiological importance of the pulsatile GnRH secretion has been shown by several groups (Yen *et al.*, 1972; Santoro *et al.*, 1988; Levine *et al.*, 1991; Rossmanith, 1991; Kotsuji *et al.*, 1992). They have reported that qualitative and quantitative differences in the pulsatile pattern, as observed between different phases of the cycle and between subjects with and without ovarian function, are essential for an accurate regulation of all kinds of events determining female fertility. For example, the number of GnRH receptors on and the LH secretion from pituitary gonadotropic cells are decreased after continuous infusion of GnRH, but increased after intermittent administration of GnRH (Pickering and Fink, 1976; De Koning et al, 1978). Thus, pulsatile secretion of GnRH can lead to a "self-priming" effect of GnRH, which appears to be necessary for induction of the preovulatory LH surge.

The pulsatile pattern of GnRH and LH secretion, reflected by frequency and amplitude of pulses, changes during the oestrous and the menstrual cycle. During the luteal phase, pulses are of low frequency and high amplitude. This pattern changes to one of high frequency and low amplitude pulses at the start of the follicular phase. At the end of the follicular phase, pulses of high frequency and high amplitude preceed and constitute the preovulatory LH surge, that induces ovulation (Genazzani *et al.*, 1992). These dynamics of LH secretion are mainly determined by the hypothalamic GnRH pulse generator (Knobil, 1990; Veldhuis, 1990). However, there is some evidence that the pituitary LH response to GnRH changes during the oestrous and the menstrual cycle (Apfelbaum, 1981; Rossmanith, 1991).

Gonadal Steroids in Regulating Pulsatile GnRH and LH Secretion

The changes in the pulsatile GnRH and LH secretion during the oestrous cycle are, amongst others, regulated by the gonadal steroids progesterone (P) and 17ß-oestradiol (E2ß) through a feedback mechanism. During the luteal phase, P is produced in large amounts by the corpus luteum, with very low production of E2ß and androgens (Franz, 1988). The major effect of P is decreasing the LH pulse frequency by decreasing the GnRH pulse frequency (Bouchard *et al.*, 1988; Couzinet and Schaison, 1993). The high LH pulse amplitude during the luteal phase is thought to be related to the low GnRH pulse frequency at the level of the hypothalamus. However, Couzinet and Schaison(1993) speculate that P might also increase LH release at the pituitary level. The significance of the very low concentration of E2ß and androgens under these conditions are not understood. The decrease of P from luteal tissue at the end of the luteal phase leading to the start of the follicular phase coincided with an

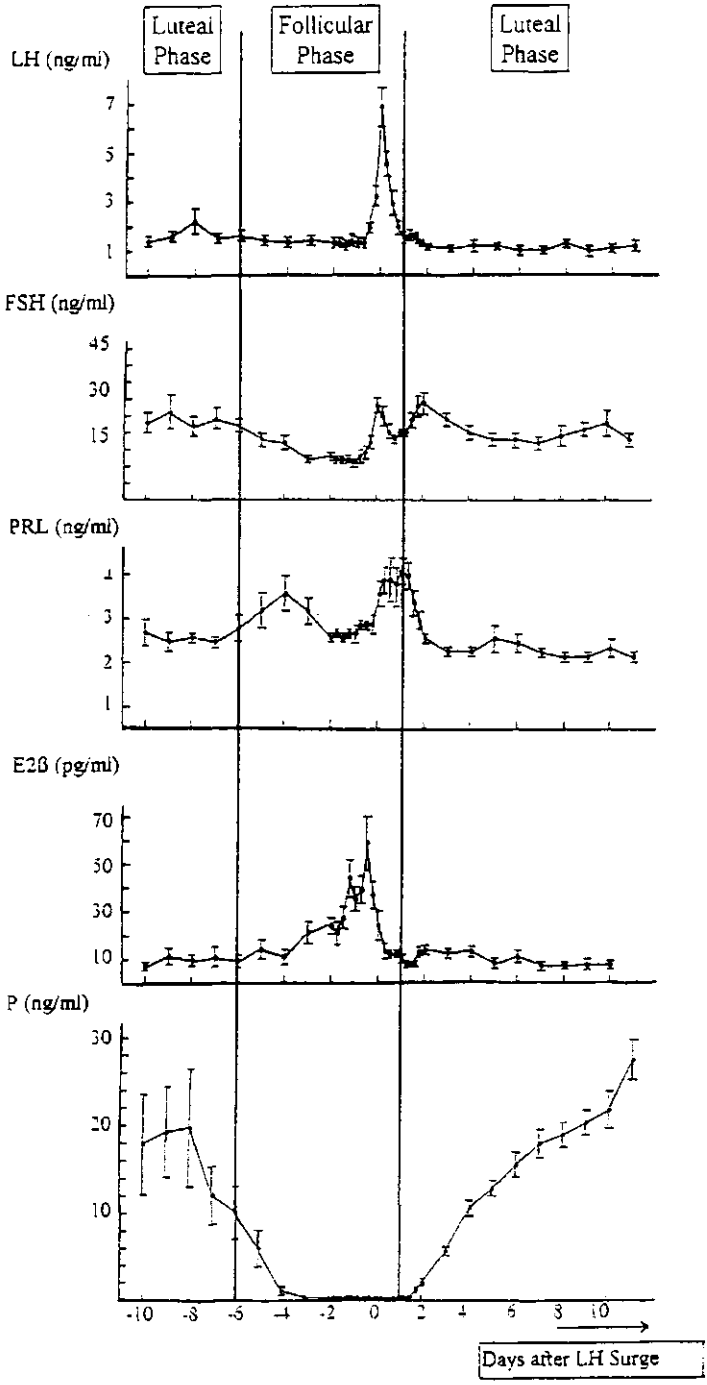

***Figure 2**: Profiles of the concentrations (mean ± SEM) of the pituitary hormones LH, FSH, PRL and the ovarian steroids E2ß and P during the follicular and luteal phase of the oestrus cycles of 19 female pigs (modified after Helmond, F.A., unpublished observations)*

increase in pituitary FSH secretion which induces the development of ovarian follicles for the next cycle and stimulates the biosynthesis of E2ß in the steroid-producing cells of the ovarian follicles (Franz, 1985; Bouchard *et al.*, 1988). E2ß is reported to have both stimulatory and inhibitory actions on the GnRH and gonadotropin secretion, with FSH having a higher sensitivity for E2ß than LH (Couzinet and Schaison, 1993). In early and mid follicular phase, E2ß exerts a negative feedback by inhibiting the LH pulse frequency, reducing multi-unit activity in the mediobasal hypothalamus, which is thought to be part of the "GnRH pulse generator" (Kesner *et al.*, 1987; Tanaka *et al.*, 1992; O' Byrne *et al.*, 1993). However, other studies also suggest a pituitary site of action for the negative feedback of oestradiol by blocking LH and FSH secretion (March *et al.*, 1981; Knobil and Hotchkiss, 1988).
During late follicular phase, E2ß switches to a "positive feedback" which is mainly exerted at the level of the pituitary as demonstrated by Knobil and Hotchkiss (1988) in ovariectomized monkeys with hypothalamic lesions. In vitro studies of rat pituitary cells in culture demonstrate an increase in the response of the pituitary to GnRH administration following administration of E2ß (Drouin *et al.*, 1976; Kamel and Krey, 1982). Furthermore, the number of GnRH receptors on gonadotropic cells, and transcription and storage of gonadotrophin subunits is increased by E2ß (March *et al.*, 1981; Bouchard *et al.*, 1988).
The follicular phase ends with the preovulatory LH surge, which occurs within hours after peak levels of E2ß have been reached. Studies in a variety of species have shown that a preovulatory LH surge can be triggered if plasma levels of E2ß are maintained high enough for a certain amount of time (dependent on the species) and as long as GnRH is present (Knobil *et al.*, 1980; Fink, 1988; Karsch *et al.*, 1992). Other studies have demonstrated that plasma P and 17OH-P start to increase 12 h before a detectable rise in LH in women, and 6-12 h before a mid-cycle rise in LH in monkeys, which might suggest that progesterone is required to establish a preovulatory surge to its full magnitude (Bouchard *et al.*, 1988; Mahesh *et al.*, 1996). Such a synergy between the "positive feedback" of P and E2ß has also been described in vitro (Karsch, 1987).
The mechanism by which gonadal steroids exert their feedback on the level of the hypothalamus is still subject of many studies. Both P and E2ß do not seem to affect the GnRH secretion by directly inhibiting the GnRH neurones in the hypothalamic nuclei, since these GnRH neurones do not posses receptors for P and E2ß (Shivers *et al.*, 1983; Herbison and Theodosis, 1992). Although, recently functional receptors for E2ß have been found in immortalised mouse GnRH neurones (GT1-7 cells; Shen *et al.*, 1998), it is likely that gonadal steroid signals are relayed to GnRH cells by other neurones. Indeed, steroid receptors have been found on a variety of neurones, ranging from gamma amino-butyric acid (GABA; Flügge *et al.*, 1986) and neurotensin neurones (Axelson *et al.*, 1992; Herbison and Theodosis, 1992) in the preoptic area, to neuropeptide Y and ß-endorphin containing neurones in the

arcuate and periarcuate region (Sar *et al.*, 1990; Lloyd *et al.*, 1991; Leshin *et al.*, 1992), the norepinephrin-containing systems originating in the brainstem (Heritage *et al.*, 1977; Zhen and Gallo, 1995) and the tubero-infundibular dopaminergic neurones (Merchenthaler *et al.*, 1995). In addition, changes in norepinephrine, dopamine and ß-endorphin secretion have been shown to be related to stage of the oestrous cycle (Di Paolo *et al.*, 1988; Lévesque *et al.*, 1989; Lloyd *et al.*, 1991; ThyagaRajan *et al.*, 1995). In particular ß-endorphin, an endogenous opioid peptide, is of interest because besides its direct inhibitory action on GnRH secreting cells in the periarcuate region (Blank *et al.*, 1985; Horton *et al.*, 1987; Rodriguez and Wise, 1989), it may inhibit GnRH secretion through direct actions on norepinephrenic neurones in the preoptic area (Mallory *et al.*, 1989; Chang *et al.*, 1993).

Endogenous Opioid Peptides

Endogenous opioid peptides (EOP) have long been implicated in the control of female reproduction, based on the clinical observation of profound disturbances in the menstrual regularity of morphine addicted women. Since Hughes *et al.* (1975) first described endogenous peptides with opiate-like actions, numerous investigations have provided evidence for the pivotal role of EOP's in the neuro-endocrine control of gonadotropin secretion in a variety of animals (Barb *et al.*, 1985; Nanda *et al.*, 1991; Currie *et al.*, 1992; Aurich *et al.*, 1995) and humans (Genazzani *et al.*, 1993). Before going into the role of EOP's in the regulation of LH and GnRH secretion, the nature and origin of these peptides will be described.

As yet, three major groups of opioid peptides have been identified (Lord *et al.*, 1977; Guillemin, 1980; Akil *et al.*, 1984; Yen, 1991): the endorphins (ß-endorphin and related peptides), the enkephalins (met- and leu-enkephalin and related peptides) and the dynorphins (dynorphin A and B and related peptides). ß-Endorphin as a 31-amino acid sequence derived from the proopiomelanocortin (POMC) precursor (Eipper and Mains, 1980) is considered the most important opioid peptide for the neuro-endocrine regulation of reproduction (Rossmanith, 1992). The pentapeptides Met- and Leu-enkephalin, originate from proenkephalin A (Lazarus *et al.*, 1976; Lowry *et al.*, 1980; Akil *et al.*, 1984), and the prodynorphin precursor peptide is cleaved into dynorphin A, dynorphin B (also known as rimorphin) and α- and ß-neo-endorphin (Akil *et al.*, 1984; Rossier, 1988). All three types of EOP's are widespread in the brain, mainly localised in hypothalamic areas. In addition, enkephalins and ß-endorphin are found in the anterior pituitary and other organs. Pharmacological investigations have permitted identification of at least five distinguishable opioid receptor types: the μ-, ϵ-, δ-, κ- and σ-receptors (Paterson *et al.*, 1983; Akil *et al.*, 1984; Ferin *et al.*, 1984; Yen *et al.*, 1991). All EOP's share similar peptide sequences, and they may therefore be functionally active at more than one of the distinct receptor types

(Rossmanith, 1992). In neuroendocrine processes, opioids thus may operate as neurotransmitters or neuromodulators. Since EOP's are locally secreted within the anterior pituitary, they may also exert paracrine effects locally (Barkin *et al.*, 1983; Rossmanith, 1992).

High densities of opioid receptors are found in a wide variety of nuclei throughout the brain (Pfeiffer *et al.*, 1982), and EOP's play a role in the regulation of various vegetative and behavioural functions and the neuroendocrine control of hormone release. Opioids have been implicated in functions such as pain, temperature perception, hunger and thirst control, sexual behaviour and adaptation to different environmental inputs (Guillemin, 1980; Akil *et al.*, 1984; Grossman, 1988; Rossmanith and Lauritzen, 1991; Armeanu, 1991). In addition, it has been observed that endorphins are activated during the response to stress (Akil *et al.*, 1984; Bloom, 1980; Grossman *et al.*, 1982; Rivier and Rivest, 1991; Dobson and Smith, 1995), defined by Selye (1973) as "a non-specific response of the body to any demand (usually noxious) or to any stimulus causing an alteration in homeostatic processes". For example, in pigs, it has been shown that tethered housing -a chronic stressor- increases adrenocortical sensitivity to ACTH, its steroidogenic capacity and plasma concentrations of cortisol and prolactin (PRL; Janssens, 1994; Janssens *et al.*, 1994; Janssens *et al.*, 1995a). Furthermore, tethered housing increases endogenous opioid activity (Janssens *et al.*, 1995b) and the number of μ-opioid receptors in the brain (Zanella *et al.*, 1996). In addition, tethered housing leads to opioid dependent stereotyped behaviour (Cronin, 1985; Schouten en Wiepkema, 1991), with a negative correlation between both μ and κ receptor densities and duration of stereotypies (Zanella *et al.*, 1996).

Opioidergic Control of Gonadotropin Release During The Oestrus Cycle

Numerous studies have shown that the expression of endogenous opioid activity plays a pivotal role for gonadal steroids feedback during the oestrous cycle in a variety of species (Haynes *et al.*, 1989; Weiland and Wise, 1990; Schwarz and Pohl, 1994; Simpkins, 1994). As ovarian steroid concentration changes during the transition from the follicular to the luteal phase, opioid tone is subsequently altered (Lloyd *et al.*, 1991; Thom *et al.*, 1996). During the P dominated luteal phase, EOP's exert a tonic inhibition on LH pulse frequency resulting in decreased plasma LH concentration as has been shown in rat (Higuchi and Kawakami, 1982), sheep (Montgomery *et al.* 1985), pig (Barb *et al.*, 1988) and humans (Rossmanith *et al.*, 1989). Treatment with the opioid receptor antagonist naloxone or naltrexone during the luteal phase, increases plasma LH concentration. The involvement of EOP's in regulating pulsatile LH release during the E2ß dominated follicular phase is not clear. It seems that EOP's are not involved in the negative feedback of E2ß on the pulse amplitude and mean plasma concentration of LH during the early follicular phase. However, during the late follicular phase and the presumed positive

feedback of E2ß increases of these parameters of LH release were found after treatment with opioid receptor antagonists in humans (Rossmanith *et al.*, 1989), rats (Piva *et al.*, 1985) and pigs (Okrasa *et al.*, 1992). Prolonged opioidergic blockade elicits a markedly enhanced LH pulse amplitude but not pulse frequency (Rossmanith *et al.*, 1989). In addition, there is increasing evidence that EOP's might play a role in the generation and timing of the preovulatory LH surge and oestrus (Massotto *et al.*, 1990; Kraeling *et al.*, 1992; Walsh and Clarke, 1996; Smith and Gallo, 1997). Armstrong *et al.* (1988) reported that chronic administration of morphine (sc) to sows for 5 days after weaning delayed the onset of oestrus, and other studies (Ziecik *et al.*, 1994; Kraeling *et al.*, 1992) reported a delayed preovulatory LH surge in E2ß primed OVX gilts after iv and ICV morphine treatment with no effect on the height of the surge.

Aims And Outline Of The Thesis

LH secretion during the oestrous or menstrual cycle, and the luteal phase in particular, has been studied in a variety of species as sheep (Whisnant *et al.*, 1991), human (Fillicori *et al.*, 1986, Rossmanith *et al.*, 1990), pig (Okrasa and Tilton, 1992) and primate (Norman *et al.*, 1994). During the luteal phase, plasma LH concentration has been shown to be decreased by endogenous opioid peptides (EOP's), mediating the negative feedback of progesterone (P; Yang *et al.*, 1988; Barb, *et al.*, 1992; Kaynard *et al.*, 1992). However, relatively few data are available on the pattern of pulsatile LH release during the E2ß dominated follicular phase, in particular with regard to pulse frequency, pulse amplitude and timing of the preovulatory LH surge, events that participate in, and possibly determine, the timing of ovulation and therefore fertility and success of fertilisation. In addition, stressful conditions have been shown to have adverse effects on LH secretion (Brann and Mahesh, 1991; Dobson and Smith, 1995) and hormones of the hypothalamus-pituitary-adrenal (HPA)-axis and EOP's released during stress have been shown to inhibit the hypothalamus-pituitary-gonadal (HPG) axis.

The aim of the present thesis was to gain insight in the neuroendocrine regulation of the LH secretion during the follicular phase, and in particular to determine whether EOP's also play a role in the pulsatile LH release and the timing of events leading to the preovulatory LH surge and oestrus. The female pig was used as experimental animal for a number of reasons. In modern intensive pig breeding where tethered housing is common practice, a large percentage of the sow population shows reduced fertility and has to be replaced. Insight in the mechanisms underlying reduced fertility could contribute to effective pig breeding. Furthermore, in pigs, like in primates and humans, opioid modulation of the LH secretion is dependent on the gonadal steroid environment which is, although predominantly at the hypothalamic level, also regulated at the level of the pituitary (Knobil, 1980). Thus the tethered pig would render an excellent animal model for studying the effects of chronic stress, and possible increased opioid activity, on LH

secretion during the oestrous cycle at the hypothalamic and pituitary level (Cronin, 1985; Schouten en Wiepkema, 1991; Janssens, 1994; Zanella *et al.*, 1996).

Since it is extremely difficult to determine the transition from the luteal into the follicular phase, the oestrous cycles of all animals that were studied during the follicular phase, were synchronised using the progesterone agonist altrenogest.

Before studying the possible effects of EOP's during the follicular phase in detail, it was investigated whether a change in housing condition, from life long tethered housing to a loose housing system in individual pens, affects the LH pulse characteristics during altrenogest treatment, the timing of the preovulatory LH surge and the LH secretion during the following oestrous cycle (Chapter II). The change in housing condition appeared to advance the preovulatory LH surge and to decrease mean plasma LH concentration, which could not be explained with differences in activation of the HPA system. The question arose whether these effects could be ascribed to a change in the reactivity of the EOP-system, which has been shown to occur in tethered housing (Janssens *et al.*, 1995b; Zanella *et al.*, 1996; Loyens, Schouten and Wiegant, unplublished observations). To address this question, we decided to use the potent and long acting opioid receptor antagonist naltrexone. This drug had not been used in pig before, and therefore we first designed a study with the objective to investigate the possible individual variability in the response of the reproductive axis by determining the increase in mean plasma LH concentration after intravenous administration of saline or 5 doses of naltrexone (Chapter III).

Subsequently, we determined the pulsatile pattern of LH release, and the possible role of EOP's in modulating this pattern, during 4 days of the follicular phase in Chapter IV. Furthermore, the effect of EOP's on the occurrence of the first day of oestrus was investigated. The results from this experiment suggested that EOP's do not inhibit the pulsatile LH secretion as such, but might affect the events leading to the timing of oestrus and possibly the preovulatory LH surge.

A pilot study, described in Chapter V, was designed to investigate changes in the responsivity of the pituitary gonadotrophs *in vitro* to GnRH changes during the follicular phase. In addition, the possible role of EOP's therein was studied.

A novel intracerebroventricular (ICV) cannulation technique for pigs was developed (Chapter VI). This technique, was used to treat freely moving intact gilts (nulliparous pigs) with repeated ICV injections of naltrexone on multiple days during the follicular phase of the oestrous cycle (Chapter VII) to determine whether the shift of oestrus (as found in Chapter IV) or the preovulatory LH surge (as found in Chapter II) was a consequence of changing opioid activity in the brain.

In Chapter VIII, the results of the present thesis are summarised and discussed.

Chapter II

Change in housing conditions alters the timing of the preovulatory LH surge in sows

Dierx, J.A.J.[*], Kemp, B.[**], Soede, N.M.[**], Helmond, F.A.[***], van der Heide, D.[*] and Wiegant, V.M.[****]

[*]Department of Human and Animal Science, Wageningen Institute of Animal Science, Agricultural University Wageningen, Haarweg 10, 6709 PJ Wageningen, The Netherlands.
[**]Department of Animal Husbandry, Wageningen Institute of Animal Science, Agricultural University Wageningen, Marijkeweg 40, 6709 PG Wageningen, The Netherlands.
[***]N.V. Organon Scientific Development Group, PO Box 20, 5340 BH Oss, The Netherlands
[****]Rudolf Magnus Institute for Neurosciences, University Utrecht, Stratenum Universiteitsweg 100 3584 CG Utrecht, The Netherlands

Abstract

The effect of housing conditions on the pulsatile LH secretion during the oestrous cycle were investigated in stress-adapted sows. Multiparous sows with a history of long term tethered housing either remained tethered housed (N=10; TETHER pigs) or were individually loose housed (N=12; LOOSE pigs). The oestrous cycles of the pigs were synchronized by altrenogest treatment during 21 days (first day following treatment: Day 0). Blood samples for hormone determinations were collected with 12 minutes intervals on Days -3, 2, 4 and 19, and every 4 hours from Day 4 until the preovulatory LH surge had occurred.
During Day -3, Day 19 (luteal phase), mean plasma LH was lower in LOOSE than in TETHER animals, but LH pulse frequency and amplitude were not different between housing conditions. The preovulatory LH surge and oestrus occurred later in LOOSE than in TETHER animals (116 ± 5 vs 91 ± 3 h, respectively 6.7 ± 0.4 vs 5.3 ± 0.2 days after terminating altrenogest treatment). LH surge height and duration of oestrus were not different between housing conditions. During the follicular phase, the LH pulse frequency was lower, and pulse amplitude and mean plasma LH were higher on Day 2 than on Day 4. Housing condition had no effect on these parameters.
The present data suggest that a change in housing condition affects LH secretion in pigs with a history of tethered housing. Conversely, this suggests that chronic stress leads to alterations in the mechanisms regulating LH secretion.

Introduction

Reproductive processes, like LH secretion, follicle development and ovulatory activity, are influenced by stressful conditions (Dobson and Smith, 1988; Brann and Mahesh, 1991). As reviewed by Brann and Mahesh (1991), the effects of stress on the reproductive axis seem to depend on whether the stressor is acute or chronic. Acute stress can stimulate LH release and enhance ovulatory activity (Higuchi *et al.*, 1986; Armario *et al.*, 1987; Briski and Sylvester, 1988), whereas chronic stress can inhibit LH secretion, cyclicity and follicle development (Gray *et al.*, 1978; Taché *et al.*, 1978; Rasmussen and Malven, 1983; Moberg, 1987). It is thought that the effects of stress on reproductive processes are brought about by altered activity of mediators of the stress response. Indeed, hormones of the hypothalamus-pituitary-adrenal (HPA)-axis and endogenous opioid peptides (EOP's) can inhibit the hypothalamus-pituitary-gonadal (HPG)-axis (Naylor *et al.*, 1990; Norman *et al.*, 1994; Akema *et al.*, 1995). In pigs, it has been shown that long term tethered housing induces symptoms typical of chronic stress that are generally thought to relate to adverse effects on physiological

functions, welfare and health of the animals. Thus, tethered housed pigs show increased adrenocortical steroidogenic capacity and sensitivity to adrenocorticotropin (ACTH), elevated plasma cortisol and prolactin concentrations and a flattened diurnal rhythm of cortisol (Janssens *et al.*, 1994, 1995a). Interestingly, there is evidence for stress-adaptive changes in long-term tethered housed pigs. These include an increase in the endogenous opioid activity that mitigates hormonal and autonomic responses to acute challenges (Janssens *et al.*, 1995b; Zanella *et al.*, 1996; Loyens, Schouten and Wiegant unpublished observations). Increased activity of endogenous opioids also underlies the behavioural stereotypies that are frequently observed in tethered pigs, particularly when the animals are aroused and that are associated with de-arousal and diminished autonomic and pituitary-adrenocortical responsivity (Cronin, 1985; Schouten en Wiepkema, 1991). Adaptive changes in regulation of behaviour and physiology become most evident under conditions of environmental demand. In commercial breeding farms, tethered housing is frequent practice, but there is no evidence sofar for impaired reproductive performance of the animals, suggesting that adaptive mechanisms counteract the detrimental effects of stress on reproductive regulation.
The present study is designed to investigate whether chronic tethered housing of sows affects the LH pulse characteristics during the oestrous cycle and the timing of the preovulatory LH surge after oestrus synchronisation with the progesterone analogue altrenogest.

Materials and methods

Animals and housing

Twenty six commercial, multiparous (parity 2-7), crossbred sows (F1, Great Yorkshire x Dutch Landrace) were obtained from a Dutch breeding company and had a history of long term tethered housing. On arrival, they were randomly assigned to tethered housing with a neckchain, or loose housing in individual pens of 6 m^2 with a concrete floor covered with woodshavings, except for a slatted dunging area at the rear of the pen. Lights were on from 7.30 h to 19.30 h and ambient room temperature ranged from 15 °C to 25 °C. At 800 hours and 1600 hours the animals were fed 1.25 kg of dry sow feed (12.2 MJ of metabolizable energy per kilogram containing 15.4% crude protein) by hand. Water was available ad libitum through a nipple drinker.

Surgery

In order to collect frequent bloodsamples, the sows were surgically fitted with a permanent jugular vein catheter (Silastic® medical grade tubing, 0.040 in. i.d., 0.085 in. o.d.; Dow Corning, Michigan U.S.A.) under sterile conditions and under general anaesthesia with

inhalation of O_2/N_2O, enflurane Ethrane®, Abott B.V., The Netherlands) as described previously (Janssens *et al.*, 1994). The animals were equipped with a harness to protect the cannula, which was externalized between the scapulae. The harness (23 cm x 20cm; polyvinyl chloride with nylon; Bizon Chemie, The Netherlands) was fixed at the back of the animals with a belt around the chest during the week before surgery in order to habituate to the harness. All animals were treated with antibiotics (12 ml of T.S. Sol®, containing trimethorprim and sulfamethoxazol, orally; Dopharma, The Netherlands) once daily from 3 days prior to 3 days after surgery. To prevent obstruction by bloodclots, the catheters were flushed with saline once weekly and filled with heparinized saline (25 IU heparin/ml of 0.9% saline; Leo Pharmaceutical Products, The Netherlands) when not in use. When catheter patency was reduced, obstructions were removed as described by Leuvenink and Dierx (1997). In short, the catheter was filled with a solution of 25000 IU kabikinase (Kabi Pharmacia, Sweden), 2500 IU heparin and 0.2 ml Ticarpen® (Beecham, England) to 25 ml sterile 25% polyvinylpyrrolidone/saline (Merck, Germany), and after 1 week, flushed with a 2% heparine/saline solution.

Experimental design

On arrival at the facilities of the Wageningen Agricultural University, 13 animals were housed tethered (TETHER) with a neck chain and 13 animals were housed loose (LOOSE) in individual pens. After 1 week, surgery was performed and the animals were allowed approximately 1 week for recovery during which they were frequently handled and habituated to the bloodsampling procedure. Then, the oestrous cycles of all animals were synchronized by daily oral administration of 20 mg of the progesterone agonist altrenogest (Regumate®) for 21 days (day 0). The day of altrenogest withdrawal was designated Day 0. On Day -3 (the 19th day of the altrenogest treatment) and at Day 2, 4 (both follicular phase) and 19 (luteal phase) after altrenogest withdrawal, frequent bloodsamples were taken (every 12 minutes during 12 hours) from 8.30 h until 20.30 h. After Day 4, bloodsamples were taken every 4 hours until the preovulatory LH surge had occurred as determined by a fast LH radioimmunoassay. Of all 22 animals the peak value of preovulatory LH surge was detected.

Bloodsampling

Blood samples were taken according to the procedure as described previously (Janssens *et al.*, 1994). Immediately after collection, the blood samples (approximately 5 ml) were transferred to ice-cooled polypropylene tubes containing 50 µl EDTA solution (144 mg EDTA/ml of saline; Triplex®III, Merck Nederland BV,The Netherlands). The tubes were shaken, kept on ice and subsequently centrifuged at 3000xg for 15 minutes at 4 °C. Plasma was collected and stored at -20 °C until hormone analysis.

Hormone analysis

LH. Plasma samples were analysed for LH using a double anti-body radioimmunoassay (RIA) as described by Niswender *et al.* (1970), using porcine LH (pLH iodination grade batch 004/3; potency, 0.85 x NIH LH-S19; UCB bioproducts, Brussels, Belgium) as a standard and for radioiodination (specific activity, 38 μCi/μg). Anti-porcine LH batch 004 (UCB bioproducts, Brussels, Belgium) was used at a final dilution of 1:360 000, which gave an initial binding of the labeled hormone of approximately 39%. The main cross reacting peptides were pFSH (2.7%), pLHa (1.1%), pTSH (0.5%) and pTSHa, pTSHß and pLHß (all<0.1%). Sac-Cel® was used as the second antibody, (donkey anti-rabbit; Wellcome Reagents, Beckenham). The minimal detectable concentration at the 90% $B:B_0$ was 0.1 ng/ml. The interassay coefficient of variation was 14.4 % and the intra-assay coefficient of variation was 7.2 %. The fast LH RIA was performed according to the same method but with slight modifications of incubation time and temperature (2 hours at 37 °C).

Cortisol. Hourly samples on Day -3 and 19, were analysed for cortisol. After extraction of plasma with dichloromethane (DCM, Merck, Darmstadt, Germany), cortisol concentration was determined using a single anti-body radioimmunoassay. For estimation of procedural losses, 500 cpm of [1,2,6,7-^{3}H]cortisol (TRK407, specific activity 80.5 Ci/mmol, Amersham Int., Amersham U.K.) was added to 1 ml plasma sample and mixed with 3 ml DCM. The organic phase of the mixture was evaporated under a stream of nitrogen and redissolved in 500 μl phosphate buffer with 1% BSA. An aliquot of 150 μl was taken to determine the recovery of [^{3}H]cortisol. Cortisol concentrations were measured in duplicate (2 aliquots of 50 μl) using a single-antibody radioimmunoassay (RIA) technique, previously described by Janssens *et al.* (1994). The main crossreacting steroids were 21-desoxycortisol (72%), cortisone (59%), prednisolone (53%), 11 desoxycortisol (43%), corticosterone (10%), progesterone (2.3%), estradiol-17ß, dexamethasone, and triamcinolone acetonide (all <0.1%). The sensitivity of the assay was 0.5 $ng.ml^{-1}$ at the 90% B/B_0 level. The intra- and inter-assay coefficients of variation were 8.2 % and 14.7 % respectively. The amount of cortisol was expressed in $ng.ml^{-1}$ after correction for procedural losses.

Detection of oestrus

Oestrus detection was performed by a back-pressure test in the presence of a vasectomized boar once daily in the morning on all days until 1 month after withdrawal of altrenogest treatment. The time of oestrus was defined as the first day the sow showed a standing response. When animals had not shown oestrus before day 7, ultrasound was performed as described by Soede *et al.* (1991) to determine whether ovulation had occurred.

LH Pulse detection

The profiles of the pulsatile LH release were analysed using the pulse analysis program of Maxima/Chromcard (Fisons Instruments,Interscience, Breda, Holland) with baseline calculated according to an allgorithm taking into account the total LH profile. A pulse was defined by a baseline-peak ratio of 0.5 or higher and a minimal area under the pulse of 6.0×10^6 ng.ml^{-1}.min.

Data analysis and statistics

Data of pulse frequency, pulse amplitude and mean plasma LH concentration of LH during and after altrenogest withdrawal, time and height of the preovulatory LH surge and the first day of oestrous, obtained after altrenogest withdrawal, were analysed using SAS statistical analysis system (1990). In addition, to determine whether effects found in pulse frequency, pulse amplitude and mean plasma LH were due to differences in the timing of the preovulatory LH surge, data were analysed relative to the day of preovulatory LH surge. By synchronising the data, the number of animals per housing condition is reduced. On Day -2 and -3, most animals had data on pulse frequency, pulse amplitude and mean plasma LH, and were used to analyse possible effects of housing conditions on pulsatile LH release relative to the preovulatory LH surge. The effect of tethering on these parameters, and on hourly samples of cortisol and on the daily mean plasma level of cortisol on Day -3 and Day 19, was tested using the GLM procedure using the model: $Y_{ij} = \mu + T_i + e_{1i} + D_j + (TxD)_{ij} + e_{2ij}$; Where Y_{ij} = value of parameter in a sow (n=22) receiving treatment i on sampling day j; μ = overall mean; T_i= fixed effect of treatment i (1,2); e_{1i}= error term 1, which represents the random effect of sow within treatment i; D_j= fixed effect of sampling day j (1,..,4); e_{2ij}= error term 2, which represents the random effect of treatment i between sampling days j. The effect of tethering treatment was tested against error term 1. The other effects were tested against error term 2. Differences were considered significant when $P<0.05$. Pulse amplitude and mean plasma LH did not have a normal distribution and were therefore subjected to non-parametric analysis using the Kruskal-Wallis test from the NPAR1WAY procedure of the SAS programme.

Results

General

Of the 13 TETHER and 13 LOOSE sows, 3 TETHER animals and 1 LOOSE animal did not show oestrous behaviour after altrenogest treatment was terminated. Ultrasound with the sows that did not show oestrous behaviour showed that 1 TETHER and 1 LOOSE animal had developed cystic ovaries. The other 2 TETHER sows had corpora lutea and were therefore

classified as having displayed silent oestrus. Data of all 4 animals were excluded from statistical analysis and, as a consequence, 12 LOOSE and 10 TETHER sows were used to assess LH pulse characteristics.

Plasma LH and cortisol concentration during the luteal phase with and without altrenogest
No differences were found in number of LH pulses (p=0.50) and LH pulse amplitude (p=0.61) between endogenous (Day 19) and exogenous (altrenogest period; Day -3) progesterone dominance (Table 1), or between LOOSE and TETHER sows (p=0.40 and p=0.83 respectively). Furthermore, no interaction between housing conditions and altrenogest treatment was found (p=0.60 and p=0.50 respectively).
Mean plasma LH was not different between Day -3 and Day 19 (p=0.57). However, a significantly higher mean plasma LH was found in TETHER than in LOOSE animals (p=0.005), with no interaction between housing condition and altrenogest treatment (p=0.80). In plasma cortisol, no differences were found (p=0.24) between LOOSE and TETHER sows, nor between Day -3 and Day 19 (p=0.09). Furthermore, no significant interaction between housing and day was found(p=0.99). In addition, there was no indication for the presence of a diurnal rhythm in cortisol in of both LOOSE and TETHER sows. The daily mean plasma concentration of cortisol of LOOSE respectively TETHER animals on these days was: 15.2 ± 2.0 ng/ml versus 20.2 ± 2.6 ng/ml (mean ± SEM; n=12 versus n=10) on Day -3 and 25.1 ± 6.5 ng/ml versus 29.1 ± 5.0 ng/ml (mean ± SEM; n=12 versus n=9) Day 19.

Table 1: Pulsatile LH release during progesterone dominance in the luteal phase and under altrenogest treatment in LOOSE and TETHER sows.

	Day -3		Day 19	
	LOOSE (n=12)	TETHER (n=10)	LOOSE (n=12)	TETHER (n=9)
number of pulses (#/12 h)	6.08 ± 0.41	6.10 ± 0.61	6.17 ± 0.53	6.78 ± 0.74
pulse amplitude (ng. ml^{-1})	8.54 ± 1.60	14.1 ± 5.60	10.5 ± 1.78	12.6 ± 4.13
mean plasma LH ($ng.ml^{-1}$)	9.44 ± 2.05	15.9 ± 2.68*	7.55 ± 0.74	15.4 ± 3.76*

Data of animals during altrenogest treatment (Day -3) and the luteal phase of the oestrous cycle (Day 19) are presented as means ± SEM.
** = significantly different from loose housed ($p<0.05$).*

Oestrus
LOOSE sows came in oestrus on average at 6.7 ± 0.4 days after termination of altrenogest treatment, which was significantly (p=0.002) later than TETHER sows that came in oestrus after on average at 5.3 ± 0.2 days. The duration of oestrus was not different (p=0.47) in LOOSE compared to TETHER sows (2.2 ± 0.2 days and 2.2 ± 0.4 days respectively).

Preovulatory LH surge

The peak value of the preovulatory LH surge in the TETHER animals (n=10) occurred on average at 91 ± 3 (mean ± SEM) hours after altrenogest withdrawal (see Figure 1). This is significantly (p=0.001) earlier than in the LOOSE animals (n=12), in which the preovulatory LH surge peaked at 116 ± 5 hours after altrenogest withdrawal. There was no significant difference in the height of the preovulatory LH surge between LOOSE and TETHER sows (36.7 ± 5.3 ng/ml versus 49.5 ± 6.3 ng/ml; mean ± SEM; p=0.16).

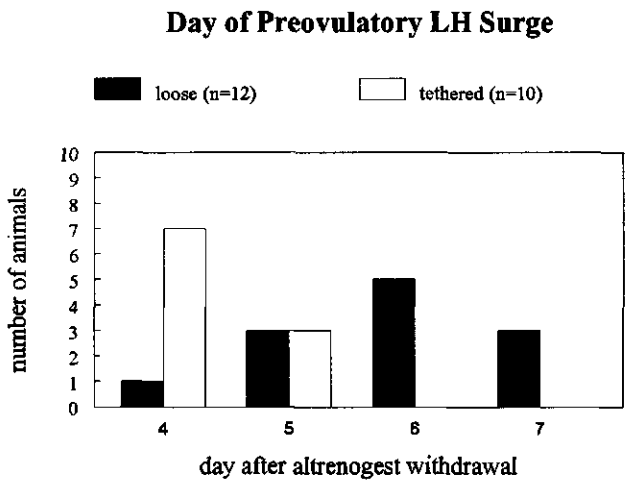

***Figure 1**: Time of the preovulatory LH surge (days after altrenogest withdrawal) in LOOSE and TETHER sows. Distribution of number of LOOSE (closed bars, total n=12) and TETHER (open bars; total n=10) animals, that have their preovulatory LH surge, over the period after altrenogest withdrawal.*

Pulsatile LH release on Day 2 and 4 after altrenogest withdrawal

In pulse frequency a significant day effect (p=0.007; Table 2) was found. Pulse frequency on Day 2 after altrenogest withdrawal was significantly higher compared to Day 4. No housing effect (p=0.40) was found, and no significant interaction between housing and day was found in pulse frequency (p=0.39).

A significant day effect (p=0.01) was found for LH pulse amplitude, with the amplitude on Day 4 being significantly higher than on Day 2 (table 2). Yet, no differences in pulse amplitude were found between LOOSE and TETHER housing (p=0.35), and there was no significant interaction between housing and day in pulse amplitude (p=0.13).

In mean plasma LH, a significant day effect (p=0.02) was found. Mean plasma LH on Day 4 was significantly higher than on Day 2. No housing effect (p=0.34) nor a significant interaction between day and housing (p=0.08) was found.

Table 2: Pulsatile LH release on Day 2 and 4 after altrenogest withdrawal in LOOSE and TETHER sows.

	Day 2 after altrenogest		Day 4 after altrenogest	
	LOOSE (n=12)	TETHER (n=10)	LOOSE (n=12)	TETHER (n=10)
pulse frequency (#/12h)	8.17 ± 0.37	8.10 ± 0.31	6.67 ± 0.40	7.30 ± 0.54
pulse amplitude (ng. ml^{-1})	5.23 ± 0.51	8.88 ± 2.89	7.58 ± 1.35	24.3 ± 5.62
mean plasma LH ($ng.ml^{-1}$)	8.25 ± 1.62	14.5 ± 2.64	10.0 ± 2.00	26.4 ± 5.04

Data are presented as mean ± SEM. A day effect was found with LOOSE and TETHER animals having significantly higher pulse frequency but lower pulse amplitude and mean plasma LH on Day 2 than on Day 4 ($p<0.05$).

Since the occurrence of the preovulatory LH surge on Day 4 in 8 out of 22 animals could have interfered with data on LH pulse frequency, pulse amplitude and mean plasma LH, data were synchronized on the day of the preovulatory LH surge (Day 0) and analysed on Day -2 and -3. As a consequence, the number of animals per treatment group decreased. No differences were found in LH pulse amplitude between Day -2 and -3 ($p=0.64$), nor between TETHER and LOOSE animals ($p=0.91$), nor an interaction between day and housing ($p=0.92$). However, LH pulse frequency tended to be higher on Day -3 than on Day -2 ($p=0.08$), with no effect between housing conditions ($p=0.75$), nor an interaction between day and housing conditions ($p=0.96$). Mean plasma LH was lower on Day -3 compared to Day -2 ($p=0.03$) relative to the preovulatory LH surge. No effect of housing nor an interaction between day and housing was found in mean plasma LH on these days ($p=0.78$; $p=0.14$).

Table 3: Pulsatile release of LH on Day -2 and -3 relative to the preovulatory LH surge in LOOSE and TETHER sows

	Day -3 relative to LH surge		Day -2 relative to LH surge	
	LOOSE (n=6)	TETHER (n=3)	LOOSE (n=6)	TETHER (n=7)
pulse frequency (#/12h)	8.00 ± 0.58	9.00 ± 0.00	6.83 ± 0.75	7.71 ± 0.36
pulse amplitude (ng. ml^{-1})	4.88 ± 0.75	5.15 ± 1.56	6.86 ± 2.35	10.5 ± 4.01
mean plasma LH ($ng.ml^{-1}$)	6.91 ± 0.73	7.79 ± 2.22	10.6 ± 4.02	17.4 ± 3.10

Data are presented as mean ± SEM. No day or housing effects are found in LH pulse frequency and pulse amplitude. Mean plasma LH in LOOSE and TETHER animals is significantly lower ($p<0.05$)on Day -3 than on Day -2 relative to the preovulatory LH surge

Discussion

Long-term tethered housing induces chronic stress in pigs, and leads to stress-adaptive changes in physiological regulation that may become apparent upon environmental challenge. In the present study, we have investigated the effect of a change in housing conditions on the hypothalamus-pituitary-gonadal (HPG) axis in multiparous stress-adapted female pigs. During the luteal phase, the LOOSE group of animals showed lower mean plasma LH levels than the THETHER animals, whereas LH pulse frequency and amplitude were not affected by housing condition. Sofar, no overt effects of chronic tethered housing on plasma LH concentration in females have been reported. Studies in rats and rhesus monkeys showed decreased plasma LH concentrations after restraint stress (Taché, *et al.*, 1978; Goncharov *et al.*, 1984; Lopez-Calderon et al, 1987), however, they used male subjects and chronic intermittent immobilisation stress.

Both groups of sows used in the present study had a history of long term tethered housing during which adaptational processes in the regulation of the hypothalamus-pituitary-adrenal (HPA) axis, autonomic nervous system and behaviour occur, rendering the pigs more reactive to changes in environmental conditions (Schouten *et al.*, 1991; Janssens *et al.*, 1994; Janssens *et al.*, 1995a; Clark *et al.*, 1997). The change in housing conditions of the LOOSE sows, that were able to move freely from approximately 6 weeks before the start of the experiment onwards, likely was a challenge for the animals, inducing changes in mean LH concentration . In a study by Rampacek *et al.* (1984), a change from confined to non-confined housing also resulted in a change in LH plasma concentration. The fact that they found an increase in plasma LH concentrations in the non-confined group, whereas our results show decreased LH levels in the LOOSE animals may be explained by the fact that, apart from methodological differences, they used prepubertal pigs, that is animals in which the regulation of LH differs considerably from that in multiparous sows as used in the present study.

LOOSE pigs showed a significant delay of the preovulatory LH surge with 25 hours. It could be argued that this was caused by a difference in activation of the HPA-axis. Janssens *et al.* (1995a) reported a flattened diurnal rhythmicity of cortisol and a hypercortisolemia during long-term tethering. These changes, however appeared to be of a transient nature, indicating development of adaptational changes in the HPA system at least in part of the animals. In the present study, plasma cortisol concentrations were not different between housing conditions, *but*, relatively high when compared to those of the loose housed controls in studies by Janssens *et al.* (1994; 1995a). In addition, there was no indication of a diurnal cortisol rhythm in both the LOOSE and the TETHER animals. Therefore, the shift in the preovulatory LH surge cannot be explained by a difference in activation of the HPA axis. Furthermore, from the present data together with those from the literature, it might be suggested that LOOSE

animals are in a transitional state, during which changes in the HPG- but not the HPA-system, induced by previous long term tethered housing, are reversed towards pretethering levels. Pulse frequency decreased while pulse amplitude and mean plasma LH increased from the early (Day 2 after altrenogest withdrawal) towards the late follicular phase (Day 4 after altrenogest withdrawal) irrespective of housing condition. When data were lined up to the day of *the* preovulatory LH surge, this pattern was still largely present with a significant time effect in mean plasma LH concentration and a trend in pulse frequency. These findings are in line with other studies showing higher pulse frequency with low amplitude in early follicular phase compared to mid- and late follicular phase (Kesner *et al.*, 1989; Messinis *et al.*, 1992; Matt *et al.*, 1993) depending on the presence of gonadal steroids.

In summary, the present study shows that a change in housing conditions delays the preovulatory LH surge and decreases the mean LH concentration during the luteal phase, but has no effect on LH pulse frequency and pulse amplitude under progesterone dominance with altrenogest and during both phases of the oestrus cycle after termination of altrenogest treatment.

Aknowledgement

The authors like to thank the Msc students Lucy d'Hoore, Joanne Maaskant, Eline Schrasser, Martine Janszen, Dorothé Steverink, Emmy Bouwman who have participated in the periods of bloodsampling and for analysing the bloodsamples under the supervision of Corrie Oudenaarden.

Chapter III

Naltrexone dose dependently increases luteinizing hormone and prolactin during the luteal phase in gilts: Individual variation in response

Dierx, J.A.J.[*], Helmond, F.A.[**], Baksh A[***], van der Heide, D.[*] and Wiegant, V.M.[***]

[*]Department of Human and Animal Science, Wageningen Institute of Animal Science, Agricultural University Wageningen, Haarweg 10, 6709 PJ Wageningen, The Netherlands.
[**]N.V. Organon Scientific Development Group, PO Box 20, 5340 BH Oss, The Netherlands.
[***]Barry Reed Oncology Lab, St. Bartholomew's Hospital, West Smithfield London EC 1A 7BE, Great Brittain.
[****]Rudolf Magnus Institute for Neurosciences, University Utrecht, Stratenum Universiteitsweg 100 3584 CG Utrecht, The Netherlands

Abstract

The response of the reproductive axis to endogenous opioid peptides (EOP) was investigated by determining the increase in mean plasma LH concentration after intravenous administration of 5 doses of the opioid antagonist naltrexone using PRL as a positive control. Cyclic gilts received iv treatment with saline or 0.125, 0.25, 0.5, 1 or 2 mg/kg naltrexone during the luteal phase of the oestrous cycle and frequent bloodsamples were taken. On average, plasma LH and PRL concentrations increased with increasing dose of naltrexone in the first hour postinjection. Plasma LH concentration was increased ($P<0.05$) after 0.25 and 2 mg/kg naltrexone compared to control, whereas plasma PRL concentration was increased after all doses used. However, a considerable variation between animals was found in the response of both hormones with 2 animals showing no response to the doses used, 2 animals responding to 0.25 mg/kg and higher, and 3 animals responding to all doses. The 5 "responders" showed increased plasma LH concentrations during the first hour postinjection after 0.25, 0.5 and 2 mg/kg naltrexone. Over the total postinjection period 2 mg/kg naltrexone increased plasma LH concentration compared to all other doses ($P<0.05$) except 0.5 mg/kg. During the first hour postinjection, the 5 "responders" showed increased ($P<0.05$) plasma PRL concentration after all doses used

In conclusion, naltrexone dose dependently increased the mean plasma LH and PRL concentration with a considerable variation between animals. Furthermore, it is suggested that the LH and PRL response differ in sensitivity to inhibition by EOP's.

Introduction

Endogenous opioid peptides (EOP's) are involved in the regulation and the pulsatile secretion of luteinizing hormone (LH). Intravenous treatment with opioid receptor antagonists has been shown to increase plasma LH concentration in primates (Mello *et al.*, 1988), sheep (Whisnant *et al.*, 1991), humans (Gindoff et la., 1988; Remorgida *et al.*, 1990), rat (Babu *et al.*, 1988) and pig (Barb *et al.*, 1985). In the pig, intravenous infusion with a met-enkephalin analog decreased mean LH concentration (Okrasa and Tilton, 1992). Furthermore, ß-endorphin and leu-enkephalin have been reported to inhibit the LH release from rat pituitaries *in vitro* (Leiva and Croxatto,1994). Studies by Barb *et al.* (1986) in pigs and by Stumpf *et al.* (1993) in ovariectomised beef cattle have shown that the reduction of plasma LH concentration by progesterone was counteracted by the administration of the opioid receptor antagonist naloxone. This strongly suggests that the inhibition of plasma LH concentration by progesterone is mediated by an opioidergic mechanism.

A technical problem in studying effects of EOP's on the plasma LH concentrations is the pulsatile pattern which might obscure the response to opioid antagonists. The concentration of PRL in plasma, like that of LH, is under inhibitory control of EOP and has been reported to increase after naloxone treatment (Snowden *et al.*, 1984; Barb *et al.*, 1986). This together with the fact that PRL release does not follow pulsatile kinetics, makes the plasma PRL concentration suitable as a positive control for the effect of drugs that interfere with the opioid regulation of LH secretion system.

A considerable variation between individual animals in the response to opioid receptor agonists and antagonists appears to exist. Deroche *et al.* (1993) showed differences in locomotor response to morphine treatment between individual rats. Martin del Campo *et al.* (1992) reported large individual differences in naloxone induced rise in plasma cortisol in humans, and Raevskaia, (1992) has shown in rabbits that the naloxone induced hyperalgesic effect depended on individual properties of the animals. Therefore, it might be useful to check whether individual variability in response to opioid agonist and antagonists is related to the plasma concentrations of the drugs used.

The aim of the present study was to investigate the response of the reproductive axis to EOP's by determining the increase in mean plasma LH concentration after intravenous administration of saline or 5 doses of naltrexone in gilts. Mean plasma PRL concentration is used as a positive control and plasma concentrations of naltrexone were determined to check adequacy of treatment.

Materials and methods

Animals

Seven crossbred cyclic gilts (Great Yorkshire X British Landrace; Pig Improvement Company, Oxfordshire, United Kingdom) which had shown two or more normal oestrous cycles were used in this study. At the start of the experiment, mean body weight of the animals was 192.6 ± 6.1 kg (mean ± SD). All animals were housed loose in individual pens with a concrete floor that was covered with woodshavings, except for a slatted dunging area at the rear of the pens. Lights were on from 7.30 h to 19.30 h and ambient room temperature ranged from 15°C to 25 °C. At 8.00 h and 16.00 h the gilts were fed 1 kg of a pelleted, dry sow feed (12.2 MJ of metabolizable energy per kilogram containing 15.4% crude protein) by hand. Water was available ad libitum through a drinking nipple.

Surgery

In order to collect frequent blood samples, the gilts were surgically fitted with a permanent jugular vein catheter (Silastic® medical grade tubing, 0.040 in. i.d., 0.085 in. o.d.; Dow Corning, Michigan U.S.A.) under sterile conditions, and under general anaesthesia with inhalation of O2/N2O, enflurane Ethrane®, Abott B.V., The Netherlands) as described previously (Janssens *et al.*, 1994) with slight modification in the attachment of the catheter to the jugular vein. The animals were equipped with a harness to protect the cannula, which was externalized between the scapulae. The harness (23 cm x 20 cm, polyvinyl chloride with nylon; Bizon Chemie, The Netherlands) was fixed at the back of the animals with a belt around the chest during the week before surgery in order to habituate to the harness. All animals were treated with antibiotics (12 ml of T.S. Sol®, containing trimethorprim and sulfamethoxazol, orally; Dopharma, The Netherlands) once daily from 3 days before surgery until 3 days after surgery. The animals were allowed to recover from surgery and anaesthesia for at least 10 days.

To prevent obstruction by bloodclots, the catheters were flushed with saline once weekly and filled with heparinized saline (25 IU heparin/ml of 0.9% saline; Leo Pharmaceutical Products, The Netherlands) when not in use. When catheter patency was reduced, obstructions were removed as described by Leuvenink and Dierx (1997). In short, a solution of 25000 IU kabikinase (Kabi Pharmacia, Sweden), 2500 IU heparin and 0.2 ml Ticarpen® (Beecham, England) was added to 25 ml sterile 25% PVP/saline (Merck, Germany) solution. After 1 week, the catheter was flushed with a 2% heparine/saline solution.

Experimental procedure

In all 7 gilts, the effect of 5 doses of naltrexone (0.125 mg/kg, 0.25 mg/kg, 0.5 mg/kg, 1 mg/kg and 2 mg/kg) on the plasma concentration of LH was tested and compared to 5 ml saline (control) with a washout period of 7 days. Each animal received all doses in a random order over a total period of 4 oestrus cycles (2 doses/ cycle) and each animal served as its own control. The first day of oestrus was designated as Day 0. On Days 7 and 14 (luteal phase), blood samples were collected every 12 minutes for 8 hours (from 9.00 h until 17.00 h) according to the procedure described previously (Janssens *et al.*, 1994). The samples of the first two hours (9.00 h - 11.00 h) were used to determine the basal LH release and at 11.00 h, saline or one of the doses naltrexone (Sigma Chemicals, St Louis, U.S.A.) was injected given as an iv. bolus via the catheter.

Blood sampling procedure

Before the experiment, the animals were frequently handled and habituated to the blood sampling procedure. Immediately after collection, the blood samples (approximately 5 ml)

were transferred to ice-cooled polypropylene tubes containing 50 ml EDTA solution (144 mg EDTA/ml of saline; Triplex®III, Merck Nederland BV, The Netherlands). The tubes were mixed, placed on ice, and subsequently centrifuged at 3000xg for 15 min at 4°C. Plasma was collected and stored at -20°C until hormone analysis.

Plasma analysis

Plasma samples were analysed using validated immunoassays for LH (Niswender *et al.*, 1970), PRL (Van Landeghem and Van der Wiel, 1978) and progesterone (Helmond *et al.*, 1980). All samples were analysed for LH, whereas PRL was determined in plasma samples of 9.24 h, 10.00 h, 10.36 h and every 12 minutes from 11.00 until 12.00 (first hour after injection). Plasma levels of progesterone were determined in 10.00 h, 12.00 h, 14.00 h and 16.00 h samples. The intra- and inter-assay coefficient of variation was 7.2 and 14.4%, 6.9 and 12.3% and was 5.5% and 12.2%, respectively, for LH, PRL and progesterone.

Plasma concentration of naltrexone was determined by a HPLC method used for detection of morphine described previously by Joel *et al.* (1988), which has been modified by using 100 mg Varian C8 extraction cartridges, and the use of an ASPEC automated sample preparation device. The samples were eluted with 15% acetonitrile instead of 10% acetonitrile to improve extraction efficiency up to 75%.

Animals that showed an increase in plasma LH and PRL concentrations of at least 150% of baseline concentration within the first hour after iv treatment with naltrexone, were qualified as responders. Animals that did not meet this criterion even at a dose of 2 mg/kg naltrexone, were qualified as non-responders.

Detection of oestrous

Detection of oestrous was performed once daily in the morning on all days of the cycle by a back-pressure test in the presence of a vasectomized boar. The time of oestrous was defined as the day the gilt showed a standing response to the back-pressure test in the presence of the boar.

Statistical analysis

LH concentration of all samples was expressed as a percentage of the average basal release. In order to describe the time course of the mean plasma LH concentration after treatment with saline or the several doses of naltrexone, the experiment days were divided in 8 Time Periods (TP's) of 1 hour. Data of mean plasma concentration of LH, PRL and progesterone were analysed using SAS statistical analysis system (1990). The effect of the several doses of naltrexone was tested by means of a F-test, using the procedure GLM and the following linear model: $Y_{ijkl} = m + A_i + N_j + TP_k + (NxTP)_{jk} + IT_l + e_{ijkl}$; Where Y_{ijkl}= value of hormone

parameter in gilt i treated with dosis j at injection time k in an oestrous cycle; m = overall mean; A_i = fixed effect in animal i (i = 1,...,7); N_j = fixed effect of dosis j (j = 1,...,6); TP_k = fixed effect of time period k (k = 1,...,8); IT_l = fixed effect of first or second injection k (k = 1,2) within an oestrous cycle; e_{ijkl} = error term, which represents the random effect of dosis j in animal i at and first or second injection l within an oestrous cycle. Differences were considered significant when $P<0.05$. Values are expressed as means and standard error of the means.

Results

Plasma hormone concentrations

In none of the parameters studied, namely plasma LH, PRL and progesterone concentration, interactions were found between iv treatment and injection time (day 7 or day 14 after first day of oestrus). Furthermore, sequence of administration of the doses naltrexone or saline had no effect on these parameters.

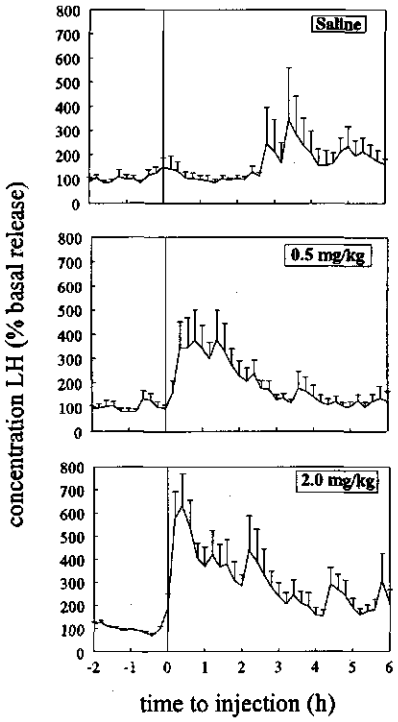

***Figure 1**: Illustrative profiles of the average LH levels of 7 gilts. Profiles of pulsatile LH release of 5 gilts (average ± SEM) treated with (A) saline, (B) 0.5 mg/kg and (C) 2 mg/kg naltrexone. Data are presented as % of basal release and relative to time of injection (t=0).*

The high level of progesterone in bloodplasma of all animals (53.6 ± 1.0 ng/ml; n=7), indicated that all the experimental days were during the luteal phase. There were no differences between plasma levels of progesterone before and after treatment with naltrexone (50.6 ± 2.5 ng/ml vs 53.2 ± 2.6 ng/ml) or saline (54.5 ± 8.5 ng/ml vs 53.9 ± 6.3 ng/ml).

Plasma concentrations of both LH and PRL during the first hour after injection increased with increasing dose of naltrexone (Figure 2), returning to preinjection levels within the sampling period except for LH after administration of the highest naltrexone dose (as illustrated in Figure 1). The peak concentrations of LH and PRL after treatment with naltrexone were found within the first hour (on average after 24 ± 12 minutes; mean ± SEM) post injection of the opioid antagonist as is illustrated by Figure 1. The doses of 0.25 mg/kg and 2 mg/kg increased plasma LH concentrations significantly ($p=0.05$ and $p=0.001$ respectively) compared to control, whereas plasma PRL concentrations were significantly ($p<0.05$) increased by all doses, except 0.125 mg/kg (figure 2).

A considerable variation between animals was found in the LH and PRL response to the several doses of naltrexone. Out of 7 animals, 2 showed neither a LH, nor a PRL response at any of the doses used and were therefore qualified as non-responders. Furthermore, 2 animals showed significant LH and PRL responses to doses of 0.25 mg/kg and higher, and 3 animals responded to all doses and might be qualified as "low responders" and "high responders" respectively.

Plasma naltrexone

Plasma concentrations of naltrexone increased proportional to the dose in all animals (Figure 2). Plasma concentrations of naltrexone after the doses of 0.5 mg/kg and 1.0 mg/kg were two respectively four times the plasma concentrations after the dose of 0.25 mg/kg naltrexone. The absence of response in 2 animals could not be attributed to the absence of naltrexone in the blood, indicating they were not responding to naltrexone. Data of the other 5 animals that did show a response in LH and PRL were subjected to further analysis.

Plasma hormone concentrations of responders

A treatment effect in mean plasma LH during the first hour post injection was found ($P<0.002$; Table 1). Compared to saline, mean plasma LH was significantly increased after treatment with the doses 0.25 mg/kg, 0.5 mg/kg and 2.0 mg/kg ($P<0.05$). Furthermore, the dose of 2.0 mg/kg, significantly increased mean plasma LH ($P<0.05$) compared to the other doses used except 0.25 mg/kg and 0.5 mg/kg (Table 1).

During the second and third hour post injection, when plasma levels were decreasing towards preinjection levels, the effect of naltrexone on LH levels was still found, but not statistically significant. During the fourth hour post injection, saline treated animals showed significantly

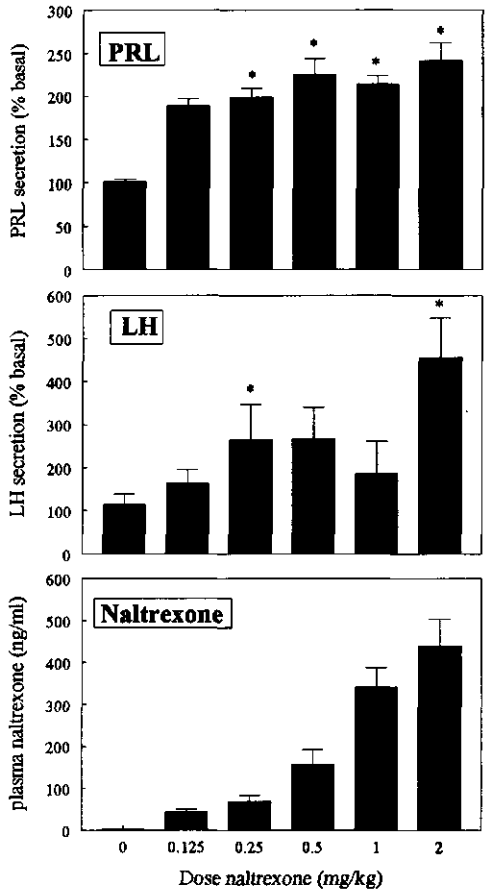

***Figure 2**: Hormone and naltrexone concentrations in plasma of 7 gilts. Data are presented as mean ± SEM. Concentration of LH (ng/ml; upper panel), PRL (ng/ml; middle panel) and naltrexone (ng/ml; bottom panel) during the first hour after iv treatment with saline or several doses of naltrexone.*
** = p<0.05 from saline (P<0.05)*

higher mean plasma LH (257 ± 124 ng/ml; mean ± SEM) than animals treated with 0.125 mg/kg (77.8 ± 5.56 ng/ml), 0.25 mg/kg (104 ± 26 ng/ml), 0.5 mg/kg (147 ± 29.6 ng/ml) and 1 mg/kg (126 ± 88.7 ng/ml) naltrexone except for the highest dose (210 ± 52.5 ng/ml). During the fifth hour PI, this pattern was similar, but the differences were not significant.

When the total post injection period (6 hours) was analysed, mean LH plasma concentration over this period showed a significant treatment effect (Table 1; P=0.01). Treatment with the 2.0 mg/kg dose of naltrexone resulted in significantly higher mean plasma LH concentration than all other doses (P<0.05) except 0.5 mg/kg.

A significant treatment effect (P=0.0001) was found in plasma PRL levels (Table 1). Plasma PRL levels were significantly (P<0.05) increased by naltrexone in all doses in the first hour post injection.

Table 1: LH release in responders following iv. administration of several doses of naltrexone.

dose	Mean plasma LH first hour PI[1] (%basal, n=5)	Mean plasma PRL first hour PI[1] (%basal, n=5)	Number of responders (n out of 5)	Mean plasma LH total period PI (%basal, n=5)
saline	104 ± 22^{a}	101 ± 3.49^{a}	0	162 ± 35^{a}
0.125 mg.kg^{-1}	167 ± 34^{ab}	177 ± 17.4^{b}	3	119 ± 15^{a}
0.25 mg.kg^{-1}	302 ± 96^{bc}	174 ± 13.3^{b}	5	191 ± 53^{a}
0.5 mg.kg^{-1}	317 ± 91^{bc}	197 ± 19.1^{b}	5	199 ± 44^{ab}
1.0 mg.kg^{-1}	220 ± 89^{ab}	188 ± 20.3^{b}	5	152 ± 52^{a}
2.0 mg.kg^{-1}	470 ± 99^{c}	201 ± 20.2^{b}	5	290 ± 63^{b}

Data are presented as means ± SEM. Numbers in columns with the same superscript letter (a,b,c) are not significantly different (P>0.05). [1]Mean plasma levels of LH and PRL during the first hour postinjection period (% of basal); Number of animals that responded to treatment with saline or the several doses naltrexone during the first hour postinjection; Mean plasma LH concentration during the 6 hours postinjection period (% of basal).

Discussion

There appeared to be a considerable individual variation in the response to naltrexone, as is reflected by the "non-responding" and "responding" animals, although all animals clearly showed increasing plasma concentrations of naltrexone with increasing dose. Within the group of "responders", there were three animals that showed an increased plasma LH concentration the first hour already after injection of the lowest dose of naltrexone (0.125 mg/kg), whereas the two other pigs showed the first increase at 0.25 mg/kg of the opioid receptor antagonist. In line with this, studies in rats (Deroche *et al.*, 1993; Morgan and Picker, 1996), humans (Martin del Campo *et al.*, 1992) and rabbits (Raevskaia, 1992), have reported a considerable individual variation in behavioural responses after treatment with the opiate drug morphine, or the opioid receptor antagonist naloxone. These and the present findings show that, when using opioid receptor anta- or agonists, data of the individual animal should be considered with the interpretation of group data.

In the responders, a dose-dependent effect of naltrexone on mean plasma LH during the first hour post injection was found. However, the dose of 1 mg/kg showed a (not significant) lower response compared to 0.25 mg/kg and 0.5 mg/kg. A similar phenomenon was reported

by Taylor *et al.* (1991), who found less attenuation of self-injurious behaviour in humans after treatment with 1 mg/kg naltrexone compared to treatment with 0.5 mg/kg. This "dip" in the dose-response could not be attributed to the dosage or the bioavailability of naltrexone since plasma concentration increased with increasing dose. At present, we were not able to find a gratifying explanation for this phenomenon.

Naltrexone increased plasma PRL levels during the luteal phase of the oestrous cycle. These results are in agreement with studies by Gold *et al.* (1979), Snowden *et al.* (1984) and Barb *et al.* (1985) that have shown increased PRL release during the luteal phase of the oestrous cycle after iv. treatment with naloxone in primates, humans and pigs respectively. The effectiveness of naltrexone to increase PRL levels over the complete dose range used, and the absence of a clear dose-response relationship for this effect, validate the use of the PRL-response as a positive control for the effectiveness of the treatment. Yet different mechanisms may underlie the naltrexone effect on PRL and LH.

Mean plasma LH concentration over the total 6 hour postinjection period increased with increasing dose of naltrexone, showing a similar pattern as during the first hour post injection period. Saline treatment, however, showed relatively high mean plasma LH concentration during this period. This was caused by an endogenous LH pulse that occurred in the third hour post injection in 6 out of 7 animals. This phenomenon was not observed after treatment with naltrexone (data not shown). It might be suggested that the administration of naltrexone increased LH release directly after injection, thereby reducing the GnRH and/or LH releasable pool and the ability of the hypothalamus and/or pituitary to respond to the endogenous rhythm of pulsatile LH release.

In summary, iv. administration of naltrexone increases the plasma concentrations of LH and PRL with PRL responding to a lower dose than LH. In addition, a considerable variation between animals in the LH and PRL response to naltrexone was observed.

Aknowledgement

The authors like to thank Corrie Oudenaarden for the hormone analysis of the bloodsamples. Special thanks for Dr. S.P. Joel for the naltrexone analysis of the bloodsamples.

Chapter IV

Pulsatile release of luteinizing hormone during the follicular phase in gilts: Possible role of endogenous opioid peptides

Dierx, J.A.J.[*], Helmond, F.A.[**], Baksh A[***], Schrama, J.W.[****] van der Heide, D.[*] and Wiegant, V.M.[*****]

[*]Department of Human and Animal Science, Wageningen Institute of Animal Science, Agricultural University Wageningen, Haarweg 10, 6709 PJ Wageningen, The Netherlands.
[**]N.V. Organon Scientific Development Group, PO Box 20, 5340 BH Oss, The Netherlands
[***]Barry Reed Oncology Lab, St. Bartholomew's Hospital, West Smithfield London EC 1A 7BE, Great Brittain.
[****]Department of Animal Husbandry, Wageningen Institute of Animal Science, Agricultural University Wageningen, Marijkeweg 40, 6709 PG Wageningen, The Netherlands.
[*****]Rudolf Magnus Institute for Neurosciences, University Utrecht, Stratenum Universiteitsweg 100 3584 CG Utrecht, The Netherlands

Abstract

The role of endogenous opioid peptides (EOP) in modulating the pulsatile LH release during the follicular phase was investigated in 5 chronically tethered and 8 loose housed gilts. Oestrous cycles of all animals were synchronised with altrenogest for 21 days. Subsequently, 3 tethered and 4 loose housed gilts were treated per os with opioid receptor antagonist naltrexone for 6 days with the remaining animals serving as controls. Frequent bloodsamples were taken on Days 2, 4, 5 and 6 after termination of altrenogest treatment. No effects of housing condition on the pulsatile LH release were found. LH pulse frequency did not differ between days in the naltrexone treated animals but was decreased ($P<0.05$) on Day 4 and 5 compared to Day 2 and 6 in control animals. LH pulse amplitude and area under the pulse (AUP) were higher ($P<0.05$) on Day 6 than on all other days in control animals. In naltrexone treated animals, LH pulse amplitude and AUP were higher on Day 5 but only significant compared to Day 4 ($P<0.05$). No effects of naltrexone treatment were found on all parameters when data were lined up to the first day of oestrus. Oestradiol concentration was lower on Day 2 compared to Day 5 and higher in control compared to naltrexone treated animals ($P<0.05$). In addition, oestradiol concentration was negatively correlated with LH pulse frequency.

In summary, after pulses of high frequency and low amplitude during early follicular phase, pulse frequency decreases until the day of the preovulatory LH surge. This pattern does not seem to be modulated by EOP's.

Introduction

LH secretion during the oestrous cycle has been studied in a variety of species as sheep (Whisnant *et al.*, 1991), human (Fillicori *et al.*, 1986; Rossmanith *et al.*, 1990), pig (Okrasa and Tilton, 1992) and primate (Norman *et al.*, 1994). Relatively few data are available, however, on the pattern of pulsatile LH release during the follicular phase, particularly with regard to pulse frequency and pulse amplitude. Reame *et al.* (1994) and Rossmanith *et al.* (1990) showed an increased pulse frequency in the late compared to the early follicular phase in humans, whereas Cagnacci *et al.* (1995) did not find significant differences in pulse frequency between these phases of the follicular phase in women.

Plasma LH release has been shown to be decreased by endogenous opioid peptides (EOP's) in a variety of species. EOP's are reported to mediate the negative feedback of progesterone during the luteal phase (Yang *et al.*, 1988; Barb, *et al.*, 1992; Kaynard *et al.*, 1992). The stimulatory effect of opioid receptor antagonists, like naloxone and naltrexone on plasma LH concentration during this phase of the oestrous cycle has been reported in numerous studies (Whisnant and Goodman, 1988;

Kaynard *et al.*, 1992; Chang *et al.*, 1993; Heisler *et al.*, 1993). However, whether EOP's play also an inhibitory role in LH secretion and/or the timing of events leading to the preovulatory LH surge and oestrus during the oestradiol dominated follicular phase, still remains to be elucidated.
Several studies have shown that the LH release is decreased by stress, suggesting an inhibitory role of EOP's, which have been shown to be activated during stress (Rivier and Rivest, 1991; Heisler *et al.*, 1993; Norman *et al.*, 1994). Furthermore, in pigs, it has been shown that tethered housing induces chronic stress. Thus, it increases adrenocortical sensitivity to ACTH, its steroidogenic capacity and plasma concentrations of cortisol and PRL (Janssens *et al.*, 1995a). Furthermore, tethering increases endogenous opioid activity (Janssens *et al.*, 1994; Schouten and Wiepkema, 1991), the number of μ-opioid receptors in the brain (Zanella *et al.*, 1996), and it leads to opioid dependent stereotyped behaviour (Cronin, 1985; Schouten and Wiepkema, 1991). However, the effects of tethered housing on the plasma LH concentration and pulsatile LH release during the oestrous cycle, and the follicular phase in particular is, are not known.
The purpose of the present study was to determine the pulsatile pattern of LH release during the follicular phase of the oestrous cycle, and to investigate the role of EOP's in modulating this pattern by treating loose or tethered housed gilts orally with the opioid receptor antagonist naltrexone.

Materials and Methods

Animals and Housing

Thirteen healthy cyclic cross-bred gilts (118-145 kg body weight; Great Yorkshire x British Landrace, Pig Improvement Company, UK) were used in this study. Five pigs were housed tethered by a 50 cm heavy-gauge neck-chain in individual tether stalls, each 65 cm wide for 5 months prior to and during the experiment. During the same time, eight gilts were housed loose in individual pens of 5 to 6 m^2 with a concrete floor that was covered with wood shavings, except for a slatted dunging area of 2.5 m^2 at the rear of the pens. In all housing conditions lights were on between 7.30 h and 19.00 h and ambient temperature ranged from 15 to 25 °C. At 8.00 h and 15.00 h the gilts were fed 1 kg of a pelleted, dry sow feed (12,2 MJ of metabolizable energy per kilogram containing 15.4% crude protein) by hand. A bell signal was given each time just before feeding in order to prevent the gilts from associating the presence of people with feeding. Water was available ad libitum through a nipple drinker.

Surgery

In order to collect frequent blood samples, the gilts were fitted surgically with a permanent jugular vein catheter (silastic® medical grade tubing, 0.040 in. i.d., 0.085 in. o.d.; Dow Corning, Michigan, U.S.A.) under sterile conditions and under general anaesthesia with inhalation of O_2/N_2O, enflurane (Ethrane®, Abott BV, The Netherlands) as described previously (Janssens *et al.*, 1994). The animals were equipped with a harness to protect the catheter, which was externalized between the scapulae. The harness (23 cm x 20 cm, polyvinyl chloride with nylon; Bizon Chemie, The Netherlands) was fixed at the back of the pigs with a belt around the chest during the week before surgery in order to habituate the animals to the harness. All animals were treated with antibiotics (12 ml of T.S. Sol®, containing trimethoprim and sulfamethoxazol, orally; Dopharma, The Netherlands) once daily from 3 days before surgery until 3 days after surgery. The gilts were allowed to recover from surgery and anaesthesia for at least 10 days before experimentation was started.

To prevent obstruction by bloodclots, the catheters were flushed with saline once weekly and filled with heparinized saline (25 IU heparin/ml in 0.9% saline; Leo Pharmaceutical Products, The Netherlands) when not in use.

Treatment

The oestrous cycle of all gilts was synchronized by daily oral administration of 20 mg altrenogest (Regumate®, Hoechst Holland, The Netherlands), a progesterone agonist, during 21 days. The day after altrenogest treatment 4 loose housed and 3 tethered animals were treated for 6 days with the opioid receptor antagonist naltrexone.HCl (Sigma Chemicals, St. Louis, U.S.A.) to investigate the involvement of EOP's in the regulation of the pulsatile LH release during the follicular phase. Naltrexone was given p.o. mixed with the feed. On day 1, 50 mg was given in the morning feed and in the afternoon feed as a loading dose. As a maintenance dose, 25 mg was given in the morning feed and 50 mg in the afternoon feed from day 2 to day 6. These doses of naltrexone were similar to those used by others in humans, to study the role of EOP's in amenorrhea induced by hyperprolactinemia and weight loss (Kremer *et al.*, 1991; de Wit *et al.*, 1991; Genazzani *et al.*, 1995). The control group of 4 loose housed and 2 tethered animals received no naltrexone treatment.

The experiments were approved by the committee on animal care and the use of the Agricultural University, Wageningen, The Netherlands.

Blood sampling

Before the experiment, the animals were frequently handled and habituated to the blood sampling procedure. Blood samples (approximately 5 ml) were collected every 12 minutes during 12 hours (from 8.36 h to 20.36 h) on Day 2, 4, 5 and 6 after termination of altrenogest according to the

procedure as described previously (Janssens *et al.*, 1994) and immediately transferred to icecooled polypropylene tubes containing 50 ml EDTA solution (144 mg EDTA/ml of saline; Triplex®III, Merck Nederland BV, The Netherlands). The tubes were mixed, placed on ice, and subsequently centrifuged at 3000g for 15 minutes at 4°C. Plasma was collected and stored at -20°C until hormone analysis.

Plasma analysis

LH. Plasma samples were analysed for LH using a double-anti body radioimmunoassay as described by Niswender *et al.* (1970), using porcine LH (pLH iodination grade batch 004/3; potency, 0.85 x NIH LH-S19; UCB bioproducts, Brussels, Belgium) as a standard and for radio-iodination (specific activity, 38 mCi/mg ^{125}I-pLH). Anti-porcine LH batch 004 (UCB bioproducts, Brussels, Belgium) was used at a final dilution of 1:360,000, which gave an initial binding of the labelled hormone of approximately 39%. Sac-Cel® was used as the second antibody, (donkey anti-rabbit; Wellcome Reagents, Beckenham). The main crossreacting peptides were pFSH (2.7%), pLHα (1.1%), pTSH (0.5%)and pTSHα, pTSHß and pLHß (all <0.1%). The minimal detectable dose at the 90% B/B_0 concentration was 0.1 ng/ml. The inter-assay coefficient of variation was 25.7% and the intra-assay coefficient of variation was 3.6%.

Oestradiol. Plasma oestradiol concentration was measured in samples collected at 10.00 h, 12.00 h, 14.00 h and 16.00 h. After extraction with dichloromethane (DCM, Merck, Darmstadt, Germany), using a single-antibody radioimmunoassay, 50 ml [2,4,6,7-^{3}H]oestradiol (NEN Chemicals, 's Hertogenbosch, The Netherlands), was added to 650 ml plasma sample prior to extraction with 3 ml DCM to estimate procedural losses. The extracts were evaporated under a stream of nitrogen and the residues were redissolved in 500 ml ethanol 96%. An aliquot of 150 ml was taken to determine the recovery of [^{3}H]oestradiol. Oestradiol concentrations were measured in 150 ml aliquots by radioimmunoassay using a specific Rabbit antiserum raised against 6-keto-oestrone 6-CMO-BSA. Oestradiol (Sigma Chemical Co., St. Louis, MO, U.S.A.) was used as a standard and [2,4,6,7-3H]oestradiol (specific activity, 95.4 Ci/mmol; NEN Chemicals) as a tracer. The main crossreacting steroids were oestrone (1.49%) and oestriol (0.21%). The antiserum was used at a final concentration of 1:125,000. The minimal detectable dose at the 90% B/B0 level was 8 pg/ml. The interassay coefficient of variation was 17.4% and the intra-assay coefficient of variation was 12.4%. The concentration of oestradiol was expressed in pg/ml after correction for the procedural losses.

Naltrexone. Plasma concentration of naltrexone was determined in samples collected at 8.36 h (30 minute after morning feeding), 15.36 h and 16.48 h (36 and 108 minutes after afternoon feeding respectively) by a HPLC method used for detection of morphine described previously by Joel *et al.* (1988), which has been modified by using 100 mg Varian C8 extraction cartridges, and the use of an ASPEC automated sample preparation device. The samples were

eluted with 15% acetonitrile instead of 10% acetonitrile to improve extraction efficiency up to 75%.

Detection of oestrus

Detection of oestrus was performed once daily in the morning on all days of the cycle by a back-pressure test in the presence of a vasectomized boar. The time of oestrus was defined as the day the gilt showed a standing response to the back-pressure test in the presence of the boar.

Pulse detection

The profiles of LH release were analysed using the pulse analysis program of Maxima/Chromcard (Fisons Instruments,Interscience, Breda, Holland) with baseline calculated according to an algorithm taken into account the total profile. A pulse was defined by a baseline-peak ratio of 0.5 or lower and a minimal area under the pulse of 6.0×10^6 ng.ml^{-1}.min. The highest value of a pulse above baseline was taken as pulse amplitude.

Statistical analysis

Data were analysed using SAS statistical analysis system (1990). The procedure GLM was used to analyse the linear model. Values are expressed as means ± standard error of the mean. The effects of naltrexone treatment, housing system, time (day after altrenogest withdrawal) and their interactions on the measured hormonal parameters were tested by means of an F-test using the following model with time data within gilts taken as repeated measurements. $Y_{ijkl} = m + N_i + H_j + (HxN)_{ij} + e_{1ijk} + T_l + (TxN)_{il} + (TxH)_{jl} + (TxNxH)_{ijl} + e2_{ijkl}$, where Y_{ijkl}: value of hormone parameter at naltrexone treatment i, housing system j , for gilt k at sampling day l; m = overall mean; N_i = fixed effect of naltrexone treatment i (i = 1,2); H_j = fixed effect of housing system j (j = 1,2); e_{1ijk} = error term 1, which represents the random effect of gilt k within naltrexone treatment i and housing system j; Tl = fixed effect of sampling day (l = 1,...,4); e_{2ijkl} = error term 2 which represents the random effect within gilts between sampling days. The effects of naltrexone treatment, housing system and their interactions were tested against error term 1. The other effects were tested against error term 2. Differences were considered significant when $P<0.05$, corrected for number of observations in the cases of interactions. The procedure CORR was used to calculate Pearson's correlation coefficients.

Results

General

Of all 13 animals included in the experiment, 7 gilts showed a preovulatory LH surge within the period of sampling, with 4 out of 6 control animals on Day 6, and 2 out of the 7 naltrexone treated animals on Day 5, and 1 animal on Day 6. On average the 13 gilts came into oestrus 6.8 ± 0.3 days after termination of altrenogest treatment. In the naltrexone treated group, there was a considerable variation among gilts in this respect (range: Day 5 to Day 9; Figure 1). With regard to the time of oestrus, no differences were found between loose and tethered housed animals.

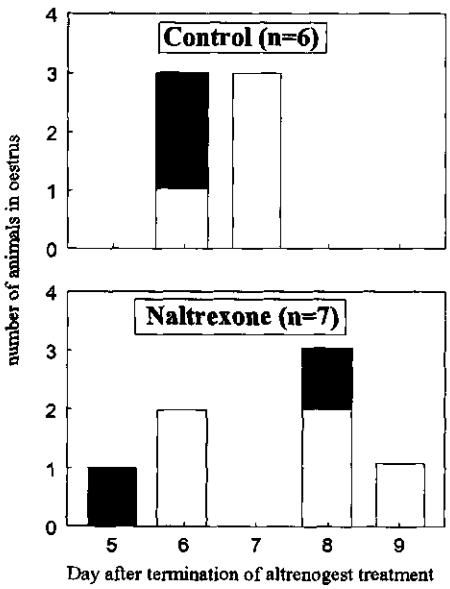

***Figure 1**: First day of oestrus in control (upper panel) and naltrexone (lower panel) treated pubertal gilts, tethered (filled bars) or loose housed (open bars), after discontinuation of treatment with altrenogest.*

All animals gained weight during the experimental period of 5 months, with weight gain being significantly higher in loose housed (24 ± 1.7 kg) than in tethered gilts (16 ± 2.4 kg) ($p=0.03$).

In all animals treated with naltrexone, plasma levels of the opioid receptor antagonist were readily detectable on Day 2 yielding a concentration of 9.6 ± 2.3 ng/ml (mean ± SEM) at 30 minutes after the morning feeding, 11.6 ± 2.1 ng/ml and 9.9 ± 2.7 ng/ml at 36 minutes respectively 108 minutes after the afternoon feeding. In most of the samples taken at comparable timepoints on Day 6, the plasma concentration of naltrexone remained below detection (<2 ng/ml). Only 2 animals showed detectable but very low naltrexone concentrations, even after the high dose of 50 mg.

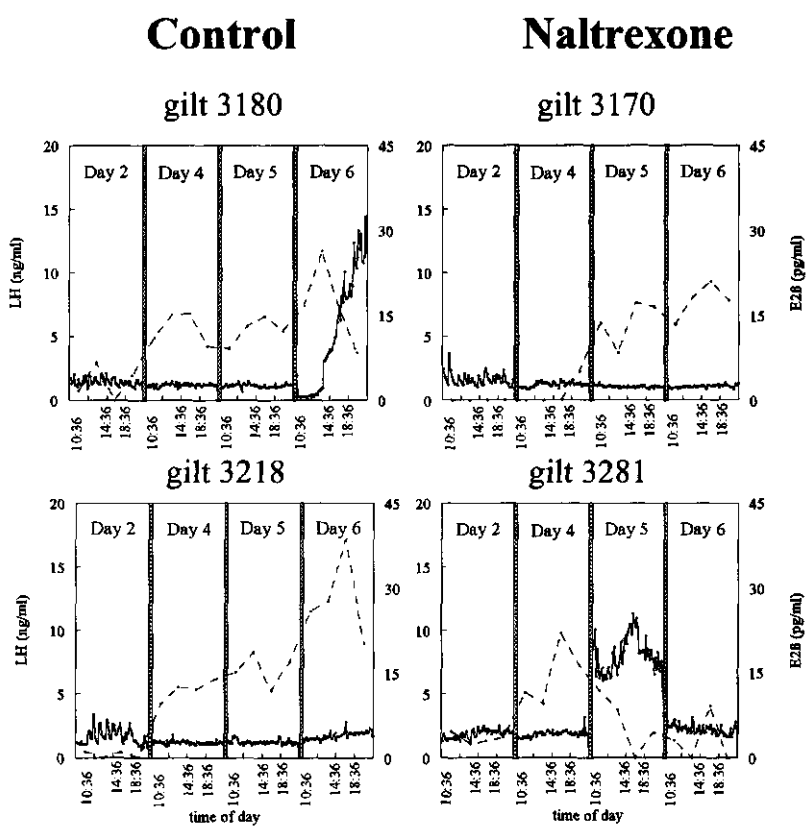

***Figure 2**: Illustrative profiles of pulsatile LH secretion and plasmalevels of oestradiol of control (left) and naltrexone (right) treated pubertal gilts on day 2, 4, 5 and 6 after synchronisation of the oestrous cycle with altrenogest.*

Plasma LH

Tethered housing did not affect pulse frequency (p=0.84), pulse height (p=0.71) and area under the pulse (AUP), nor was there an interaction effect between naltrexone treatment and housing (p=0.93; p=0.71 and p=0.40 respectively). For further analysis of the effect of naltrexone on LH pulse frequency and pulse amplitude during the follicular phase, the data of loose and tethered animals were pooled. Profiles of the pulsatile LH release are shown in Figure 2. Group data on pulse frequency and pulse amplitude are presented in Figure 3.
A significant treatment x time interaction effect was found for pulse frequency (p=0.04). LH pulse frequency on Day 4 and 5 was significantly decreased compared to Day 2 and Day 6 (the average day that the preovulatory LH surge occurred) in control animals (Figure 2 and 3), whereas in naltrexone treated animals no differences were found between Days 2, 4, 5 and

6. Furthermore, pulse frequency in the naltrexone treated group was significantly lower compared to the control group on Day 2 after termination of altrenogest treatment ($p<0.02$). For pulse amplitude and AUP, a treatment x time interaction effect was found ($p=0.0001$; Figure 2 and 3).

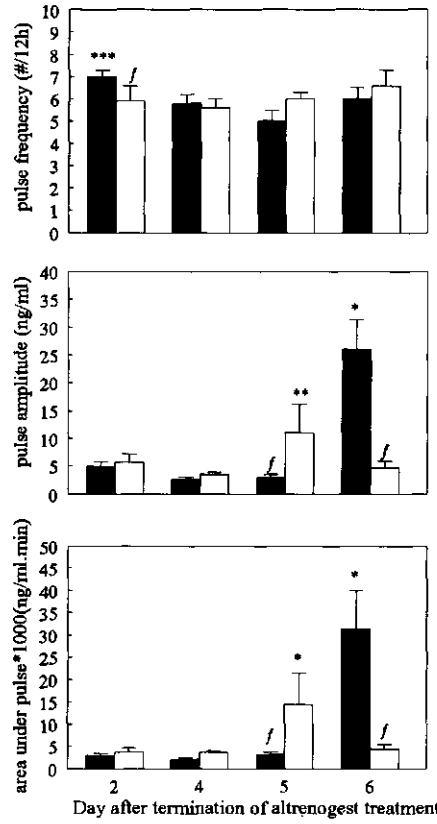

***Figure 3**: Data represent mean ± SEM of LH pulse frequency (A; number per 12 h), pulse amplitude (B; ng/ml) and area under pulse (C; ng.ml^{-1}.min) in control (closed bars) and naltrexone treated animals (open bars) during the follicular phase of the oestrous cycle.*

Time effect:

** = significantly different from all others days within treatment group ($P<0.05$).*

*** = significantly different from naltrexone treatment on Day 4 ($P<0.05$).*

**** = significantly different from control on Day 4 and 5 ($P<0.05$).*

Treatment effect:

f = significantly different between treatment on the same day ($P<0.05$).

In control animals, on Day 6 (the average day of preovulatory LH surge) pulse amplitude and AUP were significantly (both $p=0.0001$) higher compared to Day 2, 4 and 5. In naltrexone treated animals, pulse amplitude and AUP on Day 5 were significantly higher (both $p=0.04$) than on Day 4. In addition, compared to control animals, LH pulse amplitude and AUP in

naltrexone treated animals were significantly higher (both p=0.03) on Day 5 but significantly lower (both p=0.0001) on Day 6.
To exclude the possibility that the effect of naltrexone was due to the occurrence of the preovulatory LH surge, data were lined up to the first day of oestrus. The effects of naltrexone found, when determined relative to altrenogest withdrawal, were not found when lined up to the first day of oestrus. LH pulse frequency (p=0.51), pulse amplitude (p=0.18) and AUP (p=0.23) in naltrexone treated animals were not different from control animals.

Plasma oestradiol
No difference was found in the plasma level of oestradiol between tethered and loose housed gilts (p=0.21), and therefore the data from tethered and loose housed animals were pooled for further statistical analysis. A clear-cut time-effect (p=0.0009) was found with significantly lower plasma levels of oestradiol on Day 2 (3.5 ± 1.1 pg/ml) compared to Day 4 (9.4 ± 1.7 pg/ml; p=0.02), 5 (14.3 ± 2.2 pg/ml; p=0.0001) and 6 (11 ± 2.4 pg/ml; p=0.003) after termination of altrenogest treatment (see also profiles in Figure 1). In addition, on Day 4, oestradiol levels were significantly lower than on Day 5 (p=0.05). A treatment effect (p=0.03) was found, with naltrexone treated animals showing lower plasma levels of oestradiol (mean over Day 2 to 6: 7.4 ± 1.2 pg/ml) than control animals (12.3 ± 1.8 pg/ml).

Discussion

The characteristics of the release of LH during the follicular phase of the oestrous cycle and the putative role of endogenous opioid peptides (EOP) in this respect, were investigated in loose and tethered housed gilts treated with the opioid receptor antagonist naltrexone. The follicular phase was characterised by highly frequent, low amplitude LH pulses on Day 2, followed by a decrease in pulse frequency on Day 4 and 5. The preovulatory LH surge occurred within 5-9 days after termination of altrenogest treatment, and was constituted of pulses of high frequency and high amplitude. The oestradiol concentration increased during the follicular phase with peak value on average on Day 5, the day before most of the control animals display the preovulatory LH surge. Furthermore oestradiol was negatively correlated with pulse frequency, which might reflect a negative feedback on the level of the hypothalamus. This would be in line with findings of Tanaka *et al.* (1992) and O'Byrne *et al.* (1993), showing decreased multi-unit activity in the mediobasal hypothalamus of oestradiol treated ovariectomized goats and monkeys, respectively. Thus, it might be suggested that oestradiol exerts its negative feedback, at least in part, by reducing GnRH pulse frequency at the level of the hypothalamus, and consequently the number of LH pulse secreted from the pituitary.

Abundant evidence exists reporting that stress can affect LH secretion and disrupt oestrous cyclicity (Fonda *et al.* 1984; Brann and Mahesh 1991; Rivier and Rivest, 1991). In our study, long term tethered housing did not influence LH pulse frequency, pulse amplitude and mean LH plasma concentrations in gilts during the follicular phase. In accord with the notion that tethered housing represents a chronic stressor, however, it did reduce body weight gain as compared to the loose housed condition. Thus, LH secretion in gilts may be relatively resistant to this form of housing stress. Alternatively, it could be that adaptive processes had occurred that maintained normal LH secretion under these conditions. Indeed, Janssens *et al.* (1995a) have shown that tethered housed gilts initially develop hypercortisolaemia and hyperprolactinaemia, but that these stress symptoms are temporary, and disappear after 3 months of tethering in at least part of the animals. In addition, they found evidence for adaptive changes in opioid mechanisms that could be instrumental in this respect. Furthermore, in Chapter II using multiparous sows, tethered housing as such did not change the frequency and amplitude of LH pulses but increased mean plasma LH concentration and advanced the preovulatory LH surge. Therefore, it might be suggested that tethered housing is not disrupting the pulse pattern of LH during the follicular phase, but, as suggested in a previous study (Chapter II), it might affect other processes like the timing of the preovulatory LH surge.

In animals treated with the opioid receptor antagonist naltrexone, the timing of oestrus seemed to be shifted and possibly also the timing of the preovulatory LH surge. This is reflected by the lower pulse frequency and pulse amplitude in naltrexone treated pigs on Day 6, that is the day when the majority of the control animals displayed the preovulatory LH surge. This would be in support of a study by Faigon *et al.* (1987), who showed that in rats the "positive feedback" of the ovarian hormones on LH secretion is advanced by naloxone. In addition, Armstrong *et al.* (1988) reported that administration of morphine (sc) to sows for 5 days after weaning delayed the onset of oestrus, and other studies (Yearwood *et al.*, 1991; Kraeling *et al.*, 1992a) reported a delayed preovulatory LH surge in E2ß primed OVX gilts after iv and ICV morphine treatment with no effect on the height of the surge. The present findings, together with data from the literature, might indicate that EOP activity is necessary for proper timing of events leading to the preovulatory LH surge.

On Day 5, we found higher LH pulse amplitude and AUP in naltrexone treated animals compared to control animals. To determine whether this was caused by naltrexone treatment on this day or might have been due to the fact that 2 naltrexone treated animals showed the preovulatory LH surge on this day, data were lined up to the first day of oestrus. It appeared that naltrexone treatment had no effect on LH pulse amplitude and AUP, when data were lined up on the first day of oestrus. Therefore it might be suggested that naltrexone had no effect on LH pulse frequency, pulse amplitude and AUP but disrupted the timing of events leading to the preovulatory LH surge.

In the present study, repeated naltrexone administration did not result in a plateau level of naltrexone in plasma concentrations. Plasma concentrations of naltrexone decreased from detectable levels on Day 2 in all animals to concentrations below detection on Day 6 in most animals. This could not be attributed to the schedule of administration, since a plasma concentration of 3.2 ng/ml naltrexone would be expected on Day 6, using a mathematical approach. It could be suggested that plasma clearance of naltrexone is increased possibly by increased metabolic and/or renal clearance. From a study in humans by Vereby et al (1976) and Meyer *et al.*, (1984), this was not expected since they reported a steady state equilibrium ranging approximately 2.5 ng/ml from the first day of administration until 18 days after a daily oral dose of 100 mg. This might indicate a species difference in pharmacokinetics of naltrexone between pigs and humans. Furthermore it should be mentioned that, although plasma concentrations of naltrexone rapidly decrease to undetectable levels (i.e. <2 ng/ml), the opioid receptor antagonist is able to disrupt the timing of the preovulatory LH surge.
In summary, after high frequency and low amplitude pulsatile LH release in the early follicular phase, pulsatility decreases until the day of the preovulatory LH surge. Oestradiol might modulate this pulse pattern of LH through EOP's on hypothalamic level since only LH pulse frequency is negatively correlated with plasma levels of oestradiol and naltrexone treatment disturbs the normal pulse pattern.

Aknowledgement

The authors like to thank Mirjam Robert for assistance in the intensive bloodsampling and the hormone analysis of the bloodsamples under supervision of Corrie Oudenaarden. Special thanks for Dr. S.P. Joel for the naltrexone analysis of the bloodsamples.

Chapter V

Effect of oral treatment with naltrexone of cyclic gilts during the follicular phase of the oestrous cycle on the GnRH stimulated, hypophysial LH release *in vitro*: A pilot study

Dierx, J.A.J.[*], Helmond, F.A.[**], van der Heide, D.[*] and Wiegant, V.M.[***]

[*]Department of Human and Animal Science, Wageningen Institute of Animal Science, Agricultural University Wageningen, Haarweg 10, 6709 PJ Wageningen, The Netherlands.
[**]N.V. Organon Scientific Development Group, PO Box 20, 5340 BH Oss, The Netherlands
[***]Rudolf Magnus Institute for Neurosciences, University Utrecht, Stratenum Universiteitsweg 100 3584 CG Utrecht, The Netherlands

Abstract

The aim of the study was to probe whether the responsivity of the pituitary to GnRH changes during the follicular phase, and whether this might be mediated by endogenous opioid peptides (EOP's). Oestrus cycles of 16 loose housed gilts were synchronized with altrenogest for 21 days. Subsequently, 8 animals were treated per os with opioid receptor antagonist naltrexone for 6 days and 8 animals served as controls. On Days 2, 4, 5 and 6 after termination of altrenogest treatment, 2 control and 2 naltrexone treated animals were killed. Trunk blood, ovaries and pituitaries were collected. Surge like levels of LH were generally found on Day 6, but in 1 naltrexone animal on Day 5. Oestradiol concentration was highest on Day 4 and 5 and lowest on Day 6. Hemipituitaries were superfused and challenged with GnRH (10^{-6} M during 20 seconds) in vitro. In control animals, the LH response of hemipituitaries to GnRH was lowest on Day 4. In naltrexone treated animals, the LH response of hemipituitaries to GnRH was lowest on Day 2 and highest on Day 6. Furthermore, the LH response on Day 4 and 6 seemed to be higher in hemipituitaries of naltrexone treated than in control animals. Plasma LH concentrations showed a positive correlation with the hemipituitary LH response to GnRH in vitro and both parameters were negatively correlated with plasma oestradiol. It is suggested that pituitary responsivity to GnRH changes during the follicular phase and that EOP's modulate the timing of events that eventually lead to the preovulatory LH surge at the suprapituitary level.

Introduction

LH secretion during the oestrous cycle has been studied in a variety of species (Fillicori *et al.*, 1986; Whisnant *et al.*, 1991; Okrasa and Tilton, 1992; Norman *et al.*, 1994). During the luteal phase, LH pulses are of low frequency and high amplitude. This pattern changes to one of high frequency, low amplitude pulses during the early follicular phase, and then to high frequency, high amplitude pulses that constitute the preovulatory LH surge. These dynamics are thought to be determined mainly by a GnRH pulse generator at the hypothalamic level (Knobil, 1990; Veldhuis, 1990). However, there is some evidence that the responsivity of the pituitary to GnRH changes throughout the oestrous cycle in the rat (Apfelbaum, 1981), and the menstrual cycle in humans (Rossmanith, 1991). Whether such changes in the responsivity of the pituitary for GnRH occur in the pig and contribute to the dynamics of plasma LH during the oestrous cycle, particularly during the follicular phase and the preovulatory LH surge, is unclear.

Endogenous opioid peptides (EOP) have been implicated in the regulation of the pulsatile LH release, but their activity depends on the stage of the oestrous cycle (Kaynard *et al.*, 1992; Chang *et al.*, 1993; Heisler *et al.*, 1993). There is considerable evidence for a tonic inhibition of LH

secretion by EOP's during the progesterone dominated luteal phase in sheep (Yang *et al.*, 1988), pig (Barb, *et al.*, 1992) and rat (Kaynard *et al.*, 1992). Thus, it has been indicated that EOP's mediate negative feedback actions of progesterone. They do not seem to mediate, however, the negative feedback actions of oestradiol on LH release during the follicular phase (Barb *et al.* 1986; Seifer *et al.*, 1990; Okrasa and Tilton, 1992). From recent studies, using rats and gilts treated with opioid receptor antagonists (ICV; per os), it was suggested that EOP's, rather than exerting a direct inhibition of LH release throughout the follicular phase, play a permissive role: termination of EOP-activity allows the preovulatory LH surge to be generated (Roozendaal *et al.*, 1997; Chapter VII). A direct effect of naltrexone at the level of the pituitary, however, could not be excluded since it has been shown that opioid antagonists can increase LH release in pituitary cell cultures from pig, rat and cattle (Cacicedo and Franco, 1985; Blank *et al.*, 1986; Barb *et al.*, 1990).
The present study was designed as a pilot study to probe whether the responsivity of the pig pituitary to GnRH changes during the follicular phase, and whether this might be mediated by EOP's.

Materials and Methods

Animals and housing
The experiment was conducted with 16 crossbred gilts (Great Yorkshire x British Landrace; Pig Improvement Company, Oxfordshire, UK), weighing approximately 150 kg, which had shown 2 or more oestrous cycles of 19 to 21 days,. All animals were housed loose in individual pens (approximately 6 m^2) with a concrete floor that was covered with wood shavings, except for a slatted dunging area at the rear of the pens. They were housed under a 12:12 light:dark rhythm and ambient room temperature ranged from 15°C to 25°C. Pigs were fed 1 kg of a pelleted, dry sow feed (12.2 MJ of metabolizable energy per kilogram containing 15.4% crude protein) daily at 8:00 h and 16:00 h, and water was available ad libitum.

Experimental design
The oestrous cycles of all animals were synchronized by daily oral administration of 20 mg of the progesterone agonist altrenogest (Regumate®, Hoechst Holland, The Netherlands), added to the morning feeding for 21 days. Starting the day after last altrenogest treatment, 8 animals were treated twice daily for 6 days with the opioid receptor antagonist naltrexone.HCl (Sigma Chemicals, St. Louis, U.S.A.). Naltrexone was given p.o. mixed with the feed. On Day 1, 50 mg was given in the morning (8.00 h) and evening feed (16.00 h). From Day 2 to Day 6, 25 mg was given in the morning feed and 50 mg in the evening feed. The control group of 8 animals received

no naltrexone treatment. On Day 2, 4, 5 and 6 after altrenogest withdrawal, 2 control and 2 naltrexone treated animals were killed and pituitaries were quickly removed and kept on ice in culture medium. At slaughter, trunk blood was collected in cooled heparinized tubes, centrifuged at 3000xg for 15 minutes at 4 °C, and plasma was stored at -20 °C until radioimmunoassay of LH and E2ß (detection of the preovulatory LH surge and E2ß peak). The ovaries were removed for follicle counts. The experiments were approved by the Committee on Animal Care and Use of the Agricultural University, Wageningen, The Netherlands.

Superfusion

Superfusion was performed using the method described previously by Verburg-van Kemenade *et al.* (1987b) with slight modifications. After removal of the posterior lobe, anterior pituitary glands were hemisected and each half was put in an air- and water-tight superfusion chamber (452 µl volume). The superfusion chambers were placed in a temperature controlled (38 °C) container. Culture medium consisted of Dulbeco's Modified Eagles Medium Nutrient Mix F12 (1:1) containing 0.356 ‰ L-Glutamine (w/v) and 15 mM HEPES with added per liter: 3.439 g TES, 35 mg Penicillin-G, 1 g $NaHCO_3$, 200 µl 0.25x10^{-3} ‰ gentamycin-sulfate, 500 µl ethanol, 50 µl catalase, 30.4 µl 0.1% (v/v) ethanolamine, 100 µl 250 µM sodiumselenite, 100 µl 500 nM Tri-iodothyronin , 898,3 mg D-glucose, 5.0 g bovine serum albumin. In order to mimic in vivo conditions during the follicular phase, oestradiol-17ß (E2ß) was added to the superfusion medium in concentrations matching those found in blood plasma on Day 2, 4, 5 and 6 respectively in a previous experiment with animals of the same breed, weight and treatment. Furthermore, superfusion medium of pituitaries from naltrexone treated animals, was supplemented with naltrexone in a concentration of 10^{-6} M. Medium was pumped at a speed of 80 µl/minute and superfusate was collected using a fraction collector (LKB type 3401B, Sweden). The first 100 minutes of superfusion were used to allow hormone release reach baseline values, and fractions of 10 minutes collected during the following 100 minutes were used to determine basal LH release. Then hemipituitaries were superfused with culture medium containing 10^{-6} M GnRH during 20 seconds (GnRH pulse), and 8 fractions of 2.5 minutes were collected. Thereafter, 10 minute sampling was resumed and continued for another 140 minutes. Collected fractions were stored at -20 °C until hormone analysis. Basal LH release was determined by calculating the average LH concentration of the 10 fractions during the second 100 minutes of superfusion. The highest value measured during the 2.5 minute fractions following the GnRH pulse was used to calculate the LH response that was computed as % of basal. The procedure was validated for pig tissue by conducting a GnRH dose response curve and exposing the tissue to 60 mM KCl to assess residual LH releasability (Dierx *et al.*, unpublished results). In

the dose-response curve a maximal LH response was found at a GnRH concentration of 3.10^{-5} M GnRH. In the present study a submaximal dose of 10^{-6} M was used to asses pituitary sensitivity for GnRH.

Hormone analysis

LH. Superfusion samples and trunk blood plasma were analysed for LH with a double antibody radioimmunoassay (RIA) as described by Niswender *et al.* (1970), using porcine LH (pLH iodination grade batch 004/3; potency, 0.85 x NIH LH-S19; UCB bioproducts, Brussels, Belgium) as a standard and for radioiodination (specific activity, 38 μCi/μg). Anti-porcine LH batch 004 (UCB bioproducts, Brussels, Belgium) was used at a final dilution of 1:360,000 yielding an initial binding (B_0) of the labeled hormone of approximately 39%. Sac-Cel® was used as the second antibody, (donkey anti-rabbit, Wellcome Reagents, Beckenham). The minimal detectable concentration at the 90% B/B_0 level was 0.1 ng/ml. The interassay coefficient of variation was 14.4 % and the intra-assay coefficient of variation was 7.2 %.

Oestradiol. Concentrations of E2ß in trunk blood plasma were measured after extraction with dichloromethane (DCM, Merck, Darmstadt, Germany) using a single anti body radioimmunoassay. For estimation of procedural losses, 500 cpm of [2,4,6,7-^{3}H]E_2ß (NEN Chemicals, 's Hertogenbosch, The Netherlands) was added to 1 ml plasma sample and mixed with 3 ml DCM. The organic phase of the mixture was evaporated under a stream of nitrogen and the residue redissolved in 500 μl phosphate buffer with 1% BSA. An aliquot of 150 μl was taken to determine the recovery of [^{3}H]E2ß (57.7 ± 2.2%; mean ± SEM). Aliquots of 150 μl were used to measure E2ß concentrations in duplicate by RIA using a specific rabbit antiserum against 6-keto-estrone (6-CMO-BSA, UCB bioproducts, Brussels, Belgium) in a final dilution of 1:125,000. The mean crossreacting steriods were oestrone (1.49%) and oestriol (0.21%). E2-17ß (Sigma Chemical Co., St. Louis, MO, U.S.A.) was used as a standard and [2,4,6,7-^{3}H]E_2 (specific activity 95.4 Ci/mmol^{-1}, NEN Chemicals, 's Hertogenbosch, The Netherlands) as a tracer. The minimal detectable dose at the 90% B/B_0 level was 8 pg.ml^{-1}. The intra- and the inter-assay coefficient of variation 12.4 % and 17.4 % respectively. The concentration of E2ß was expressed in pg.ml^{-1} after correction for procedural losses.

Detection of oestrous

Oestrus detection was performed once daily in the morning after withdrawal of altrenogest treatment by a back-pressure test in the presence of a vasectomized boar. The time of oestrous was defined as the first day the sow showed a standing response.

Statistics

The procedure CORR of the SAS statistical analyis system (1990) was used to calculate Pearson's correlation coefficients.

Results

General

All 4 animals that were killed on Day 6 (2 control and 2 naltrexone treated animals) had shown oestrous behaviour on day 5. The ovaries of animals of the control group (N=8) were not different in weight (9.8 ± 2.7 g; P=0.67) or number of follicles (20 ± 1.4; P=0.46) compared to animals of the naltrexone treated group (N=8; 8.9 ± 0.69 g and 22 ± 1.4 respectively).

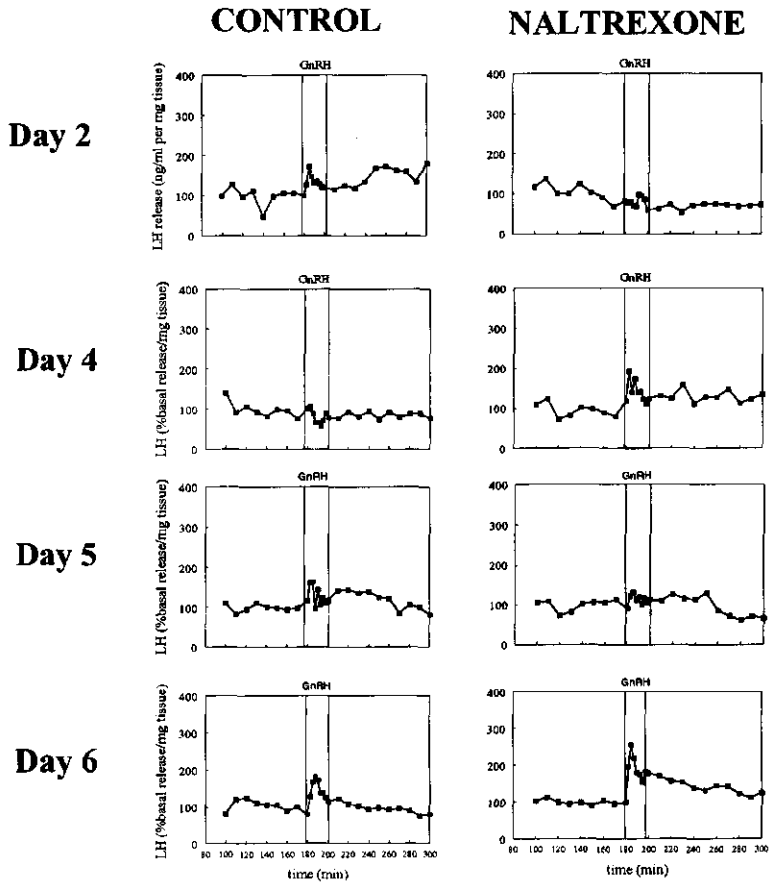

Figure 1. Pituitary LH response to a GnRH pulse in superfused hemipituitaries. Data are presented as the mean value (% basal release/mg tissue) of 2 hemipituitaries from control (left panels) or 2 hemipituitaries from naltrexone (right panels) treated animals on Day 2, 4, 5 and 6 after termination of altrenogest treatment.

Plasma hormone concentrations in vivo

The plasma E2ß concentration, determined in trunk blood, in general reached the highest levels on Day 4 or 5 and were lowest on Day 6 (Table 1). On Day 2, 1 naltrexone treated animal had a relatively high plasma oestradiol concentration (89.9 pg/ml) and had follicles that were in developmental stage of Day 4.

The LH concentration, determined in trunk blood, on average was higher in animals on Day 6 than on Day 2, 4 and 5 (Table 1). One naltrexone treated animal already showed preovulatory surge-like LH concentrations on Day 5 (25.7 ng/ml). On Day 6 plasma concentrations of LH seemed to be lower in gilts of the naltrexone group compared to gilts in the control group.

Table 1: Hormone parameters *in vivo* and *in vitro* in control and naltrexone treated animals

Day after altrenogest	treatment	plasma LH	plasma E2ß	LH response
2	C	7.8	21	129
	C	1.6	<8	227
	N	2.7	90	85
	N	4.8	32	128
4	C	2.5	44	109
	C	1.5	95	100
	N	2.5	41	121.0
	N	5.1	92	271
5	C	2.2	85	139
	C	3.2	81	190
	N	4.3	118	122
	N	25.7	13	169
6	C	15.1	<8	248
	C	19.9	<8	161
	N	11.5	<8	204
	N	6.6	<8	324

Data represent plasma LH concentration (ng/ml) in vivo, plasma E2ß concentration (pg/ml) in vivo, and pituitary LH response (% basal) to a GnRH pulse in vitro in individual control (C) and naltrexone (N) treated animals on Days 2, 4, 5 and 6 after termination of altrenogest treatment.

LH response to GnRH pulse in vitro

Data of the LH response of superfused hemipituitaries to GnRH are presented in Table 1 and illustrated by Figure 1. In control gilts the LH response seemed to be lowest on Day 4. In naltrexone treated gilts, the LH response seemed to be lowest on Day 2 and highest on Day 6. Furthermore, the LH response of hemipituitaries on Day 2 seemed to be lower in naltrexone

treated animals than in control animals. On Day 4 and 6, the LH response of hemipituitaries from naltrexone treated animals seemed to be higher compared to those from control animals.

Correlations between in vivo and in vitro data

The plasma LH concentrations showed a positive correlation with the pituitary LH response to GnRH (P=0.06; r^2=0.48), and both parameters showed a negative correlation with the plasma E2ß concentrations (P=0.04; r^2=-0.52 and P=0.02; r^2=-0.57 respectively). No correlations were found between the basal LH secretion *in vitro* and the plasma concentration of LH (P=0.56; r^2=-0.16), the plasma concentration of E2ß (P=0.79; r^2=0.073), or the GnRH-induced LH response *in vitro* (P=0.27; r^2=-0.29).

Discussion

The present study was designed as a pilot study to assess whether the LH response to a pulse of GnRH changes during the follicular phase and whether this is mediated by EOP's. The response of pituitaries of control animals seemed lowest on Day 4, and highest on Day 6, the day of the preovulatory LH surge as indicated by the high plasma LH concentration. Pituitaries of naltrexone treated animals showed an increase in LH response from Day 2 reaching the highest response on Day 6. In view of the submaximal concentration of GnRH used, it is therefore suggested that, independent of the treatment with naltrexone, the responsivity of the pituitary increases from the follicular phase onwards reaching a maximum at the day of the preovulatory LH surge. This is in accord with studies from others that reported increased pituitary sensitivity to GnRH around the preovulatory LH surge in rats (Clayton *et al.*, 1980; Apfelbaum, 1981) and humans (Rosemberg *et al.*, 1974) and with data from the literature showing that E2ß enhances the sensitivity of the pituitary for GnRH (Drouva *et al.*, 1983; Koiter *et al.*, 1987; Gregg *et al.*, 1990), but inhibiting the LH secretion from the pituitary gonadotrophs (March *et al.*, 1981; Knobil and Hotchkiss, 1988). Indeed, the negative correlations between the plasma E2ß concentration and on the one hand plasma LH concentration and on the other hand the pituitary LH response *in vitro* in both treatment groups correspond with the presumed negative feedback by E2ß reported in a variety of species *in vivo* (Helmond *et al.*, 1986; Kesner *et al.*, 1989; Messinis *et al.*, 1992; Matt *et al.*, 1993).

Naltrexone treatment seemed to affect the pituitary LH response to GnRH during the follicular phase of the oestrus cycle. The lowest GnRH induced LH response was found on Day 4 in control animals, but already on Day 2 in naltrexone treated animals. Together with the data on follicle maturation on Day 2 and surge like LH levels on Day 5 of 2 other

naltrexone treated gilts also suggests that the naltrexone treatment had advanced the timing of events during the follicular phase. The inhibitory actions of EOP's could be directly on the pituitary or indirectly through the GnRH pulse generator. Chao *et al.*, (1986) found no effect of naloxone (added in vitro) on GnRH stimulated LH secretion from bovine pituitary cells, suggesting that EOP's do not modulate LH secretion at the level of the pituitary. In a previous study, we reported an advanced termination of the preovulatory LH surge after repeated ICV treatment with naltrexone during the follicular phase (Chapter VII), supporting a central site of action. This notion is in line with the results of studies in intact or E2ß primed mature OVX gilts (Armstrong *et al.*, 1988; Yearwood *et al.*, 1991; Kraeling *et al.*, 1992) and intact sheep (Currie *et al.*, 1991), showing a delayed preovulatory LH surge after iv or ICV treatment with the opiate agonist morphine. Thus, one could speculate that EOP's exert an inhibitory action on the sequence of processes that lead to the preovulatory LH surge and that interruption of EOP activity permits generation of the surge.
In summary, the results from the present pilot study suggest that responsivity of the pituitary to GnRH changes during the follicular phase, and that EOP's modulate the timing of events that eventually lead to the preovulatory LH surge by an inhibitory action at the suprapituitary level.

Aknowledgement

The authors like to thank the MSc students Hinke ten Hulscher and Regis Lamberts for their assistance in the development of the superfusion system. Furthermore we like to thank Corrie Oudenaarden for the analysis of the superfusion samples

Chapter VI

A new technique for implantation of a chronic cannula in the lateral brain ventricle of postpubertal, freely moving pigs

J.A.J. Dierx[*] and F.J.C.M. van Eerdenburg[**]

[*]Department of Human and Animal Physiology, Wageningen Agricultural University, Haarweg 10, 6709 PJ Wageningen, The Netherlands.
[**]Department of Herd Health and Reproduction, Faculty of Veterinary Medicine, Utrecht University, Yalelaan 7, 3584 CL Utrecht, The Netherlands.

Abstract

Intracerebroventricular (ICV) cannulation in adolescent and adult pigs has some unique problems. Placement of the cannula is very difficult due to large individual variability in size and structure of the skull and frontal sinus. Furthermore, the rough behaviour of the animals makes it necessary to restrain the animal for ICV treatment, which is disadvantageous when studying responses to stress. In the present study, we developed and employed a cannulation system for pig brain, based on landmarks on the neurocranium, that enabled us to perform ICV treatment in freely moving post pubertal animals for a long period of time. Furthermore, plasma cortisol concentrations have been determined in freely moving pigs treated ICV with a-CSF or naltrexone to validate the present ICV method for effectivity and stressfulness.

Introduction

Intracerebroventricular (ICV) cannulation has facilitated investigation of the role of neuropeptides in modulating pituitary function and measuring concentrations of neuropeptides and/or neurotransmitters in the cerebrospinal fluid (CSF) in the rat (Rivest *et al.*, 1993; Bonavera *et al.*, 1994), sheep (Conover *et al.*, 1993) and primate (Meyers, 1977). A method for cannulation of the lateral ventricles in prepubertal pigs has been described by Poceta *et al.* (1981) and has been used with some modifications by Barb and co-workers in a number of studies (Barb *et al.*, 1989; Estienne *et al.*, 1990; Barb *et al.*, 1991; Barb *et al.*, 1993). However, as reported by Poceta *et al.* (1981), ICV cannulation in the pig has some unique problems due to individual variability in skull structure and the absence of a detailed atlas of the brain with stereotactic co-ordinates. Furthermore, the development of the frontal sinus, the rough behaviour of pigs, including investigative activity, that could dislodge or damage the anchoring system, the difficulty of CSF-sampling or drug-injection in freely moving animals and the potentially dirtiness of the pig house, makes it very difficult to design a reliable and reproducible method of ICV-treatment in growing and freely moving pigs. The main purpose of this paper is to report a novel ICV cannulation technique for pigs which was developed utilising a stereotaxic apparatus affixed to the head and the injection device inserted into the frontal sinus. In addition, a pilot experiment was conducted to determine whether the injection method was stressful to the animal by measuring plasma cortisol. Furthermore, plasma cortisol response to ICV treatment with the opioid receptor antagonist naltrexone was used to validate the technique, since the opioid receptor antagonist naloxone has been shown to increase the plasma cortisol concentration (Voklavka *et al.*, 1979; Barb *et al.*, 1986).

Materials and Methods

Stereotactic Apparatus

A stereotaxic apparatus (Figure 1, panel I, II, III) was developed that was attached to the head by means of a mouth piece at the front and two bone screws at the back of the skull. The apparatus was adjustable to every (adult) headsize. The base consists of two metal bars orientated in a medial-lateral direction (A) containing the attachment points to the head, connected by two parallel anterior-posterior sliding bars (B). By means of a tube-sliding mechanism over the two anterior-posterior bars, a micromanipulator unit (C) with a medial-lateral orientation is attached to the base by two metal stands. Lateral movement of the micromanipulator can be made by a tube-sliding mechanism over two parallel bars, that can also be rotated 60°in total (30° to either side) in the vertical anterior-posterior plane. The micromanipulator unit itself was attached to the apparatus by means of an aluminium disc (Figure 1, panel II D and III D), that could be rotated 50° in total in the vertical medial-lateral plane. In both the anterior-posterior and medial-lateral direction, scales in millimetre were attached for measurement of cannula placement.

ICV Injection Device

The injection device (Figure 2 panel I, II, III) consisted of a stainless steel cup (A) with a luer lock cavity (B) of stainless steel ending in a stainless steel tube on the outside of the cup. The cup was closed by a Teflon® (PTFE=polytetrafluorethene) lid (C) that was locked with a bayonet catch. By fastening the socket cap screw (D), containing a Teflon lid, the orifice of the luer-lock cavity is closed and the bayonet catch is ultimately secured. This construction was wrapped in a Teflon® hull (E) to reduce the noise when unlocking and to prevent an inflammatory reaction and skin overgrowth.

A sterile flexible PVC tubing (Figure 3G) (PVC Perfusor® tubing, B. Braun Melsungen A.G., Melsungen, Germany) was attached to the tip of the stainless steel luer-lock canula tube that connected the injection device to a stainless steel cannula designed for placement within the lateral ventricle (Figure 3 H). This cannula was kept in place by a perforated stainless steel collar that was fixed together with the cannula to the neurocranium using dental cement (Simplex Rapid®, Kendent Works, Purton Swindon, U.K.).

Using this construction, growth and the further development of the frontal sinus does not affect the co-ordinates of the ICV cannula since the distance between neurocranium and the lateral ventricle remains constant (Table 1) during development around and after puberty, as was found in pigs of several crossbreeds and ages. An increase in the distance between the skull bone and neurocranium is compensated for by the flexible tubing.

Determination of cannula-placement and point of entry

The exact caudal-ventral and lateral co-ordinates of the site of cannula-placement on the neurocranium were determined empirically in 17 saggitally and coronally cut heads of female pigs of the different crossbreeds and age from an abattoir. To determine caudal-ventral co-ordinates, the distance between the neurocranium and the lateral ventricle in a perpendicular line on the neurocranium were measured (Table 1). In addition, the distance between the caudal bone rim and most frontal dip in the neurocranium (Table 1, A), i.e. the most frontal part of the brain, and the distance from the dip in the neurocranium to the lateral ventricle (Table 1, B) were determined using a sliding calliper, to calculate a multiplication factor ($f = B/A$; $f = 0.55$), which was used to determine the rostral-caudal co-ordinates (Figure 4 panel I and II). These co-ordinates were then measured from the anterior calliper point, i.e. the dip in the neurocranium. The point of entry was then determined at 5 mm lateral to the medial bone septum from this point; the drill was angled at 5° caudally.

Accuracy of the calculated co-ordinates and the technique of cannulation was verified using 8 heads of sows of different crossbreeds and age obtained from the abattoir by verification of placement with methylene blue. After injection of the dye, brains were removed and site of injection was determined.

Intracerebroventricular cannulation of the lateral ventricle

From three days before until three days after surgery, gilts (great Yorkshire x British Landrace; weight range 100-150 kg) were given 12 ml of the antibiotic Methoxasol-T® (20 mg Trimethorprim/ml, 100 mg sulfamethoxasol/ml, AUV, Cuijk, The Netherlands) orally once a day. Animals were fasted overnight and approximately 45 minutes prior to surgery they were premedicated by i.m. administration of 6 mg azaperone kg^{-1} body weight (Stresnil®; Janssen Pharmaceutica BV, Tilburg, The Netherlands). During general anaesthesia, which was induced and maintained using a mixture of O_2/N_2O, enflurane (Ethrane®, Abott, B.V., The Netherlands), heart rate and respiration were monitored. Surgery was carried out under standard sterile conditions and the animals underwent surgical procedures in the ventral recumbency. A circular piece of skin tissue, subcutis and underlying periosteum, 50 mm in diameter centered on the midline of the head, was excised. A piece of frontal-parietal bone 40 mm in diameter was excised using a circular sawblade (Figure 3 A) and the frontal sinus was exposed. The circular bone tissue and the remaining spongeous bone within the 40 mm perimeter were removed from the neurocranium using a chisel. The exact caudal-ventral and lateral co-ordinates of the site of cannula-placement on the neurocranium were determined as described earlier, using the dip in the neurocranium and the most caudal bone rim as landmarks (Figure 4 panel I and II). The stereotaxic apparatus was affixed to the

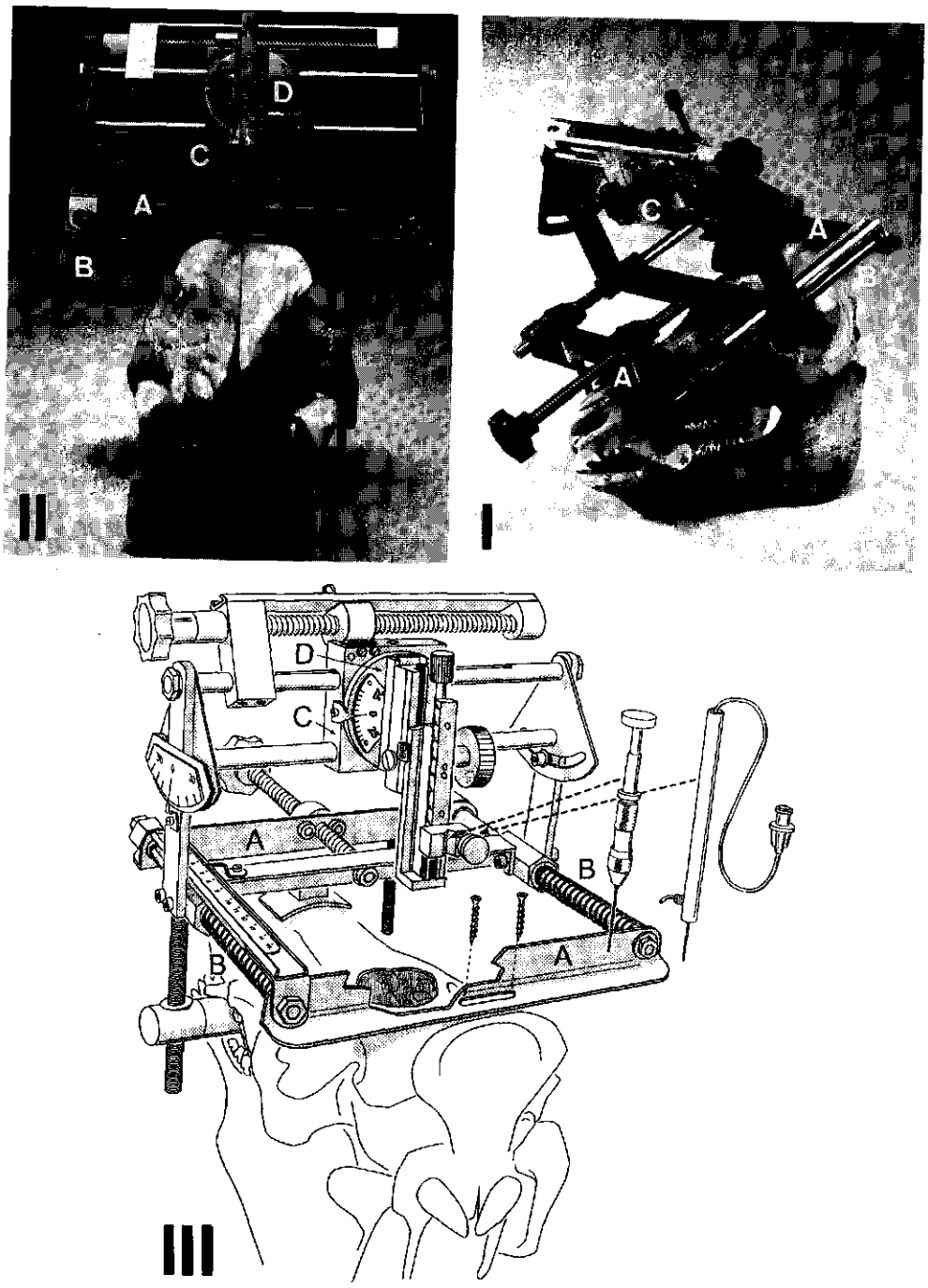

***Figure 1**: Stereotaxic apparatus. Frontal lateral side (I), caudal (II) view and illustrative drawing (III) of the stereotaxic apparatus with the base consisting of two medial-lateral bars (A) and two parallel anterior-posterior bars (B). The micromanipulator is attached to a disk (D) that is mounted on a block (C) for medial-lateral movement.*

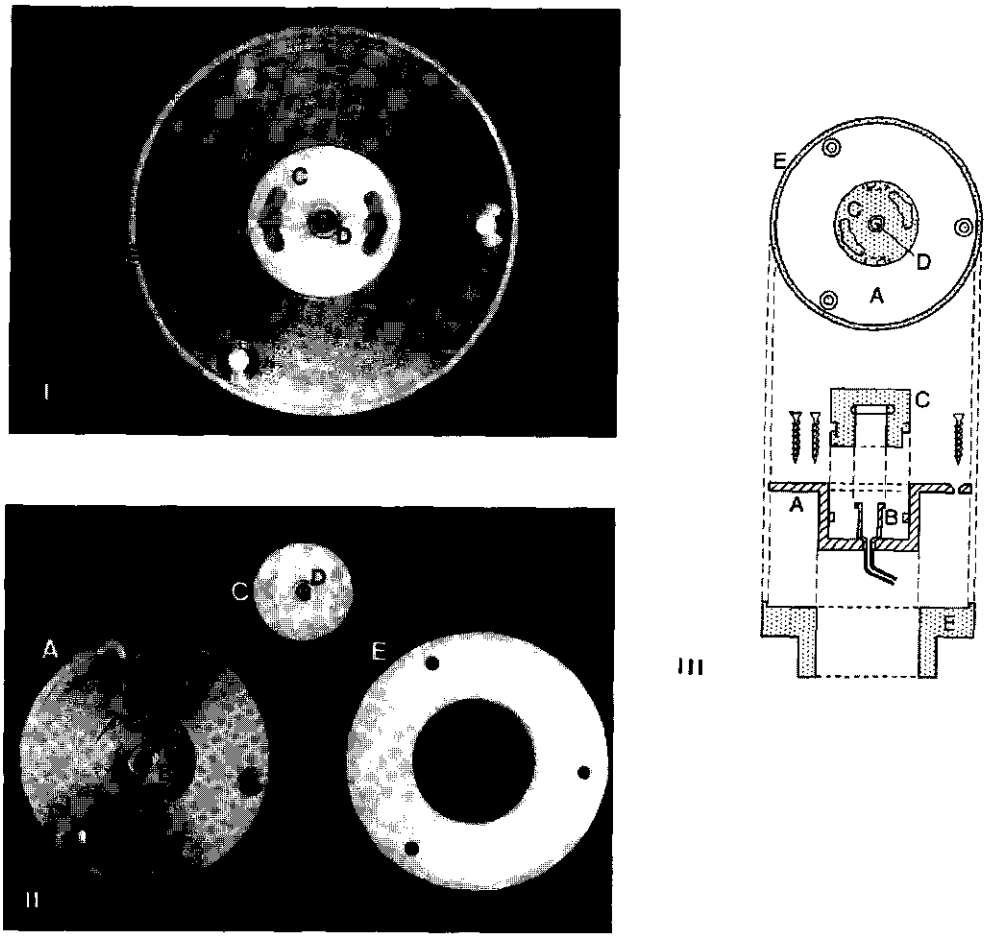

Figure 2: The ICV injection device. The intact (I), the separate parts (II) and a schematic drawing of the device (III). The stainless steel cup (A) with the Luer lock mouthpiece (B). This is protected from the external environment by a Teflon® lid (C), closed by a bayonet catch and secured by a socket cap screw (D). Inside the Teflon® lid (C), a piece of rubber is mounted to securely close the Luer lock mouthpiece (B). The total construction is wrapped in a Teflon® hull (E).

skull by means of a bit in the mouth and 2 bone screws at the back of the head (Figure 1 panel I, II, III).Three 2 mm diameter holes were drilled (Figure 3 B) in the neurocranium near the site of cannula placement, 2 mm in depth for placement of screws. A hole, 2 mm in diameter was drilled that penetrated the neurocranium at the chosen site for placement of the cannula. The dura mater was punctured carefully before lowering the cannula. The injection cannula was attached to a 250 mm long tube (Figure 3G; PVC Perfusor® tubing, B. Braun Melsungen A.G., Melsungen, Germany) filled with 0.9% saline and vertically held above the guide tube. The cannula and PVC tube were led through a guide tube (Figure 3 C) and fixed with a screw

at the cannula-tube connection to assure rigidity between guide tube and injection cannula. This unit was attached to the stereotaxic apparatus. When the cannula, equipped with a perforated collar, was placed on the dura, the syringe with 0.9% saline was detached from the PVC tubing with the meniscus at the top of the tubing, approximately 150 mm above the guide tube. Subsequently, the cannula was slowly lowered stereotaxically by means of the guide tube until an influx of fluid was observed indicating entrance into ventricular space (Buxton, 1988). The perforated collar was fixed with dental cement (Simplex Rapid®, Kendent Works, Purton Swindon, U.K.) around the cannula on the neurocranium. The tube connected to the cannula was cut to a length of approximately 50 mm and connected to the tube connector (Figure 2 panel III and Figure 3 D) on the bottom of the cup, which fitted precisely in the 40 mm hole of the outer skull. Before attachment of the injection device to the skull with three vitallium screws (diameter 2.7 mm, length 20 mm, Instruvet, Amerongen, The Netherlands), 10 ml of the antibiotic Ritriprim® (per 9.5 g gel, 300 mg rifamycine and 200 mg trimethoprim, Dopharma, Raamsdonksveer, The Netherlands) was injected into the frontal sinus in order to prevent infection. A silicone layer was placed between the skin and injection device.

Cannulation of the jugular vein

Gilts were fitted with a jugular vein catheter immediately after the ICV cannulation, as described by Janssens *et al.* (1994). An indwelling catheter (Silastic® Medical Grade Tubing, 0.040 in. i.d., 0.085 in. o.d.; Dow Corning, Michigan U.S.A.) was implanted into the external jugular vein pointing towards the cranial vena cava. The catheter was passed s.c. and externalized between the scapulae. A one-way luer-lock stopcock (Vygon BV, Veenendaal, The Netherlands) was secured to the end of the catheter. The catheter was covered in a harness of 23 cm x 20 cm PVC/Nylon (Bizon Chemie, Goes, The Netherlands) that was fixed on the back of the animal by means of chest belts.

The catheter was flushed with saline once a week and filled with heparinized saline (25 IU heparin/ml of 0.9% saline; Leo Pharmaceutical Products, The Netherlands) when not in use. When catheter patency was reduced, obstructions were removed as described by Leuvenink and Dierx (1997). In short, a solution of 25000 IU Kabikinase® (Kabi Pharmacia, Sweden), 2500 IU heparin and 0.2 ml Ticarpen® (Beecham, England) was added to 25 ml sterile 25% polyvinylpropylene (PVP)/saline (Merck, Germany) solution. After 1 week the catheter was flushed with a 2% heparine/saline solution.

ICV Injection Procedure

Before injection, the lid was removed using an Allan wrench (Figure 3 E) to loosen the socket cap screw (Figure 2D) followed by the use of a bipointed wrench (Figure 3 F) inserted into

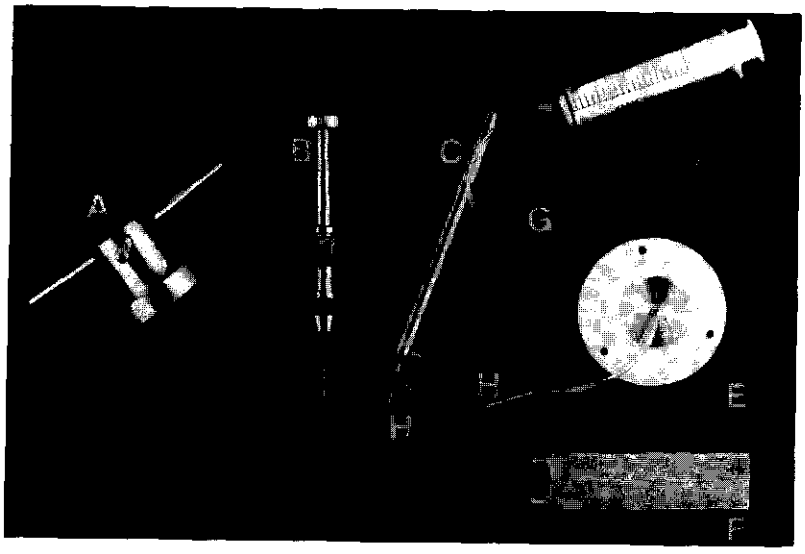

***Figure 3**: ICV surgery tools. Bone from the skull is excised with the circular sawblade (A) and the holes for the screws and cannula are drilled using a manual drill (B). With the guide tube (C), the cannula and perforated collar (H) are positioned and attached to a PVC tube (G), filled with 0.9% saline. After fixing the cannula, the tube is cut and attached to the tube connector (D) of the ICV injection device. The wrench (E) and bipointed wrench (F) are used to loosen the screw and the lid respectively.*

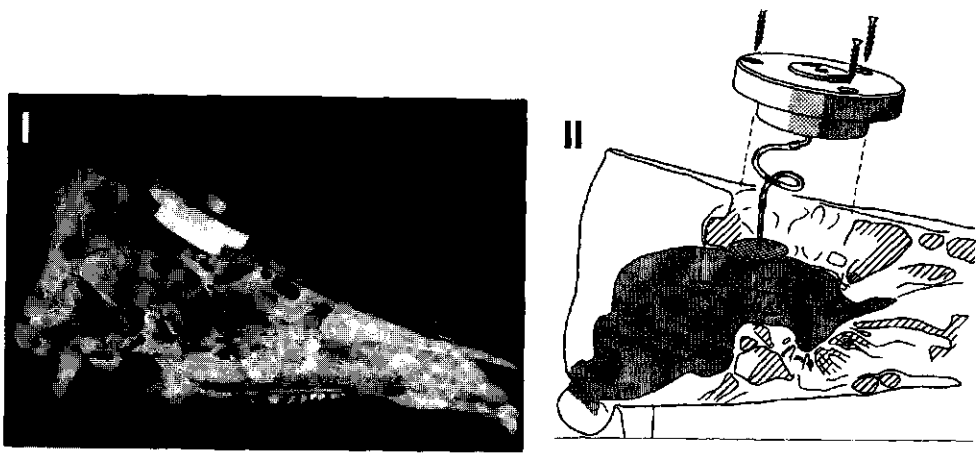

***Figure 4**: Positioning of the cannula. Skull model (I) and schematic drawing (II) of cannula positioning. The cannula was placed at the point 5 mm lateral to the medial bone septum. Dorsal-ventral position was determined by multiplying the distance from the front of the neurocranium to the most caudal bone rim by a factor of 0.55, which was then measured off from the anterior calliper point angling at 5° caudally.*

semicircular grooves in the lid (Figure 2 III) to unlock the bayonet catch and extract the lid. This lid was kept in a 70% alcohol solution during the injection procedure. A piece of 1 meter long PVC tube (Perfusor® tubing, B. Braun Melsungen, Melsungen, Germany) with a luer lock stopcock two-way valve (Vygon BV, Veenendaal) containing a 1 mg naltrexone (Sigma Chemicals, St. Louis, U.S.A.) in 500 µl artificial cerebrospinal fluid (a-CSF, per liter solution: 166 mg $CaCl_2$, 240 mg $MgSO_4$, 134 mg KCl, 170 mg KH_2PO_4, 2,184 mg $NaHCO_3$ and 7,247 mg NaCl, pH=7.6) was connected to the injection device with the luer-lock stopcock and the solution was injected followed by 500 µl of a-CSF to overcome the dead volume in the system. After injection, the lid was replaced and locked.

Experimental Procedures

As a pilot experiment, 7 crossbred gilts (Great Yorkshire x British Landrace; Pig Improvement Company, Oxfordshire, U.K.; weight range 100-150 kg) which had shown 2 or more oestrous cycles of 19 to 21 days were used to determine the effect of the ICV injection procedure on plasma cortisol concentrations by ICV treatment with a-CSF or the EOP antagonist naltrexone. The gilts were housed in individual pens (approximately 6 m^2) with a concrete floor that was covered with wood shavings, except for a slatted dunging area at the rear of the pens. Gilts were exposed to 12:12 light:dark cycle and ambient room temperature ranged from 15 °C to 25 °C. Pigs were fed at 8.00 h and 16.00 h 1 kg of a pelleted, dry sow feed (12.2 MJ of metabolizable energy per kilogram containing 15.4 % crude protein) and water was available ad libitum. Cannulation of the lateral ventricle and the jugular vein was performed, as described earlier. After a recovery period of 7 days, pigs were treated ICV with 500 µl of a 2 mg/ml naltrexone solution (N=2) or 500 µl a-CSF (control; N=5). From naltrexone treated animals, blood samples were taken every 12 minutes for 1 hour before, 3, 8, 14, 20 and 26 minutes after ICV treatment and every 12 minutes for 2 additional hours. From control animals, blood samples were taken every 12 minutes during 8 hours. Blood samples (approximately 5 ml) were collected in tubes containing 50 µl EDTA solution (144 mg/ml of saline; Triplex®III, Merck Nederland, The Netherlands) to prevent clotting. Contents of the tubes were mixed and placed on ice, subsequently centrifuged at 3000 x g for 15 minutes at 4 °C and stored at -20 °C until analysis for cortisol using a specific radioimmunoassays (RIA's) as described by Janssens *et al.* (14). After 6 weeks, the naltrexone treated animals were killed to check cannula placement by cutting the heads saggitally and coronally. The experiments were approved by the Committee on Animal Care and Use of the Agricultural University, Wageningen, The Netherlands

Statistical Analysis

For statistical analysis, the means of cortisol concentration at 9:00 h (the hour before treatment) were compared to means of the cortisol concentration of 13:00 h (the first hour after treatment). The effect of ICV treatment with naltrexone or a-CSF on plasma cortisol concentrations was analysed using a two tailed student T-Test.

Table 1: Data of distances between anatomical structures in pig skull

	Distances (in mm) between:			factor
	NC - LV	BR - DNC (A)	DNC - LV (B)	(f=B/A)
Mean ± SEM	20.1 ± 0.4	87 ±1.9	47 ± 0.9	0.55 ± 0.008
Range	18 - 22	70 - 102	41 - 56	0.51- 0.62

Data are presented as mean ± SEM and the range. Data were determined in 17 sows of different crossbreeds and age that were obtained from an abattoir. NC = neurocranium, LV = lateral ventricle, BR = most caudal bone rim, DNC = dip in neurocranium.

Results

Recovery

Animals exhibited normal eating behaviour, which reappeared within 24 hours after surgery. The skin healed within 3-4 days with minor inflammation. One animal exhibited temporary paralysis of the left hindleg after surgery, due to the positioning of the leg during surgery. However, this had healed by 24 hours post surgery. Post mortem examination revealed that all animals had a fungal infection in the frontal sinus. However, none of the animals showed any behavioural or physical signs of discomfort.

Solidity of the ICV injection device and cannula patency

Although, the pigs frequently rubbed their heads during the first days post surgery, none of the ICV injection devices were dislodged or damaged by their rough behaviour. In addition, the ICV injection devices were not damaged or dislodged by the biting behaviour of neighbouring pigs. Furthermore, in the 5 animals that were not killed to check cannula placement, patency of ICV cannulae remained at least up to 6 months, as checked by flushing with a-CSF.

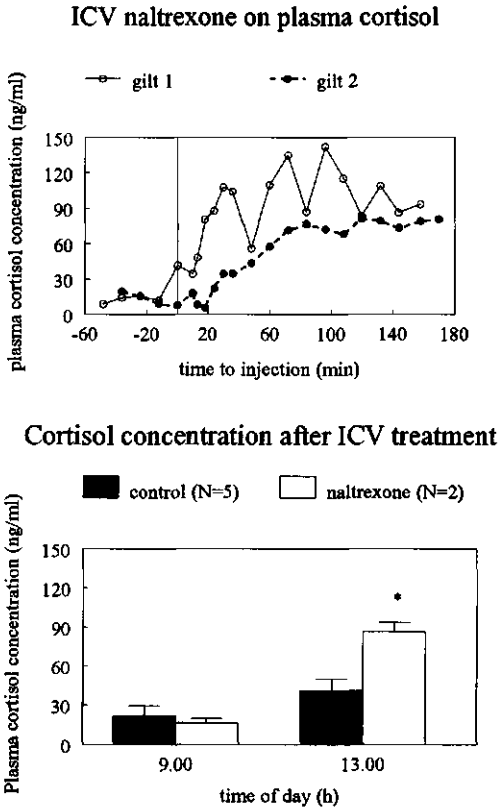

Figure 5: Plasma cortisol concentrations in animals treated ICV with a-CSF or naltrexone. Top panel shows the profiles of the cortisol concentrations of gilts (N=2) treated with ICV naltrexone injection relative to time of injection (t=0). Bottom panel presents data of plasma cortisol concentrations at 9:00 h and 13:00 h of animals treated ICV with a-CSF (N=5) or naltrexone (N=2) at 11:00 h.
** = significantly different ($p<0.05$)*

Cannula-placement

The data obtained from the 17 sows showed that the distance from the dip in the neurocranium, i.e. the most rostral part of the brain, to the most caudal bone rim, respectively the lateral ventricle, showed no considerable individual variation (Table 1). In addition, no considerable individual variation was found in the distance from the neurocranium to the lateral ventricle when measured in a perpendicular line to the neurocranium (Table 1).
The position and orientation of the cannula in the skull and cavity of the brain is shown in Figure 4 (panel I, II). After injection of the dye, methylene blue was found in the lateral ventricle of the brains of all 8 sows, with the point of entry of the cannula in the body of the lateral ventricle dorsally from the body of the caudate nucleus. In all animals of the pilot experiment it was found that, when entering ventricular space, an influx of a-CSF from the PVC tubing was observed and CSF could be aspirated. Post mortem check of cannula

placement in 2 gilts from the pilot experiment, showed the same point of entry as the sows that were injected with dye. In these pigs, it was found that the tip of the cannula had penetrated the corpus callosum, protruding in the ventricle over a length of 2 mm.

Plasma cortisol

Pre-treatment cortisol concentration was not different between a-CSF and naltrexone treatment (Figure 5 bottom panel). ICV treatment with a-CSF had no significant effect on plasma cortisol concentration. ICV treatment with naltrexone increased plasma cortisol concentration significantly compared to preferment cortisol concentrations (Figure 5). Plasma cortisol concentrations remained increased and were significantly higher ($p<0.05$) in naltrexone compared to control pigs at 3 hours post treatment (Figure 5 bottom panel).

Discussion

The ICV cannulation as described in the present study differs from the method used by others. First, in the method as presented in this study, the stereotaxic apparatus is placed on the skull without the use of ear bars. Therefore, there is no risk of damage to the eardrums. The use of ear bars has shown some adverse effects on the eardrums (Barb, personal communication).

Second, the orientation for cannula placement takes place on the neurocranium instead of the frontal-parietal bone together with the interaural axis. There is considerable variation in skull structure between breeds of pig and individual pigs. As shown in the present study, the neurocranium has considerable variation with regard to rostral-caudal position and distance relative to the lateral ventricle. By computing factor f, this individual variation between animals and thereby variability in cannula placement is minimised.

Third, in the present method, the cannula that is fixed on the neurocranium (inner table of the frontal-parietal bone) is connected by means of flexible PVC tubing to the injection device on the frontal-parietal bone, in contrast to methods used by others (Poceta *et al.*, 1981; Barb *et al.*, 1993), where the cannula is fixed directly on outer table of the frontal-parietal bone of the skull. When studying central mechanisms, during development, a problem may arise, since theouter table of the frontal-parietal bone shifts in a rostro-dorsal direction due to the development of the frontal sinus. This shift might pull the cannula out of the ventricle as has been reported by Poceta *et al.* (1981). By attaching the cannula to the neurocranium and connecting it to an injection device at the frontal-parietal bone with a flexible PVC tube, the outgrowth of the frontal sinus during growth of the animal does not affect the position of the

cannula. The present method opens the possibility to study maturation processes in the brains from the age of puberty until adulthood.

Fourth, another difficulty in other methods for ICV cannulation and injection in pigs is the anchoring system. Due to the rough explorative behaviour of pigs it is difficult to maintain external cannulas in place. Furthermore, it is very difficult to maintain a sterile environment. By internalizing the site of injection in an injection device that is located in the frontal sinus, as described in this paper, the site is protected against dislodgement and damage. None of the implanted ICV injection devices were damaged or dislodged by the animal itself or neighbouring animals. In addition, by cleaning the injection site with alcohol prior to covering it with an alcohol sterilised Teflon® lid, sterility is maintained. Although, all animals were treated with anti biotics, we could not prevent a fungal infection in the frontal sinus. However, none of the animals used in the pilot study, and only 1 out of 12 animals used in a following study (Chapter VII), showed an infection of the brain or meningitis. How fungal infection in the frontal sinus can be prevented in future studies, is currently under investigation and the use of an antiseptic drain or frequently flushing the frontal sinus with sterile saline and/or antibiotics could be considered.

When studying mechanisms that are influenced by stress, it is important to minimise stress, such as restraining animals for ICV treatment. Using the injection device and an Infusor® tubing as described in the present study, animals are not restrained for ICV treatment. Plasma cortisol concentrations were not increased by ICV treatment with a-CSF, suggesting that animals are subjective to no or minimal stress using the present ICV technique. ICV treatment with 1 mg naltrexone increased plasma cortisol concentrations. These findings support studies in pigs (Barb *et al.*, 1986) and human (Voklavka *et al.*, 1979), reporting increased plasma cortisol concentrations preceded by increased ACTH release after treatment with naloxone. In view of the route of administration and the relatively low dose of naltrexone used (Barb *et al.*, 1989), it is likely that naltrexone exerted its action within the brain. Since the present study is only a preliminary experiment, further research is needed to substantiate this suggestion.

In summary, the present study reports a modified ICV technique that enables ICV injection in freely moving, large, growing pigs. This technique should prove to be useful for studies during pubertal development, since this technique prevents dislodgement or damage of the anchoring system or the cannula growing out of the ventricle. Thus far we have been able to follow animals for a longer (6 month) period of time.

Aknowledgement

The authors would like to thank Mr. Gradus Leenders and Mr. Dick van Kleef for their technical assistance during stereotaxic surgery and Dr. C.Richard Barb and Dr. Colin L. Gilbert for useful comments on the manuscript. Furthermore, we would like to thank Mr. Piet Kostense for the schematic drawings and Duotone Wageningen for the pictures.

Chapter VII

Repeated intracerebroventricular treatment with naltrexone during the follicular phase advances the termination of the preovulatory LH surge in cyclic, freely moving gilts

J.A.J. Dierx[*] and F.J.C.M. van Eerdenburg[**], van der Heide, D.[*] and Wiegant, V.M.[***]

[*]Department of Human and Animal Physiology, Wageningen Agricultural University, Haarweg 10, 6709 PJ Wageningen, The Netherlands.
[**]Department of Herd Health and Reproduction, Faculty of Veterinary Medicine, Utrecht University, Yalelaan 7, 3584 CL Utrecht, The Netherlands.
[***]Rudolf Magnus Institute for Neurosciences, University Utrecht, Stratenum Universiteitsweg 100 3584 CG Utrecht, The Netherlands.

Abstract

Endogenous opioid peptides (EOP's) have been shown to inhibit the pulsatile LH secretion during the luteal phase at the level of the hypothalamus. The involvement of EOP's in regulating pulsatile LH secretion during the E2ß dominated follicular phase was investigated by treating freely moving cyclic gilts intracerebroventricular twice daily for 6 days of with 1 mg of the opioid receptor antagonist naltrexone (N=4) or a-CSF (N=5) after oestrus synchronisation with altrenogest. Blood samples were taken frequently (every 12 minutes during 8 hours) on Day 2, 4, 5 and 6, and every 6 hours on Days 1, 3 and from Day 6 onwards. Pulse frequency, pulse amplitude and mean plasma concentration of LH was lowest on Day 2 and 4 reaching highest values on Day 6 (the average day of the preovulatory LH surge and first day of oestrus). Mean LH concentration returned to baseline values after the preovulatory LH surge significantly sooner in naltrexone than control animals. Naltrexone had no effect on pulse frequency and mean LH concentration, whereas it increased pulse amplitude on Day 4.

In summary, the results show that LH pulse frequency remains constant during the follicular phase, whereas LH pulse amplitude decreases from early to mid follicular phase, and increases from mid to late follicular phase. Both, LH pulse amplitude and pulse frequency, reach peak values at the day of the preovulatory LH surge. It is hypothesised that EOP's delay the timing of the preovulatory LH surge by decreasing pulse amplitude during the negative feedback phase of E2ß in early and mid follicular phase.

Introduction

The role of endogenous opioid peptides (EOP) in the regulation of the pulsatile LH release has been investigated in a variety of species (Whisnant and Goodman, 1988; Kaynard *et al.*, 1992; Chang *et al.*, 1993; Heisler *et al.*, 1993). Opioid receptor antagonists, like naloxone and naltrexone, are reported to increase the LH release (Muraki *et al.*, 1979; Remorgida *et al.*, 1990; Barb *et al.*, 1992; Chang *et al.*, 1993; Genazzani *et al.*, 1993). The effect of these non selective opioid ligands, depends on the presence of gonadal steroids. During the progesterone dominated luteal phase, EOP's exert a tonic inhibition on plasma LH by decreasing LH pulse frequency and pulse amplitude as has been shown in rat (Higuchi and Kawakami, 1982), sheep (Montgomery *et al.* 1985), pig (Barb *et al.*, 1988) and humans (Rossmanith *et al.*, 1989). In the oestradiol (E2ß) dominated follicular phase, the involvement of EOP's in regulating pulsatile LH release is not clear. It seems that EOP's are not involved in the negative feedback of E2ß on the pulse amplitude and mean plasma concentration of LH during the early follicular phase (Rossmanith *et al.*, 1989;

Okrasa *et al.*, 1992). However, during the late follicular phase and the presumed positive feedback of E2ß, increases of these parameters of LH release were found after systemic treatment with opioid receptor antagonists in humans (Rossmanith *et al.*, 1989), rats (Piva *et al.*, 1985) and pigs (Okrasa *et al.*, 1992). In view of recent studies, the question arises whether EOP's act as an intermediate in the negative and/or positive feedback of oestradiol, or play a permissive role in the regulation of the pulsatile LH release in the generation and timing of the preovulatory LH surge (Massotto *et al.*, 1990; Kraeling *et al.*, 1992; Walsh and Clarke, 1996; Smith and Gallo, 1997).
It is thought that opioids exert their possible inhibitory action on the pulsatile LH release mainly at the level of the hypothalamus. This opioidergic inhibition is highly associated with plasma concentration of progesterone during the luteal phase (Ferrer *et al.*, 1997). Studies in sheep (Whisnant *et al.*, 1991), rat (Kubo *et al.*, 1983), primate (Pau *et al.*, 1996), humans (Kawahara, 1991) and pig (Asanovich *et al.*, 1998) show that opioid receptor antagonists are able to increase pulse frequency, pulse amplitude and mean plasma concentration of LH by disinhibiting the GnRH pulse generator. However, whether EOP's play a role in the feedback effects of E2ß during the follicular phase, and if so whether they have a central site of action, is still not known.
The aim of the present study was to investigate whether EOP's in the brain are involved in the regulation of the pulsatile LH release on multiple days during the follicular phase of the oestrous cycle by treating freely moving intact gilts with repeated ICV injections of naltrexone.

Materials and Methods

Animals and housing

The experiment was conducted with 12 crossbred gilts (Great Yorkshire x British Landrace; Pig improvement company, Oxfordshire, UK), which had shown 2 or more oestrous cycles of 19 to 21 days. Upon arrival, the animals, weighing approximately 130 kg, were housed loose in individual pens (approximately 6 m^2) with a concrete floor that was covered with wood shavings, except for a slatted dunging area at the rear end of the pens. The gilts were kept under 12:12 light:dark and ambient room temperature ranged from 15°C to 25°C, and fed 1 kg of a pelleted, dry sow feed (12.2 MJ of metabolizable energy per kilogram containing 15.4% crude protein) at 8:00 h and 16:00 h. Water was available ad libitum.

Surgery

All animals were fitted with an intracerebroventricular (ICV), and a jugular vein cannula as described by Dierx *et al.* (1998, submitted) and Janssens *et al.* (1994) respectively. In short, animals were given 12 ml of the antibiotic Methoxasol T® (20 mg Trimethorprim/ml, 100 mg sulfamethoxasol/ml, AUV, Cuijk, The Netherlands) orally once a day, from three days before

until three days after surgery. Animals were fasted overnight, and approximately 45 minutes prior to surgery they were premedicated by i.m. administration of 6 mg azaperone kg^{-1} body weight (Stresnil®; Janssen Pharmaceutica BV, Tilburg, The Netherlands). General anaesthesia was induced and maintained using enflurane (Ethrane®, Abott BV, The Netherlands) and a mixture of O_2/N_2O. The ICV cannula was implanted in the left lateral ventricle under guidance using a stereotactic apparatus that was attached to the head by means of a mouthpiece and a screw in the back of the head. The ICV cannula was connected, by means of a PVC tubing, to an injection device that was mounted in the skull. The luer-lock stopcock inside the device, the actual site of a-CSF or naltrexone injection, was covered by a lid. The jugular vein catheter (Silastic® Medical Grade Tubing, 0.040 in. i.d., 0.085 in. o.d.; Dow Corning, Michigan U.S.A.) was implanted into the external jugular vein with the tip pointing towards the superior vena cava. The catheter was passed s.c. and externalised between the scapulae. A one-way luer-lock stopcock (Vygon BV, Veenendaal, The Netherlands) was secured to the end of the catheter. The catheter was covered in a harness of 23 cm x 20 cm PVC/Nylon (Bizon Chemie, Goes, The Netherlands) that was fixed on the back of the animal by means of chest belts. The animals were allowed to recover from surgery for a minimum of 7 days. Following surgery, animals were treated with the 10 cc of the analgesic finadyn twice daily for two days and with 10 cc of the antibiotic depocillin.

The ICV cannula was flushed once during the recovery period with 500 µl sterile artificial cerebrospinal fluid (a-CSF, per liter solution: 166 mg $CaCl_2$, 240 mg $MgSO_4$, 134 mg KCl, 170 mg KH_2PO_4, 2.15 g $NaHCO_3$ and 7.25 g NaCl, pH 7.6). The venous catheter was flushed once a week with sterile saline and filled with heparinized saline (25 IU heparin/ml of 0.9% saline; Leo Pharmaceutical Products, The Netherlands) when not in use. When catheter patency was reduced, obstructions were removed as described by Leuvenink and Dierx (1997). In short, a solution of 25000 IU Kabikinase® (Kabi Pharmacia, Sweden), 2500 IU heparin and 0.2 ml Ticarpen® (Beecham, England) was added to 25 ml sterile 25% polyvinylpyrrolidone (PVP/saline, Merck, Germany) and injected in the catheter. After 24 hours, the catheter was flushed with heparinized saline.

Experimental design

Before the experiment, the animals were handled daily for 3 weeks and habituated to the ICV injection and blood sampling procedures. After surgery and the recovery period, the oestrous cycles of all animals were synchronised by daily oral administration of 20 mg of the progesterone agonist altrenogest (Regumate®) in the morning feed for 21 days. The last day that animals received altrenogest treatment, was designated as Day 0. Starting on Day 1, 6 animals received 1 mg naltrexone ICV twice a day at 8:00 h and 20:00 h for 6 days and 6 animals received a-CSF (controls). On Day 2, 4, 5 and 6 after altrenogest withdrawal,

bloodsamples were taken every 12 minutes during 8 hours from 9.00 h until 17.00 h, according to the procedure described previously (Janssens *et al.*, 1994). On Day 1, 3 and from Day 6 onwards, bloodsamples were taken every 6 hours until one day after first day of oestrus. Gilts that had not shown oestrous behaviour on Day 8, were scanned with ultrasound (Soede *et al.*, 1990). Immediately after collection, the blood samples (approximately 5 ml) were transferred to ice cooled polypropylene tubes containing 50 µl EDTA solution (144 mg EDTA/ml of saline; Triplex®III, Merck Nederland BV,The Netherlands). The tubes were shaken and placed on ice and subsequently centrifuged at 3000 x g for 15 minutes at 4 °C. Plasma was collected and stored at -20 °C until hormone analysis. The experiments were approved by the Committee on Animals Care and Use of the Agricultural University, Wageningen, The Netherlands.

Hormone analysis

LH. Plasma samples were analysed for LH using a double antibody radioimmunoassay (RIA) as described by Niswender *et al.* (1970), using porcine LH (pLH iodination grade batch 004/3; potency, 0.85 x NIH LH-S19; UCB bioproducts, Brussels, Belgium) as a standard and for radioiodination (specific activity, 38 µCi/µg). Anti-porcine LH batch 004 (UCB bioproducts, Brussels, Belgium) was used at a final dilution of 1:360,000, which gave an initial binding of the labeled hormone of approximately 39%. The minimal detectable concentration at the B/B_0=0.9 was 0.1 ng/ml. The interassay coefficient of variation was 14.4 % and the intra-assay coefficient of variation was 7.2 %.

Oestradiol. Plasma concentrations of oestradiol were measured in samples of 2.00 h, 8.00 h, 14.00 h and 20.00 h on Day 1, 3 and 7, and in samples collected at 10.00 h, 12.00 h, 14.00 h and 16.00 h on Day 2, 4, 5 and 6. After extraction of plasma with dichloromethane (DCM, Merck, Darmstadt, Germany), oestradiol concentration was determined using a single anti-body radioimmunoassay. For estimation of procedural losses, 500 cpm of [2,4,6,7-^{3}H]E_2 (NEN Chemicals, 's Hertogenbosch, The Netherlands) was added to 1 ml plasma sample and mixed with 3 ml DCM. The organic phase of the mixture was evaporated under a stream of nitrogen and redissolved in 500 µl phosphate buffer with 1% BSA. An aliquot of 150 µl was taken to determine the recovery of [^{3}H]E_2 . E_2 concentrations were measured in duplicate (2 aliquots of 150 µl) using a specific rabbit antiserum against 6-keto-estrone (6-CMO-BSA). The antiserum was used in final dilution of 1:125,000. Oestradiol-17ß (Sigma Chemical Co., St. Louis, MO, U.S.A.) was used as a standard and [2,4,6,7-^{3}H]E_2 (specific activity 95.4 Ci/.mmol^{-1}, NEN Chemicals, 's Hertogenbosch, The Netherlands) as a tracer. The main crossreacting steroids were oestrone (1.49%) and oestriol (0.21%). The minimal detectable dose at the 90% B/B_0 level was 8 pg.ml^{-1}. The intra- and inter-assay coefficients of variation were 12.4 % and 17.4 % respectively. The amount of E_2 was expressed in pg.ml^{-1} after correction for procedural losses, and daily means were calculated.

Detection of oestrous

Oestrus detection was performed by a back-pressure test in the presence of a vasectomized boar once daily in the morning on all days after withdrawal of altrenogest treatment. The time of oestrous was defined as the first day the sow showed a standing response.

Pulse detection

The profiles of LH release were analysed using the pulse analysis program of Maxima/Chromcard (Fisons Instruments,Interscience, Breda, The Netherlands) with baseline calculated according to an algorithm taken into account the total profile. A pulse was defined by a baseline-peak ratio of 0.5 or lower and a minimal area under the curve of 50 $ng.ml^{-1}.min$. The highest value of a pulse above baseline was taken as pulse amplitude.

Statistical analysis

Data of pulse frequency, pulse amplitude and mean plasma LH concentration, time of return to baseline levels after the preovulatory LH surge and the time of oestrus, were analysed using SAS statistical analysis system (1990). The effect of naltrexone on these parameters was tested using the GLM procedure by means of a F-test to analyse the linear model: $Y_{ij} = \mu + T_i + e_{1i} + D_j + (TxD)_{ij} + e_{2ij}$; Where Y_{ij} = value of parameter in a gilt (n=11) receiving treatment i on sampling day j; μ = overall mean; T_i= fixed effect of treatment i (1,2); e_{1i}= error term 1, which represents the random effect within treatment i; D_j= fixed effect of sampling day j (1,..,4); e_{2ij}= error term 2, which represents the random effect of treatment i between sampling days *j*. The effect of naltrexone treatment was tested against error term 1. The other effects were tested against error term 2. Differences were considered significant when $P<0.05$. The parameters pulse amplitude, time of return to baseline levels after the preovulatory LH surge and the time of oestrus had no normal distribution and were therefore analysed with a Kruskal-Wallis test using the procedure Npar1way of the statistical analysis system SAS (1990).

Results

General

During the experiment, 1 control and 1 naltrexone treated animal died of meningitis and of intestinal torsion respectively. In addition, 1 naltrexone treated animal showed neither oestrous behaviour nor a preovulatory LH surge. The data of this animal were excluded from analysis, since no ovarian follicles were detected when scanned with ultrasound. Furthermore, 1 control animal had a silent oestrus, showing a preovulatory LH surge but no oestrous

behaviour. All other animals had the first day of oestrus on day 6 after termination of the altrenogest treatment.

The preovulatory LH surge

Five control and 4 naltrexone treated animals showed a preovulatory LH surge. In all cases, return of LH to baseline levels after the preovulatory surge could be accurately detected. In the naltrexone group LH returned to baseline values on average at 138 ± 4.9 h (N=4) after termination of the altrenogest treatment, which was significantly (P=0.01) sooner than in animals in the control group that reached baseline values on average at 166 ± 6.2 h (N=5) after altrenogest withdrawal.

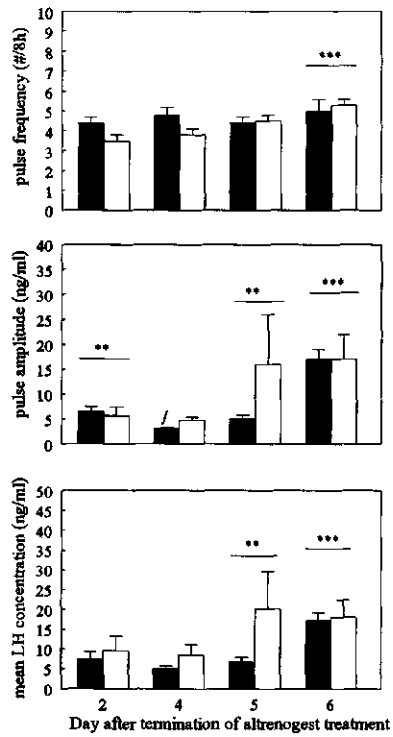

***Figure 1**: Data represent mean ± SEM of LH pulse frequency (A; number per 8 h), pulse amplitude (B; ng/ml) and mean LH concentration (C; ng/ml) in control (closed bars; n=5) and naltrexone (open bars; n=4) treated animals during the follicular phase of the oestrous cycle.*

Time effects:

** = significantly different from Day 4 (P<0.05).*

*** = significantly different from Day 2 and 4 (P<0.05).*

****= significantly different from Day 2, 4 and 5 (P<0.05).*

Treatment effects:

ƒ = significantly different between a-CSF and naltrexone treatment (P<0.05).

Pulsatile LH release

Data on plasma LH concentrations are presented in Table 1. No treatment effects were found in LH pulse frequency (P=0.08) and mean LH concentration (P=0.18) on Days 2, 4, 5 and 6 after discontinuation of altrenogest treatment. LH pulse amplitude was significantly (P<0.05) higher in the naltrexone treated group compared to the control group on Day 4.

An effect of time was found in LH pulse frequency (P=0.03), pulse amplitude (P=0.0004) and mean LH concentration (P=0.0025). LH pulse frequency was significantly higher on Day 6 than on Day 2 and Day 4 (P<0.05). LH pulse amplitude was increased on Day 6 compared to Days 2, 4 and 5, and LH pulse amplitude was higher on Days 2 and 5 than on Day 4 (P<0.05). Mean LH concentration was significantly increased on Day 6 compared to Day 2, 4 and 5 (P<0.05) with a significantly higher mean LH concentration on Day 5 than on Day 4 (P<0.05).

Oestradiol

Data on daily mean oestradiol concentrations are presented in Table 2. Plasma concentrations of oestradiol were not significantly affected by naltrexone treatment (P=0.33), but a time-effect was found (P=0.0003). Mean oestradiol concentrations were higher on Day 5 than on Day 2, 4 and 6, with Day 4 showing higher mean oestradiol concentrations compared to Day 2 and 6 (P<0.05).

Table 2: Mean oestradiol concentration on Day 2, 4, 5 and 6 after altrenogest withdrawal.

day after altrenogest	control (n=5)	naltrexone (n=4)
2[a]	7.6 ± 3.5	13 ± 2.0
4[b]	38 ± 8.6	46 ± 7.5
5[c]	67 ± 5.8	64 ± 13
6[a]	38 ± 21	6.6 ± 4.5

Data represent mean ± SEM of mean oestradiol concentration (pg/ml) in control (n=5) and naltrexone (n=4) treated groups of animals during the follicular phase of the oestrous cycle. Different superscript letters (a,b,c) in a column represent significant differences (P<0.05) in time.

Discussion

In the present study, repeated ICV treatment of pigs with the opioid receptor antagonist naltrexone was used to investigate the involvement of endogenous opioid activity in the brain in the regulation of LH release during the follicular phase in ovary-intact gilts. In naltrexone treated animals, plasma LH concentrations returned to basal significantly earlier after the preovulatory LH surge than in a-CSF treated controls. In view of the route of administration

and the relatively low dose of naltrexone used (Barb *et al.*, 1989), it is likely that naltrexone exerted its action within the brain. Thus the data strongly suggest that activation of opioid receptors in the brain by EOP's participates in the regulation of the preovulatory LH surge in pigs.

It remains unclear whether the premature cessation of the LH surge in the naltrexone treated gilts reflected a time-shift of the peak LH value or the complete surge (including onset), or was the result of an effect on the height of the surge or even on LH clearance kinetics, since the design of our study did not allow exact determination of these parameters. There is ample evidence from studies with rats (Allen and Kalra, 1986; Berglund *et al.*, 1988), sheep (Currie *et al.*, 1991) and humans (Snowden *et al.*, 1984), however, indicating that tonic inhibitory activity of brain opioid systems precludes the occurrence of a LH surge during the late follicular phase, and that cessation of this inhibitory activity permits a surge to occur (Barkan *et al.*, (1983; Okrasa *et al.*, 1992; Roozendaal *et al.*, 1997). A similar involvement of EOP's in the regulation of surge timing in pigs, is supported by data from Armstrong *et al.* (1988) who found that chronic administration of morphine (sc) to sows for 5 days after weaning delayed the onset of oestrus, and data from others (Yearwood *et al.*, 1991; Kraeling *et al.*, 1992a) who reported a delayed preovulatory LH surge in E2ß primed OVX gilts after IV or ICV morphine treatment with no effect on the height of the surge.

The frequency and amplitude of LH pulses and mean LH concentration during the follicular phase showed significant time effects. LH pulse amplitude and mean LH concentration initially decreased to lowest values on Day 4 after termination of altrenogest treatment, and thereafter gradually increased during the late follicular phase. All LH parameters of study were highest on Day 6, which for most of the animals was the first day of oestrus and preovulatory LH surge. These dynamics of LH during the follicular phase may be explained from the plasma concentration of E2ß. E2ß increases during early follicular phase and exerts a "negative feedback" on pulse amplitude and therefore on mean LH plasma concentration. During the late follicular phase, E2ß reaches peak values on the day before the preovulatory LH surge, positively influencing LH pulse amplitude and mean plasma LH concentration (Helmond *et al.*, 1986; Kesner *et al.*, 1989; Messinis *et al.*, 1992; Matt *et al.*, 1993).

Naltrexone treatment in the present study had no effect on LH pulse frequency and mean LH concentration during the follicular phase of the oestrous cycle, but enhanced the pulse amplitude on Day 4 yielding values on that day that were comparable to those on Day 5 in control animals. It could thus be that the negative feedback of E2ß influences the LH pulse amplitude, leaving pulse frequency unaffected. In line with this, studies in sheep (Whisnant and Goodman, 1988; Whisnant *et al.*, 1991) reported that only pulse amplitude was increased by treatment with an opioid receptor antagonist during the high E2ß levels of the follicular phase. From this, together with the abovementioned earlier return of LH to basal

concentrations after the preovulatory surge, we hypothesise that EOP's in the brain are involved in the timing of the preovulatory LH surge by decreasing LH pulse amplitude during the negative feedback of E2ß.

In summary, the results show that LH pulse frequency remains constant during the follicular phase, whereas LH pulse amplitude decreases when going from early to mid follicular phase and increase when going from mid to late follicular phase. Both, LH pulse amplitude and pulse frequency, reach peak values at the day of the preovulatory LH surge. Furthermore, the data suggest that EOP's delay the timing of the preovulatory LH surge possibly by decreasing LH pulse amplitude during the negative feedback phase of E2ß in the early and mid follicular phase.

Akowledgement

The authors like to thank the MSc students Anite Mulder and Yvonne van Hierden and Dr. R. Oonk for their assistance in the intensive bloodsampling and the analysis of the bloodsamples under supervision of Corrie Oudenaarden.

CHAPTER VIII

Summary and General discussion

SUMMARY AND GENERAL DISCUSSION

LH secretion during the oestrous or menstrual cycle, and the luteal phase in particular, has been studied in a variety of species as sheep (Whisnant *et al.*, 1991), human (Fillicori *et al.*, 1986; Rossmanith *et al.*, 1990), pig (Okrasa and Tilton, 1992) and primate (Norman *et al.*, 1994). During the luteal phase, plasma LH concentration has been shown to be decreased by endogenous opioid peptides (EOP's), mediating the negative feedback of progesterone (P; Yang *et al.*, 1988; Barb, *et al.*, 1992; Kaynard *et al.*, 1992). Yet, relatively few data have been available on the pattern of pulsatile LH release during the oestradiol (E2ß) dominated follicular phase of the oestrous or menstrual cycle, and whether EOP's also play an inhibitory role in LH secretion and/or the timing of events leading to the preovulatory LH surge and oestrus.
In the present thesis, we have first described the pulse pattern of LH secretion during multiple days of the follicular phase of the oestrous cycle using the female tethered housed pig as a model. Second, we have demonstrated that EOP's seem to be involved in the timing of events leading to the preovulatory LH surge, but not in the regulation of the pulse pattern. Therefore, in this chapter, these processes will be described separately and possible functional implications of EOP's in the regulation of female reproduction are discussed.

Stress and the Preovulatory LH Surge

In Chapter II and IV, the tethered pig was used as an animal model to study the effects of chronic stress on the HPG-axis. This model was chosen because tethered housing leads to changes in neuroendocrine regulation and behaviour in which altered activity of endogenous opioids in the brain is an important feature (Cronin, 1985; Schouten and Wiepkema, 1991; Janssens *et al.*, 1995b; Zanella *et al.*, 1996; Loyens, Schouten and Wiegant, unpublished observations). It was found that the preovulatory LH surge and the expression of oestrus were delayed when long-term tethered pigs were released from their tether (Chapter II). This delay was likely not caused by loose or tethered housing per se, but rather by the change in housing condition that apparently represented a major challenge for the animals. One may speculate that such changes in reproductive parameters may occur independent of the exact nature of the stimulus and can be relevant for reproductive performance in pig breeding. Indeed, there is ample evidence that tethered housing renders the animals more reactive to environmental stimuli, although to a certain extend pigs can adapt to the chronic stress of tethered housing (Janssens *et al.*, 1995b). In Chapter IV, no differences were found between long-term (6 month) tethered housed pigs and loose housed controls regarding the onset of oestrus, possibly because animals had adapted to the tethered housing condition, which has been shown to occur within a few months after start of tethered housing (Janssens *et al.*, 1995a). Previous reports indicate that in long-term tethered housed ("stress-adapted") pigs the activity

of EOP's is increased (Cronin, 1985; Schouten *et al.*, 1991; Zanella *et al.*, 1996). Although, oral treatment with the opioid receptor antagonist naltrexone led to a shift in the onset of oestrus, and possibly also of the preovulatory LH surge, we found no evidence for differential EOP activity in the regulation of reproductive processes between tethered and control pigs. It should be kept in mind, however, that our pigs had been tethered for more than 5 months, and the adaptive increase in EOP-activity may cease to be functional after several months (Janssens *et al.*, 1995a). An interesting observation was the large variation in onset of oestrus in the naltrexone treated group of pigs. The individual variability in the response to naltrexone, described in Chapter III, may be relevant in this respect.

Opioidergic Regulation of the Preovulatory LH Surge

The data from Chapter II and IV suggested a possible role of stress and EOP's in the timing of the preovulatory LH surge and the onset of oestrus. Whether EOP's exert their actions at the level of the pituitary or the hypothalamus, or both, was investigated in Chapters V and VII. In a pilot study, described in Chapter V, we obtained data suggesting that the pituitary responsivity to GnRH, as assessed *in vitro*, changes during the follicular phase. Maximal responsivity was found around the day of the preovulatory LH surge. It was concluded that treatment with naltrexone seemed to advance the maximal pituitary responsivity and the preovulatory LH surge. To investigate whether EOP's might act at the level of the hypothalamus, a new intracerebroventricular (ICV) injection technique was developed as described in Chapter VI. Using this technique, we showed in Chapter VII that the preovulatory LH surge was terminated sooner after ICV treatment with naltrexone. Together with data on pulse amplitude, showing higher values after ICV naltrexone treatment on Day 4 after termination of altrenogest treatment, this observation supports the hypothesis that EOP's delay the timing of events leading to the preovulatory LH surge, most likely at the hypothalamic level. This would be in line with studies by Armstrong *et al.* (1988), who reported that chronic administration of morphine (sc) to sows for 5 days after weaning delayed the onset of oestrus and studies by Yearwood *et al.* (1991) and Kraeling *et al.* (1992) showing a delay of the preovulatory LH surge in E2ß primed OVX gilts after iv and ICV morphine treatment.

The Role of EOP's in the Pulsatile LH Secretion during the Follicular Phase

In Chapters IV and VII it is shown that the early follicular phase (approximately Days 1 and 2) is characterised by highly frequent, low amplitude LH pulses, followed by a decrease in pulse frequency during mid-follicular phase (approximately Days 3-5), and that the late follicular phase with the preovulatory LH surge (approximately Days 6-7) is constituted of pulses of high frequency and high amplitude. In addition, pituitary responsivity to GnRH appeared to change

during the follicular phase with maximal responsivity on the day of the preovulatory LH surge (Chapter V). These dynamics of LH during the follicular phase and pituitary responsivity are similar to those found in humans, primates and rats (Clayton *et al.*, 1980; Kesner *et al.*, 1989; Genazzani *et al.*, 1992; Rossmanith, 1995) and may largely be explained from the plasma concentration of E2ß. E2ß starts to increases during early follicular phase, when it exerts a "negative feedback" on pulse amplitude and mean LH plasma concentration but enhances pituitary sensitivity for GnRH (Drouva *et al.*, 1983; Koiter *et al.*, 1987). Peak levels of E2ß are reached at the end of the mid-follicular phase, when LH pulse amplitude and mean plasma concentration are positively influenced (Helmond *et al.*, 1986; Kesner *et al.*, 1989; Messinis *et al.*, 1992; Matt *et al.*, 1993).

As found in Chapters IV and VII, the pulse pattern of LH during the follicular phase did not seem to be affected by EOP's, supporting other studies in humans (Genazzani and Petraglia, 1989; Genazzani *et al.*, 1993; Cagnacci *et al.*, 1995) and sheep (Currie *et al.*, 1992). Although naltrexone treated animals had higher LH pulse amplitudes than control animals on only 1 day of the follicular phase (Day 5 in Chapter IV and Day 4 in Chapter VII) this difference was no longer present when data were synchronised on the first day of oestrus (Chapter IV). Therefore, it was suggested that naltrexone did not change the dynamics of the LH secretion during the follicular phase but affects the timing of the preovulatory LH surge. The results from the pilot study *in vitro* (Chapter V) support this notion, as the pattern of pituitary responsivity to GnRH and plasma concentration of LH during the follicular phase seems to be shifted by naltrexone treatment. Thus, in contrast to the luteal phase where pulse frequency, pulse amplitude and mean plasma concentration of LH are inhibited by EOP's (Barb *et al.*, 1986; Genazzani *et al.*, 1993; Rossmanith, 1995), this is not the case during the follicular phase.

Putative Mechanism for Opioid Modulation during the Follicular Phase

The data presented indicate that EOP's do not only play an inhibitory role on the LH secretion during the luteal phase, but are also involved in the timing of the preovulatory LH surge in the pig. From Chapter II it might be suggested that, during the transition from the luteal to the follicular phase, P decreases rapidly, thus reducing EOP activity and leaving the GnRH pulse generator "free running". This results in an increased LH pulse frequency during the early follicular phase compared to the luteal phase as was found in humans by Rossmanith (1992) and Genazzani *et al.* (1993). Furthermore, P has been shown to exert a negative feedback on the LH secretion through EOP activation (Yang *et al.*, 1988; Barb, *et al.*, 1992; Kaynard *et al.*, 1992) by reducing pulse frequency of the GnRH pulse generator (Bouchard *et al.*, 1988; Couzinet and Schaison, 1993). Treatment with an opioid antagonist (Chapter IV and VII) pharmacologically accelerates the decline in EOP tone caused by decreasing plasma P concentrations, thus advancing the start of LH dynamics associated with the follicular phase.

During the mid-follicular phase, E2ß secretion starts to increase (Chapter IV and VII; Bouchard *et al.*, 1988; Genazzani *et al.*, 1992) due to maturing follicles. The increase in circulating E2ß subsequently decreases LH pulse amplitude during the mid-follicular phase by inhibiting the GnRH pulse generator (Chapter IV, V, VII). Since E2ß has been shown to stimulate EOP release in the hypothalamus (Frautschky and Sarkar, 1995) and to decrease electrical activity of the GnRH pulse generator (Kesner *et al.*, 1987), it could be hypothesised that E2ß acts through an opioid mechanism at the level of the hypothalamus. In addition, E2ß increases the transcription of gonadotropin subunits but inhibits the secretion of LH (March *et al.*, 1981; Knobil and Hotchkiss, 1988), thus loading the pituitary with LH. Moreover, E2ß has been reported to increase the number of GnRH receptors on pituitary gonadotrophs (Clayton and Catt, 1981; March *et al.*, 1981; Bouchard *et al.*, 1988), and thereby the responsivity of the pituitary to GnRH (Drouin *et al.*, 1976; Kamel and Krey, 1982). When plasma concentrations of E2ß start to decrease in the late follicular phase, EOP activity and thereby the "EOP-clamp" on the GnRH pulse generator and the "E2ß-block" of the LH release at the level of the pituitary might be reduced. Thereby, LH pulse frequency and pulse amplitude would be increased and the preovulatory LH surge would be permitted to occur. Indeed, blocking opioid receptors with an opioid antagonist advances the preovulatory LH surge (Chapter IV and VII). However, further research is needed to prove the validity of this model and to further elucidate the neuroendocrine processes that contribute to succesful reproduction.

References

References

Akesson TR and Micevych PE (1991) Endogenous opioid-immunoreactive neurons of the ventromedial hypothalamic nucleus concentrate estrogen in male and female rats *Journal of Neuroscience Research* **28** 359-366

Akil H, Watson SJ, Young E, Lewis ME, Khatchaturian H, and Walker JM (1984) Endogenous opioids: Biology and function *Annual Reviews of Neuroscience* **7** 223-255

Allen LG and Kalra SP (1986) Evidence that decrease in opioid tone may evoke preovulatory luteinizing hormone release in the rat *Endocrinology* **118** 2375-2381

Apfelbaum (1981) Basal and GnRH-induced release and synthesis of LH and FSH from incubated pituitary glands throughout the 4-day estrous cycle of the rat *Hormone Research* **15** 109-121

Armario A, Restrepo C, Hildalgo J, and López-Calderón A (1987) Differences in prolactin and LH response to acute stress between peripubertal and adult male rats *Journal of Endocrinology* **112** 9-13

Armeanu MC (1991) Opioid antagonists and gonadotrophin secretion *PhD thesis Elinkwijk Utrecht*

Armstrong JW, Kraeling RR, and Britt JH (1988) Morphine suppresses luteinizing hormone concentrations in transiently weaned sows and delays onset of estrus after weaning *Journal of Animal Science* **66** 2216-2223

Asanovich KM, Johnson B, Chang WJ, Barb CR, Rampacek GB, and Kraeling RR (1998) Delay of estradiol-induced surge secretion of LH in gilts by intracerebroventricular injection of morphine *Domestic Animal Endocrinology* **15** 45-53

Aurich C, Daels PF, Ball BA, and Aurich JE (1995) Effects of gonadal steroids on the opioid regulation of LH and prolactin release in ovariectomized pony mares *Journal of Endocrinology* **147** 195-202

Axelson JF, Shannon W, and Van Leeuwen FW (1992) Immunocytochemical localisation of estrogen receptors within neurotensin cells in the rostral preoptic area of the rat hypothalamus *Neuroscience Letters* **136** 5-9

Babu GN, Marco J, Bona-Gallo A, and Gallo RV (1988) Absence of steriod-dependent, endogenous opioid peptide suppression of pulsatile luteinizing hormone release between diestrus 1 and diestrus 2 in the rat estrous cycle *Neuroendocrinology* **47** 249-258

Barb CR, Barrett JB, Wright JT, Kraeling RR, and Rampacek GB (1990) Opioid modulation of LH secretion by pig pituitary cells *in vitro Journal of Reproduction and Fertility* **90** 213-219

Barb CR, Estienne MJ, and Rampacek GB (1993) Technique for the cannulation of the lateral ventricle of the brain in swine *Kopf® Carrier* **35** 1-5

Barb CR, Kineman RD, Kesner JS, Rampacek GB, and Kraeling RR (1989) Luteinizing hormone secretion following intracerebroventricular administration of morphine in the prepubertal gilt *Life Sciences* **45** 691-696

Barb CR, Kraeling RR, and Rampacek GB (1991) Opioid modulation of gonadotropin and prolactin secretion in domestic farm animals *Domestic Animal Endocrinology* **22** 39-46

Barb CR, Kraeling RR, and Rampacek GB (1992) Opioid modulation of FSH, growth hormone and prolactin in the prepubertal gilt *Journal of Endocrinology* **133** 13-19

Barb CR, Kraeling RR, Rampacek GB, and Whisnant CS (1985) Opioid inhibition stimulates luteinizing hormone and prolactin secretion in the gilt *Domestic Animal Endocrinology* **2** 93-98

Barb CR, Kraeling RR, Rampacek GB, and Whisnant CS (1986) Influence of stage of the estrous cycle on the endogenous opioid modulation of luteinizing hormone, prolactin and cortisol secretion *Biology of Reproduction* **35** 1162-1167

Barb CR, Rampacek GB, Kraeling RR, Estienne MJ, Taras E, Estienne CE, and Whisnant CS (1988) Absence of brain opioid peptide modulation of luteinizing hormone secretion in the prepubertal gilt *Biology of Reproduction* **39** 603-609

Barkan A, Regiani S, Duncan J, Papavasiliou S, and Marshall JC (1983) Opioids modulate pituitary receptors for gonadotropin-releasing hormone *Endocrinology* **101** 134-142

Berglund LA and Simpkins (1988) Alterations in brain opioid receptor mechanism on proestrous afternoon *Neuroendocrinology* **48** 394-400

Blank MS Ching M, Catt KJ, and Dufau ML (1985) Inhibition of gonadotropin-releasing hormone release as the basis of pituitary-gonadal dysfunction during treatment with 4-amino-pyrazolo-(3,4-*d*)-pyrimidine *Endocrinology* **116** 1778-1783

Blank MS, Fabri A, Catt J, and Dufau ML (1986) Inhibition of luteinizing hormone release by morphine and endogenous opiates in cultured pituitary cells *Endocrinology* **118** 2097-2101

Bloom FE (1980) The endorphins: Natural peptides for pains, pleasure, and other purposes *Psychopharmacological Bulletin* **16** 51-52

Bonavera JJ, Sahu A, Kalra SP, and Kalra PS (1994) The hypothalamic peptides, ß-endorphin, neuropeptide K and the interleukin-1ß, and the opiate morphine, enhances the excitatory amino acid-induced LH release under the influence of gonadal steroids *Journal of Neuroendocrinology* **6** 557-564

Bouchard Ph, Wolf JP, and Hajri S (1988) Inhibition of ovulation: Comparison between the mechanism of action of steroids and GnRH analogues *Human Reproduction* **3** 503-506

Brann DW and Mahesh VB (1991) Role of corticosteroids in female reproduction *FASEB Journal* **5** 2691-2698

Briski KP and Sylvester PW (1988) Effects of specific acute stressors on luteinizing hormone release in ovariectomized and ovariectomized estrogen-treated female rats *Neuroendocrinology* **47** 194-202

Buxton DF (1988) Stereotaxic implantation of cannulae into sheep hypothalamus based on ventriculography *Anatomical and Histological Embryology* **17** 362-363

Cacicedo L and Franco FS (1985) Direct action of opioid peptides and naloxone on gonadotropin secretion by cultured rat anterior pituitary cells *Life Sciences* **38** 617-625

Cagnacci A, Paoletti AM, Soldani R, Tuveri F, and Melis GB (1995) Prolonged opioid blockade does not influence luteinizing hormone modifications of the follicular and luteal menstrual phases *Journal of Clinical Endocrinology and Metabolism* **80** 860-863

Cagnacci A, Soldani R, Paoletti AM Falqui, and Melis GB (1994) Prolonged opioid blockade with naltrexone and luteinizing hormone modifications in women with polycystic ovarian syndrome *Fertility and Sterility* **62** 269-272

Carmel PW, Araki S, and Ferin M (1976) Pituitary stalk portal blood collection in Rhesus monkeys: Evidence for pulsatile release of gonadotropin-releasing hormone (GnRH) *Endocrinology* **99** 243-248

Chang WJ, Barb CR, Kraeling RR, Rampacek GB, and Leshin LS (1993) Involvement of the central noradrenergic system in opioid modulation of luteinizing hormone and prolactin secretion in the pig *Biology of Reproduction* **49** 176-180

Chao CC, Moss GE, and Malven PV (1986) Direct opioid regulation of pituitary release of bovine luteinizing hormone *Life Sciences* **39** 527-534

Clark JD, Rager DR, and Calpin JP (1997) Animal well being: II. Stress and distress *Laboratory Animal Science* **47(6)** 571-579

Clayton RN and Catt KJ (1981) Gonadotropin-releasing hormone receptors: Characterisation, physiological regulation, and relationship to reproductive function *Endocrine Reviews* **2** 186-209

Clayton RN, Solano AR, Garcia-Vela A, Dufau ML, and Catt KT (1980) Regulation of pituitary receptors for gonadotropin-releasing hormone during the rat estrous cycle *Endocrinology* **107** 699-706

Conover CD, Kuljis RO, Rabii J, and Advis J-P (1993) Beta-endorphin regulation of luteinizing hormone-releasing hormone release at the median eminence in ewes: Immunocytochemical and physiological evidence *Neuroendocrinology* **57** 1182-1195

Couzinet B and Schaison G (1993) The control of gonadotrophin secretion by ovarian steroids *Human Reproduction* **8 (supplement 2)** 97-101

Cronin GM (1985) The development and significance of abnormal stereotyped behaviours in tethered sows *PhD thesis Wageningen Agricultural University*

Currie WD, Joseph IBJK, and Rawlings NC (1991) Morphine, naloxone and the gonadotropin surge in ewes Journal of Reproduction and Fertility **92** 407-414

Currie WD, Ravindra J, Kingsbury DL, and Rawlings NC (1992) Independence of progesterone blockade of the luteinizing hormone surge in ewes from opioid activity at naloxone-sensitive receptors *Reproduction and Fertility* **95** 249-255

de Koning J, van Dieten JAMJ, and van Rees GP (1978) Refractoriness of the pituitary gland after continuous exposure to luteinizing hormone-releasing *hormone Journal of Endocrinology* **79** 311-318

Deroche V, Piazza PV, Le Moal M and Simon H (1993) Individual differences in the psychomotor effects of morphine are predicted by reactivity to novelty and influenced by corticosterone secretion *Brain Research* **623** 341-344

Di Paolo T, Falardeau P, and Morissette M (1988) Striatal D-2 dopamine agonist binding sites fluctuate during the rat estrous cycle *Life Sciences* **43** 665-672

Dobson H and Smith RF (1995) Stress and reproduction in farm animals *Journal of Reproduction and Fertility Supplement* **49** 451-461

Drouin J, Lagace L, and Labrie F (1976) Estradiol induced increase of the LH responsiveness to LHRH in rat anterior pituitary cells in culture *Endocrinology* **99** 1477-1481

Drouva SV, Laplante E, and Kordon C (1983) Effects of ovarian steroids on in vitro release of LHRH from mediobasal hypothalamus *Neuroendocrinology* **37** 336-341

Eipper BA and Mains RE (1980) Structure and biosynthesis of proadreno-corticotropin/endorphin and related peptides *Endocrine Reviews* **1** 1-27

Estienne MJ, Kesner JS, Barb CR, Kraeling RR, Rampacek GB, and Estienne CE (1990) Gonadotropin and prolactin secretion following intraventricular administration of morphine in gilts *Proceedings of the Society of Expermintal Biology and Medicine* **193** 92-97

Faigon MR, Szwarcfarb B, Scacchi P, and Moguilevsky JA (1987) Effect of naloxone on the development of the positive feed-back action of oestrogen-progesterone on LH secretion in rats *Acta Endocrinologica* **115** 16-20

Ferin M, van Vugt DA, and Wardlaw S (1984) The hypothalamic control of the menstrual cycle and the role of endogenous opioid peptides *Recent Progress in Hormone Research* **40** 441-485

Ferrer J, MinezGuisasola J, Diaz F, Alonso F, Guerrero M, and Marini B (1997) Plasma levels of beta-endorphin during the menstrual cycle *Gynaecological Endocrinology* **11** 75-82

Filicori M, Santoro N, Merriam GR, and Crowley WC, Jr (1986) Characterisation of the physiological pattern of episodic gonadotropin secretion throughout the human menstrual cycle *Journal of Clinical Endocrinology and Metabolism* **62** 1136-1144

Fink G (1988) Gonadotropin secretion and its control In: *The Physiology of Reproduction* Knobil E and Neill J (eds) *Raven Press Ltd., New York* 1349-1377

Flügge G, Oertel WH, and Wuttke W (1986) Evidence for estrogen-receptive GABAergic neurons in the preoptic/anterior hypothalamic area of the brain *Neuroendocrinology* **43** 1-5

Fonda ES, Rampacek GB, and Kraeling R (1984) The effect of adrenocorticotropin or hydrocortisone on serum luteinizing hormone concentrations after adrenalectomy and/or ovariectomy in the prepuberal gilt *Endocrinology* **114** 268-273

Franz WB (1988) Basic Review: Endocrinology of the normal menstrual cycle *Primary Care* **15** 607-616

Frautschy SA and Sarkar DK (1995) Estrogen augments hypothalamic ß-endorphin secretion and activates an inhibitory ß-endorphin short-loop feedback system *Endocrine* **3** 273-275

Genazzani AR, Gastaldi M, Bidzinska B, Mercuri N, Genazzani AD, Nappi RE, Segre A, and Petraglia F (1992) The brain as a target organ of gonadal steroids *Psychoneuroendocrinology* **17** 385-390

Genazzani AR, Genazzani AD, Volpogni C, Pianazzi F, Li GA, Surico N, and Petraglia F (1993) Opioid control of gonadotropin secretion in humans *Human Reproduction* **8** 151-153

Genazzani AR and Petraglia F (1989) Opioid control of luteinizing hormone secretion in humans *Journal of Steroid Biochemistry* **33** 751-755

Gindoff PR, Jewelewicz R, Hembree W, Wardlaw S and Ferin M (1988) Sustained effects of opioid antagonism during the normal human luteal phase *Journal of Clinical Endocrinology and Metabolism* **66** 1000-1004

Goncharov NP, Tavadyan DS, Powel JE, and Stevens VC (1984) Levels of adrenal and gonadal hormones in rhesus monkeys during chronic hypokinesia *Endocrinology* **115** 129-135

Gold MS, Redmond DE and Donabedian RK (1979) The effects of opiate agonist and antagonist on serum prolactin in primates: Possible role for endorphins in prolactin regulation *Endocrinology* **105** 284-289

Gray GE, Smith ER, Ehrenkrantz JR, and Davidson JM (1978) Neuroendocrine mechanisms mediating the suppression of circulating testosterone in male rats following chronic stress *Neuroendocrinology* **25** 247-256

Gregg DW, Allen MC, and Nett TM (1990) Estradiol-induced increase in number of gonadotropin-releasing hormone receptors in cultured ovine pituitary cells *Biology of Reproduction* **43** 1032-1036

Grossman A, Gaillard AC, McCartney P, Rees LH, and Besser GM (1982) Opiate modulation of pituitary adrenal axis: Effect of stress on circadian rhythm *Clinical Endocrinology* **17** 279-286

Grossman A (1988) Opioids and stress in man *Journal of Endocrinology* **119** 377-381

Guillemin R (1980) Beta-lipotropin and endorphins: implication of current knowledge In Krieger DT and Hughes JC (eds) *Neuroendocrinology Sinauer Sunderland* 67-74

Halász B, Kiss J, and Molnár J (1989) Regulation of the gonadotropin-releasing hormone (GnRH) neuronal system: Morphological aspects *Journal of Steroid Biochemistry* **33** 663-668

Haynes NB, Lamming GE, Yang K-P, Brooks AN, and Finnie AD (1989) Endogenous opioid peptides and farm animal reproduction In Milligan SR (eds) *Oxford Reviews of Reproductive Biology* 111-145

Heisler LE, Pallotta CM, Reid RL, and van Vugt DA (1993) Hypoglycaemia-induced inhibition of luteinizing hormone secretion in the Rhesus monkey is not mediated by endogenous opioid peptides *Journal of Clinical Endocrinology and Metabolism* **76** 1280-1285

Helmond F, Aarnink A and Oudenaarden C (1986) Periovulatory hormone profiles in relation to embryonic development and mortality in pigs In: Sreenan JM, Diskin MG (eds) Embryonic mortality in farm animals *Martinus Nijhoff Publishers* 119-125

Herbison AE and Theodosis DT (1992) Localisation of oestrogen receptors in preoptic neurons containing neurotensin but not tyrosine hydroxylase, cholecystokinin or luteinizing hormone-releasing hormone in the male and female rat *Neuroscience* **50** 283-298

Herbison AE, Robinson JE, and Skinner DC (1993) Distribution of estrogen receptor-immunoreactive cells in the preoptic area of the ewe: Co-localisation with glutamic acid decarboxylase but not luteinizing hormone-releasing hormone *Neuroendocrinology* **57** 751-759

Heritage A, Grant L, and Stumpf W (1977) ^{3}H-estradiol in catecholamine neurons of rat brain stem. Combined localisation by autoradiography and formaldehyde-induced fluorescence *Journal of Comparative Neurology* **176** 607-630

Higuchi T, Honda K, and Negoro H (1986) Influence of oestrogen and noradrenergic afferent neurones on the response of LH and oxytocin to immobilisation stress *Journal of Endocrinology* **110** 245-250

Higuchi T and Kawakami M (1982) Changes in the characteristics of pulsatile luteinizing hormone secretion during the oestrous cycle and after ovariectomy and oestrogen treatment in female rats *Journal of Endocrinology* **94** 177-182

Horton RJE, Cummins JT, and Clarke IJ (1987) Naloxone evokes large-amplitude GnRH pulses in luteal-phase ewes *Journal of Reproduction and Fertility* **81** 277-286

Hughes J, Smith W, and Kosterlitz HW (1975) Identification of two related pentapeptides from the brain with potent opiate agonist activity *Nature* **258** 577-579

Jacobson W and Kalra SP (1989) Decreases in mediobasal hypothalamic and preoptic area opioid ([^{3}H]naloxone) binding are associated with the progesterone-induced luteinizing hormone surge *Endocrinology* **124** 199-206

Janssens CJJG (1994) Chronic stress and pituitary-adrenal function in female pigs *PhD thesis Wageningen Agricultural University*

Janssens CJJG, Helmond FA, and Wiegant VM (1994) Increased cortisol response to exogenous adrenocorticotropic hormone in chronically stressed pigs: Influence of housing conditions *Journal of Animal Science* **72** 1771-1777

Janssens CJJG, Helmond FA, and Wiegant VM (1995a) The effect of chronic stress on plasma cortisol concentrations in cyclic female pigs depends on the time of day *Domestic Animal Endocrinology* **12** 167-177

Janssens CJJG, Helmond FA, Loyens LWS, Schouten WGP, and Wiegant VM (1995b) Chronic stress increases the opioid-mediated inhibition of the pituitary-adrenocortical response to acute stress in pigs *Endocrinology* **136** 1468-1473

Jarry H, Leonardt S, Schwarze T and Wuttke W (1995) Preoptic rather than mediobasal hypothalamic amino acid neurotransmitter release regulates GnRH secretion during the estrogen-induced LH surge in the ovariectomized rat *Neuroendocrinology* **62** 479-486

Joel SP, Osborne RJ, and Slevin ML (1988) An improved method for the simultaneous determination of morphine and its principal glucoronide metabolites *Journal of Chromatography* **430** 394-399

Kamel F and Krey LC (1982) Gonadal steroid modulation of LHRH stimulated secretion by pituitary cells in culture *Molecular and Cellular Endocrinology* **26** 151-164

Karsch FJ (1987) Central actions of ovarian steroids in the feedback regulation of pulsatile secretion of LH *Annual Reviews in Physiology* **49** 365-382

Karsch FJ, Moenter SM, and Caraty A (1992) The neuroendocrine signal for ovulation *Animal Reproduction Science* **28** 329-341

Kawahara Y (1991) Regulation of gonadotropins, prolactin and LH-RH secretion by endogenous opioid petides *Medical Journal of Kagoshima University* **43** 98-108

Kaynard AH, Low KG, and Melner MH (1992) Differential regulation of anterior pituitary prodynorphin and gonadotropin-subunit gene expression by steroid hormones *Molecular and Cellular Endocrinology* **88** 67-75

Kesner JS, Price-Taras EA, Kraeling RR, Rampacek GB, and Barb CR (1989) Negative feedback as an obligatory antecedent to the estradiol-induced luteinizing hormone surge in ovariectomized pigs *Biology of Reproduction* **41** 409-413

Kesner JS, Wilson RC, Kaufman JM, Hotchkiss J, Chen Y, Yamamoto H, Bardo RR, and Knobil E (1987) Unexpected responses of the hypothalamic GnRH pulse generator to cytological estradiol inputs in the absence of the ovary *Proceedings of the National Academy of Science of the USA* **84** 8745-8749

Kesner JS, Price-Taras EA, Kraeling RR, Rampacek GB, and Barb CR (1989) Negative feedback as an obligatory antecedent to the estradiol-induced luteinizing hormone surge in ovariectomized pigs *Biology of Reproduction* **41** 409-413

Knobil E (1980) The neuroendocrine control of the menstrual cycle *Recent Progress in Hormone Research* **36** 53-88

Knobil E (1990) The GnRH pulse generator *American Journal of Obstetrics and Gynaecology* **163** 1721-1727

Knobil E and Hotchkiss J (1988) The menstrual cycle and its neuroendocrine control In Knobil E and Neill J (eds) *The Physiology of Reproduction Raven Press New York* 1971-1994

Koiter TR, van der Schaaf-Verdonk GC, Pols-Valkhof N, and Schuiling GA (1987) Minor interference of low-dose pulses of GnRH with the positive effect of oestradiol on the pituitary gland *Acta Endocrinologica Copenhagen* **116** 399-404

Kotsuji F, Hosokawa K and Tominaga T (1992) Daily administration of gonadotrophin-releasing hormone increases pituitary gonadotroph number and pituitary gonadotrophin content, but not serum gonadotrophin levels, in female rats on day 1 of dioestrus *Journal of Endocrinology* **132** 395-400

Kraeling RR and Barb CR (1990) Hypothalamic control of gonadotrophin and prolactin secretion in pigs *Journal of Reproduction and Fertility Supplement* **40** 3-17

Kraeling RR, Barb CR, Leshin LS, and Rampacek GB (1992) Central nervous system peptide and amino acid modulation of luteinizing hormone and prolactin secretion in the pig *Journal of Physiology and Pharmacology* **43(4 suppl 1)** 79-103

Kubo K, Kiyota Y, and Yukunaga S (1983) Effects of third ventricular injection of beta-endorphin on luteinizing hormone surges in female rat: sites and mechanisms of opioid actions in the brain *Endocrinology Japan* **30** 419-433

Lazarus LH, Ling N, and Guillemin R (1976) Beta-lipotropin as a prohormone for the morphinomimetic peptides endorphins and enkephalins *Proceedings of the National Academy of Science of the USA* **73** 2156-2159

Lehman MN and Karsch FJ (1993) Do gonadotropin-releasing hormone, tyrosine hydroxylase-, and ß-endorphin-immunoreactive neurons contain estrogen receptors? A double-label immunocytochemical study in the Suffolk ewe *Endocrinology* **133** 887-895

Leiva LA and Croxatto HB (1994) Comparison of the effect of hypothalamic neuropeptides upon luteinizing hormone secretion by cultured rat anterior pituitary cells *Biological Research* **27** 113-121

Leshin LS, Rund LA, Kraeling RR, Crim JW, and Kiser TE (1992) Distribution of ß-endorphin immunoreactivity in the arcuate nucleus and median eminence of postpartum anestrus and luteal phase cows *Neuroendocrinology* **56** 436-444

Leuvenink HGD and Dierx JAJ (1997) Effective streptokinase treatment of blocked catheters in pigs and sheep *Laboratory Animals* **31** 184-185

Lévesque D, Gagnon S, and Di Paolo T (1989) Striatal D_1 dopamine receptor density fluctuates during the rat estrous cycle *Neuroscience Letters* **98** 345-350

Levine JE, Bauer-Dantoin AC, Besecke LM, Conaghan LA, Legan SJ, Meredith JM, Strobl FJ, Urban JH, Vogelsong KM, and Wolfe AM (1991) Neuroendocrine regulation of the luteinizing hormone-releasing hormone pulse generator in the rat *Recent Progress in Hormone Research* **47** 97-153

Levine JE, Pau KYF, Ramirez VD, and Jackson GL (1982) Simultaneous measurement of luteinizing hormone-releasing hormone and luteinizing hormone release in unanaesthetized, ovariectomized sheep *Endocrinology* **111** 1449-1455

Lloyd JM, Scarabough K, Weiland NG, and Wise PM (1991) Age-related changes in proopiomelanocortin (POMC) gene expression in the periarcuate region of ovariectomized rats *Endocrinology* **129** 1896-1902

Lord JAH, Waterfield AA, Hughes J, and Kosterlitz HW (1977) Endogenous opioid peptides: Multiple agonists and receptors *Nature* **267** 495-497

Lowry PJ, Gillies G, Hope J, and Jackson S (1980) Structure and biosynthesis of peptides related to corticotrophin and lipotrophin *Hormone Research* **13** 201-210

Mahesh VB, Brann DW, and Hendry LB (1996) Diverse modes of action of progesterone and its metabolites *Journal of Steroid Biochemistry and Molecular Biology* **56** 209-219

Mallory DS, Bona-Gallo A, and Gallo RV (1989) Neurotransmitter involvement in naloxone-induced stimulation of pulsatile LH release on day 8 of pregnancy in the rat *Brain Research Bulletin* **22** 1015-1021

March CM, Marrs RP, Goebelsmann U, and Mishell DR Jr (1981) Feedback effects of estradiol and progesterone upon gonadotropin and prolactin release *Obstetrics and Gynaecology* **58** 10-16

Martin del Campo AF, McMurray RG, Besser GM and Grossman A (1992) Effect of 12 hour infusion of naloxone on mood and cognition in normal male volunteers *Biological Psychiatry* **32** 344-353

Masotto C, Sahu A, Dube MG, and Kalra SP (1990) A decrease in opioid tone amplifies the luteinizing hormone surge in estrogen-treated ovariectomized rats: Comparisons with progesterone effects *Endocrinology* **126** 18-25

Matt DW, Dahl KD, Sarkissian A, and Sayles TE (1993) Apparent absence of negative feedback in middle-aged persistent-estrous rats following luteinizing hormone-releasing hormone agonist treatment: Relation to plasma inhibin and 17ß-Estradiol *Biology of Reproduction* **48** 333-339

Mello NK, Mendelson JK, Bree MP and Skupny A (1989) Naltrexone effects on pituitary and gonadal hormones in male and female Rhesus monkeys *Pharmocology Biochemistry & Behaviour* **31** 683-691

Merchenthaler I, Lennard DE, Cianchetta P, Merchenthaler A, and Bronstein D (1995) Induction of proenkephalin in tuberoinfundibular dopaminergic neurons by hyperprolactinemia: The role of sex steroids *Endocrinology* **136** 2442-2450

Messinis IE, Mademtzis I, Zikopoulos K, Tsahalina E, Seferiadis K, and Tsolas O and Templeton AA (1992) Positive feedback effect of oestradiol in superovulated women *Human Reproduction* **7** 469-474

Meyers RD (1977) Chronic Methods: Intraventricular infusion, cerebrospinal fluid sampling, and push-pull perfusion *Methods in Psychobiology* **3** 281-315

Montgomery GW, Martin GB, and Pelletier J (1985) Changes in pulsatile LH secretion after ovariectomy in Ile-de-France ewes in two seasons *Journal of Reproduction and Fertility* **73** 173-183

Morgan D and Picker MJ (1996) Contribution of individual differences to discriminative stimulus, antinociceptive and rate-decreasing effects of opioids: Importance of the drug's relative intrinsic efficacy at the mu receptor *Behavioural Pharmacology* **7** 261-275

Muraki T, Nakadate H, Tokunaga Y, Kato R, and Makino T (1979) Effect of narcotic analgesics and naloxone on proestrous surges of LH, FSH and prolactin in rats *Neuroendocrinology* **28** 241-247

Nanda AS, Ward WR, and Dobson H (1991) Opioid involvement in the LH release during the negative feedback effects of oestradiol and progesterone in dairy cows *Reproduction and Fertility Development* **3** 709-714

Naylor AM, Porter DWF, and Lincoln DW (1990) Central administration of corticotropin-releasing factor in the sheep: Effects on secretion of gonadotropins, prolactin and cortisol *Journal of Endocrinology* **124** 117-126

Niswender GD, Reichert LE, and Zimmerman DR (1970) Radioimmunoassay of serum concentrations of luteinizing hormone throughout the estrous cycle in pigs *Endocrinology* **87** 576-580

Norman RL, McGlone J, and Smith CJ (1994) Restraint inhibits luteinizing hormone secretion in the follicular phase of the menstrual cycle in rhesus Macaques *Biology of Reproduction* **50** 16-26

Okrasa S, Kalamarz H, Tilton JE, and Ziecik AJ (1992) Influence of opioids on LH secretion in gilts during the estrous cycle *Journal of Physiology and Pharmacology* **43(4 suppl 1)** 105-116

Okrasa SC and Tilton JE (1992) Concentrations of prolactin and LH after administration of Met-enkephalin analogue (FK 33-824) to gilts during the follicular phase *Animal Reproduction Science* **27** 195-207

Paterson SJ, Robson LE, and Kosterlitz HW (1983) Classification of opiate receptors *British Medical Bulletin* **39** 31-40

Pau KYF and Spies HG (1986) Oestrogen-dependent effects of norepinephrine on hypothalamic gonadotropin-releasing hormone release in the rabbit *Brain Research* **399** 15-23

Piva F, Maggi R Limonta P, Motta M, and Martini L (1985) Effect of naloxone on luteinizing hormone, follicle stimulating hormone, and prolactin secretion in the different phases of the estrous cycle *Endocrinology* **117** 766-772

O'Byrne KT, Chen M-D, Nishihara M, Williams CL, Thalabard J-C, Hotchkiss J, and Knobil E (1993) Ovarian control of gonadotropin hormone-releasing hormone pulse generator activity in the Rhesus monkey: Duration of the associated hypothalamic signal *Neuroendocrinology* **57** 588-592

Pfeiffer DG, Nikolarakis KE, and Pfeiffer A (1984) Chronic blockade of opiate receptors: Influence on reproduction and body weight in female rats *Neuropeptides* **5** 279-282

Pfeiffer A, Pasi A, Mehrein P, and Herz A (1982) Opiate binding sites in human brain *Brain Research* **248** 87-96

Pickering AJMC and Fink G (1976) Priming effect of luteinizing hormone releasing factor: in-vitro and in-vivo evidence consistent with its dependence upon protein and RNA synthesis *Journal of Endocrinology* **69** 373-378

Piva F, Maggi R Limonta P, Motta M, and Martini L (1985) Effect of naloxone on luteinizing hormone, follicle stimulating hormone, and prolactin secretion in the different phases of the estrous cycle *Endocrinology* **117** 766-772

Poceta JS, Hamlin MN, Haack DW, and Bohr DF (1981) Stereotaxic placement of cannulae in cerebral ventricles of the pig *Anatomical Record* **200** 349-356

Quigley ME and Yen SSC (1980) The role of endogenous opiates on LH secretion during the menstrual cycle *Journal of Endocrinology and Metabolism* **51** 179-181

Rabin D, Gold PW, Margioris AN and Chrousos GP (1988) Stress and reproduction: Physiologic and pathophysiologic interactions between the stress and reproductive axis *Advances in Experimental and Medical Biology* **245** 377-387

Raevskaia OS (1992) Nociceptive sensitivity of rabbits in varying localisation of pain stimuli and naloxone administration *Patol. Fiziol. Eksp. Ter* **(5-6)** 7-9

Rampacek GB, Kraeling RR, Fonda ES, and Barb CR (1984) Comparison of physiological indicators of chronic stress in confined and non-confined gilts *Journal of Animal Science* **58** 401-408

Rasmussen DD (1991) The interaction between mediobasohypothalamic dopaminergic and endorphinergic neuronal systems as a key regulator of reproduction: an hypothesis *Journal of Endocrinological Investigations* **14** 323-352

Rasmussen DD and Malven PV (1983) Effects of confinement stress on episodic secretion of LH in ovariectomized sheep *Neuroendocrinology* **36** 392-396

Reame N, Sauder SE, Kelch RP, and Marshall JC (1984) Pulsatile gonadotropin secretion during the human menstrual cycle: Evidence for altered frequency of gonadotropin-releasing hormone secretion *Journal of Clinical Endocrinology and Metabolism* **59** 328-337

Remorgida V, Venturini PL, Anserini P, Salerno E, and De Cecco L (1990) Naltrexone in functional hypothalamic Amenorrhea and in the normal luteal phase *Obstetrics and Gynaecology* **76** 1115-1120

Rivier C and Rivest S (1991) Effect of stress on the activity of the hypothalamic-pituitary-gonadal axis: Peripheral and central mechanisms *Biology of Reproduction* **45** 523-532

Rivest S, Plotsky PM, and Rivier C (1993) CRF alters the infundibular LHRH secretory system from the medial preoptic area of female rats: Possible involvement of opioid receptors *Neuroendocrinology* **57** 236-246

Rodriguez RE and Wise ME (1989) Ontogeny of pulsatile secretion of gonadotropin-releasing hormone in the bull calf during infantile and pubertal development *Endocrinology* **124** 248-256

Roozendaal MM, Swarts JJM, van Maanen JC, Wiegant VM, and Mattheij JAM (1997) Inhibition of the LH surge in cyclic rats by stress is not mediated by opioids *Life Sciences* **60** 735-742

Roozendaal MM, Swarts JJM, Wiegant VM, and Mattheij JAM (1995) Effect of restraint stress on the preovulatory LH profile and ovulation in the rat *European Journal of Endocrinology* **113** 347-353

Rosemberg E, Purohit P, and Takekida H (1974) Variation in pituitary responsiveness to synthetic gonadotropin releasing hormone (GnRH) during different phases of the menstrual cycle *Reproduccion* **1** 241-251

Rossier J (1988) Biosynthesis of opioid peptides *Annals of Endocrinology Paris* **49** 371-373

Rossmanith WG (1991) Circadian and ultradian rhythms in gonadotropin secretion: Regulation via ovarian steroids *Geburtshilfe und Frauenheilkunde* **51** 585-594

Rossmanith WG (1992) Endogenous opioid regulation of luteinizing hormone secretion in women In Negri M, Lotti G and Grossman A (eds) *Clinical Perspectives of endogenous opioids production John Wiley & Sons Ltd England* 117-144

Rossmanith WG (1995) Gonadotropin secretion during ageing in women: Review article *Experimantal Gerontology* **30** 369-381

Rossmanith WG and Lauritzen C (1991) Endogenous opioid peptides in physiology and pathology of the menstrual cycle *TW Gynäcologie* **4** 223-233

Rossmanith WG, Liu CH, Laughlin GA, Mortola JF, Suh BY, and Yen SSC (1990) Relative changes in LH pulsatility during the menstrual cycle: using data from hypogonadal women as a reference point *Clinical Endocrinology* **32** 647-660

Rossmanith WG, Wirth U, Sterzik K, and Yen SSC (1989) The effects of prolonged opioidergic blockade on LH pulsatile secretion during the menstrual cycle *Journal of Endocrinological Investigation* **12** 245-252

Santoro N, Butler JP, Filicori M, and Crowley WF Jr (1988) Alterations of the hypothalamic GnRH interpulse interval sequence over the normal menstrual cycle *American Journal of Physiology* **225** E696-E701

Sar M, Sahu A, Crowley WR, and Kalra SP (1990) Localisation of neuropeptide-Y immunoreactivity in estradiol-concentrating cells in the hypothalamus *Endocrinology* **127** 2752-2756

SAS/STAT (1990) *User's Guide* SAS Institute, Cary, NC

Schivers BD, Harlan RE, Morrell JI, and Pfaff DW (1983) Absence of oestradiol concentration in cell nuclei of LHRH-immunoreactive neurones *Nature* **304** 345-347

Schouten WGP, Rushen J, and De Passilé AMB (1991) Stereotypic behaviour and heart rate in pigs *Physiology and Behaviour* **50** 617-624

Schouten WGP and Wiepkema PR (1991) Coping styles of tethered sows *Behavioural Proceedings* **25** 125-132

Schwarz S and Pohl P (1994) Steroids and opioid receptors *Journal of Biochemistry and Molecular Biology* **48** 391-402

Seifer DB and Collins RL (1990) Current concepts of ß-endorphin physiology in female reproductive dysfunction *Fertility and Sterility* **54** 757-771

Selye H (1973) The evolution of the stress concept *American Scientist* **61** 692-699

Shen ES, Meade EH, Perez MC, Deecher DC, NegroVilar A, and Lopez FJ (1998) Expression of functional estrogen receptors and galanin messenger ribonucleic acid in immortalised luteinizing hormone-releasing hormone neurons: Estrogenic control of galanin gene expression *Endocrinology* **139** 939-948

Simpkins JW (1994) Effects of age and ovarian steroids on responses to opiates in the female rat *Neurobiology of Aging* **15** 545-552

Smith MJ and Gallo RV (1997) The effect of blockade of kappa-opioid receptors in the medial preoptic area on the luteinizing hormone surge in the proestrous rat *Brain Research* **768** 111-119

Snowden EU, Khan-Dawood FS and Dawood MY (1984) The effect of naloxone on endogenous opioid regulation of pituitary gonadotropins and prolactin during the menstrual cycle *Journal of Clinical Endocrinology and Metabolism* **59** 298-302

Soede NM, Helmond FA, Schouten WGP, and Kemp B (1997) Oestrus, ovulation and peri-ovulatory hormone profiles in tethered and loos-housed sows *Animal Reproduction Science* **46** 133-148

Soede NM, Van der Lende, Kemp B, and De Koning M (1991) Assessment of the duration of ovulation in sows using ultrasonography *ARTA* **2** 128-129

Soules MR, Steiner RA, Clifton DK, Cohen DK, Aksel SS, and Bremmer WJ (1984) Progesterone modulation of pulsatile LH secretion in normal women *Journal of Clinical Endocrinology and Metabolism* **58** 378-383

Stumpf TT, Roberson MS, Wolfe MW, Hamernik DL, Kittok RJ, and Kinder JE (1993) Progesterone, 17ß-oestradiol, and opioid neuropeptides modulate pattern of luteinizing hormone in circulation of the cow *Biology of Reproduction* **49** 1096-1101

Taché Y, DuRuisseau P, DuCharne JR, and Colu R (1978) Pattern of adenohypophyseal hormone changes in male rats following chronic stress *Neuroendocrinology* **26** 208-219

Tanaka T, Mori Y, and Hoshino K (1992) Hypothalamic GnRH pulse generator activity during the estradiol-induced LH surge in ovariectomized goats *Neuroendocrinology* **56** 641-645

Taylor DV, Hetrick WP, Neri CL, Touchette P, Barron JL and Sandman CA (1991) Effect of naltrexone upon self-injurious behaviour, learning and activity: A case study *Pharmacology Biochemistry & Behaviour* **40** 79-82

Thom B, Canny BJ, Cowley M, Wright PJ, and Clarke IJ (1996) Changes in the binding characteristics of the μ, δ and κ subtypes of the opioid receptor in the hypothalamus of the normal cyclic ewe and in the ovariectomised ewe following treatment with ovarian steroids *Journal of Endocrinology* **149** 509-518

ThyagaRajan S, MohanKumar PS, and Quadri SK (1995) Cyclic changes in the release of norepinephrine and dopamine in the medial basal hypothalamus: effect of aging *Brain Research* **698** 122-128

Urbanski HF, Pickle RL, and Ramirez VD (1988) Simultaneous measurement of gonadotropin-releasing hormone, luteinizing hormone, and follicle-stimulating hormone in the orchidectomized rat *Endocrinology* **123** 413-419

Van Landeghem AAJ and Van de Wiel DFM (1978) Radioimmunoassay for porcine prolactin. Plasma levels during lactation, suckling and weaning, and after TRH administration *Acta Endocrinologica (Copenhagen)* **88** 653-667

Veldhuis JD (1990) The hypothalamic pulse generator: The reproductive core *Clinical Obstetrics and Gynaecology* **33** 538-550

Verburg-van Kemenade BML, Jenks BG, Cruijsen PMJM, Dings A, Tono M-C, and Vaudry H (1987) Regulation of MSH release from the neurointermediate lobe of Xenopus leavis by CRF-like peptides *Peptides* **8** 1093-1100

Voklavka J, Cho D, Mallya A, and Bauman J (1979) Naloxone increases ACTH and cortisol levels in man *New English Journal of Medicine* **300** 1056-1057

Walsh JP and Clarke IJ (1996) Effects of central administration of highly selective opioid mu-, delta- and kappa-receptor agonists on plasma luteinizing hormone (LH), prolactin, and the estrogen-induced LH surge in ovariectomized ewes *Endocrinology* **137** 3640-3648

Weiland NG and Wise PM (1990) Estrogen and progesterone regulate opiate receptor densities in multiple brain regions *Endocrinology* **126** 804-808

Whisnant CS, Havern RL, and Goodman RL (1991) Endogenous opioid suppression of luteinizing hormone pulse frequency and amplitude in the ewe: hypothalamic sites of action *Neuroendocrinology* **54** 587-593

Whisnant CS and Goodman RL (1988) Effects of an opioid antagonist on pulsatile luteinizing hormone secretion in the ewe vary with changes in steroid negative feedback *Biology of Reproduction* **39** 1032-1038

Yang K, Haynes NB, Lamming GE, and Brooks AN (1988) Ovarian steroid hormone involvement in endogenous opioid modulation of LH secretion in mature ewes during the breeding and non-breeding seasons *Journal of Reproduction and Fertility* **83** 129-139

Yearwood HA, Rzucidlo SJ, and Zięcik AJ (1991) Naloxone, morphine and the estradiol-induced LH surge in gilts Journal of *Reproduction and Fertility* **8** 54

Yen SSC (1991) The human menstrual cycle: Neuroendocrine regulation. In Yen SSC and Jaffe RB (eds) *Reproductive Endocrinology Saunders Philadelphia* 273-308

Yen SSC, Tsai CC, Naftolin F, Vandenberg G, and Ajabor L (1972) Pulsatile patterns of gonadotropin release in subjects with and without ovarian function *Journal of Clinical Endocrinology* **34** 671-675

Zanella AJ, Broom DM, Hunter JC, and Mendl MT (1996) Brain opioid receptors in relation to stereotypies, inactivity, and housing in sows *Physiology & Behaviour* **59** 769-775

Zhen S and Gallo RV (1995) The role of norepinephrine in mediating luteinizing hormone release in response to blockade of κ-opioid receptors in the medial preoptic area *Brain Research* **698** 121-129

Zięcik AJ, Yearwood HA, and Espana-Espana F (1994) Effect of naloxone and morphine on the estradiol-induced LH surge in gilts *Archivos de Zootecnia* **43** 5-14

Publications & Abstracts

- **Cools AR, Dierx JAJ, Coenders C, Heeren D, Ried S, Jenks BG and Ellenbroek B** (1993) Apomorphine-susceptible and apomorphine-unsusceptible Wistar rats differ in novelty-induced changes in hippocampal dynorphin B expresion and two-way active avoidance: A new key in the search for the role of the hippocampal-accumbens axis *Behavioral Brain Research* **55(2)** 213-221.
- **Neefjes JJ, Dierx JAJ and Ploegh HL** (1993) The effect of anchor residue modifications on the stability of major histocompatibility complex class I-peptide interactions *European Journal of Immunology* **23(4)** 840-845
- **Dierx JAJ, Helmond FA and Wiegant VM** (1994) Pulsatile LH release during follicular phase in gilts: Effects of restraint and endogenous opioids *European Journal of Endocrinology* **130** p. 173
- **Helmond FA, Kemp B, Soede NM and Dierx JAJ** (1994) Pulsatile LH release during the lactation period in relation to the metabolic status and the time of first ovulation in sows *European Journal of Endocrinology* **130** p. 174
- **Dierx JAJ, Helmond FA and Wiegant VM** (1995) Endogenous opioid regulation of the pulsatile LH release during the follicular phase in gilts *Neuroscience Research Communications* **17(1)** p. 52
- **Dierx, JAJ, Helmond FA and Wiegant VM** (1995) Effect of opioid antagonist naltrexone on pulsatile LH release in the luteal phase in gilts: A dose response curve *Neuroscience Research Communications* **17(1)** p. 52
- **ten Hulscher HI, Lamberts RR, Dierx JAJ, Helmond FA and Wiegant VM** (1995) The effect of CRF on the release from superfused pig pituitary depends on the presence of the neurointermediate lobe *Neuroscience Research Communications* **17(1)** p. 52
- **Dierx JAJ, Helmond FA and Wiegant VM** (1997) Endogenous opioid peptides modulate the response of the GnRH induced LH release by the pituitary *in vitro* *Neurochemical Research Communications* **30(3)**

Samenvatting

Samenvatting

Inleiding

Het doel van dit proefschrift is meer inzicht te krijgen in de neuro-endocriene regulatie van het voortplantingshormoon luteïniserend hormoon uit de hypofyse tijdens de folliculaire fase van de oestrische cyclus van het vrouwelijk varken. In het bijzonder is bestudeerd of stress en endogene opioïde peptiden dit systeem beïnvloeden, en zo ja hoe deze factoren dat doen. Alvorens in te gaan op de experimenten en de resultaten, is het noodzakelijk om kennis te hebben van een aantal vaak terugkerende begrippen.

De Hypothalamus-Hypofyse-Gonade As

Het vermogen zich voort te planten wordt voor een belangrijk deel bepaald door de aanwezigheid van de voortplantingsorganen die de zogenaamde *hypothalamus-hypofyse-gonade (HHG) as* vormen. De *zenuwcellen of neuronen* in het hersengebied "de hypothalamus", geven het *gonadotropine releasing hormoon (GnRH)* af aan de portale bloedvaten die naar de *hypofyse* leiden. Dit GnRH stimuleert in de gonadotrope cellen van de hypofyse de vorming en afgifte van de gonadotrofines: het *luteïniserend hormoon (LH)* en het *follikel stimulerend hormoon (FSH)*. Deze gonadotrofines stimuleren in de *geslachtsklieren of gonaden* de vorming en afgifte van de *steroïde hormonen* testosteron, oestrogenen (E2ß) en progesteron (P) respectievelijk in het mannelijk dier en vrouwelijk dier. Verder stimuleren ze de ontwikkeling van geslachtscellen en, in het vrouwelijk dier, de *eisprong of ovulatie*. De steroïd hormonen koppelen op hun beurt direct of indirect terug *(feedback)* op de afgifte van GnRH in de hypothalamus en op de gonadotrope cellen van de hypofyse. In dit proefschrift is alleen aandacht besteed aan de hormonale regulatie in het vrouwelijk individu zodat de verdere beschrijving van de LH secretie alleen gericht is op het vrouwelijke individu.

De Oestrische of Menstruele Cyclus

De hormonale profielen vertonen periodieke veranderingen, en worden de zogenaamde *menstruele* (in de mens en de niet-menselijke primaat) *of oestrische cycli* (andere zoogdieren) genoemd. Tijdens zo'n cyclus kunnen grofweg 2 fasen worden onderscheiden. De *luteale of secretoire fase* is de tijdsperiode die ligt tussen het moment van ovulatie en de menstruele bloeding. De tijdsperiode tussen het einde van de menstruele bloeding tot het moment van ovulatie wordt de *folliculaire of proliferatie fase* genoemd.

De hormonen GnRH, LH en FSH worden afgegeven in de vorm van regelmatige pulsen. Dit pulsatiel patroon is essentieel voor een goede regulatie van allerlei gebeurtenissen die de vrouwelijke vruchtbaarheid voor een groot deel bepalen. Tijdens de luteale fase zorgt een hoge P concentratie in het bloed voor een *lage puls frequentie* maar *hoge puls amplitude* van

LH door de GnRH-neuronen te remmen. Tijdens de vroeg-folliculaire fase daalt de P concentratie en neemt de LH puls frequentie toe terwijl de LH puls amplitude afneemt. Tijdens de folliculaire fase stijgt de E2ß concentratie in het bloed die ervoor zorgt dat de puls frequentie van GnRH, en daardoor ook van LH, weer afneemt tijdens de mid-folliculaire fase ("negatieve feedback"). Tijdens de laat folliculaire fase heeft E2ß een "positieve feedback" die voor een kortstondig verhoogde afgifte van GnRH en vervolgens ook van LH zorgt. Door deze LH-piek wordt in het ovarium de ovulatie gestart, reden waarom deze hormoonpiek de *preovulatoire LH piek* wordt genoemd.

Stress en Voortplanting

Stress is een alomtegenwoordig verschijnsel in het leven en de reactie (respons) op stress wordt omschreven als "...een niet-specifieke reactie van het lichaam op een willekeurige stimulus (meestal schadelijk) die een verandering in het interne evenwicht veroorzaakt". Hoewel stress en de respons op stressvolle gebeurtenissen een belangrijke rol spelen bij de overleving van een individu, kan het ook een gevaar voor het welzijn en de gezondheid van het individu zijn. Ook de voortplanting wordt door stress beïnvloed. Dit kan op meerdere manieren gebeuren. In het algemeen leidt stress tot een onderdrukking van de processen die bijdragen aan de voortplanting, vermoedelijk om energie te sparen voor processen die voor de overleving van het individu zorgen. In de moderne intensieve varkensfokkerij, waar de dieren veelal aangebonden in nauwe, individuele boxen worden gehuisvest en er daardoor sprake is van chronische stress, treedt er aanzienlijke uitval van dieren op mede door gereduceerde vruchtbaarheid en zelfs onvruchtbaarheid. Welke mechanismen hierbij een rol spelen is vooralsnog niet duidelijk. Gedacht wordt onder andere aan een langdurig verhoogde activiteit van endogene opioïde peptiden, die betrokken zijn bij de respons op stress.

Opioïderge Controle van de Gonadotrofine Afgifte tijdens de Oestrische Cyclus

De expressie van *endogene opioïde peptiden (EOP)* speelt een centrale rol in de negatieve feedback van steroïd gonadale hormonen tijdens de oestrische cyclus. De opioïde activiteit verandert met de veranderde concentratie van ovariële steroïden tijdens de cyclus. Tijdens de P-gedomineerde luteale fase remmen EOP de LH puls frequentie continu waardoor de LH concentratie in het bloed laag blijft. Of EOP ook betrokken zijn bij de feedback door E2ß tijdens de folliculaire fase is vooralsnog onduidelijk. Gegevens uit de literatuur wijzen erop dat EOP's niet betrokken zijn bij de negatieve feedback van E2ß tijdens de vroeg- en mid-folliculaire fase. Echter bij de "positieve feedback" tijdens de laat-folliculaire fase lijken EOP's een remmende invloed te hebben op de LH-afgifte. Verder lijken EOP's betrokken te zijn bij de timing en het optreden van de preovulatoire LH piek.

Dit Proefschrift

In dit proefschrift is ten eerste het patroon van de pulsatiele LH afgifte tijdens meerdere dagen van de folliculaire fase van de oestrische cyclus beschreven, waarbij het aangebonden vrouwelijke varken als modelsysteem werd gebruikt. Ten tweede hebben we onderzocht of EOP's betrokken zijn bij de regulatie van het patroon van de pulsatiele LH afgifte en/of bij de timing en het optreden van de preovulatoire LH piek. Hoofdstuk I geeft een korte beschrijving van de reproductieve as, de betrokken organen, de hormonale regulatie en de interactie tussen de verschillende hormonen die leiden tot de oestrische of menstruele cyclus. Wanneer dieren die langduring aangebonden gehuisvest zijn geweest worden vrijgelaten, worden de preovulatoire LH piek en het moment van oestrus wordt vervroegd (Hoofdstuk II). Deze vervroeging wordt waarschijnlijk niet veroorzaakt door aangebonden of vrije huisvesting als zodanig maar meer door de verandering in huisvesting. Die verandering is waarschijnlijk een belangrijke prikkel. Immers, in Hoofdstuk IV werd er geen verschil gevonden in het moment van oestrus tussen langdurig (6 maanden) aangebonden varkens en vrij gehjuisveste controle varkens. Dat wijst erop, dat de aangebonden dieren waren geadapteerd aan hun huisvestingsconditie. Hoewel in dit Hoofdstuk de toediening van de opioïd receptor antagonist naltrexon leidde tot een verschuiving van het moment van oestrus, en mogelijk ook de preovulatoire LH piek, hebben we geen bewijs gevonden voor een verschil tussen aangebonden en vrije dieren in de betrokkenheid van EOP bij de regulatie van de reproductieve processen. Een interessante waarneming was de grote variabiliteit in het moment van oestrus in de met naltrexon behandelde varkens. Die zou, gegeven de bevindingen in Hoofdstuk III, verklaard kunnen worden door een aanzienlijke individuele variabiliteit in de respons op naltrexon.

Deze resultaten suggereren een mogelijke betrokkenheid van stress en EOP's bij de timing van de preovulatoire LH piek en het moment van oestrus. Of EOP aangrijpen op het niveau van de hypothalamus en/of de hypofyse is onderzocht in de Hoofdstukken V en VII. De maximale responsiviteit van de hypofyse voor GnRH wordt bereikt op de dag van de preovulatoire LH piek en dit moment van maximale responsiviteit lijkt, samen met de preovulatoire LH piek, door behandeling met naltrexon vervroegd te worden (Hoofdstuk V). Om de mogelijke effecten van naltrexon op het niveau van de hypothalamus *in vivo* te kunnen bestuderen, hebben we een nieuwe methode ontwikkeld die het mogelijk maakt om bij varkens farmaca direct in de laterale ventrikel van de hersenen te kunnen injiceren (Hoofdstuk VI). Met behulp van deze methode hebben we aangetoond dat injecties van naltrexon in de laterale ventrikel gedurende de folliculaire fase een vervroegd einde van de preovulatoire LH piek veroorzaakten. Samen met de hogere puls amplitude die tijdens de midfolliculaire fase (Dag 4) bij naltrexon behandelde dieren werd gevonden, ondersteunt dit

de hypothese dat EOP activiteit in de hersenen, waarschijnlijk op het niveau van de hypothalamus, het te vroeg optreden van de preovulatoire LH piek voorkomt.
Het patroon van de pulsatiele afgifte van LH bestaat uit hoog-frequente pulsen met een lage amplitude tijdens de vroeg-folliculaire fase (ongeveer Dag 1 en 2). Tijdens de mid-folliculaire fase (ongeveer Dag 3-5) daalt de LH pulsfrequentie, terwijl de laat-folliculaire fase met de preovulatoire LH piek (ongeveer Dag 6-7) wordt gekenmerkt door hoog-frequente pulsen met een hoge amplitude (Hoofdstuk IV en VII). De responsiviteit van de hypofyse voor GnRH verandert gedurende de folliculaire fase met een maximale responsiviteit op de dag van de preovulatoire LH piek (Hoofdstuk V). Dit patroon wordt waarschijnlijk niet beïnvloed door EOP's. Dieren die met naltrexon behandeld waren vertoonden weliswaar hogere LH puls amplitudes dan controle dieren op 1 dag tijdens de folliculaire fase (Dag 5 in Hoofdstuk IV en Dag 4 in Hoofdstuk VII), maar dit verschil was niet langer aanwezig als de data werden opgelijnd op het moment van oestrus, derhalve suggererend dat het pulspatroon van LH tijdens de folliculaire fase niet wordt beïnvloed door naltrexon.

Uit de experimenten van dit proefschrift blijkt dat langdurige aanbindstress leidt tot adaptieve veranderingen in de reproductieve as, en dat stress en EOP's tijdens de folliculaire fase, in tegenstelling tot de luteal fase, de pulsatiele afgifte van LH niet beïnvloeden. Daarentegen zijn EOP's tijdens de folliculaire fase betrokken bij de timing van de preovulatoire LH piek en het moment van oestrus.

Dankwoord

Tja, het kost wat maar dan heb je ook wat. Nu komt het terugkijken en evalueren. Zo ben ik erachter gekomen dat begeleiding in de kwaliteit zit en niet zozeer in de kwantiteit. Ik wil dan ook in de allereerste plaats mijn beide promotoren bedanken. Victor en Daan, jullie kritische blik heeft mij meerdere malen tot wanhoop gedreven. Maar zonder die blik was het nooit zover gekomen. Gelukkig weet ik nu precies wat er voor nodig is om een wetenschapper te zijn. Ook wil ik Frans Helmond hierbij bedanken voor de gaatjes die hij nog wist te creëren in zijn vaak overvolle agenda.
Maar wat is het FMD schip zonder bemanning? Een bemanning bestaande uit hard werkende analisten, dierverzorgers, "techneuten", secretaresses, docenten/onderzoekers. Allen met gouden handjes en harten. Mensen het was een voorrecht om met jullie gewerkt en gelachen te hebben. Sorry voor degene die te vaak het slachtoffer waren van Henri's en mijn vrije associatie uurtjes.
Eindelijk kan ik dan ook de dierverzorgers van "De Haar" bedanken. Met Jan Hagens als ploegleider, fietsten Ben, Ries, André, Peter, Truus en Jack mee en deden veel meer dan van hun gevraagd werd. Ook bedankt namens mijn varkens 3249, 3385, 4707, Babs....
Collega AIO's en Eline (!), vanaf het AIO weekend kon het echt niet meer stuk. Bedankt voor alle gezelligheid en overblijfetentjes op de maandagen dat er gevolleybald moest worden en de overige gelegenheden. Henri, ik zal ons "geouwehoer" erg missen.
Dan de mensen waar ik nauw mee heb samengewerkt. Bas, Nicoline, dr. Bob (Frank van Eerdenburg), Corrie, Dick Bongers, Gradus, Wim en Dick van Kleef naast de studenten Miriam, de "stress" crew van het jaar 1994, Yvonne, Anite en verder alle vrienden en kennissen die mijn wel en wee de afgelopen 6 jaar van dichtbij hebben meegemaakt. Bedankt voor jullie geduld en steun. Ik kan jullie nooit beschrijven hoeveel jullie eigenlijk gedaan hebben.
De "Magnificent Seven" Ingrid, Guido, Marit, Regis, Hinke, Ard en Koen. Zeven namen die bij mij heel diep in het hart gegrift zijn. Bedankt, en we spreken elkaar nog.
Familie is iets ongelofelijk belangrijks in iemands leven. Mam, Christinette, Tanja, Ger, Yvonne, Roy, Peter en de afgelopen twee jaar ook Piet, Maria en Pauline. Jullie maken het verschil tussen leven en **LEVEN**.
Toch is er maar één persoon die mijn leven *is*. Liefste Lein, jij hebt "de vos getemd". Met de liefde, de rust en de kracht die je mij geeft kan ik bergen verzetten en zelfs een proefschrift schrijven. Bedankt voor de warmte die je elke dag weer uitstraalt, voor de gemoedelijke schouder waar ik op uit kan huilen, en voor alles waar nog geen woorden voor zijn uitgevonden.
Als laatste wil ik hem bedanken die helaas niet meer onder ons is maar waar ik nog elke dag van leer: Mijn Vader. Leeve pap, geej het meej aaltiêd en overaal in gesteund. Dit werk draag ik daan ók aan oow op. Bedankt veur aal daat geej meej het geliërd en het laote zeen. Aliën joamer daat toen ow oëge zich vur iëwig slote, die vaan meej pas ôpe gingen. Bedankt en ut giët oow good.

John

Curriculum Vitae

Josephus Antonius Jacobus (John) Dierx werd geboren op 15 juli 1967 te Horst (L). Hij ging naar de lagere school "De Weisterbeek" te Horst. Zijn middelbare schooltijd heeft hij doorgebracht op het "Boschveldcollege" te Venray. Na de brugklas volgde hij het gymnasium. Hij behaalde zijn VWO-diploma in 1985 en startte met de studie Biologie aan de Katholieke Universiteit van Nijmegen. Met een tussenstap van 1 jaar naar de studie Geneeskunde behaalde hij in 1992 zijn doctoraal diploma in de Biologie. Tijdens zijn studie is hij 1 jaar actief geweest als penningmeester van de Nijmeegse Biologenvereninging "BeeVee". Van 1 oktober 1992 tot 1 oktober 1996 was hij als AIO verbonden aan de vakgroep Fysiologie van Mens en Dier van de Landbouwuniversiteit te Wageningen. Het huidige proefschrift is het resultaat van het onderzoek dat hij heeft uitgevoerd. Tijdens deze AIO-periode was hij één van de AIO-vertegenwoordigers binnen het vakgroepbestuur. Thans volgt hij de 8 maanden durende opleiding "Gezondheidsvoorlichting en Opvoeding" aan de Rijksuniversiteit te Utrecht die zal worden afgerond op 29 april 1999.