STRESS AND THE HYPOTHALAMUS-PITUITARY-GONADAL AXIS IN THE CYCLIC RAT

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Proefschrift

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Stellingen

1. Stress kan de pre-ovulatoire LH-piek verstoren.
(dit proefschrift)

2. Er is relatief weinig LH nodig om Graafse follikels te laten ovuleren.
(dit proefschrift)

3. Onder stress-omstandigheden is de pre-ovulatoire LH-piek afhankelijk van vasopressine.
(dit proefschrift)

4. CRH, endogene opioiden en GABA spelen geen belangrijke rol bij de invloed van stress op de pre-ovulatoire LH-piek.
(dit proefschrift)

5. Substitutie van oestradiol en progesteron na ovariëctomie maakt van een rat nog geen goed model voor onderzoek naar de invloed van stress op de pre-ovulatoire LH-piek.
(Salisbury et al. (1980), Endocrinology 101:1194-1202)
(Kubo et al. (1983), Endocrinol. Jpn. 30:419-433)

6. Het ontstaan van "luteinized unruptured follicles" kan niet verklaard worden als veroorzaakt door een te lage LH-afgifte alleen.
(Mattheij & Swarts (1995), Eur. J. Endocrinol. 132:91-96)

7. Alleen een grote bedreiging van hun welzijn zal dieren ervan weerhouden om zich voort te planten.
(Moberg G.P. (1985). In: Animal stress, The Williams & Wilkins Company, Baltimore, blz 245)

8. Bij het induceren van ovulatie in de vrouwelijke meerval zijn feromonen betrokken.
(proefschrift J.W. Resink (1988), Utrecht)

9. Mannelijke watervlooien onstaan pas als het voor vrouwelijke moeilijk wordt om te overleven.

10. De voorgeschiedenis van het proefdier is van invloed op de uitkomsten van de dierproef.

11. Hersenen zijn gecompliceerder als je denkt.

12. Omdat "verwacht rendement" en "risico" subjectieve begrippen zijn, zullen de beurskoersen altijd beïnvloed worden door sentimenten.

13. Een geaccepteerde hypothese blijft een hypothese en laat dus vele vragen onbeantwoord.

14. Wachtgeld betekent soms "wacht op je geld".

15. De besluitvorming in de Tweede Kamer wordt versluierd door het gebruik van voor leken onduidelijke afkortingen.

16. Hoewel het geslacht van een ongeboren baby doorgaans niet bekend is wordt ze in het spraakgebruik altijd aangeduid als "hij".

Marjolijn M. Roozendaal
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Wageningen, 25 november 1997

Contents

Chapter 1

General introduction

1 Introduction

The concept of homeostasis was first formulated by Bernard (1). He recognized the interaction between the external environment ("milieu extérieur") that surrounds the organism and its internal environment ("milieu intérieur"). An organism is constantly subjected to changes in the external environment. By maintaining the internal environment within fixed limits, organisms have evolved to become more independent from changes in the outer world. This maintenance of a relative stability of the internal environment was termed homeostasis by Cannon (2). Fluctuations in the internal environment are tolerated but are kept within limits. When external influences alter a physiological variable (e.g. temperature, blood pressure) so that a deviation from a physiological setpoint is detected, feedback mechanisms will be activated, that re-adjust the variable towards the physiological setpoint. Activation of feedback systems in order to reinstate homeostasis is termed "adaptation".

In a constantly changing external environment, maintenance of a relative stability of the internal environment (homeostasis) is necessary for an organism to survive. An environmental condition or factor that threatens or disturbs homeostasis is usually referred to as a stressor. Activation of behavioural, autonomic and endocrine mechanisms, the stress response, aims at protecting the organism against (potential) damaging conditions.

2 Stress

Stress is an ubiquitous feature of life and the concept of stress has been widely and frequently discussed. After many decades of stress research, an exact definition of the phenomenon stress is still not established. For an animal to survive, it must maintain its vital variables within preset limits. Thus, specific, routinely operating homeostatic mechanisms, consisting of behavioural and physiological programmes, control the vital functions and protect homeostasis. Any condition that could lead to a change in such functions beyond the capacity

of these mechanisms - and thus threatens homeostatic control - triggers a general defence mechanism, the stress response, that includes behavioral, autonomic and neuroendocrine adaptations aiming at removal of the threat and reestablishment of homeostasis. Whether or not a stimulus represents a significant threat to control (and is a stressor), is assessed in the central nervous system (CNS). This not only depends on qualities of the stimulus condition itself (e.g. intensity, frequency, duration), but also on characteristics of the animal such as genetic constitution (e.g. species, selection line, gender), pre- and postnatal development, prior experiences and skills (e.g. history of the individual, learning, memory), and the physiological (normal or pathological) and psychological state of the animal at that particular moment (Fig. 1).

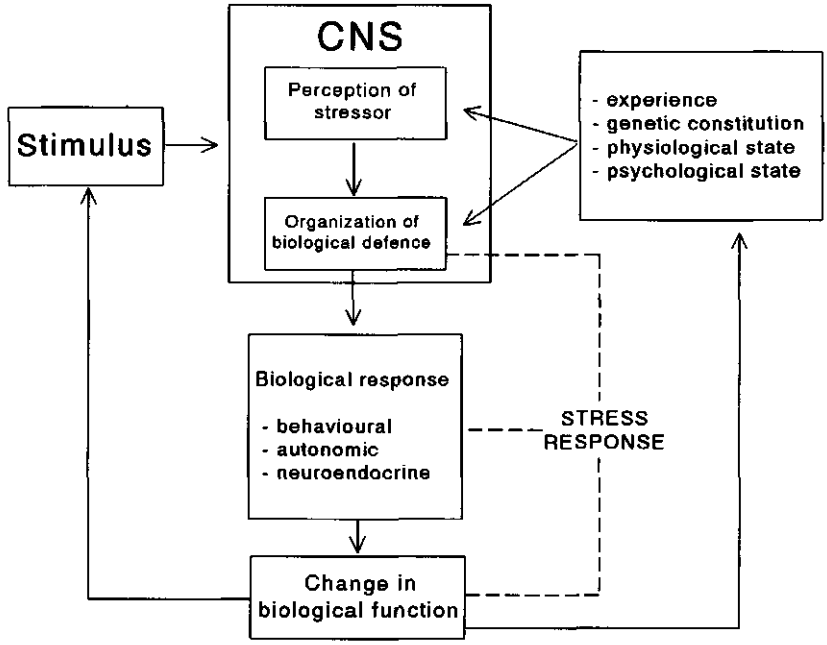

Fig. 1. Schematic representation of the response to a potential stressful event and examples of factors that influence this response (adapted from Moberg [3])

The stress response comes in several modes. Active behaviour can be used to approach and fight or to flee from the stressor (fight-flight response). When an active response is not possible or available, behavioural inactivity is displayed.

Each mode is parallelled by a different set of physiological reactions, that basically thought to concern mobilization and redistribution of energy to allow or prepare for the behavioural response to be generated (4). Thus, energy is mobilized from stores, and storage of energy is blocked. Anabolic processes are deferred, and physiological functions which normally cost energy and are not needed to survive the stressful situation, are suppressed (such as digestion, growth and repair, immunity, and reproduction). Thus, the stress response comes with costs for the organism, and during prolonged stress it can therefore become as damaging as the stressor itself. If the stress experience lasts too long or the stress response can not be appropriately terminated at the end of stress, stress-related disturbances or diseases can emerge. These disturbances actually concern physiological systems that are affected during the stress response. It is generally thought, that they arise as a consequence of biochemical/cellular changes induced by mediators of the stress response (hormones, neuropeptides, neurotransmitters).

The neuroendocrine response to stress has been the subject of extensive research. A major focus of these studies has been on the hypothalamus-pituitary-adrenal (HPA) axis. With the onset of stress, corticotropin-releasing hormone (CRH) is secreted from the hypothalamus into the hypophysial portal circulation (see review 5). CRH then triggers the release of adrenocorticotropic hormone (ACTH) from the pituitary, which in turn triggers the release of glucocorticoids from the adrenal cortex (6-9). Activation of the HPA axis blocks energy storage, helps in mobilizing energy from storage sites, and inhibits anabolic processes such as growth, immunity and reproduction. Therefore, a relationship is suggested between hormones of the HPA axis (which are released during stress) and those of the hypothalamic-pituitary-gonadal (HPG) axis (which are also influenced by stress).

3 The reproductive cycle of the female rat

3.1 Ovarian follicular cycle

In the adult laboratory rat, the ovarian cycle continues throughout the year and has a length of 4 or 5 days. The stages of the oestrous cycle in the rat can be determined by microscopical rating of the cell types that appear in the vaginal smear (see review 10). Pro-oestrus is characterized by a predominance of

nucleated epithelial cells. On oestrus, the dominant cell is the nonnucleated, cornified squamous epithelial cell. During metoestrus, leucocytes appear along with a significant number of cornified squamous epithelial cells. The stage of dioestrus is characterized by a predominance of small leucocytes interspersed by a few nucleated epithelial or cornified squamous epithelial cells.

At birth the ovaries contain approximately 35000 oocytes. The oocytes have entered into the prophase of meiosis I which is progressed to the dictyate or "resting stage" 4 days after birth (11). Only a small fraction of these viable oocytes will develop into mature ova. The oocyte is surrounded by a single layer of flattened epithelium-derived granulosa cells; the combined structure is called a primordial follicle. After transformation of the follicular epithelial cells into a single layer of cuboidal cells, the composite structure is referred to as a primary follicle. During each oestrous cycle a random group of primary follicles develops into secondary follicles; a fully developed mature follicle is known as a graafian follicle. After rupture of the mature follicle and liberation of the ovum during ovulation, the cells of the follicle are luteinized which leads to formation of the corpus luteum.

3.2 Hormonal regulation of the ovarian cycle (see reviews 10,12)

Luteinizing hormone and follicle stimulating hormone

The ovaries perform two basic functions: production and release of gametes and production and secretion of steroids. Both functions are under control of gonadotropic hormones i.e. luteinizing hormone (LH) and follicle stimulating hormone (FSH). Hypothalamic release of the gonadotropin releasing hormone (GnRH) induces the secretion of these gonadotropins from the anterior pituitary into the systemic circulation. The secretion of LH and FSH during the ovarian cycle of the rat is maintained at low levels on metoestrus, dioestrus, and the morning of pro-oestrus (Fig. 2). This basal gonadotropin secretion is regulated by gonadal steroids (oestrogen, progesterone and testosterone) through negative feedback action on hypothalamus and pituitary levels. On pro-oestrus this negative feedback action converts into a positive feedback by rising concentrations of circulating oestrogens, which are secreted by ripening follicles, and subsequently a surge of LH and FSH is induced (Fig. 2). The LH surge induces resumption of meiosis of the oocyte, triggers ovulation on the ensuing morning, and induces

transformation of the ovulated follicle into a corpus luteum (luteinization). Precocious follicle luteinization can lead to luteinized unruptured follicles (LUF). Mattheij et al. (14,15) showed that an injection of a small amount of LH in the morning of pro-oestrus causes LUF. The FSH surge is of influence upon the development of graafian follicles of the following oestrous cycle.

It has been known for many years that the preovulatory rise of oestrogen together with a daily signal generated by the biological clock are the primary determinants of GnRH secretion and the subsequent LH and FSH surge (16). It has been clearly established that the preovulatory surge of LH and FSH in intact rats occurs at a fixed time relative to the light-dark cycle. The onset of the pro-oestrous LH and FSH surge can be predicted from the known light-dark schedule i.e. approximately half of the light period plus two hours.

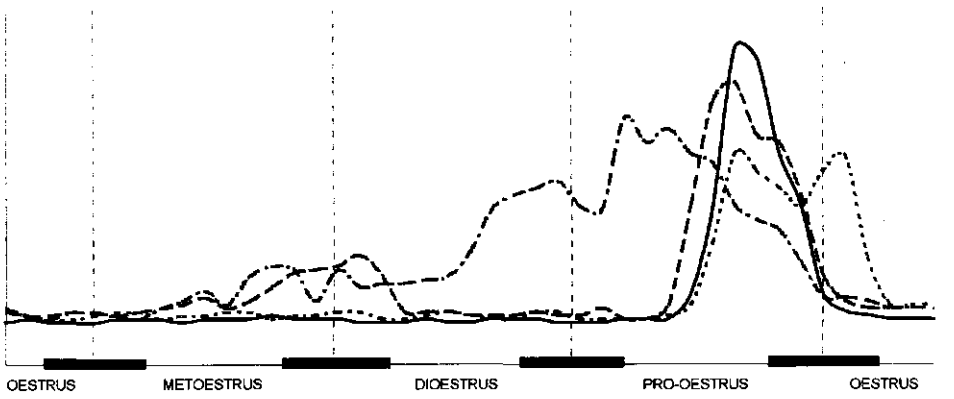

Fig. 2. Schematic diagram of the hormone profiles of LH (—), FSH (-----), oestradiol (— . —) and progesterone (— —) during the oestrous cycle of the rat. Black bar represents the dark period of the lighting cycle. (adapted from Smith et al. [13])

Gonadotropin-releasing hormone

GnRH provides a humoral link between the brain and the reproductive system. GnRH neurons in the rat are located in the diagonal band of Broca (DBB), the medial nucleus of the septum, the medial preoptic area (MPOA), and the anterior hypothalamic area (AHA), and also in the retrochiasmatic area of the medial hypothalamus (see reviews 10,17). In the rat, tracer studies showed that vir-

tually (>90%) all GnRH neurons project to the median eminence (ME) irrespective of sex, age or gonadal status of the animal (18). The GnRH neurons that project to the ME are concentrated basally in the midline regions from the MPOA rostrally and through the retrochiasmatic area caudally. GnRH is released in a pulsatile manner from the ME into the portal system and then transported to the anterior pituitary, where it stimulates release of LH and FSH.

GnRH neurons are regulated by oestrogens (and progesterone) but because these GnRH neurons do not have oestrogen receptors (19), it is believed that they are controlled by interneurons containing oestrogen receptors. At pro-oestrus there is a shift from a negative to a positive feedback action of oestrogen on the HPG axis (see reviews 10,12,20). A steady rise of oestrogens on pro-oestrus entrains the biological clock with neurosecretory events in peptidergic systems that participate in the induction of the LH and FSH surge. This positive feedback action of oestrogens requires the synergistic participation of progesterone (21). Increments of progesterone facilitate the neural event leading to hypersecretion of GnRH from the ME nerve terminals. Additionally, the increased levels of progesterone also prevent the recurrence of a LH and FSH surge the following day.

It was suggested that on pro-oestrus the biological clock operates in two stages to induce two distinct neurosecretory events (22). Initially, the clock disrupts the inhibitory influence of hypothalamic signals (including opioids) on the morning of pro-oestrus. A gradual restraint on the hypothalamic inhibitory systems may stimulate an increase in the rate of posttranslational processing leading to accumulation of GnRH in ME nerve terminals. The evidence that prevention of this disinhibition interrupted the accumulation of GnRH in the medial basal hypothalamus (MBH) is in accord with this suggestion (23). Subsequently, the clock may act again to augment neurochemical signalling for accelerated release of GnRH into the portal system.

The peptidergic pathways that propagate and transmit impulses for the GnRH surge reside in the suprachiasmatic nucleus (SCN) - medial preoptic nucleus (MPN) - arcuate nucleus (ARC) - MPOA - ME neural complex. The timely initiation of the impulses is entrained to the photoperiodic input reaching the SCN (biological clock). An increase in ME levels of GnRH is the most consistent earliest event, which occurs 1-2 h before the onset of the LH surge on pro-oestrus (24). This is observed in adult cyclic (25-27) and prepubertal rats (28).

The occurrence of this ME GnRH response is closely tied to the onset of a rise in portal GnRH levels. After stimulation of the MPOA on pro-oestrus the GnRH content of the ME is decreased (29), and GnRH release into the portal system is increased compared to other days of the cycle (30).

On pro-oestrus there is an increased pulsatile release of GnRH but also the anterior pituitary is sensitized to the actions of GnRH (31-33). The rapid increase in pituitary responsiveness to GnRH that occurs on pro-oestrus has been attributed to the acute self-priming action of GnRH, in combination with a positive effect of oestrogen at the level of the pituitary. Indeed, exposure of the pituitary to GnRH sensitizes the pituitary to further injections of GnRH (34). The sensitivity is also augmented by direct actions of progesterone (35,36) and there is evidence that the neuromediators galanin (37), pituitary adenylate cyclase activating peptide (38) and neuropeptide Y (39,40) enhance the responsiveness of gonadotropes to stimulation of GnRH. There is no evidence that the GnRH pulse frequency is increased during the initiation of the LH surge but the increase in GnRH release appears to take the form of pulses of increased amplitude (see review 41). Opioids may be involved in the increase of the amplitude of GnRH release that results from the positive feedback action of steroids (42).

A major difficulty when studying the GnRH neuronal system is the distribution of GnRH neurons and the high number of factors (e.g. classical neurotransmitters, neuropeptides, steroids, etc.) that have been reported to modulate directly or indirectly GnRH neurons at the level of the cell bodies or at their axonal endings in the ME (Fig. 3). There is extensive literature on the potential role of various neuromediators (neurotransmitters and neuropeptides) in the regulation of LH release (see reviews 20,44). Neurotransmitters involved in the regulation of LH release are for instance norepinephrine, epinephrine, dopamine, serotonin, histamine, gamma aminobutyric acid, and glutamate. Also neuropeptides are involved in the regulation of LH secretion, i.e. neuropeptide Y, galanin, oxytocin, vasopressin, vasoactive intestinal polypeptide, CRH and endogenous opioids. The modulation of GnRH release depends on complex interactions between several of these factors within a hypothalamic network. Depending on the steroid environment, these neuromediators may play facilitory or inhibitory roles in GnRH secretion.

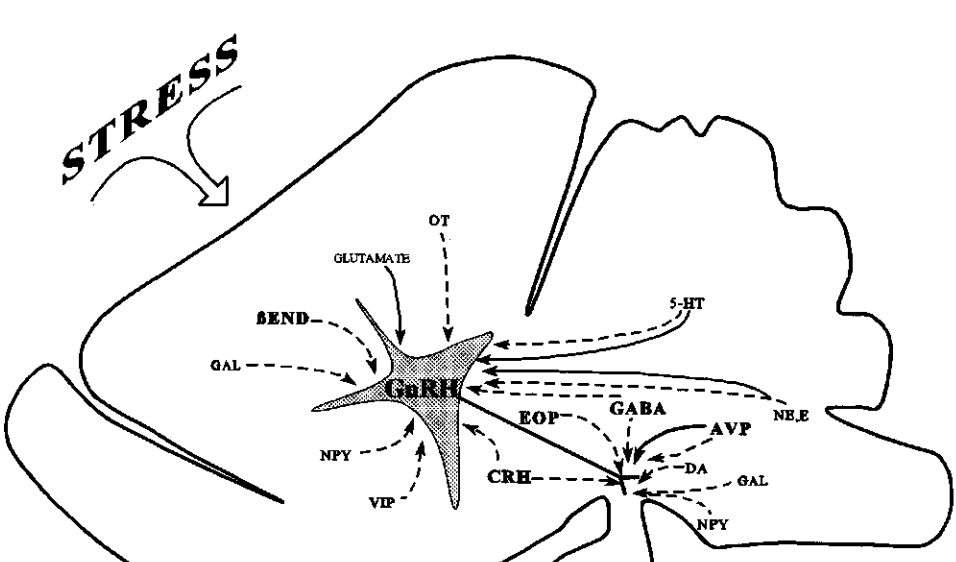

Fig. 3. Summary of factors which modulate directly or indirectly GnRH neuronal activity from either cell bodies or nerve terminals. Solid arrows, stimulatory effect; dashed arrows inhibitory effect. AVP, arginine vasopressin; ßEND, ß-endorphin; CRH, corticotropin-releasing hormone; DA, dopamine; E, epinephrine; EOP, endogenous opioid peptides; GABA, gamma aminobutyric acid; GAL, galanin; 5-HT, serotonin; NE, norepinephrine; NPY, neuropeptide Y; OT, oxytocin; VIP, vasoactive intestinal peptide. (modified after Rivest and Rivier [43])

4 Stress and reproduction

A well known consequence of stress is disruption of fertility. In animals, one of the earliest studies discussing the relationship between stress and reproduction, was based on the effects of overcrowding in both natural and captive animal populations (45). Undoubtedly reproduction in male and female is vulnerable to the effects of stress. A primary mechanism by which stress can disrupt reproduction is by altering the control of gonadotropin secretion (Fig. 3). In most studies an inhibiting effect of stress on gonadotropin secretion has been found

(table 1) but shortly after application of a stressor a small transient rise of basal LH release can be induced (table 1a,b: 46-49). During the oestrous cycle of the female rat stressors appear to have their greatest impact just prior to ovulation, when precise neuroendocrine control is most essential for reproductive success. However, the exact mechanism through which stress alters reproductive function remains largely unknown.

Many investigators have studied the effects of various stressors on LH and to a lesser extent FSH secretion in the rat (table 1a,b). Most of the studies have been performed with male rats or with ovariectomized or ovariectomized steroid-primed female rats. Only a few studies have used intact cyclic female rats to investigated the influence of stress (table 1a: 50,51) on the surge of gonadotropins. The effect of administration of potential stress-related neuromediators in the intact cyclic rat has been studied more detailed (table 2a,b). Stress can influence reproductive functions at the three levels of the HPG axis; at the hypothalamus to alter GnRH secretion, at the pituitary to interfere with GnRH-induced gonadotropin release, and at the gonads to alter the stimulatory effect of gonadotropins on steroid secretion.

The pathways via which stress influences reproductive functions have been subject of intense research for many years. Investigation of this relationship is complicated by the variety of responses an animal can display under stress and the complex regulation of the processes of reproduction. Selye (52) was the first who suggested a possible relationship between hormones and neuropeptides of the HPA axis, which are liberated during stress, and those involved in the HPG axis. Apart from glucocorticoids, indeed, CRH, the opioid β-endorphin (βEND) and vasopressin (AVP) may play a role in the effect of stress on reproductive functions (table 3, and sections below). Additionally, administration of CRH, AVP and βEND can inhibit the release of gonadotropins and ovulation (table 2a,b). Besides these hormones and peptides, various other factors may be involved in stress-induced inhibition of reproductive functions. Among these, the major inhibitory neurotransmitter of the brain, gamma aminobutyric acid (GABA) is a likely candidate. GABA can alter the activity of the HPA and HPG axis; GABA metabolism and binding and function of its receptors are influenced by several stressors (see review 53 and table 3).

Table 1a: Effect of stress on gonadotropin secretion and ovulation in the rat

Reference	Stressor	Compound	Gender	Site	Duration / Dose	Effect
50	immobilization		♀		3-4 h	ovulation ↓
	+ surgery				2 h	ovulation ↓
133	immobilization		♀		8 h/day 10 d	LH ↓, FSH = (plasma)
						GnRH = (hypothalmus)
46	ether		♀, ♂		3 min	LH ↑ (transient), FSH =
47	immobilization		♀ OVX		1 h	LH ↓
			♀ OVX + EB		1 h	LH ↑ (transient)
134	novelty		♂		30 min	LH ↑ (transient)
					30 min/day 10 d	LH =
	restraint				15 min	LH ↑ (transient)
					15 min/day 10 d	LH =
76	footshocks		♂ castr.		2 h	LH ↓
135	ether		♀ OVX		1 min	LH =
		+ CRF-Ab		iv		LH =
		+ CRF-Ab		icv		LH =
73	footschocks		♂ castr.		3 h	LH ↓
		+ α-helical CRH		icv	100 μg	LH =
48	ether		♀ OVX		1 min	LH ↑ (ns)
		+ AVP-Ab		icv		LH ↓
89	histamine		♂ Brattleboro	ip	6 mg/kg	LH =
			♂			LH ↓
51	footshocks		♀		3 h	LH surge ↓, ovulation ↓
		+ naloxone		iv	5 mg/kg	LH surge =, ovulaton =
111	immobilization		♀ + PMSG		3 h	ovulation ↓
		+ naloxone		ip	5 mg/kg	ovulation =
109	hyperprolactemia		♀ OVX			GnRH ↓
						LH ↓
		+ naloxone		sc	10 mg/kg	GnRH ↑, LH ↑

Table 1b: Effect of stress on gonadotropin secretion and ovulation in the rat

Reference	Stressor	Compound	Gender	Site	Duration / Dose	Effect
110	restraint		♂		8 h/day	LH ↓
					8 h/day 14 d	LH =
		+ naltrexone		iv	2 mg/kg	LH =
49	ether		♂		2 min	LH ↑ (transient)
		+ morphine		sc	5 mg/kg	LH =
	ether				15 min	LH ↓
		+ naltrexone		sc	2 mg/kg	LH =
	immobilization				8 h	LH ↑ (15'-60'), LH ↓ (6-8 h)
		+ naltrexone		sc	2 mg/kg	LH ↑↑
	food deprivation				5 days	LH ↓
		+ naltrexone			2 mg/kg 3x/day	LH =

OVX, ovariectomized; EB, estradiol benzoate; castr., castrated; Ab, antibody; α-helical CRH, CRH receptor antagonist; d, day; ns, non-significant; iv, intravenous; icv, intracerebroventricular; ip, intraperitoneal; sc., subcuteneous; PMSG, pregnant mare serum gonadotropin; ↑, increase, stimulation; ↓, decrease, inhibition; =, no change

Table 2a: Effect of stress-related neuropeptides on gonadotropin secretion, ovulation and βEND release in the rat

Reference	Compound	Gender	Site	Dose	Effect
71	CRH	♀	icv	7.5 μg	LH surge ↓, ovulation ↓
70	CRH	♀	icv	0.1-5 nmol	GnRH surge ↓, LH ↓
		♀ OVX		5 nmol	GnRH ↓, LH ↓
		♀ OVX + EB		5 nmol	GnRH ↓, LH ↓
72	CRH	♀ OVX	icv	5 μg	LH ↓
			iv		LH =
69	CRF	♂ castr.	icv	2 nmol	LH ↓
	CRF + βEND-Ab		icv		LH =
137	CRH	♂	sc	0.75 nmol/h 7 d	LH ↓, FSH =
76	CRH	♂	icv	0.3-30 ng	βEND ↑ (plasma)
					βEND = (CSF)
6	CRH	♂	iv	0.01-10 μg	βEND ↑ (plasma)
138	CRH	♂	in vitro	0.03-380 nmol	GnRH ↓
97	CRH	♂	in vitro	0.4 μmol	βEND ↑↑
	AVP			0.09 pmol-11 μmol	βEND ↑↑
	CRH + AVP-Ab			9,6 μmol	βEND ↑
76	AVP	♂	icv	10-1 pg	βEND = (plasma)
					βEND ↑ (CSF)
91	AVP	♀	iv	0.1 μg (4x)	LH surge =
				1 μg (4x)	LH surge ↓, ovulation ↓
92	AVP	♀	icv	1 or 5 μg (6x)	LH surge ↓
93	AVP	♀ OVX + EB + P	iv	1 μg (4x)	presurge LH ↑, LH surge ↓

Table 2b: Effect of stress-related neuropeptides on gonadotropin secretion and ovulation in the rat

Reference	Compound	Gender	Site	Dose	Effect
105	morphine	♀	sc	5, 8 or 40 mg/kg	LH surge ↓, FSH surge ↓
	+ naloxone		sc	10 mg/kg	LH surge =, FSH surge =
106	morphine	♀	ip	60 mg/kg	LH surge ↓, FSH surge ↓ ovulation ↓
	+ naloxone		sc	10 mg/kg	LH surge =, FSH surge = ovulation =
107	morphine	♀	iv	10 mg/kg	LH surge ↓, FSH surge =
	+ naloxone		iv	0.2 mg/kg	LH surge =
108	βEND	♀	icv	10 μg (3x)	LH surge ↓, ovulation ↓
	+ naloxone		sc	2 mg/kg	LH surge =, ovulation =
139	βEND	♀	icv	1 μg (2x)	LH surge ↓
		♀ OVX + EB		1 μg (2x)	LH ↓
		♀ OVX + EB + P		2 μg	LH surge ↓
123	GABA	♀	MPOA	10 μM	LH surge ↓
122	GABA	♀	icv	10 μg	LH surge ↓, ovulation ↓
	+ bicuculline		ip	6 mg/kg	LH surge =, ovulation =
124	baclofen	♀ + EB	icv	2 and 5 μg	LH surge ↓
125	muscimol	♀ + EB	icv	1 and 10 μg	LH surge ↓
126	muscimol	♀ + EB	MPN	50 ng	LH surge ↓
	+ bicuculline			30 ng	LH surge =

OVX, ovariectomized; EB, estradiol benzoate; P, progesterone; castr., castrated; Ab, antibody, d, day; iv, intravenous; icv, intracerebroventricular; ip, intraperitoneal; sc., subcuteneous; CSF, cerebrospinal fluid; MPOA, medial preoptic area; MPN, medial preoptic nucleus; ↑, increase, stimulation; ↓, decrease, inhibition; =, no change

Table 3: Effect of stress on ACTH, ßEND, CRH, AVP and GABA release in the rat

Reference	Stressor	Compound	Gender	Site	Duration / Dose	Effect
135	ether		♀ OVX		1 min	ACTH ↑↑
		+ CRF-Ab		iv		ACTH =
		+ CRF-Ab		icv		ACTH ↑
110	hyperprolactemia		♀ OVX			βEND ↑ (portal)
136	restraint		♂		2 or 5 min	CRH ↑, AVP = (plasma)
	ether				1 min	CRH ↑, AVP ↑ (transient)
127	pain		♂			GABA ↓ (hypothalamus)
128	immobilization		♂		3 h	GABA ↑ (hypothalamus)
129	restraint		♂		5 min	GABA = (hypothalamus)
130, 131	ether		♂		30 sec/day 20 d	GABA ↑ (hypothalamus)

Ab, antibody; *d, day*; ↑, *increase, stimulation*; ↓, *decrease, inhibition*; =, *no change*

4.1 Corticotropin-releasing hormone

In 1955, the first convincing demonstrations were provided of the existence of a factor derived from the hypothalamus that could elicit ACTH secretion from the pituitary (54, 55). This factor was named corticotropin-releasing factor (CRF) because of its ability to stimulate secretion of ACTH. In 1981, Vale et al. (7) succeeded in determining the amino acid sequence of ovine CRF and since then this factor is also indicated as corticotropin-releasing hormone (CRH). CRH is a 41-amino acid hormone derived from a 196-amino acid precursor. The amino acid sequence of this hormone has been determined in sheep, man, rat, pig, goat and cattle; in all it shows a high homology in structure (56). In the hypothalamus, it is synthesized in parvocellular neurons of the paraventricular nucleus (PVN) where it is colocalized with a variety of peptides, e.g. oxytocin and vasopressin (8,57-60). Paraventricular neurons project to the ME, and to the pituitary. In addition to its hypothalamic localization, CRH-containing cell bodies and binding sites have been identified in many extra-hypothalamic sites including the neocortex, areas of the limbic system involved in emotion and stress responses, such as the amygdala, nucleus accumbens, and hippocampus and regions involved in the regulation of the autonomic nervous system (locus ceruleus, nucleus of the solitary tract, olfactory bulb, cerebral cortex and the spinal cord) (5,59-62). The wide distribution of CRH and its binding sites in the CNS has implicated the hormone in the regulation of endocrine, autonomic and behavioural processes.

CRH is the major endogenous corticotropin releasing hormone, although a number of other factors (e.g. AVP, oxytocin (OXT) and endogenous opioids) participate in the regulation of ACTH release (62-64). ACTH subsequently elicits the secretion of glucocorticoids from the adrenal cortex which exert a negative feedback on hypothalamus and pituitary sites (66,67). Activation of the HPA axis is considered to be characteristic of the stress response and serves to counteract disturbances of homeostasis.

In addition to its ability to activate the HPA axis, central CRH may function as neurotransmitter in the brain. Indeed, central administration of CRH has indicated that the hormone has a variety of endocrine, physiological, neurochemical and behavioural activities that are not shared with ACTH and corticosterone. It has been recognized that a lot of the effects of CRH resembled those observed during stress, suggesting that CRH may be an endogenous mediator of such

responses (table 4).

The observation that intracerebroventricular (icv) injection of CRH suppresses the activity of the HPG axis in castrated male, and in intact and ovariectomized female rats (table 2a: 69-72) indicates that CRH plays a role in stress-induced inhibition of reproduction. In the pro-oestrous intact female rat, central administration of CRH attenuates LH secretion by inhibiting GnRH release into the hypophysial portal system (70). During stress conditions, central administration of the CRH antagonist α-helical CRH prevented the inhibition of LH release induced by footshocks in castrated male rats (73).

Table 4: Responses following CRH administration that resemble those observed during stress (adapted from Dunn and Berridge (67))

Endocrine	decreases LH (GnRH) secretion
	decreases growth hormone (growth-releasing hormone) secretion
	initiates the hypothalamic-pituitary-adrenal response
Physiological	increases large bowel transit and fecal excretion
	decreases gastric acid secretion, decreases gastric emptying and small intestinal transit
Electrophysiological	activates the EEG; seizures at higher doses
Neurochemical	increases the activity of cerebral noradrenergic and dopaminergic neurons
Behavioural	decreases sexual behaviour in males and females
	increases grooming
	decreases feeding
	increases locomotor activity
	decreases responding in a conflict test
	decreases social interaction
	enhances footshock-induced freezing

The origin of CRH neurons innervating GnRH neurons remains unclear (43). Also the mechanisms through which endogenous CRH can influence GnRH release are not fully understood. MacLusky et al. (74) demonstrated a direct anatomical connection between CRH axon terminals and dendrites of GnRH-

secreting neurons. However, the mechanisms through which CRH influences GnRH release are likely to involve the activation of other pathways, such as those dependent on vasopressin and/or endogenous opioids (69,75-77).

4.2 Vasopressin

In 1953, Du Vigneaud et al. isolated two hormones from the pituitary, AVP (arginine vasopressin) and OXT (78,79). AVP and OXT are peptides made up of nine amino acids. The sequence of AVP differs in only two positions of OXT. These differences are sufficient to markedly alter the biological activities, although AVP and OXT can act on each others' receptors due to chemical similarity in structure. AVP is synthesized in the supraoptic nucleus (SON) and PVN. Two distinct populations of neurons supply AVP to the anterior pituitary: parvicellular neurons from the PVN which terminate in the ME, and magnocellular neurons from the PVN and SON which terminate in the posterior pituitary (80). In addition, AVP is also distributed in extrahypothalamic structures (81-83).

A well-known effect of AVP on the pituitary is the stimulation of ACTH release and the potentiation of CRH effects. AVP modulates the effect of CRH on ACTH secretion and appears to take part in mediating the ACTH response to stress (see review 84). However, little attention has been paid on a role of AVP in stress-induced alterations of the HPG axis. Several studies suggest that the release of CRH and AVP in the portal system is increased during stress (85-88) but only a few studies are focused on a possible role for AVP in control of LH secretion during stress. In ovariectomized (OVX) rats, basal LH tended to increase following ether stress. LH levels were significantly lower, however, in rats pretreated with an AVP-antiserum than in rats pretreated with normal rabbit serum (49). In AVP deficient male Brattleboro rats, histamine stress had no inhibiting effect on LH whereas in control parent strain rats (Long Evans) basal LH was suppressed (89). In the OVX rhesus monkey, vasopressin antagonism prevented hypoglycemia-induced LH suppression (90). In addition, administration of high doses of AVP caused suppression of the LH surge and ovulation in intact and OVX steroid-primed female rats (table 2a: 91-93).

The actions of AVP, like those of CRH, are probably exerted at the level of the hypothalamus because AVP does not affect LH secretion from isolated pituitary tissue in vitro (49). Anatomical studies indicate that CRH neurons, which also contain AVP (94), lie in close apposition with β-endorphinergic neurons

within the hypothalamus (95). Moreover, AVP, like CRH (96,97), has been shown to stimulate the release of βEND from hypothalamic tissue in vitro (98) and in vivo (76). Burns et al. (77) demonstrate that CRH acts via AVP to release βEND from hypothalamic slices of male rats in vitro, and there is evidence that endogenous opioid peptides can mediate the CRH-induced inhibition of LH secretion (69, 70, 99, 100).

4.3 Endogenous opioid peptides

The first endogenous opioid peptides (EOP), the enkephalins, were isolated from brain tissue and sequenced in 1975 (101). At present three families of EOP are known. Each family is derived from a separate precursor: βEND is derived from pro-opiomelanocortin (POMC), enkephalins are derived from proenkephalin and dynorphins are derived from prodynorphin. Peptides derived from POMC include the opioid βEND and the non-opioid hormones ACTH and α-melanocyte stimulating hormone (α-MSH). All three families of EOP have been identified in the brain, each with a characteristic distribution within particular neural networks (see reviews 20,44) .

The multiple opioid effects are mediated by multiple opioid receptors. Opioid receptors have been subdivided into three "main" classes: μ, δ and κ receptors (102-104). Each of the three opioid peptide precursors generates predominantly ligands to one of the three opioid receptor types. Pro-enkephalin derived enkephalins bind preferentially to the δ-receptors, while pro-dynorphin derived opioids are all κ-ligands, and POMC derived opioids bind to the μ-receptors. This categorization was initially based on distinct behavioural syndromes correlated to different classes of opiate drugs. EOP and their receptors have also been localized in the hypothalamus suggesting that EOP may play a role in reproductive endocrinology. Research in this area during the last years has revealed many aspects of the role of EOP in regulation of reproduction which is reviewed by several authors, (see reviews 20,41,44). In summary, it has been demonstrated that EOP neurons tonically inhibit secretion of LH in the rat through intrahypothalamic inhibition of GnRH release, except for a brief interval during the pro-oestrous LH surge in the female or during the period of a progesterone-induced LH surge in the oestrogen-primed OVX rat. Using selective μ opioid agonists and antagonists it was demonstrated that mainly the μ-receptor is involved in the inhibition of hypothalamic GnRH secretion. Administration of the opioid receptor

antagonist naloxone stimulates LH release and can reverse the exogenous opioid induced suppression of LH secretion (105-108). A prevailing theory holds that the tonic EOP inhibition of GnRH release may be important in transmission of the negative feedback of gonadal steroids. The activation of the EOP system is, at least partly, dependent on gonadal steroids. Opioid agonists usually inhibit, and opioid antagonists stimulate LH secretion, but they fail to evoke an LH response in long-term OVX rats; the response is restored after substitution with oestradiol, or with the combination of oestradiol and progesterone. The primary action of EOP in the rat appears to involve suppression of the amplitude of GnRH release while gonadal steroids decelerate the rate of pulse generation. Therefore, it seems that opioids may play an important role in GnRH release that results from the positive feedback of gonadal steroids (41,42).

As GnRH neurons do not contain steroid receptors, the action of steroids must be mediated by other neurons. Opioids appear to modulate the regulation of GnRH directly (see reviews 41,44) at the hypothalamic level and/or by multiple interactions with other central regulatory mechanisms. Within the hypothalamus, opioids can affect the action of dopamine, noradrenaline, adrenaline, serotonin, GABA, neuropeptide Y, CRH, excitatory amino acids, etc.

Several studies have suggested a role for EOP in the effects of stress on reproductive parameters. Experimental evidence exists that stress-related inhibition of LH secretion is mainly mediated by μ opioid receptors. Pretreatment with naloxone or naltrexone (opioid receptor antagonists) which have a high affinity for μ-receptors, reverses inhibitory effects on LH by various stressors in mainly male rats and ovariectomized female rats (table 1a,b: 49,51,109-111). In the intact female rat, it was demonstrated that footshock stress alters reproductive function by inhibiting LH secretion through mediation of EOP (51). In addition, as mentioned above, opioids may be involved in the mediation of the inhibitory actions of stress-induced CRH and AVP release on GnRH secretion.

4.4 Gamma aminobutyric acid

GABA was discovered in the nervous system in 1950 (112). GABA is synthesized in neural tissues through decarboxylation of L-glutamic acid. The reaction is catalyzed by L-glutamic acid decarboxylase (GAD). GABA is degraded in the CNS by GABA-a-oxoglutarate transaminase (GABA-T). First evidence of the general inhibitory role of GABA was obtained from the observation that the

enzyme GAD was found in high concentration in inhibitory axons of the crayfish stretch receptor system, and not in excitatory axons (113). Later GABA was also localized in areas of the vertebrate nervous system with known inhibitory functions. From biochemical and electrophysiological studies, GABA has been shown to be widely distributed throughout the mammalian nervous system (see review 114). Neurochemical changes affecting the GABAergic system are judged by measurements of i.e. GABA concentrations, GABA turnover, or the activity of the enzyme GAD.

There are at least two kinds of receptor classes, $GABA_A$ (chloride channel-activating) and $GABA_B$ receptors (guanosine triphosphate coupled, also an auto-receptor to control the release of GABA itself from nerve endings) (115,116). The primary effect of central $GABA_B$ receptor activation is a diminution in membrane K^+ and Ca^{++} conductance. The $GABA_A$ receptor is a complex entity; in addition to the GABA recognition site it consists several other binding sites: a benzodiazepine receptor, receptor sites for convulsant and anticonvulsant drugs such as picrotoxin and barbiturates, and a receptor binding site for steroids (see reviews 53,115,116). Not all GABA receptor complexes contain all the components of the receptor complex as described above.

GABA appears to have a significant influence on pituitary hormone secretion, primarily through direct hypothalamic effects. It has been proposed that GABA neurons in the MPOA mediate the negative feedback action of oestrogen on GnRH (117,118). GAD-containing neurons were shown to have oestrogen receptors (118) and to synapse on GnRH-containing neurons (119). Additionally, GABA release in the preoptic hypothalamic region decreased coincident with the oestrogen-induced LH surge in OVX rats (117,120,121). Central administration of GABA in the intact female rat suppressed the LH surge and ovulation. Administration of the $GABA_A$ agonist muscimol or the $GABA_B$ agonist baclofen in the steroid-primed female rat caused also a suppression of the steroid-induced LH surge (table 2b: 122-126).

GABA inhibits the activity of the HPA axis, and therefore influences the stress response (see review 53). Many studies have been done on the effect of a variety of stressors on GABAergic functions in several brain regions. For instance, in the hypothalamus of male rats, pain reduced GABA levels whereas 5 min of restraint did not alter GABA levels. Three hours of immobilization stress or 30 sec/day of ether stress (during 20 days) induced increased hypothalamic GABA levels (table 3: 127-131). The effect of stress on the GABA content in

the hypothalamus seems to depend on the type of stressor applied and the stress protocol. Various stressors can also induce alterations in $GABA_A$ receptor binding and function, especially at the benzodiazepine binding site, which have been the subject of several studies (see review 53). However, little attention has been paid to the involvement of GABA as inhibitory neurotransmitter in the altered gonadotropin secretion during stress. In women with stress-related anovulation, treatment with alprozalam, a benzodiazepine, restored LH pulsatility, and increased mean LH pulse frequency and amplitude (132). In the latter, activation of the GABA receptor seemed to stimulate GnRH release by inhibition of the stress-induced CRH release. To our knowledge no studies have been done on the role of GABA as a mediator of the stress-induced inhibition of GnRH release in the female rat .

5 Outline of this thesis

Stress of physiological or emotional origin can influence the release of GnRH and subsequently that of LH and FSH. In the intact female rat, the LH surge on the day of pro-oestrus induces meiotic resumption of the oocyte, triggers ovulation and induces luteinization of the follicle. An inadequate LH surge may induce premature luteinization in graafian follicles and/or subsequently prevent ovulation. Very little research has been done with respect to the effect of stress on the pro-oestrous LH and FSH surge and subsequent effects on ovarian function in the intact female rat. It has been reported that footshock stress results in an inhibition of the pro-oestrous LH surge and ovulation (51) and that restraint (immobilization) stress on pro-oestrus inhibits ovulation (50,111).

In view of the scarce literature on the effect of stress on reproduction in the intact cyclic female rat, we first investigated in this thesis the effect of different periods of restraint stress on pre-ovulatory surge profiles of gonadotropins in the 5-day cyclic female rat and concomitant effects on ovarian histology (Chapter 2). Additionally, experiments were performed to gain more insight in the mechanism underlying the restraint-induced inhibition of the surge of gonadotropins. Therefore, the role of several neuropeptides known to be released in the brain during conditions of stress was investigated. First, the effect of central administration of exogenous CRH in pro-oestrous rats was studied (Chapter 3). Icv injections of CRH were given just before the presumed onset of the LH surge.

Also the effect was studied of infusions with CRH which were started before the presumed onset of the LH surge and continued until the beginning of the dark period thereby covering most of the period during which the surge appears. Because a role for AVP in mediating the effect of CRH on LH secretion is suggested, rats were pretreated with an AVP-antiserum before administration of CRH. Subsequently, the role of CRH and AVP in the restraint-induced suppression of the pro-oestrous LH surge was studied (Chapter 4). Therefore, restrained rats were pretreated with the CRH antagonist, α-helical CRH or with AVP-antiserum.

A role for endogenous opioids as mediator in the inhibitory effect of stress on basal and pro-oestrous LH release has also been suggested. In Chapter 5, we studied the effect of pretreatment with the opioid antagonist, naloxone and its longer acting analog naltrexone on the inhibitory effect of restraint on the LH surge.

Finally, GABA appears to be involved as inhibitory neurotransmitter in the regulation of GnRH release. During the application of several stressors the GABAergic system is altered. Since an interaction between the HPG axis and GABAergic modulation of the stress response have been hardly investigated, a possible involvement of GABA in the restraint-induced inhibition of the LH surge was studied by pretreatment with a $GABA_A$ and $GABA_B$ antagonist (Chapter 6).

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Chapter 2

Effect of restraint stress on the preovulatory LH profile and ovulation in the rat

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Abstract

Plasma profiles of luteinizing hormone (LH) and follicle stimulating hormone (FSH) were measured during restraint stress on the day of pro-oestrus; these profiles were considered in relation to ovulation rate on the next day. Rats bearing a permanent jugular vein cannula were subjected to restraint which was started 0, 1 or 2 h before the presumed onset of the LH surge and ended just before the beginning of the dark period. Exposure to restraint resulted in a suppression of the secretion of both gonadotropins on the day of pro-oestrus. Suppression of the LH surge was virtually complete (plasma LH $\leq$ 0.2 ng/ml) in 15 out of 32 stressed rats, and the ovaries of these rats contained graafian follicles with oocytes in germinal vesicle stage. In these rats, the LH surge did not occur 24 h later. In the remaining 17 rats, restraint resulted in a considerable suppression of the LH surge. Of these rats, 5 had an ovulation rate of 100% and 4 ovulated partially. In unruptured follicles of the latter, the oocyte had not resumed meiosis and the follicle wall was not luteinized. In the remaining 8 rats with a reduced LH surge, ovulations had not occurred and graafian follicles were unaffected. The results of this study indicate that during pro-oestrus restraint stress suppresses and does not delay the release of preovulatory gonadotropins. Partial suppression of LH by restraint does not result in induction of meiotic resumption without subsequent ovulation or in luteinized unruptured follicles.

Introduction

Stress of physical or emotional origin may interfere with reproductive functions (1,2). Stress can influence the release of gonadotropin releasing hormone (GnRH) (3) and so of luteinizing hormone (LH) and follicle stimulating hormone (FSH). Most studies have been performed in male or in ovariectomized female animals (4-6). Little research has been done into the effect of stress on the pro-oestrous LH and FSH surge in intact female rats. In adult cyclic rats, unpredictable footshocks applied on pro-oestrus caused a partial inhibition of the LH surge and of subsequent ovulation (7). After exposure to immobilization stress on the day of pro-oestrus ovulation was blocked and delayed for one day, implying also a delay of the LH surge by one day (8).

In the intact female rat, the LH surge on the day of pro-oestrus triggers ovu-

lation. LH also induces meiotic resumption of the oocyte and can induce premature luteinization in graafian follicles. In graafian follicles, meiotic resumption and luteinization have been shown to start after a dose of LH smaller than needed for ovulation (9,10). If restraint stress partially suppresses the LH surge, it may conceivably induce either meiotic resumption without subsequent ovulation or luteinized unruptured follicles (LUF). In the present study, we investigated the effect of restraint stress on preovulatory surge profiles of gonadotropins in rats. Different starting times of restraint on pro-oestrus were chosen with the aim of obtaining a variety of moderately to strongly reduced LH surge profiles. In these rats, we wanted to investigate whether a partially suppressed LH surge might induce meiotic resumption and/or luteinization without ovulation. To that end the ovaries were collected for histology the day after restraint. Occurrence of ovulation, meiotic resumption without subsequent ovulation and LUF were studied in relation to the LH profiles.

Materials and Methods

Rats, housing and surgery

The experiments were performed with female F1 hybrids (6-8 months of age, 200-250 g body weight) of two Wistar substrains (U-inbred males and R-inbred females) from the University breeding colony. They were maintained in a controlled temperature environment (22 ± 1 °C) with lights on from 0000 to 1400 h. With this light regimen these rats have 5-day cycles and the onset of the gonadotropin surge is at around 0900 h, i.e. 2 h after the middle of the light period (11). The rats were individually housed and received standard food pellets and tap water ad libitum. A dim light was left on during the dark period to facilitate blood sampling.

Rats were provided with a jugular vein cannula according to the method of Steffens (12) with some modifications (13) under ketamine (40-60 mg/kg intraperitoneal) and xylazine (210 µl subcutaneous of a 2% solution diluted with 2 volumes of saline) anaesthesia. Starting one day after surgery, the rats were handled daily to minimize stress during blood sampling. Oestrous cyclicity was assessed each day by inspection of vaginal smears and observation of lordosis behaviour induced after introduction of a male rat for a brief period in the cage. Rats that exhibited at least two consecutive 5-day cycles following surgery,

were used for experimentation.

Restraint procedure

After being connected to a blood sampling cannula, individual rats were placed in a perspex cylinder of 4.8 cm inner diameter. One end of the cylinder was cone-shaped and provided with perforations to facilitate air supply to the rat. The other end of the cylinder contained outlet-drains for urine and an opening for the tail of the rat. A slit (0.7 cm wide) running along the length of the cylinder enabled fixation of a partition-wall to adjust the interior length of the cylinder to the size of the rat. The blood sampling cannula was exteriorized via the slit.

Experimental protocol

The experiments were conducted after approval by the University Committee on Animal Care and Use (DEC).

Rats (2-5 rats at a time) were transferred to a novel experimental room and immediately placed in a restraint cylinder. The restraint was started at 0, 1 or 2 h before the onset of the LH surge, which was anticipated to begin at approximately 0900 h. The rats were released and returned to their home cage shortly before the onset of the dark period at 1400 h. After a recovery period of two hours, oestrous behaviour (lordosis) was assessed by allowing a male into the cage. Blood samples were taken hourly from 0900 h up to 1700 h and also on the next day at 1100, 1200 and 1300 h. After the 1300 h sample the rats were anaesthetized by ether and killed by cervical dislocation. Ovaries were collected for histological examination. Controls were cannulated rats in pro-oestrus of which blood samples were taken hourly in their home cage from 0900 to 1700 h and they were killed the next day at 1300 h.

Radioimmunoassay

Blood samples (150 μl) were collected in ice-cooled heparinized tubes. After centrifugation plasma samples (60 μl) were diluted with 3 volumes of phosphate-saline buffer (pH 7.5) containing 0.1% bovine serum albumin (Sigma RIA-grade) and stored at -20°C. LH and FSH levels were measured, using kits provided by the NIDDK. rLH-RP-2 and rFSH-RP-2 diluted in serum of hypophysectomized rats were used as reference materials. The second antibody was donkey-anti-rabbit (Sac-Cel®, Wellcome Reagents, Beckenham, GB). Determina-

tions were performed in triplicate. Quality control sera with low, medium and high LH or FSH concentrations were included in each RIA. Assay sensitivity at 90% B/B_0 was 0.05 ng/tube for LH and 0.4 ng/tube for FSH. In the diluted plasma samples, concentrations lower than 0.2 ng LH/ml and lower than 1.6 ng FSH/ml were considered as baseline levels. The intra-assay coefficients of variation for LH and FSH were 4.3% and 5.7%, respectively.

Histology

Ovaries were fixed in Bouin's solution and embedded in paraffin wax. Serial sections (8-10 μm) were stained with hematoxylin (Gurr, GB) and eosin and mounted in DePeX (Gurr, GB). In both ovaries of each rat, graafian follicles with or without meiotic resumption, luteinized unruptured follicles and ruptured follicles were counted; the latter were compact structures containing numerous dark-staining nuclei and these structures could easily be distinguished from corpora lutea of preceding cycles (for details see Mattheij et al., (9)).

Statistical analysis

LH and FSH peak values were based on the value from zero to maximum LH respectively FSH value. To compare the response curves of LH and FSH to restraint stress the individual data of the surge between 0900 and 1700 h were expressed as the area under the curve (AUC). The AUC is the integrated area between the baseline and the LH/FSH response above the baseline. The AUC of LH and FSH, the peak values of LH and FSH, and ovulation rate were analyzed by the Kruskal-Wallis test (one-way analysis of variance), with nonparametric comparisons made, using the Mann-Whitney U-test when trends were found to be significant. For statistical analyses the Statistical Program System for the Social Sciences (SPSS/PC+ V2.0, SPSS, Inc., Chicago, IL) was used. Significance was defined at the 0.05 level. Values are reported as means ± SEM.

Results

Figure 1 illustrates the effect of restraint on plasma LH during pro-oestrus. In individual control rats, the peak value of LH ranged between 7.5 and 10.5 ng/ml and was reached between 1100 and 1400 h. In restraint rats, the highest plasma LH levels ranged between basal and 4.3 ng/ml. Exposure to restraint

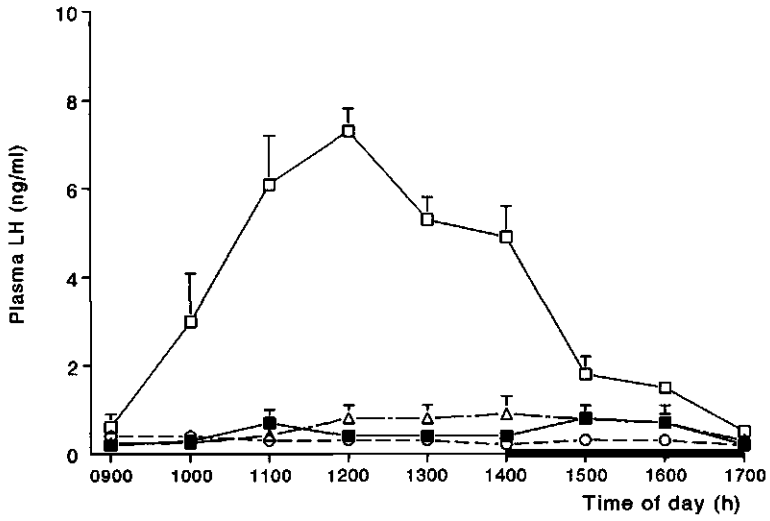

Fig. 1. Profiles of rat plasma LH during pro-oestrus: (□) controls (N = 8); (○) restraint 0900-1400 h (N = 10); (△) restraint 0800-1400 h (N = 11); (■) restraint 0700-1400 h (N = 11). Black horizontal bar represents dark period; data are given as mean ± SEM.

resulted in a significant inhibition of the LH response (peak value and AUC; both $P < 0.01$ compared to controls) in all experimental groups. No significant overall differences were observed between the three groups with different starting times of restraint.

A considerable variation of individual LH profiles was observed in all restraint groups. Restraint completely blocked the LH surge (plasma LH ≤ 0.2 ng/ml) in 15 out of 32 rats. In 10 rats, maximum LH levels varied between 0.5 and 1.7 ng/ml. Maximum LH levels between 2.1 and 4.3 ng/ml were reached in 7 rats. Figure 2 illustrates LH profiles of 6 individual rats, in which restraint did not completely suppress the LH surge. In stressed rats suppressed maximum LH levels were reached at any time point between 0900 and 1600 h. The rat in figure 2b shows an advanced onset of the LH surge. In all restraint-stressed rats LH levels were low (0.2-0.4 ng/ml) on the next day at 1100, 1200 and 1300 h. This indicates that the restraint had not delayed the surge of LH by 24 h.

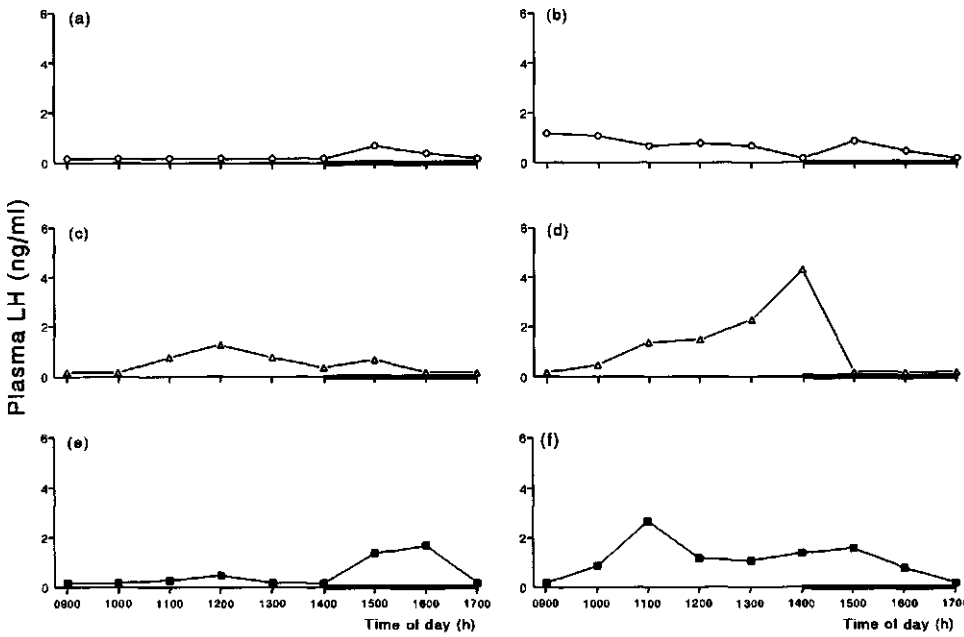

Fig. 2. Release pattern of LH in six individual rats of which restraint suppressed the LH surge partially: (O; a, b) 5-h restraint; (Δ; c, d) 6-h restraint; (■; e, f) 7-h restraint. Black horizontal bar represents dark period.

As shown in figure 3, in control rats plasma FSH increased gradually on the day of pro-oestrus and restraint significantly suppressed this gradual FSH rise ($P < 0.01$). FSH was 2.9 ± 0.1 ng/ml at 1100, 1200 and 1300 h the day after pro-oestrus. No correlation was observed between the peak and AUC values of LH and FSH.

In Table 1 the data on the ovaries, peak LH concentration in the plasma, relative amount of LH released during LH surge (AUC) and ovulation rate are given of controls and stressed rats. FSH data are not given because no correlation was seen between ovulation rate and peak or AUC values of FSH. In control rats, virtually 100% ovulation had occurred. Ovulation rate was significantly reduced in the restraint groups ($P < 0.05$). No significant differences were found between the three restraint groups with regard to ovulation rate, so they were considered as one group. In the 15 rats which had LH levels ≤ 0.2 ng/ml, the graafian follicles were unaffected, i.e. they contained an oocyte with germinal vesicle and showed no luteinization. In 7 rats with a maximum LH level between

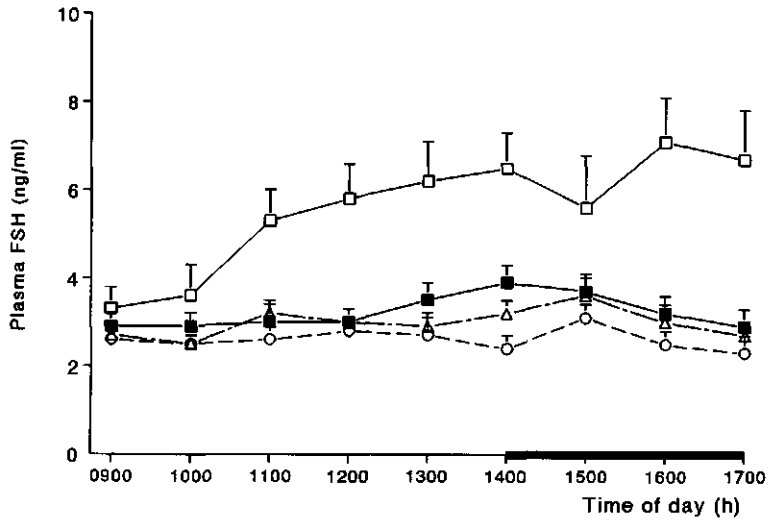

Fig. 3. Profiles of rat plasma FSH during pro-oestrus: (□) controls (N = 8); (○) restraint 0900-1400 h (N = 10); (△) restraint 0800-1400 h (N = 11); (■) restraint 0700-1400 h (N = 11). Black horizontal bar represents dark period; data are given as mean ± SEM.

0.5 and 1.7 ng/ml and an AUC between 0.4 and 3.1, the graafian follicles were also apparently unaffected. Exceptions were 2 rats with a maximum LH level of 0.5 and 1.3 ng/ml and an AUC of 0.8 and 3.0 in which respectively 100% and 17% of the graafian follicles had ovulated. Rats with a peak LH level > 2 ng/ml or an AUC of ≥ 4.0 generally showed a high ovulation rate, with the exception of one rat with a peak level of 2.8 ng/ml and an AUC of 4.0 which had not ovulated. In rats with partial ovulation, most of the unruptured graafian follicles were unaffected. Very few luteinized unruptured follicles were observed; they contained an oocyte in metaphase-II stage.

A noteworthy trend was that the number of rats that had a relatively high LH peak, AUC and ovulation rate, was greater in the 6-h and 7-h restraint group than in the 5-h restraint group.

On pro-oestrus all control rats and 70% of the stressed rats showed lordosis when a male was introduced in their home cage at 1600 h.

TABLE 1. Effect of restraint applied during pro-oestrus on plasma LH and subsequent ovulation in the rat

	N	Plasma LH during "surge time"		Number of large follicles (GF + RF + LUF)	Number of Graafian follicles (GF)	Number of ruptured follicles (RF)	Number of luteinized unruptured follicles (LUF)	Ovulation rate (%)
		Peak height (ng/ml)	Area under curve (arbitrary units)					
Control	8	8.4 ± 0.4	27.5 ± 1.6	12.4 ± 0.4	0.8 ± 0.4	11.9 ± 0.6	0	96 ± 3.4
Restraint 5 h	5	0.2 ± 0.0	0.0	13.2 ± 0.5	13.2 ± 0.5	0	0	0
(0900-1400 h)	1	0.5	0.4	12	12	0	0	0
	1	0.5	1.1	12	12	0	0	0
	1	0.6	0.5	12	12	0	0	0
	1 (a)	0.7	0.7	12	11	0	1	0
	1 (b)	1.2	4.0	14	1	12	1	86
Restraint 6 h	4	0.2 ± 0.0	0.0	12.5 ± 1.0	12.5 ± 1.0	0	0	0
(0800-1400 h)	1	0.5	0.8	12	0	12	0	100
	1	0.6	1.8	13	13	0	0	0
	1 (c)	1.3	3.0	12	9	2	1	17
	1	1.4	3.0	13	13	0	0	0
	1	2.8	4.3	11	11	0	0	0
	1	3.5	13.3	14	4	10	0	71
	1 (d)	4.3	9.0	13	0	13	0	100
Restraint 7 h	6	0.2 ± 0.0	0.0	12.5 ± 1.0	12.5 ± 1.0	0	0	0
(0700-1400 h)	1 (e)	1.7	3.1	12	12	0	0	0
	1	2.1	3.1	12	0	11	1	92
	1	2.4	4.1	12	0	12	0	100
	1	2.6	6.0	12	0	12	0	100
	1 (f)	2.7	8.3	13	0	13	0	100

Means ± SEM are given for control rats and for stressed rats with completely suppressed LH surge; individual data are given for rats with partially suppressed LH surge. "Surge time" is the period of the day of pro-oestrus at which the LH surge was presumed to occur. Ovulation rate is the number of RF versus the number of large follicles. Character between brackets refers to corresponding LH profile in figure 2.

Discussion

In male and ovariectomized female rats LH secretion is suppressed and the FSH secretion has been found not to be affected by various stressors (5,14-17). In the present study, exposure of cyclic rats to restraint on the day of pro-oestrus resulted in a strong inhibition of both the LH and the FSH surge. Hulse and Coleman (7) observed a partial suppression of the LH surge in cyclic rats subjected to inescapable footshocks for 3 h during the surge on pro-oestrus. To the authors' knowledge, a suppressive effect of stress on the FSH surge of cyclic rats has not been reported before. A reduced FSH surge may conceivably cause a decrease of the number of ovulations in the next cycle.

Transferring the rats to a novel experimental room before restraint may be an additional stressor. With respect to restraint stress the effect of novel environment on the LH surge is probably small because we observed no effect of moving the rats to a novel experimental room in pilot studies.

Restraint totally blocked the LH surge in 15 rats. These rats did not have elevated LH and FSH levels the next day, indicating that the restraint had not delayed the surge of gonadotropins by one day. The complete absence of ovulations and unruptured follicles containing an oocyte in metaphase indicates that in these rats no substantial gonadotropin secretion had occurred between the period of blood sampling on pro-oestrus and the sampling period on the next day. This corroborates the view that in the rat the preovulatory surge of LH occurs in a fixed period of the day relative to the light-dark cycle (18).

In contrast to our results, Yonetani et al. (8) reported that forced immobilization for 4 h starting at the beginning of the LH surge delayed the LH surge by one day. These discrepant results may be due to a strain-difference or may reflect a difference between the stress-model used, i.e. restraint in a cylinder versus tying up in a supine position. The preovulatory LH surge is blocked and delayed by 24 h in 4- and 5-day cyclic rats after injection of Nembutal on the day of pro-oestrus (19,20). After injection of Nembutal on pro-oestrus, LH and FSH are suppressed but oestradiol levels remain relatively high; apparently oestradiol remains sufficiently high to evoke an LH surge on the day after pro-oestrus (21). In the present study, the absence of an LH surge on the day after restraint is hard to explain. It may have been caused by restraint-stimulated corticosterone secretion (22); increased corticosterone may inhibit FSH-induced aromatase activity and consequently oestrogen production in granulosa cells

(23). However the corticosterone increase during restraint is transient (24); it is unlikely that a relative brief rise of corticosterone caused a lasting suppression of oestrogen synthesis. Stress-induced suppression of gonadotropins might have caused atresia of follicles and so a decrease of oestrogen synthetic capacity. This is also unlikely because after Nembutal-induced suppression of gonadotropins an LH and FSH surge occurred the next day (21).

Ovulation is initiated by a gonadotropin surge on the day of pro-oestrus; the magnitude of the LH surge is considerably larger than needed to cause full ovulation. Greig and Weisz (25) reported that approximately 14% of the peak LH value is sufficient to induce ovulation which agrees with the results of the present study. In the present study, it appears that also approximately 14% of the control AUC value is sufficient to trigger ovulation. The LH surge induces meiotic resumption of the oocyte in graafian follicles. In Nembutal-anesthesized rats, a dose-related effect of LH on meiotic resumption, luteinization and ovulation was reported (9,10): injection of 1 μg LH caused meiotic resumption but no ovulation; 2 μg LH caused ovulation of some follicles and meiotic resumption plus luteinization but no ovulation of the other graafian follicles; 4 μg LH caused more ovulations. Based on these data we expected that no ovulations but meiotic resumption with or without luteinization should have been induced in the rat with a small rise of LH during pro-oestrus. However the present data show that after a considerably reduced LH surge either all graafian follicles ovulated, or the graafian follicles appeared unaffected; they contained an oocyte in germinal vesicle stage and were not luteinized. The explanation of the discrepancy between Mattheij et al. (9,10) and the present results may be that in the former studies injection of LH caused a steep and short-lasting (less than 60 minutes) increase of LH in the plasma whereas in the present study the rats with a partially suppressed LH surge had a slightly increased LH level for several hours. The discrepancy may also be due to the fact that Mattheij et al. (9,10) injected LH 8 h before the presumed onset of the LH surge. Conceivably at that time fewer LH receptors were present in the granulosa layer of the graafian follicles than at the beginning of the presumed onset of the LH surge (26).

In the present study, 70% of the stressed rats showed lordosis 2 h after the end of restraint while they had a reduced or totally suppressed LH surge. In the cyclic rat, oestradiol and progesterone (27,28) together with GnRH (29) initiate and intensify oestrous behaviour. In 15 rats, restraint completely suppressed the LH surge. So an increase of the GnRH secretion in the hypophyseal portal sys-

tem and a consequent increase of ovarian progesterone did presumably not occur. Yet 13 of these rats showed lordosis. The elevated plasma oestrogen on pro-oestrus together with a stress-induced increase of the production of adrenal progesterone (27) or other adrenal steroids (30) may have induced lordosis in these rats.

We conclude that restraint stress inhibited and did not delay gonadotropin secretion on pro-oestrus. Partial inhibition of LH secretion by restraint is not followed by induction of meiotic resumption without subsequent ovulation or by luteinized unruptured follicles.

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Chapter 3

Effect of CRH on the preovulatory LH and FSH surge in the cyclic rat: a role for arginine vasopressin?

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Abstract

The effect of intracerebroventricular (icv) injection or infusion of various doses of corticotropin-releasing hormone (CRH) on the LH and FSH surge was studied in pro-oestrous rats supplied with a jugular vein and an icv cannula. Additionally, we investigated if arginine vasopressin (AVP) was involved in the CRH-induced alterations to the surge of gonadotropins. Icv injection of 10 μg CRH given 5 min before the presumed onset of the LH surge caused a strong inhibition of the LH surge and a slight inhibition of the FSH surge. Three to four h after CRH injection, its inhibitory effect diminished. A 6 h icv infusion of CRH started 1 h before the presumed onset of the LH surge, caused a dose-related inhibition of the LH and FSH surge. Infusion of 1 μg/h CRH did not suppress the surge of both hormones while infusion of 5 or 10 μg/h CRH inhibited the LH surge. Infusion of 10 μg/h CRH caused a strong suppression of plasma LH during the first 3 h of the LH surge. Despite continuation of CRH infusion, the inhibitory effect disappeared and plasma LH increased to similar levels as in controls at corresponding points of time of the LH surge. The FSH surge was also suppressed by infusion of 10 μg/h CRH. The surge of LH and FSH was not affected by a 9 h infusion of 10 μg/h CRH started 4 h before the presumed onset of the LH surge. This observation also indicates that the inhibitory effect of CRH may last for only 3-4 h. The surge of LH and FSH was not affected by icv injections of AVP-antiserum. However, pretreatment with AVP-antiserum prolonged the inhibitory effect of CRH on the LH surge. In conclusion, CRH can inhibit the pro-oestrous LH and to a lesser extent the FSH rise for only 3-4 h after the beginning of CRH administration. AVP may play a role in limiting the inhibitory effect of CRH on LH to 3-4 h.

Introduction

An important component of the adaptive response to stress is the activation of the hypothalamic-pituitary-adrenal (HPA) axis (1). A major neurohormonal regulator of adrenocorticotropic hormone (ACTH) secretion from the pituitary is CRH. CRH is widely distributed within the central nervous system (2-4), indicating that it is involved in brain functions other than the hypothalamic regulation of ACTH secretion (5). CRH also plays a role in modulating stress-induced inhi-

bition of the hypothalamic-pituitary-gonadal (HPG) axis (6,7). Icv administration of CRH to male or ovariectomized (OVX) rats resulted in inhibition of luteinizing hormone (LH) secretion (8-10), but not of follicle stimulating hormone (FSH) (8, 9,11). Prolonged icv CRH administration decreased LH but not FSH secretion in male rats (12,13). Long-term administration of CRH can cause downregulation of pituitary CRH receptors (14,15) and also of brain CRH receptors (16). So, it is conceivable that continuous exposure of the brain to high CRH levels may alter the effect of CRH on the pro-oestrous LH and FSH surge.

The central mechanisms mediating the CRH-induced suppression of gonadotropin secretion are only partly understood. Endogenous opioid peptides are involved in the inhibition of LH secretion by CRH (10,17-19). AVP may mediate the CRH-evoked β-endorphin release from the hypothalamus (20). Additionally, intravenous (21) or icv (22) administration of AVP resulted in an inhibition of the pro-oestrous LH surge in cyclic rats. On the other hand, AVP may also contribute to downregulation of CRH receptors (23, 24).

The present study aimed to assess to which extent icv injection or infusion of CRH can suppress the pro-oestrous surge of LH and FSH in cyclic rats. Also the effect of relatively long CRH infusion on the LH and FSH surge was examined. In addition, we investigated if an AVP-antiserum could modify the effect of CRH injection or prolonged CRH infusion on the pro-oestrous surge of LH and FSH.

Materials and Methods

Rats, housing and surgery

The experiments were performed with female F1 hybrids (6-8 months of age, 200-250 g body weight) of two Wistar substrains (U-inbred males and R-inbred females) from the University breeding colony. They were maintained in a controlled temperature environment ($22 \pm 1°C$) and at a photoperiod of 14 hours light per day (lights on at 0000 h). At this light regimen most of these rats have 5-day cycles and the onset of the gonadotropin surge is at approximately 0900 h (25). The rats were individually housed with free access to standard food pellets and drinking water. A dim light was left on during the dark period to facilitate blood sampling.

Preceding surgery, rats were anaesthetized with ketamine (40-60 mg/kg intraperitoneal) and xylazine (Rompun®, 1.4 mg/rat subcutaneous). They were fitted

with a silastic jugular vein cannula according to the method of Steffens (33) with some modifications (34), and an icv stainless steel cannula (23-gauche) in the right lateral ventricle (35). Starting two days after surgery, the rats were handled every day to minimize stress during blood sampling. In addition, oestrous cyclicity was monitored daily by vaginal lavage and observation of oestrous behaviour after introduction of a male rat in the cage. Oestrous cyclicity was assessed between 1200 and 1300 h preceding and during the experiments. Rats that exhibited at least two consecutive 5-day cycles following surgery, were used for experimentation. Correct location of the icv cannula was routinely checked after each experiment by injection of 3 μl Evans blue solution (10% in saline) and macroscopic inspection of the ventricular system after decapitation. Only data obtained from rats with a correctly placed icv cannula were included in the analysis.

Chemicals

The compounds used were: Synthetic rat/human CRH (1-41); Sigma Chemical Company, St Louis, USA; MW: 4757.5; Arginine vasopressin (AVP)-antiserum (W3) raised in a rabbit at the Rudolf Magnus Institute, University of Utrecht, The Netherlands. It had no cross-reaction with oxytocin and des-glycinamide-vasopressin, 6% with lysine vasopressin and 120% with vasotocin. These data were obtained under RIA conditions with iodinated AVP as competitor; AVP-antiserum (W3) was used at a concentration of 1:70.000.

Saline (0.9%) served as solvent and control icv solution. AVP-antiserum and normal rabbit serum (NRS) used in the experiments were diluted with one volume of saline. All injections and infusions were given icv. Injections were given at a volume of 3 μl using a 10 μl Hamilton syringe; icv infusion rate was 26 μl/h using a Precidor® micro-infusion pump.

Experimental Procedures

The experiments were conducted after approval by the University Committee on Animal Care and Use (DEC) of the Wageningen Agricultural University.

Experiment 1: Effect of a single icv injection of CRH on the LH and FSH surge in pro-oestrous rats.

CRH 0.1 or 10 μg was administered in conscious, freely moving rats 5 min before the presumed onset of the LH surge (at 0900 h). Rats injected with saline

and untreated rats served as control. Blood samples were taken every half hour from the beginning of the LH surge to 30 min before the dark period.

Experiment 2: Effect of icv infusion of CRH on the LH and FSH surge in pro-oestrous rats.

A 6-hour infusion of 1, 5 or 10 μg/h CRH was given from 0800 to 1400 h. Blood samples were taken hourly from 0900 to 1600 h. Controls received a 6-hour infusion of saline. In addition, a group of rats without infusion was included.

Experiment 3: Effect of pretreatment with AVP-antiserum on the CRH-induced suppression of the plasma LH and FSH surge on pro-oestrus.

This experiment consisted of 5 groups. The experimental design for this study is depicted in Table 1. Blood samples were obtained at 1 h intervals during the LH surge period (from 0900 to 1700 h).

TABLE 1. Experimental Design of Experiment 3

Group	icv pretreatment at t=-24 h* and t=-0.5 h*	icv injection at t=-5 min*
1	saline	saline
2	NRS	saline
3	AVP-antiserum	saline
4	NRS	10 μg CRH
5	AVP-antiserum	10 μg CRH

* time before the presumed onset of the LH surge at 0900 h

Experiment 4: Effect of 9 h icv infusion of CRH preceded by treatment with AVP-antiserum on the LH and FSH surge on pro-oestrus.

Two injections of AVP-antiserum or NRS were given. The first injection was given 24 h and the second injection 4.5 h before the onset of the LH surge. 30 Min after the second injection, a 9 h infusion of 10 μg/h CRH was started. The control group received two injections of saline but not a subsequent 9-hour infusion of saline. In pilot experiments we had found that prolonged infusion of

saline did not influence the surge of gonadotropins. Blood samples were obtained at 1 h intervals during the LH surge period.

Radioimmunoassay

Blood samples (160 μl) were collected in heparinized tubes at 4°C. After centrifugation, plasma samples (70 μl) were diluted with 3 volumes of phosphate-saline buffer (pH 7.5) containing 0.1% bovine serum albumin (Sigma RIA-grade) and stored at -20°C. LH and FSH levels were measured, using materials provided by NIDDK. rLH-RP-2 and rFSH-RP-2 diluted in serum of hypophysectomized rats were used as reference materials. The first antibodies NIADDK-anti-rLH-S-9 and NIADDK-anti-rFSH-S-11 were used at a final concentration of 1:20.000 and 1:80.000, respectively. The second antibody was donkey-anti-rabbit (Sac-Cel®, IDS, Boldon, Type and Wear, England). Determinations were performed in triplicate. Quality control sera with low, medium and high LH or FSH concentrations were included in each RIA. Assay sensitivity of the diluted samples at 90% B/B_0 was 0.2 ng/ml for LH and 1.6 ng/ml for FSH. The intra-assay coefficients of variation for LH and FSH were 6.1% and 5.4%, respectively. The inter-assay coefficients of variation for LH and FSH were 5.1% and 11.5%, respectively.

Statistical analysis

The response curves of plasma LH and FSH were plotted separately for each rat. The peak height of a LH surge was calculated as the value from zero to the highest LH level measured. Plasma LH and FSH values were analyzed using a two-way analysis of variance with repeated measurements (MANOVA). Post hoc analyses (Bonferroni) were performed, if a positive interaction was found. For statistical analysis the Statistical Program System for the Social Sciences (SPSS/PC+ V2.0, SPSS, Inc., Chicago, IL) was used. A probability of less than 0.05 was considered significant. Values are reported as means ± SEM.

Results

Experiment 1. Effect of a single icv CRH injection on the LH and FSH surge in pro-oestrous rats.

Fig. 1 shows the plasma LH and FSH surge and their time course following injection of saline or CRH. A dose of 0.1 or 10 μg CRH or saline was injected 5

minutes before the presumed onset (at 0900 h) of the surge of gonadotropins. In saline-treated rats, the highest plasma LH value occurred at 1200 h with a maximal value of 11.0 ± 1.0 ng/ml (Fig. 1a). The LH surge of untreated rats was similar in amplitude and duration (data not shown). After injection of 0.1 μg CRH, the LH surge was similar as in saline-treated controls. Injection of 10 μg CRH caused an inhibition of the LH surge ($P<0.01$) and peak height ($P<0.01$). LH was suppressed at most time points but at 1330 h plasma LH was similar as in the controls.

In saline-treated control rats and in untreated rats (data not shown), plasma FSH increased gradually on the day of pro-oestrus (Fig. 1b). Injection of 0.1 μg CRH or 10 μg CRH did not affect this FSH rise.

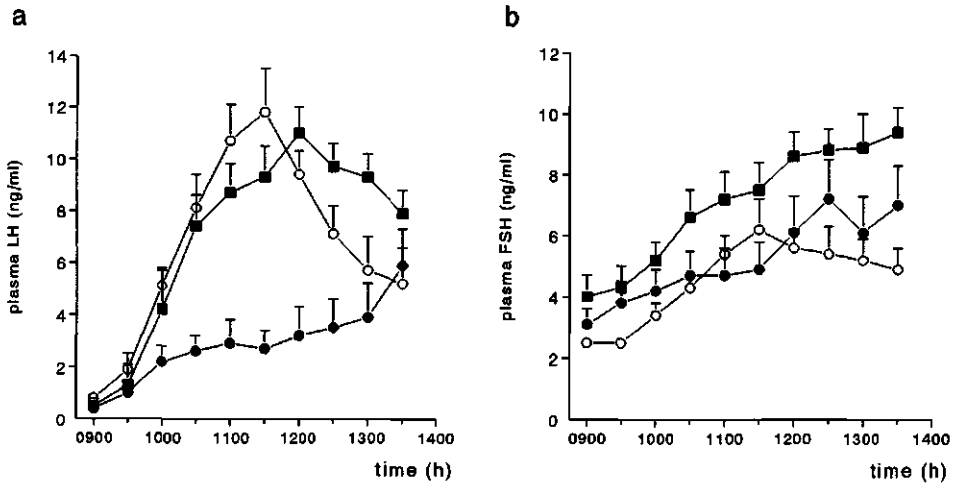

Fig. 1. Effect of 0.1 or 10 μg CRH injected icv on the pro-oestrous LH (a) and FSH (b) surge in cyclic rats. Injections were given 5 min before the presumed onset (at 0900 h) of the LH surge. (■) saline (N = 10); (○) 0.1 μg CRH (N = 6); (●) 10 μg CRH (N = 10). Values are expressed as means ± SEM.

Experiment 2: Effect of icv infusion of CRH on the LH and FSH surge in pro-oestrous rats.

Fig. 2a illustrates the effect of infusion of saline or 1, 5 or 10 μg/h CRH from 0800 to 1400 h on the LH surge. After infusion of saline the LH surge was similar as in untreated rats (data of untreated rats not shown). The LH surge was not influenced by infusion of 1 μg/h CRH. CRH infusion of 5 μg/h did not

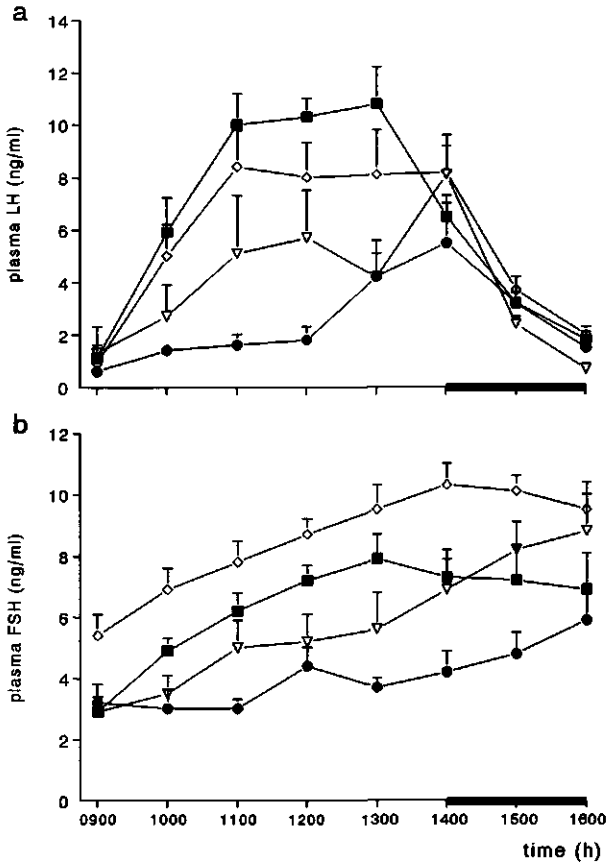

Fig. 2. Effect of icv infusion of various doses of CRH on the pro-oestrous LH (a) and FSH (b) surge in cyclic rats. Infusion was given during 6 hrs started 1 h before the presumed onset of the LH surge. (■) saline (N=6); (◊) 1 μg/h CRH (N=7); (▽) 5 μg/h CRH (N=7); (●) 10 μg/h CRH (N=7). Values are expressed as means ± SEM. The black horizontal bar represents the dark period.

alter peak height but the total LH secretion during the surge was suppressed ($P<0.01$). Infusion of 10 μg/h CRH strongly inhibited LH secretion ($P<0.01$); also peak height of the surge was suppressed ($P<0.01$). The inhibition of LH during infusion of 10 μg/h CRH lasted till 1200 h; subsequently plasma LH increased to control surge levels at corresponding points of time of the LH surge. The profile of the LH surge after icv infusion of 10 μg/h CRH (Fig. 2a) is

comparable to the profile of the LH surge after single injection of 10 μg CRH (Fig. 1a and also Fig. 3a).

As shown in Fig. 2b, in control rats plasma FSH increased gradually during infusion of saline. In untreated rats, the FSH rise during pro-oestrus was similar (data not shown). Infusion of 1 μg/h CRH induced an increased FSH secretion ($P<0.05$). Infusion of 5 μg/h CRH did not affect FSH levels. Infusion of 10 μg/h CRH caused a suppression of the FSH rise ($P<0.01$).

Experiment 3. Effect of pretreatment with AVP-antiserum on the suppression of the LH and FSH surge induced by icv injection of 10 μg CRH.

The LH surge of the control group which received injections of normal rabbit serum (NRS) 24 h and 0.5 h before the onset of the LH surge, and saline at 0855 h, had a profile of similar amplitude and duration (Fig. 3a) as the saline-treated group of experiment 2 and as the saline-treated group of experiment 3 (data not shown). After injections of AVP-antiserum instead of NRS, a similar LH surge profile was measured. In NRS-treated rats, injection of 10 μg CRH (at 0855 h) caused a strong suppression of the LH rise during the first 3 hours of the surge ($P<0.01$). Thereafter LH increased to a similar level as in controls at corresponding points of time of the LH surge. In rats pretreated with AVP-antiserum, an injection of 10 μg CRH induced also a strong suppression of the plasma LH surge ($P<0.01$) and peak height ($p<0.01$). After pretreatment with AVP- antiserum, the inhibitory action of CRH on the LH surge was prolonged. The AVP-antiserum plus CRH treatment resulted in lower mean levels of LH at 1400 and 1500 h compared to saline plus CRH injected rats.

In the NRS-saline group, plasma FSH showed the expected gradual rise after 0900 h on pro-oestrus (Fig. 3b). AVP-antiserum induced an inhibition of FSH levels ($P<0.05$). In NRS-pretreated rats, 10 μg CRH caused a suppression of the FSH rise ($P<0.01$). This suppression was not altered after pretreated with AVP-antiserum.

Experiment 4: Effect of 9 h icv infusion of 10 μg/h CRH preceded by treatment with AVP-antiserum on the LH and FSH surge.

In experiment 2, we had found that during continuous infusion of 10 μg/h CRH the LH-suppressive effect of CRH was attenuated 3-4 h after the start of infusion. To confirm this observation in experiment 4, a 9 h CRH infusion was

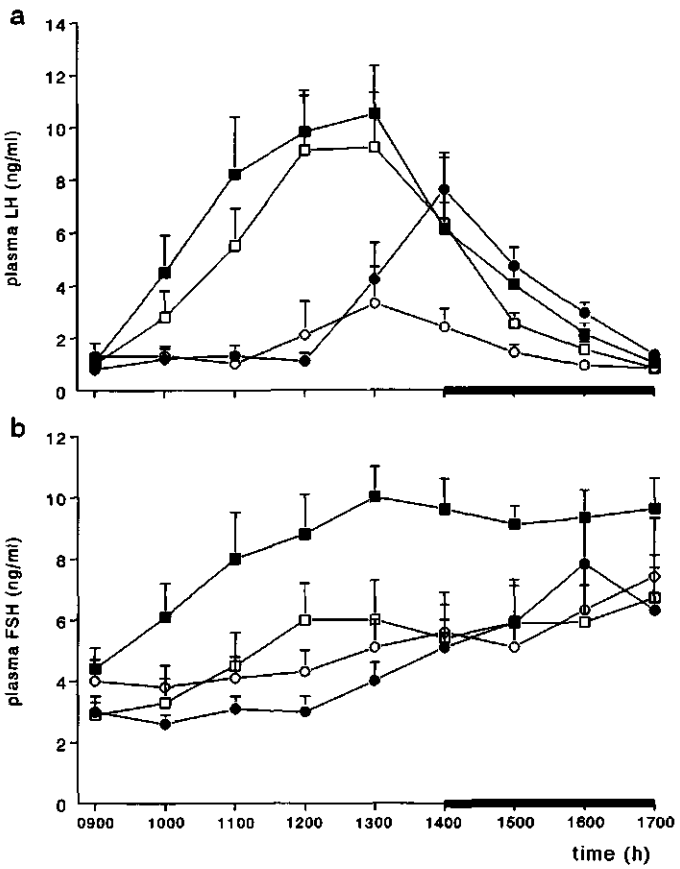

Fig. 3. Effect of pretreatment with AVP-antiserum on the CRH-induced suppression of the pro-oestrous LH (a) and FSH (b) surge in cyclic rats. Injections of AVP-antiserum or NRS were given icv 24 h and 0.5 h before the presumed onset of the LH surge; 10 μg CRH or saline was given icv 5 min before the onset of the LH surge. (■) NRS + saline (N = 7); (□) AVP-antiserum + saline (N = 6); (●) NRS + 10 μg CRH (N = 7); (○) AVP-antiserum + 10 μg CRH (N = 8). Values are expressed as means ± SEM. The black horizontal bar represents the dark period.

an LH surge as in the control group despite continued infusion until 1400 h. Fig. 4a illustrates that indeed the LH surge was not suppressed, if the 9 h infusion of CRH was started at 0500 h. Treatment with AVP-antiserum preceding CRH infusion caused a considerable suppression of the LH surge compared to the control ($P < 0.01$) and the CRH-infusion group ($P < 0.01$). While plasma LH was strongly

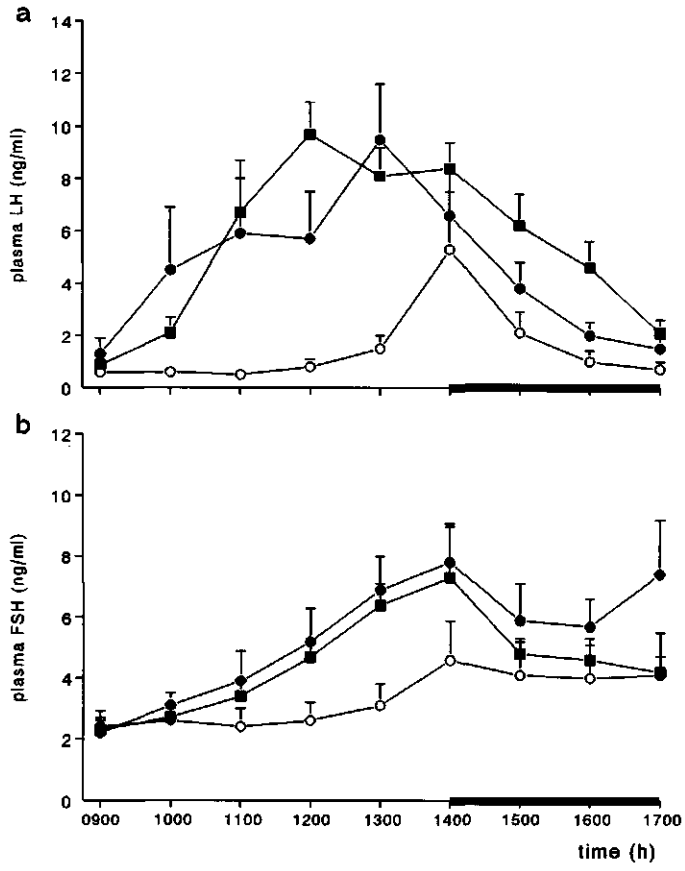

Fig. 4. Interaction of AVP-antiserum and icv infusion of 10 μg/h CRH on the pro-oestrous surge of LH (a) and FSH (b) in cyclic rats. Saline or AVP-antiserum was icv injected 24 h and 4.5 h before the presumed onset of the LH surge. 10 μg/h CRH was infused during 9 hrs, starting at 0500 h. (■) saline (N = 7); (●) saline + 10 μg/h CRH infusion (N = 6); (○) AVP-antiserum + 10 μg/h CRH infusion (N = 5). Values are expressed as means ± SEM. The black horizontal bar represents the dark period.

suppressed during the first hours of the surge, at 1400 h plasma LH had increased to the control level of 1400 h.

Fig. 4b shows that the pro-oestrous FSH rise was neither altered by prolonged infusion of 10 μg/h CRH nor by CRH infusion after pretreatment with AVP-antiserum.

Discussion

Various stressors inhibit the secretion of gonadotropins (6,25,26). A role for CRH in inhibiting reproductive functions during stress has been suggested (6,7). Icv injection of CRH caused an inhibition of plasma LH in male and OVX rats (8-10,12,13). Also in pro-oestrous rats, LH levels were inhibited by icv CRH (8, 10). The present study confirms this and demonstrates that central administration of CRH induced a dose-dependent inhibition of the plasma LH surge on the day of pro-oestrus. In addition we found that the suppressive effect of CRH on the LH surge was limited to 3-4 hrs. It is conceivable that in previous studies in pro-oestrous rats (8,10) plasma LH levels were not measured long enough after icv CRH administration to observe a possible decrease of the inhibitory effect of CRH.

In experiment 1, the diminishing inhibitory effect of CRH on plasma LH 3-4 h after CRH injection could possibly be due to degradation of the administered CRH. To verify this hypothesis we gave an icv infusion of various doses of CRH (experiment 2). Despite continuation of CRH infusion until 1400 h, a release from CRH inhibition of plasma LH occurred after 3-4 h of infusion.

In experiment 2, 3 and 4, blood sampling was extended into the dark period. In this way we could assess the descending phase of the LH surge in controls, and also if a postponed LH surge of normal magnitude occurred after release from CRH inhibition. The data indicate that a postponed LH surge of normal magnitude did not occur. This corroborates the view that in the rat the pro-oestrous LH surge can occur only during a limited period of the day (25,27).

The present results show that during pro-oestrus the inhibiting effect of CRH on the LH surge was restricted to 3-4 h. This may conceivably due to down-regulation of brain CRH receptors by continuous high local concentrations of CRH. Recently, Hauger *et al.* (16) reported that high central levels of CRH resulted in downregulation of CRH receptors in the amygdala of male rats. Support for the hypothesis that brain CRH receptors are downregulated, is further provided by the observation of experiment 4 that a 9 h CRH infusion started 4 h before the presumed onset of the LH surge did not interfere with the LH and FSH surges. However, other studies reported that the number of CRH receptors in the central nervous system was not changed if high central CRH levels were induced (14,24,28). Another possibility for the release from CRH inhibition could be that an unknown stimulatory pathway is activated which overrules the CRH

inhibition. Considering the results of the present study, AVP may be involved in this stimulatory pathway.

As far as the FSH data are concerned, conclusions on the effects of CRH on the pro-oestrous FSH rise were difficult to draw. This is partly due to the long half-life of plasma FSH (29). In addition, the FSH rise on pro-oestrus is not very steep and unexplainably plasma FSH was relatively high in some experimental groups at the start of the FSH rise. Generally CRH tended to inhibit the FSH rise of pro-oestrus. In experiment 2, however during infusion of 1 μg/h CRH plasma FSH was higher than in controls. We do not have an explanation for this observation. Whereas AVP antiserum alone did not influence plasma LH, FSH levels were suppressed, suggesting a differential action of AVP on both gonadotropins. However, the difference between plasma FSH levels in saline- and AVP antiserum-treated rats could also be explained by the relatively high FSH levels at the presumed beginning of the FSH rise in saline-treated rats.

In the present study, pretreatment with an AVP-antiserum did not prevent the CRH-induced inhibition of the LH and FSH surge. This result does not confirm our hypothesis that the inhibitory effect of CRH on the activity of the HPG axis is mediated by AVP. Our hypothesis was based on the observation of Burns et al. (20) that AVP mediated the effect of CRH on β-endorphin release from the hypothalamus; opioids are involved in the CRH-induced inhibition of the HPG axis in male (17-19), OVX and OVX oestrogen-treated rats (10). The discrepancy with Burns et al. (20) can be due to the fact that they studied slices of male hypothalamic tissue, while we studied intact cyclic female rats on the day of pro-oestrus. In addition, as far as we know an involvement of AVP in the CRH-induced inhibition of the pro-oestrous LH and FSH surge of cyclic rats has hitherto not been described.

After pretreatment with AVP-antiserum the CRH induced inhibition of the LH surge lasted longer than 3-4 h. Additionally, in rats pretreated with AVP-antiserum the LH surge was suppressed during prolonged CRH infusion started 4 h before the presumed onset of the LH surge. AVP-antiserum injections *per se* had no effect on the LH and FSH surge. These results might indicate a possible stimulatory effect of AVP on GnRH but only during conditions of high central CRH levels. This is supported by the observation that icv administered AVP-antiserum prevented ether stress-induced LH release in OVX rats (30). On the other hand, there is evidence for an inhibitory effect of AVP on LH secretion. Icv or intravenous (iv) administration of high doses of AVP has been reported to cause

suppression of the LH surge in cyclic rats (21,22), while iv infusions of AVP suppressed the steroid-induced surge of LH in OVX rats (31). However, these studies were performed under conditions with conceivably low central CRH release. Another explanation for our results is that pretreatment with AVP-antiserum prevented the downregulation of brain CRH receptors which possibly is induced by high central CRH levels. Various studies reported a contribution of AVP in the CRH-induced downregulation of CRH receptors in the pituitary (23, 24,32). It is possible that AVP also stimulates CRH-induced downregulation of CRH receptors in the brain. So, *mutatis mutandis*, AVP-antiserum may have prevented the CRH-induced downregulation of CRH receptors. According to this hypothesis, LH should remain suppressed as long as CRH was infused into rats pretreated with AVP-antiserum. However, in experiment 4 LH was rising during the last hour of CRH infusion. We have no plausible explanation for this observation. The suppression may be overriden by a not-AVP-mediated stimulatory mechanism.

In summary, we have shown that icv injection or infusion of CRH suppressed the LH and FSH surges on the day of pro-oestrus. This inhibitory effect of CRH is not mediated by AVP. On the contrary, administration of AVP-antiserum seemed to prolong the LH-suppressive effect of CRH.

Acknowledgements

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Chapter 4

Evidence for a role of arginine vasopressin and not of corticotropin-releasing hormone in the restraint-induced inhibition of the LH surge in the cyclic rat

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submitted

Abstract

The present study was designed to investigate the role of corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) in the inhibitory effect of restraint stress on the LH surge in intact, 5-day cyclic rats. Groups of rats were subjected to restraint stress for 5 or 6 hrs starting 5 or 60 min prior to the presumed onset of the surge. In restraint rats, the surge was partially inhibited as compared to non-stressed controls. Intracerebroventricular (icv) pretreatment with 10 or 30 μg of the CRH antagonist α-helical CRH (αhCRH) at 15 min before the application of restraint for 5 hrs did not affect LH levels in restraint and control rats during the period of the surge, but partially prevented the plasma corticosterone response induced by restraint. Pretreatment of rats (icv) with AVP-antiserum 24 h before the onset of the LH surge and 0.5 h before application of restraint potentiated the restraint-induced suppression of pro-oestrous LH levels, but did not influence the LH surge in non-stressed control rats. An intravenous (iv) injection with 100 ng Ovalyse®, a GnRH agonist, during restraint resulted in a steep and transient rise of plasma LH levels indicating that the pituitary was not rendered refractory to GnRH.
From these data, we conclude that CRH pathways that are activated during restraint stress do not mediate the stress-induced inhibition of the pro-oestrous LH surge in the intact, cyclic rat. In addition, the results suggest that the residual LH surge observed during restraint is dependent on stress-induced vasopressin activity in the brain.

Introduction

Stress-induced endocrine responses include activation of the hypothalamus-pituitary-adrenal (HPA) axis and inhibition of the hypothalamus-pituitary-gonadal (HPG) axis. Evidence for stress-induced inhibition of the HPG axis mainly derives from studies on male and ovariectomized female rats (1-4), but it has been shown that footshock (5) and, more recently, restraint stress (6) can suppress the pro-oestrous LH surge in intact cyclic female rats.

The mechanisms responsible for stress-induced suppression of the surge of the HPG axis are not clearly understood. Observations that CRH is released during stress (7) and that central administration of CRH largely mimics the

behavioral and physiological effects observed during stress (8) suggest that CRH may be a mediator of this stress response. Indeed, there is experimental evidence that CRH, acting within the hypothalamus, inhibits the HPG axis (9-13).

We have shown recently that administration of CRH before the onset of the pro-oestrous LH surge, can suppress the surge in cyclic female rats (14). Even with high doses of CRH, however, this inhibition was only partial and restricted to the first phase of the surge. Moreover, we found that icv pretreatment with AVP-antiserum extended the inhibitory effect of CRH (14). From this, it was concluded that during conditions of high central CRH activity, vasopressin pathways are activated that mitigate the inhibition of LH secretion. Indeed, several studies suggest a role of AVP in control of LH secretion during stress (15-17).

In the present study we investigated the role of CRH and AVP in the restraint stress-induced inhibition of the pro-oestrous LH surge in intact cyclic female rats.

Materials and Methods

Rats, housing and surgery

All experiments were carried out on adult female F1 rats of two Wistar substrains (U-inbred males and R-inbred females), weighing 200-250 g. The rats were individually housed under a 14:10 h light-dark schedule (lights on at 00:00 h) in a temperature-regulated (22 ± 1°C) room. They had free access to standard food pellets and tap water. A dim light was left on to facilitate blood sampling during the dark period. Under these circumstances most of these rats have 5-day oestrous cycles and the onset of the pro-oestrous LH surge is approximately at 0900 h (6).

Rats were provided with a silastic jugular vein cannula (18) and a stainless steel icv cannula (23 gauche) into the right lateral ventricle (19). For surgery, rats were anaesthetized with ketamine (40-60 mg/kg ip) and xylazine (Rompun®, 1.4 mg/rat sc). Starting two days after surgery, the rats were handled daily to minimize stress during blood sampling. In addition, oestrous cyclicity was monitored every day by vaginal lavage, and by observation of oestrous behaviour after introduction of a male rat. Rats that exhibited at least two consecutive 5-day cycles were used in the experiments. On termination of the experiments,

correct icv cannula placement was checked after injection of 3 μl solution of Evans blue (10% in saline) and macroscopical inspection of the brain. Data obtained from rats with an incorrectly placed icv cannula were discarded.

Drugs

The compounds used were α-helical CRH (9-41), a CRH receptor antagonist (Sigma Chemical Company, St Louis, USA), Ovalyse® (des^{10}-GnRH-ethylamide), a GnRH agonist (gift from Upjohn, Ede, the Netherlands) and AVP-antiserum (code W3). The antiserum displayed no cross-reaction with oxytocin, 6% with lysine vasopressin and 120% with vasotocin (tested under radioimmunoassay conditions).

Saline (0.9%) served as solvent and control solution. AVP-antiserum was diluted with one volume of saline. A previous experiment had shown that there was no difference between icv injections with saline and normal rabbit serum (NRS) on the LH surge. The icv injections with saline, αhCRH or AVP-antiserum were given in a volume of 3 μl using a 10 μl Hamilton syringe. Ovalyse® was given iv in a volume of 0.2 ml.

Experimental Procedures

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

Restraint procedure

On the day of pro-oestrus, rats were connected to a blood sampling cannula approximately 1 h before application of restraint stress. They were transferred to the experimental room just before the onset of restraint. Restraint was accomplished by placing individual rats in a perspex cylinder (see for more details ref. 6) and was started at 0855 or 0800 h, i.e. 5 or 60 min before the presumed onset of the LH surge at 0900 h. The rats were released and returned to their home cage after taking the blood sample at 1400 h.

Experimental design

Experiment 1

Pro-oestrous rats were subjected to restraint stress from 0855 h to 1400 h. 15 Min before the onset of restraint, they were icv injected with 10 or 30 μg αhCRH, or saline. Non-stressed rats received 30 μg αhCRH or saline. Blood

samples of 160 µl were taken hourly during the period of the LH surge, i.e. from 0900 to 1700 h.

To investigate pituitary responsiveness to GnRH during restraint, a group of stressed rats was included which received an iv injection with 100 ng Ovalyse® after withdrawal of the blood sample at 1200 h.

Experiment 2

Rats were placed in a restraint cylinder for 5 hrs (from 0855 h to 1400 h). They received an icv injection with either AVP-antiserum or saline 24 h before the onset of the presumed LH surge and a second injection 0.5 h before the start of restraint. This protocol was repeated with rats subjected to 6 hrs of restraint (from 0800 to 1400 h). Non-stressed rats received injections with saline or AVP-antiserum. Blood samples (160 µl) were taken hourly from 0900 to 1700 h.

Radioimmunoassays

LH assay

Blood samples were collected in heparinized tubes on ice. After centrifugation, plasma samples (70 µl) were diluted with 3 volumes of phosphate-saline buffer (pH 7.5) containing 0.1% bovine serum albumin (Sigma RIA-grade) and stored at -20°C. LH levels were measured, using material provided by NIDDK. rLH-RP-2 diluted in serum of hypophysectomized rats was used as reference material. The second antibody was donkey-anti-rabbit (Sac-Cel®, IDS, Boldon, Type and Wear, England). Determinations were performed in triplicate. Quality control sera with low, medium and high LH concentrations were included in each RIA. In the assays, the least detectable amount of LH in the diluted samples was 0.2 ng/ml. The intra-assay and inter-assay coefficients of variation were 5.5% and 6.1%, respectively.

Corticosterone assay

Corticosterone was extracted from 10 µl of the diluted plasma samples with dichloromethane, dried over night, resuspended in ethanol (96%) and stored at -20°C. Before determination, the samples were dried under nitrogen and dissolved in phosphate-saline buffer (pH 7.4). Corticosterone levels were measured using a corticosterone standard (Sigma C-2505) as reference, the anti-corticosterone i906 (UCB-bioproducts, Veenendaal, The Netherlands) which displayed

no cross-reaction with progesterone, and [1,2,6,7-H^3] corticosterone (Amersham TRK 406) as tracer. The assay sensitivity was 2.8 μg/100 ml, and coefficients of variation within and between assays were 4.5% and 15.2%, respectively.

Statistical analysis

For statistical analysis the Statistical Program System for the Social Sciences (SPSS/PC+ V2.0, SPSS, Inc., Chicago, IL) was used. LH and corticosterone data were analyzed using a MANOVA (two- or three-way analysis) with repeated measurements, which was followed by post hoc comparisons (Bonferroni) if a positive interaction was found. Comparisons between individual groups for LH peak heights (i.e. the highest LH level measured), LH and corticosterone levels at different points of time were assessed by ANOVA and the Mann Whitney rank sum test. A probability of less than 0.05 was considered significant. Values are reported as means ± SEM.

Results

The effect of pretreatment with αhCRH on the restraint-induced suppression of the LH surge in pro-oestrous rats is shown in Fig. 1a. Comparison of the experimental groups, revealed a treatment effect ($F=8.3$; $P<0.01$) and a treatment x time interaction ($F=1.8$; $P<0.05$).

The LH surge of non-stressed rats pretreated with 30 μg αhCRH was similar to the LH surge of saline-pretreated rats. Restraint stress for 5 hrs (starting at 0855 h) partially inhibited LH levels during the period of the LH surge ($P<0.01$). Pretreatment with 10 μg αhCRH did not affect LH levels in restraint rats. However, the plasma LH profile of restraint rats pretreated with 30 μg αhCRH differed from those of saline-pretreated restraint rats ($P<0.01$). LH levels of restraint rats pretreated with 30 μg αhCRH were lower than those of saline-pretreated restraint rats at 1000 h ($P<0.01$) and 1100 h ($P<0.05$). Thereafter they increased until 1500 h at which they were significantly higher than in saline-pretreated restraint rats ($P<0.05$). When during restraint an iv injection of Ovalyse® was given (at 1200 h) a steep, robust and transient rise of plasma LH was observed (Fig. 1b). Plasma LH levels were increased at 1300 and 1400 h ($P<0.01$) compared to untreated restraint rats.

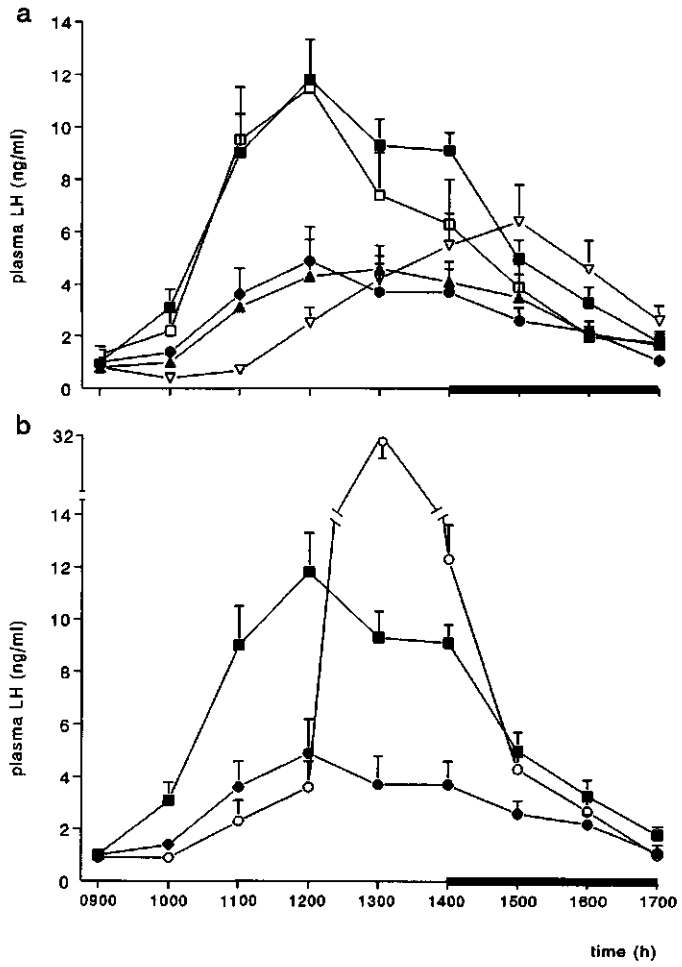

Fig. 1. Effect of αhCRH (a) and Ovalyse® (b) on the restraint-induced suppression of the pro-oestrous LH surge. Rats were pretreated (icv) with saline or αhCRH at 0840 h, and were subjected to restraint from 0855 until 1400 h. Ovalyse® was iv injected in restraint rats at 1200 h. (■) saline (n = 14); (□) 30 μg αhCRH (n = 6); (●) saline + restraint (n = 14); (▲) 10 μg αhCRH + restraint (n = 7); (▽) 30 μg αhCRH + restraint (n = 8); (○) restraint + 100 ng Ovalyse® (n = 6). Values are expressed as means ± SEM. Black horizontal bar represents the dark period of the lighting cycle.

Fig. 2 illustrates the effect of pretreatment with αhCRH on corticosterone levels in restraint rats. Comparison of the experimental groups, revealed a treatment x time interaction ($F=2.5$; $P<0.05$). Restraint stress induced an increase of corticosterone during the first 2 hrs after the start of restraint compared to non-stressed rats ($P<0.05$). In restraint rats pretreatment with 10 or 30 μg αhCRH partially prevented this response. At 1000 and 1100 h corticosterone levels in restraint rats pretreated with the CRH antagonist were neither different from those of non-stressed saline-pretreated rats nor saline-pretreated restraint rats. In non-stressed rats, administration of 30 μg αhCRH had no effect on corticosterone levels as compared to saline pretreatment.

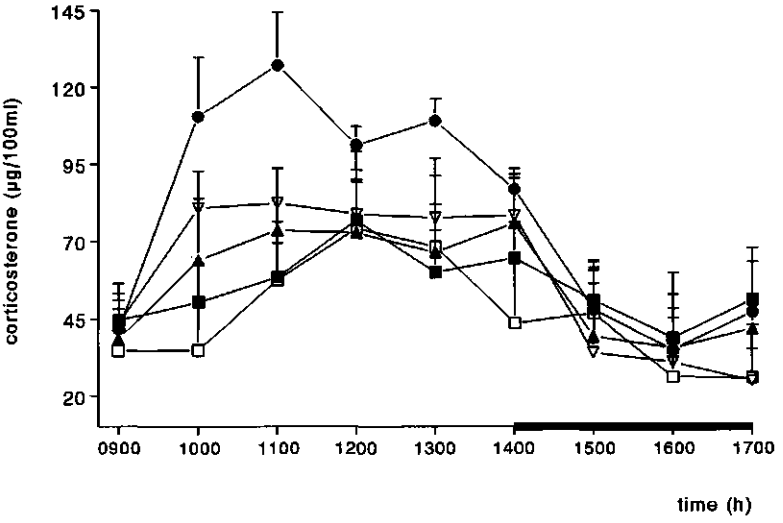

Fig. 2. Effect of αhCRH on the restraint-induced rise of plasma corticosterone. Rats were icv pretreated with saline or αhCRH at 0840 h, and were subjected to restraint from 0855 until 1400 h. (■) saline ($n=7$); (□) 30 μg αhCRH ($n=6$); (●) saline + restraint ($n=7$); (▲) 10 μg αhCRH + restraint ($n=7$); (▽) 30 μg αhCRH + restraint ($n=8$). Values are expressed as means ± SEM. Black horizontal bar represents the dark period of the lighting cycle.

Fig. 3 illustrates the effect of pretreatment with AVP-antiserum on the restraint-induced suppression of the LH surge. The three-way analysis revealed a significant restraint effect ($F=49.5$; $P<0.01$), a restraint x time interaction

(F = 7.7; P < 0.01), and an AVP-antiserum x time interaction (F = 3.0; P < 0.05). The LH surge of non-stressed rats which received injections of saline or AVP-antiserum 24 and 0.5 h before the presumed onset of the LH surge did not differ from those of saline-pretreated rats (Fig. 3). As in experiment 1, restraint suppressed LH levels during the period of the surge (P < 0.01). Pretreatment with AVP-antiserum led to a further reduction of plasma LH levels compared to saline-pretreated restraint rats (P < 0.05 from 1200 to 1500 h).

Previous experiments had shown that restraint for 6 hrs starting at 0800 h was less effective in suppressing LH levels during the period of the LH surge than restraint for 5 hrs starting at 0855 h. Also in rats subjected to this protocol pretreatment with AVP-antiserum potentiated the restraint-induced suppression of the LH surge (P < 0.05; data not shown).

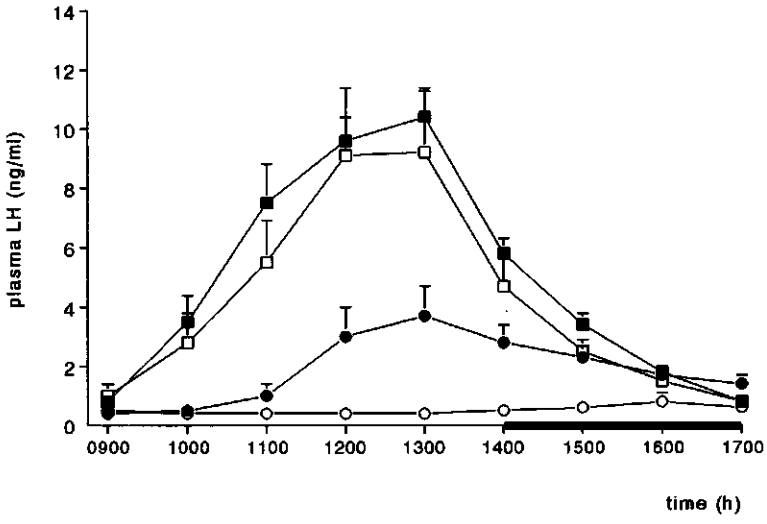

Fig. 3. Effect of pretreatment with AVP-antiserum on the restraint-induced suppression of the pro-oestrous LH surge. Rats were subjected to restraint from 0855 to 1400 h. Pretreatment consisted of icv injections of saline or AVP-antiserum at 24 h before the presumed onset of the LH surge and at 0.5 h before the start of restraint. (■) saline (n = 13); (□) AVP-antiserum (n = 6); (●) saline + restraint (n = 10); (○) AVP-antiserum + restraint (n = 7). Values are expressed as means ± SEM. Black horizontal bar represents the dark period.

Discussion

The present data that restraint stress can suppress the pro-oestrous LH surge in intact cyclic female rats corroborate previous findings from our own group (6, 14,20) and others (5). Treatment of the rats, during restraint, with an iv bolus of the GnRH agonist Ovalyse® induced a marked plasma LH response, indicating that the stress had not rendered the pituitary refractory to GnRH. This accords with data from others who, in addition, have provided evidence that central mechanisms regulating GnRH secretion are crucially involved in the effects of stress on the HPG axis in male (21-23) and intact female rats (24,25). Taken together, it is therefore most likely that the inhibitory effect of restraint stress on the LH surge is brought about via neural pathways that affect the secretion of GnRH into the portal system. In view of literature data on the role of CRH in stress responses (8,13,26-28), and our own previous results that neither endogenous opioid (20) nor GABA (Roozendaal et al., unpublished) systems mediate the effect of restraint, we considered the CRH system the prime candidate in this respect.

Contrary to our expectations, however, pretreatment of rats with αhCRH (10 or 30 μg, icv) did not antagonize the inhibitory effect of restraint on the LH surge, suggesting that endogenous CRH does not play a crucial role in this effect. One could argue, however, that the relevant site(s) in the brain had not been reached by sufficient amounts of the antagonist, but for various reasons this does not seem to be very likely. Firstly, the stress-induced corticosterone response of the same rats was partly antagonized, indicating that under the experimental conditions and in the doses used, αhCRH had been centrally effective indeed. Secondly, αhCRH is a peptide closely related to CRH, and icv administration of CRH, in doses similar to or even considerably lower than those used here for the antagonist, suppresses the LH surge (14). Thirdly, the highest dose of αhCRH (30 ug) did have an effect in stressed rats, but potentiated rather than antagonised the initial effect of restraint stress. Since icv administration of CRH itself suppresses the LH-surge (14), this potentiation might be explained by some agonistic activity that has been shown to reside in the antagonist (29; Dr.E.Ronken, personal communication). Finally, there are numerous studies reporting on effects of icv administration of αhCRH, often in doses lower then those used in this study, on a wide variety of behavioral and physiological responses involving sites throughout the brain (8,30). Therefore we conclude

that not CRH, but other factors in the brain likely mediate the effect of restraint stress on the pro-oestrous LH surge in the intact female rat.

Rivier et al. (13) have shown that footshock stress suppresses LH levels in castrated male rats, and that this effect is antagonized by icv pretreatment with 100 μg αhCRH. This apparent discrepancy with our present results could be due to differences in the stress protocol employed, or in hormonal regulation and state between the rats used, i.e. castrated male versus intact cyclic female rats. An alternative explanation, however, could be that αhCRH shares with CRH the ability to activate vasopressinergic mechanisms in the brain that can enhance LH secretion (14), and thereby counteracted the stress-induced inhibition. Such 'agonistic' properties most likely become overt when high doses are used.

In saline-pretreated rats subjected to restraint stress a residual LH surge was observed. In rats pretreated with an AVP-antiserum, however, restraint stress completely abolished the surge. This strongly suggests, that the residual LH surge is maintained by an action of endogenous AVP that is released during stress. There is considerable evidence, indeed, that various stressors, including acute and repeated restraint, induce vasopressin mRNA-expression, peptide synthesis, storage and secretion in hypothalamic neurons. These concern not only parvocellular neurons in the paraventricular nucleus (PVN) (31-37), but most likely also magnocellular vasopressin neurons in PVN and the supra-optic nucleus (35, 38, 39). In addition, the enhanced release of AVP into the circulation that occurs following restraint is probably of magnocellular origin (40). Sofar, however, the exact type and location of the vasopressin neurons, as well as the site of release and action of the vasopressin that is involved in the present effect on LH secretion remain to be elucidated. Yet, it is of interest in this respect, that we have recently obtained first lightmicroscopical immunocytochemical evidence for the presence of a vasopressin-containing fibre input on a small subset of GnRH containing neurons in the medial preoptic and anterior hypothalamic area, that may thus provide an anatomical substrate for vasopressin interaction with the hypothalamus-pituitary-ovarium axis (van der Beek and Wiegant, unpublished). Whatever its origin and locus of action during stress in this respect, vasopressin apparently does not play an important role in maintaining the surge under normal, non-stressed conditions, since pretreatment with the AVP-antiserum did not affect the surge in non-stressed control rats.

In conclusion, the present data indicate that the partial inhibition of the pro-oestrous LH surge induced by restraint stress in intact cyclic female rats is likely

not mediated by CRH. In addition, the results provide first evidence that the residual surge maintained during restraint is dependent on stress-induced vasopressin activity in the brain. Since such a residual LH-surge appears to be sufficient for triggering ovulation (6), the activation of vasopressin may be an adaptive mechanism to safeguard reproductive functions in the female rat during stress and hardship.

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Chapter 5

Inhibition of the LH surge in cyclic rats by stress is not mediated by opioids.

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Abstract

The effects of intravenous (iv) administration of the opioid antagonists naloxone and naltrexone on the restraint-induced suppression of the pro-oestrous LH surge were studied in cyclic female rats. To minimize stress during repeated blood sampling, the rats were provided with a jugular vein cannula. Restraint stress for 6 hrs starting at t=-1 h (the onset of the LH surge being at t=0 h) caused a suppression of LH levels (including peak height) during the period of the LH surge. Repeated naloxone injections, given 3 h (1 mg), 4 h (0.5 mg) and 5 h (0.5 mg) after the onset of the LH surge, did not affect the restraint-induced inhibition neither did pretreatment with 1 mg naloxone at t=-75 min (i.e. 15 min before application of restraint). Naltrexone (2 mg) administered at t=-15 min induced higher plasma LH levels at t=-6 min. When rats were subsequently subjected to restraint for 5 hrs starting at t=-5 min, the restraint-induced inhibition of surge levels of LH was not affected. The results indicate that withdrawal of opioid activity in cyclic female rats before the presumed onset of the LH surge results in a premature rise of LH levels. This is in accordance with the notion that LH levels prior to the surge are under tonic inhibition of endogenous opioid peptides (EOP). In addition, the data show that opioid receptor antagonism during or before application of restraint does not alter the restraint-induced suppression of the LH surge. It is therefore concluded that EOP do not mediate the inhibitory effect of restraint stress on the LH surge in cyclic rats.

Introduction

In cyclic rats, the preovulatory LH and FSH surge occurs on the afternoon of pro-oestrus as a consequence of increased hypothalamic GnRH secretion into the hypophysial portal system. The role of EOP in the control of GnRH secretion has been studied extensively (1-3). Administration of morphine or β-endorphin inhibits the spontaneous or induced preovulatory surge of gonadotropins in female rats, the effect being more pronounced for LH than FSH secretion (4-8). In addition, a decrease of the inhibitory EOP tone on the afternoon of pro-oestrus plays a role in the induction of pre-ovulatory GnRH secretion in the rat (9,10). The β-endorphin concentration in hypothalamic tissue and pituitary portal blood varies during the oestrous cycle with low levels at the time of the LH

surge (9,10). Moreover, blockade of EOP receptors by administration of the opioid receptor antagonist naloxone before the onset of the LH surge elicits a premature LH surge (11).

Several studies have suggested a modulatory role for EOP in the effects of stress on reproductive parameters but only a few have used intact female rats. Naloxone administered 15 min before the onset of restraint can reverse the anti-ovulatory action of restraint in immature female rats primed with pregnant mare serum gonadotropin (PMSG) (12). Footshock stress applied during the period of the LH surge results in inhibition of LH release which can be antagonized by naloxone administration 5 min prior to application of the stressor (13). In a previous study we demonstrated that on the day of pro-oestrus restraint stress can suppress the LH surge in intact cyclic rats (14). This suppression is believed to result from an impairment of hypothalamic hypophysiotropic function, since pituitary responsiveness to GnRH is not affected by restraint (submitted for publication).

The aim of the present study was to distinguish between a possible role of opioids in mediating the restraint-induced inhibition of the pro-oestrous LH surge vs. the role of endogenous opioids in the cyclic control of LH secretion in the intact female rat.

Materials and methods

Rats, housing and surgery

Female F1 of U-male and R-female substrains of Wistar rats from the University breeding colony were used. They were 6-8 months of age and had a body weight of 200-250 g. The rats were individually housed in standard conditions of temperature (22 ± 1 °C) and lighting (14L:10D). During the dark period a dim light was left on to facilitate blood sampling. Food pellets and drinking water were provided ad libitum. Most of our rats have 5-day cycles and the onset of the LH surge is approximately 2 h after the middle of the light period (14); this time was defined as $t = 0$ h.

Preceding surgery, rats were anaesthetized with ketamine (40-60 mg/kg injected ip) and xylazine (Rompun®, 1.4 mg/rat injected sc). The rats were provided with a silastic jugular vein cannula (15). Starting two days after surgery, the rats were handled daily to minimize stress during blood sampling. Daily

vaginal lavages were obtained and oestrous behaviour was observed after introduction of a male rat in the cage. Only rats exhibiting at least 2 consecutive 5-day cycles before experimentation were included in the study.

Chemicals

The opioid receptor antagonists, naloxone hydrochloride (Sigma Chemical Company, St Louis, USA; MW 363.8) and naltrexone (Sigma Chemical Company, St Louis, USA; MW 377.9) were given iv. Naloxone and naltrexone were dissolved in 0.2 ml saline (0.9%); controls received 0.2 ml saline.

Experimental Procedures

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

Restraint procedure

At pro-oestrus, rats were connected to a blood sampling cannula and 1 h later they were transferred to the experimental room. Rats were subjected to restraint stress by placing them individually in a perspex cylinder at t=-1 h (exp 1 and 2) or at t=-5 min (exp 3), i.e. 1 h and 5 min respectively before the presumed onset of the LH surge at t=0 h (for more details see ref. 14). The rats were released and returned to their home cages at t=5 h.

Exp 1. Effect of naloxone administration during restraint on the LH surge.

Rats subjected to restraint from t=-1 to t=5 h received naloxone or saline iv at t=3 h (1 mg) and at t=4 and t=5 h (0.5 mg). Non-stressed rats receiving saline served as controls. Blood samples (160 μl) were taken hourly during the LH surge period from t=0 to t=8 h.

Exp 2. Effect of pretreatment with naloxone on the restraint-induced suppression of the LH surge.

Rats received 1 mg naloxone or saline iv at t=-75 min (i.e. 15 min preceding the onset of restraint). The rats were subjected to 6 hrs of restraint stress starting at t=-1 h. Non-stressed rats receiving saline served as controls. Blood samples were taken hourly from t=0 to t=7 h.

Exp 3. Effect of naltrexone on the restraint-induced suppression of the LH surge.

In this experiment we tried to minimize the influence of opioid antagonism on the pro-oestrous GnRH-releasing mechanism. To extend the duration of the opioid receptor blockade and to avoid a premature onset of the LH surge, naltrexone (2 mg/rat) or saline was iv injected at t=-15 followed by restraint for 5 h which was started at t=-5 min. Non-stressed rats received naltrexone or saline at t=-15 min. A blood sample was taken just before application of restraint to determine a premature rise of plasma LH and subsequently blood samples were taken hourly from t=1 to t=8 h.

Radioimmunoassay

Blood samples were collected in heparinized tubes on ice. After centrifugation, plasma samples (70 μl) were diluted with 3 volumes of phosphate-saline buffer (pH 7.5) containing 0.1% bovine serum albumin (Sigma RIA-grade) and stored at -20°C. Plasma LH values were expressed as ng/ml in terms of LH-RP-2 which was diluted in serum of hypophysectomized rats. NIADDK-anti-rLH-S-9 antisera was used at a final concentration of 1:20.000. Donkey-anti-rabbit (Sac-cel, IDS, Boldon, Type and Wear, England) was used as second antibody. Determinations were performed in triplicate. Quality control sera with low, medium and high LH concentrations were included in each RIA. The detection level of the LH assay for diluted blood samples was 0.2 ng/ml at 90% B/B_0 level. The intra-assay and inter-assay variation for LH were 5.5% and 10.0%, respectively.

Statistical analysis

The response curves of plasma LH were plotted separately for each rat. LH data were analyzed using a MANOVA (two- or three-way analysis) with repeated measurements followed by post hoc analyses (Bonferroni) if a positive interaction was found. Each experimental group was defined as a separate treatment. One-way analyses of variance followed by the Mann Whitney-U test, if appropriate, was used for comparing values of peak height (i.e. the highest LH level measured) and values of different groups at given times. For statistical analysis the Statistical Program System for the Social Sciences (SPSS/PC+ V2.0, SPSS, Inc., Chicago, IL) was used. Differences were considered significant at a level of $P<0.05$. Values are reported as means ± SEM.

Results

Fig. 1 shows the effect of iv naloxone or saline injections given during restraint. In non-stressed rats the onset of the LH surge was at t=0 h and a maximum LH level of 9.0 ± 1.0 ng/ml was reached at t=3 h, after which LH levels decreased to basal at t=8 h. Comparison of the three experimental groups revealed a treatment effect ($F=7.5$; $P<0.01$) and a treatment x time interaction ($F=3.7$; $P<0.01$). Post hoc analysis showed, that LH levels (including peak height) during the entire period of the LH surge in saline- and naloxone-treated restraint rats were suppressed compared to non-stressed controls ($P<0.01$). No significant differences were found between saline- and naloxone-treated restraint groups.

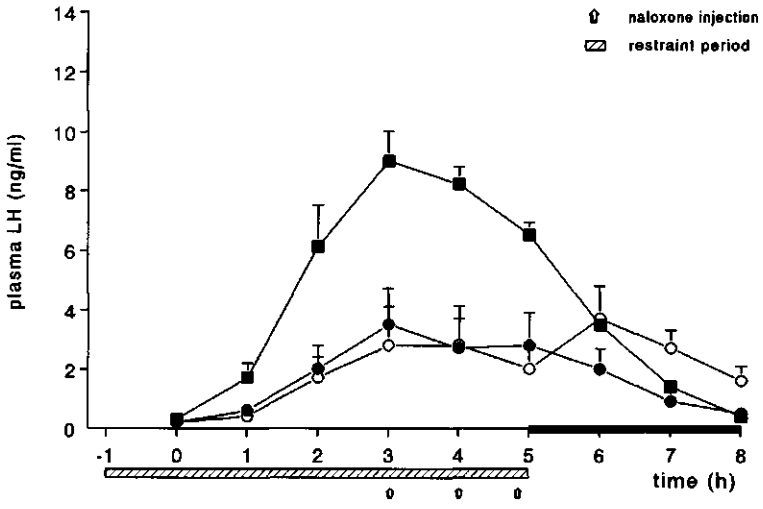

Fig. 1. Effect of naloxone administration during restraint on the LH surge in pro-oestrous rats. During restraint from t=-1 to t=5 h, naloxone or saline was iv injected at t=3 h (1 mg naloxone) and at t=4 and t=5 h (0.5 mg naloxone). (■) saline (N=10); (●) restraint + saline (N=7); (○) restraint + naloxone (N=10). Values are expressed as means ± SEM. The black horizontal bar represents the dark period.

Fig. 2 illustrates the effect of naloxone administered 15 min prior to application of restraint for 6 h starting at t=-1 h. A significant treatment effect ($F=5.1$; $P<0.05$) and a treatment x time interaction were observed ($F=2.2$; $P<0.05$). Saline pretreated restraint rats had reduced LH levels (including peak

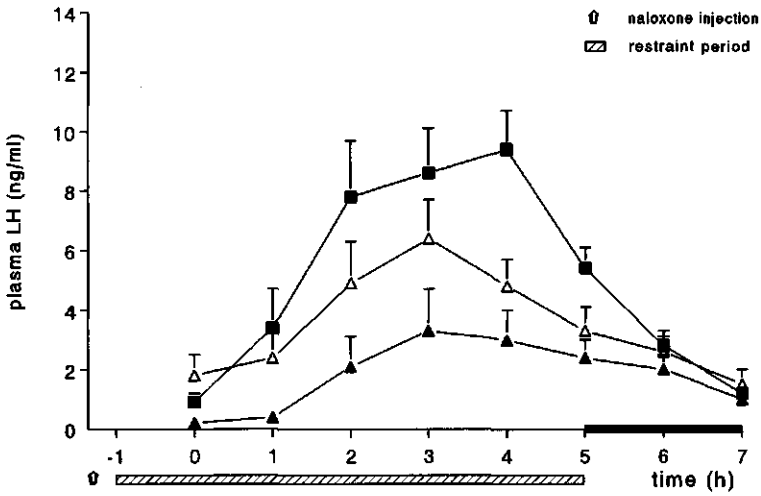

Fig. 2. Effect of pretreatment with naloxone (1 mg) or saline at t=-75 min on the restraint-induced suppression of the LH surge. Rats were subjected to restraint for 6 hrs starting at t=-1 h. (■) saline (N=10); (▲) saline + restraint (N=9); (Δ) naloxone + restraint (N=7). Values are expressed as means ± SEM. The black horizontal bar represents the dark period.

height) during the entire surge period as compared with non-stressed controls ($P<0.01$). LH levels in naloxone pretreated restraint rats differed from those of control rats only at t=4 h ($P<0.01$) and t=5 h ($P<0.05$), but not from saline pretreated restraint rats.

The effect of pretreatment with naltrexone on the restraint-induced suppression of the LH surge is depicted in Fig. 3. A significant effect of restraint ($F=19.6$; $P<0.01$) and a restraint x time interaction ($F=4.8$; $P<0.01$) were found. Restraint for 5 hrs starting at t=-5 min induced a suppression of LH levels (including peak height) during the entire period of the LH surge as compared to controls ($P<0.01$). Pretreatment with naltrexone at t=-15 min did not significantly affect the LH surge in non-stressed controls and restraint rats, although in both naltrexone-treated groups the LH levels during the first hours appeared higher as compared to their saline counterparts. Naltrexone pretreatment induced significantly higher LH levels at t=-6 min as compared to saline-treated rats (1.7 ± 0.2 vs 1.0 ± 0.1; $P<0.01$).

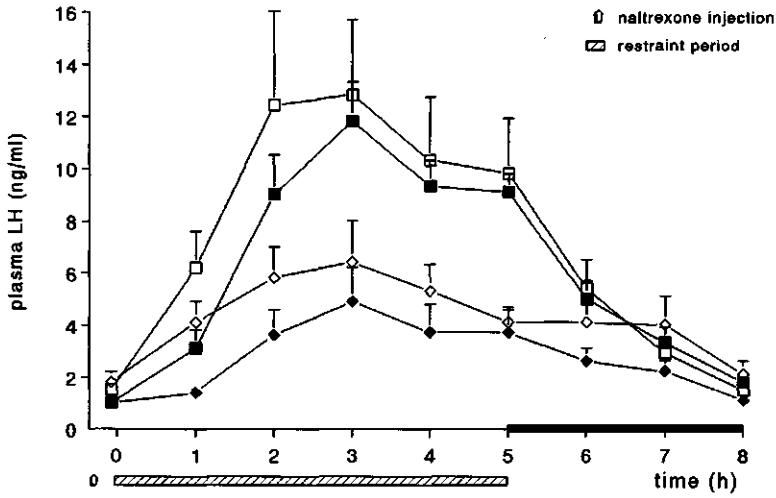

Fig. 3. Effect of pretreatment with naltrexone (2 mg) or saline at t=-15 min on the restraint-induced suppression of the LH surge. Rats were subjected to restraint for 5 hrs starting at t=-5 min. The first blood sample was taken at t=-6 min. (■) saline (N=14); (◆) saline + restraint (N=14); (□) naltrexone (N=6); (◇) naltrexone + restraint (N=8). Values are expressed as means ± SEM. The black horizontal bar represents the dark period.

Discussion

In the present study, we have examined the influence of the administration of opioid antagonists on the LH response to restraint stress. In agreement with previous studies (14,16,17), exposure to restraint resulted in an inhibition of the pro-oestrous LH surge in cyclic female rats. The inhibitory effect of restraint was not antagonized by iv injections of naloxone from 4 hrs after the initiation of restraint. Thus, the inhibition of the LH surge by restraint may not be due to activation of opioid systems, or could no longer be counteracted by naloxone injections given after the onset of restraint. A decline in opiate sensitivity during the LH surge period has been suggested since morphine (8) and naloxone (18) administration were not effective when given after initiation of the oestrogen progesterone-induced LH surge in OVX rats. Morphine or β-endorphin injections given before the onset of the LH surge, however, inhibit the spontaneous or

steroid-induced LH surge (4-8) suggesting that EOP exert an inhibitory action before the onset of the surge. Therefore, naloxone and naltrexone were subsequently given before the onset of restraint stress.

The results demonstrate that pretreatment with naltrexone induced elevated LH levels before the presumed onset of the LH surge. This confirms the results of other studies which report an immediate release of LH after administration of naloxone or naltrexone (11,18-20). It is generally assumed that in cyclic rats except for the period of the LH surge, EOP exert a tonic inhibitory influence on GnRH and LH secretion throughout the oestrous cycle (9,11,20). On the day of pro-oestrous, a premature decrease in opioid tone by naloxone administration can initiate an LH surge (11). In restraint rats pretreated with naloxone, the LH levels during the first 3 hrs of the surge were not different from those of controls whereas those of saline pretreated restraint rats were. This may be attributed to a premature rise of plasma LH or to a partial blockade of the restraint effect on the surge. The former possibility seems most likely since pretreatment with naltrexone (experiment 3), like naloxone, did not significantly affect the restraint-induced suppression of the LH surge but raised LH levels significantly at t=-6 min (i.e. at a time point before the stress was applied), and appeared to elevate LH levels during the first phase of the surge.

Many studies have suggested an opioidergic involvement in stress-related inhibition of gonadotropins in male and ovariectomized rats (21-25). The few available studies using intact female rats, have also suggested a role of opioids in the stress-induced inhibition of reproductive function in the intact female rat. Naloxone pretreatment reversed the anti-ovulatory effect of restraint in PMSG-primed female rats (12), and temporarily antagonized the footshock stress-induced inhibition of LH surge levels in pro-oestrous rats (13). It can not be excluded, however, that naloxone may have evoked a premature rise of LH by blockage of the inhibitory opioid tone also in these studies, because it was administered 15 and 5 min respectively before the presumed onset of the LH surge. Moreover, it is known that even slightly elevated plasma LH levels on pro-oestrus can induce ovulation (14). In accord with the present data, the results cited above may thus be explained by a premature LH surge induced by the naloxone treatment, rather than by antagonism of the effects of increased EOP release induced by stress.

In summary, the present study shows that the inhibitory effect of restraint on the surge of LH was not affected by pretreatment with naloxone or naltrexone.

This indicates that opioids are not involved as mediators in the inhibitory effect of restraint stress on the LH surge in the cyclic rat. In addition, withdrawal of EOP inhibition before the presumed onset of the LH surge resulted in elevated LH levels, suggesting an involvement of EOP in the initiation of the surge.

Acknowledgements

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Chapter 6

Inhibition of the LH surge by restraint stress in cyclic rats: studies on the role of $GABA_A$ and $GABA_B$ receptors

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Stress (accepted for publication)

Abstract

There is evidence that stress can alter the activity in the brain of gamma-aminobutyric acid (GABA), a neurotransmitter that has been implicated in the regulation of LH secretion. In the present study the role of GABA in the restraint stress-induced inhibition of the LH surge was investigated in the intact cyclic rat. Intracerebroventricular (icv) administration of the $GABA_A$ receptor agonist muscimol (0.1, 0.5 or 1 μg) 5 min before the presumed onset of the pro-oestrous LH surge (at 0900 h) caused a dose dependent suppression of the surge. A single dose of the $GABA_B$ receptor agonist baclofen (1 μg; icv) injected at 0855 h postponed the onset of the LH surge, and repeated injections at 0855 and 1130 h suppressed the surge. These data indicate that GABAergic activity in the brain can inhibit the LH surge in the cyclic rat via $GABA_A$ and $GABA_B$ receptors. Pro-oestrous rats were subjected to 5 hrs of restraint starting at 0855 h. Pretreatment with the $GABA_A$ receptor antagonist bicuculine (1 μg; icv) at 0840, 0940 and 1040 h or pretreatment with the $GABA_B$ receptor antagonist phaclofen (10 μg; icv) at 0840 h were ineffective in preventing the restraint-induced inhibition of the LH surge. The results suggest that $GABA_A$ and $GABA_B$ receptors are not involved in the inhibitory effect of restraint stress on the LH surge.

Introduction

Restraint stress suppresses the surge of gonadotropins in intact female rats (1-3). Stressors can induce significant alterations in the activity of various neurotransmitter systems in the central nervous system (4), but the mechanisms underlying this effect of restraint have not been resolved sofar.

GABA is a major inhibitory neurotransmitter in the brain. Under non-stress conditions, it plays an important role in the regulation of the GnRH release from the hypothalamus (5-8). GnRH neurons are under negative feedback regulation by oestrogen, but they do not contain oestrogen receptors (9). It has been proposed that GABA neurons, located in the medial preoptic area mediate this negative feedback action (10-12), since they contain oestrogen receptors (8,11-15). Indeed, the actions of GABA depend on the steroidal milieu and the phase of the oestrous cycle (16-18). In addition, a decrease in GABAergic activity precedes

the oestradiol-induced LH surge in ovariectomized rats (19), and GABA release in the preoptic hypothalamic region decreases coincident with the oestrogen-induced LH surge in ovariectomized rats (11,20,21).

Several studies using male rats have reported an involvement of GABA in behavioural changes induced by stress (22,23). Additionally, various stressors can induce alterations in the GABA content and/or turnover in several areas of the brain (4,23-26). An increase in hypothalamic GABA content has been reported after immobilization or ether stress (4,23,25). Little notice has been paid to the involvement of GABA in the effects of stress on the activity of the hypothalamus-pituitary-gonadal axis.

In the present study the effect of icv administration of the specific $GABA_A$ receptor agonist muscimol and $GABA_B$ receptor agonist baclofen on the pro-oestrous LH surge in intact female rats was determined. In addition, the effect of pretreatment with bicuculline, a specific $GABA_A$ receptor antagonist, and with phaclofen, a specific $GABA_B$ antagonist on the restraint-induced suppression of the pro-oestrous LH surge was investigated.

Materials and Methods

Rats, housing and surgery

Adult female F1 rats (200-250 g) of U-male and R-female substrain Wistar rats from the University breeding colony were used. They were individually housed under controlled conditions of temperature (22 ± 1°C) and lighting (14L:10D; lights on at 0000 h). Food and water were available ad libitum. At this light regimen most of our rats have 5-day cycles and the onset of the LH surge is approximately at 0900 h (1). During the dark period a dim light was left on to facilitate blood sampling.

Under ketamine and xylazine anaesthesia, cyclic rats were provided with a silastic jugular vein cannula and a stainless steel icv cannula into the right lateral ventricle (2,27,28). After surgery, the rats were handled daily to minimize stress during blood sampling. Daily, oestrous cyclicity was determined by microscopical rating of vaginal smears and by observation of oestrous behaviour after introduction of a male rat. Only rats showing at least 2 regular 5-day cycles were used for the experiments. After the experiments, icv cannula placement was examined by injection of 3 μl solution of Evans blue (10% in saline) and ma-

croscopical inspection of the brain after decapitation. Only data obtained from rats with a correctly placed icv cannula were used for analysis.

Chemicals

Muscimol, bicuculline, baclofen and phaclofen were purchased from Sigma Chemical Company, St Louis, USA. The drugs were dissolved in 0.9% saline and administered icv in a volume of 3 μl.

Experimental Procedures

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

Restraint procedure

Pro-oestrous rats were connected to a blood sampling cannula and transferred to the experimental room 1 h later. The rats were then individually subjected to restraint stress for 5 hrs in a perspex cylinder starting at 0855 h (for more details see ref. 1). The rats were released and returned to their home cages at 1400 h.

Effect of GABA agonists

The following treatment groups were studied: (1) control rats injected icv with saline at 0855 h; (2) rats injected icv with 0.1, 0.5 or 1.0 μg muscimol at 0855 h; (3) rats injected icv with 1 μg baclofen at 0855 h, or at 0855 and 1130 h. Blood samples were taken at regular time intervals during the period of the LH surge (from 0900 to 1400 h or to 1700 h).

Effect of restraint stress and pretreatment with GABA antagonists

Rats received icv 1 μg bicuculline or 10 μg phaclofen at 0840 h, followed by restraint stress from 0855 to 1400 h. Bicuculline (1 μg) injections were repeated at 0940 and 1040 h. Non-stressed rats receiving saline, bicuculline or phaclofen served as controls. Blood samples were taken hourly during the LH surge period (from 0900 to 1700 h).

Radioimmunoassay

Blood samples (160 μl) were collected in heparinized tubes on ice. After centrifugation, plasma samples (70 μl) were diluted with 3 volumes of phosphate-

saline buffer (pH 7.5) containing 0.1% bovine serum albumin (Sigma, RIA-grade) and stored at -20°C. LH levels were measured by radioimmunoassay, using materials provided by NIDDK. rLH-RP-2 diluted in serum of hypophysectomized rats was used as reference material. The second antibody was donkey-anti-rabbit (Sac-Cel®, IDS, Boldon, Type and Wear, England). Determinations were performed in triplicate. Quality control sera with low, medium and high LH concentrations were included in each assay. The detection level of the assay for diluted blood samples was 0.2 ng/ml at 90% B/B_0 level. The intra-assay and inter-assay variation for LH were 5.8% and 5.9%, respectively.

Statistical analysis

For statistical analysis the Statistical Program System for the Social Sciences (SPSS/PC+ V2.0, SPSS, Inc., Chicago, IL) was used. LH data were analyzed using a MANOVA (two-way or three-way analysis) with repeated measurements followed by post hoc analyses (Bonferroni) if a positive interaction was found. Each experimental group was defined as a separate treatment. One-way analysis of variance followed by the Mann Whitney-U test, if appropriate, was used for comparing values of peak height (i.e. the highest LH level measured) and values of different groups at given times. Differences were considered significant at a level of $P<0.05$. Values are reported as means ± SEM.

Results

Effect of icv administration of GABA agonists on the pro-oestrous LH surge

Fig. 1 illustrates the effect of icv administration of different doses of the $GABA_A$ agonist muscimol on the LH surge. Comparison of the four experimental groups revealed a treatment effect ($F=11.8$; $P<0.01$) and a treatment x time interaction ($F=2.2$; $P<0.01$). In the group of rats treated with 0.1 μg muscimol mean LH levels showed a partial inhibition of the LH surge ($P<0.05$). Notably, two rats in this group showed a normal LH surge. The other three showed complete inhibition of LH secretion until at least 1300 h. In the group treated with 0.5 μg muscimol, the inhibition was complete in all rats until 1200 h ($P<0.01$), after which plasma LH levels started to increase. This delayed onset of the LH surge varied from 1200 to 1400 h. After 1 μg muscimol the LH surge was suppressed in all rats during the entire sampling period ($P<0.01$).

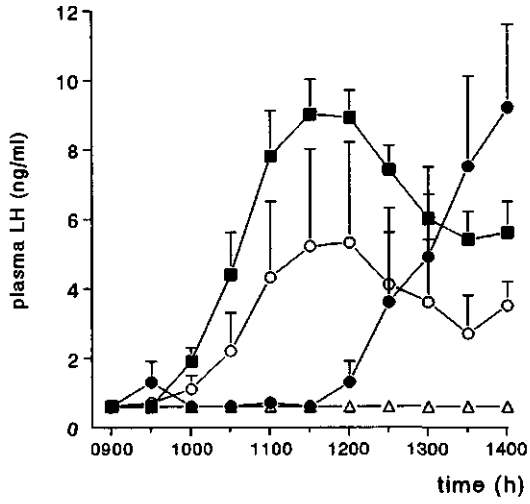

Fig. 1. Effect of icv injection of graded doses of the $GABA_A$ receptor agonist muscimol at 0855 h on the pro-oestrous LH surge. (■) saline (n = 11); (○) 0.1 μg muscimol (n = 5); (●) 0.5 μg muscimol (n = 7); (Δ) 1 μg muscimol (n = 8). Values are expressed as means ± SEM.

The effect of icv treatment with the $GABA_B$ agonist baclofen, on the LH surge is represented in Fig. 2. A significant treatment effect ($F = 6.1$; $P < 0.05$) and a treatment x time interaction ($F = 2.6$; $P < 0.05$) was found. Administration icv of a single dose of 1 μg baclofen at 0855 h suppressed LH surge levels until 1200 h ($P < 0.01$); subsequently plasma LH increased to control surge levels, resulting in a delayed surge with LH levels that were higher than controls at 1500 h ($P < 0.05$). Repeated injections with 1 μg baclofen at 0855 and 1130 h suppressed the LH surge ($P < 0.01$).

Effect of restraint stress and icv pretreatment with GABA antagonists on the pro-oestrous LH surge

Groups of rats were icv pretreated with saline, bicuculline or phaclofen and subjected to restraint from 0855 to 1400 h (Fig. 3). Comparison of the experiental groups revealed a significant treatment effect ($F = 9.9$; $P < 0.01$) and a treatment x time interaction ($F = 2.4$; $P < 0.01$). In non-stressed rats the LH surge after saline-treatment was similar to the surge of bicuculline- or phaclofen-

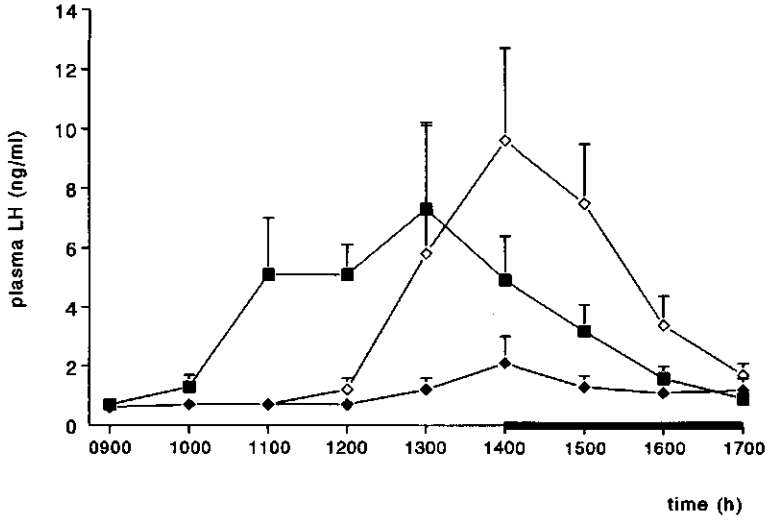

Fig. 2. Effect of icv injection(s) of 1 μg baclofen ($GABA_B$ receptor agonist) on the pro-oestrous LH surge. (■) saline at 0855 h (n=7); (◊) baclofen at 0855 h (n=4); (◆) baclofen at 0855 and 1130 h (n=8). Values are expressed as means ± SEM. The black horizontal bar represents the dark period of the lighting cycle.

treated rats (Fig.3; phaclofen data not shown). Saline-pretreated restraint rats had reduced LH levels during the period of the surge as compared with non-stressed controls ($P<0.01$). Neither pretreatment with the $GABA_A$ antagonist bicuculline, nor pre-treatment with the $GABA_B$ antagonist phaclofen did alter the restraint-induced inhibition of the LH surge in the intact female rat.

Discussion

First aim of the present study was to determine whether activation of central GABA mechanisms during the pro-oestrous LH surge can inhibit the surge in the intact female rat. The data show that the LH surge can be postponed or inhibited by administration of the $GABA_A$ receptor agonist muscimol, and the $GABA_B$ receptor agonist baclofen as well. These data accord with those obtained by others in ovariectomized or ovariectomized/steroid-primed females that were icv treated with muscimol or baclofen (29-33). Moreover, they are in

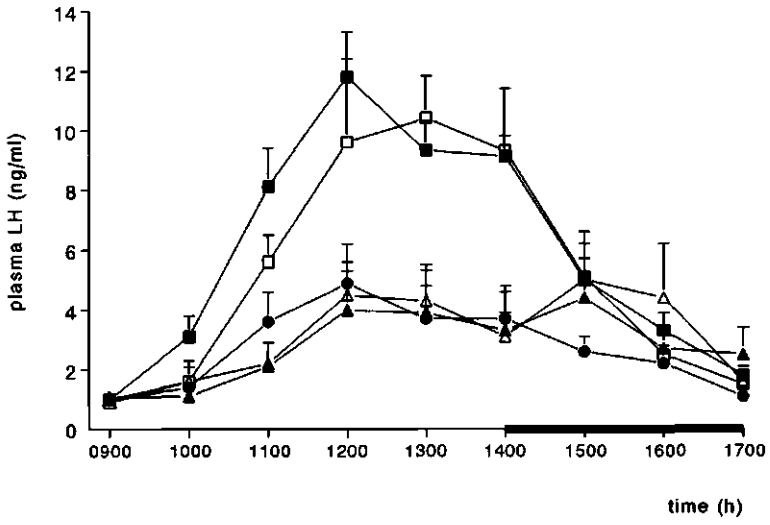

Fig. 3. Effect of icv pretreatment with bicuculline ($GABA_A$ receptor antagonist) or phaclofen ($GABA_B$ receptor antagonist) on the restraint-induced suppression of the pro-oestrous LH surge. Rats received saline at 0840 h, 1 μg bicuculline at 0840, 0940 and 1040 h, or 10 μg phaclofen at 0840 h, and were subjected to restraint stress from 0855 to 1400 h. (■) saline (n = 14); (□) bicuculline (n = 6); (●) saline + restraint (n = 14); (Δ) bicuculline + restraint (n = 7); (▲) phaclofen + restraint (n = 7). Values are expressed as means ± SEM. The black horizontal bar represents the dark period of the lighting cycle.

line with the notion that a decrease in GABAergic activity is a prerequisite for initiation of the LH surge (19).

In rats treated with low doses of muscimol (0.1 and 0.5 μg), LH levels were initially inhibited and thereafter started to increase, indicating a delay in the onset of the LH surge. Similarly, a single injection with baclofen resulted in a delayed onset of the surge. This temporary inhibition cannot be explained by a shift during the 'surge period' in the sensitivity of GABA mechanisms regulating gonadotropin release, since a higher dose of muscimol and repeated injections of baclofen completely inhibited LH secretion during the entire sampling period. The temporary effects thus likely relate to the biological half-life of the drugs. Yet, kinetic data to substantiate this notion have not been reported to our knowledge.

Since GABA is a major inhibitory neurotransmitter in the brain and plays an important role in the regulation of the GnRH release, we hypothesized that it might be involved in the restraint-induced suppression of the LH surge. The present study showed, however, that neither pretreatment with the $GABA_A$ receptor antagonist bicuculline, nor with the $GABA_B$ receptor antagonist phaclofen, antagonized the inhibitory effect of restraint on the LH surge. This strongly suggests that the restraint-induced inhibition of the LH surge is not mediated by $GABA_A$ receptors nor by $GABA_B$ receptors.

It has been suggested (7) that oscillating GABA activity in the period before the onset of the surge may be the synchronizing signal which triggers GnRH release into the portal vessels. In accord with this notion, it has been shown that in ovariectomized rats hypothalamic GABA concentrations fall before the onset of a steroid-induced LH surge (11,20). In addition, a 3 h infusion of the GABA antagonist bicuculline from 4 to 1 h before the presumed onset of the surge advanced the onset of the pro-oestrous LH surge, whereas a 3 h infusion started 5 h before the presumed onset of the surge, did not affect the onset of the surge (21). From these data it is inferred that during a critical period of 2-1 h before the onset of the LH surge, GABA activity prevents a premature increase of LH levels. Indeed, in the present study, non-stressed rats pretreated with bicuculline (or phaclofen) 20 min before the presumed onset of the surge, showed no premature LH surge. Thus, our results can not be explained from interference of the pretreatment with GABA antagonists with GABA activity involved in the cyclic control of the LH surge.

It has been proposed that, like GABA, endogenous opioids are also involved in the negative feedback action of oestrogen on GnRH release (34-37). Indeed, we have recently found that withdrawal of endogenous opioid activity in cyclic female rats before the presumed onset of the LH surge results in a premature rise of LH levels (3). In addition, several studies have suggested a role of endogenous opioids in the inhibitory effect of stress on reproduction but only a few studies have been performed with intact female rats (38,39). However, we have recently demonstrated that (pre)treatment with the opioid receptor antagonists naloxone or naltrexone did not affect the inhibitory effect of restraint on the pro-oestrous LH surge in intact cyclic rats, indicating that endogenous opioids are not crucially involved in this effect (3).

We conclude that, although the pro-oestrous LH surge can be inhibited by exogenous administration of $GABA_A$ and $GABA_B$ agonists, it is unlikely that

activation of GABA receptors mediates the inhibitory effect of restraint stress on the pro-oestrous LH surge.

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Chapter 7

Summary and conclusions

The influence of stress on reproductive functions has been subject of much research. Various kinds of stress are known to affect reproductive functions. In females, the complex regulation of the ovarian cycle relies on a series of neuroendocrine events whose temporal relationship is so critical that any disruption in the orderly sequence may jeopardize reproductive success. During the oestrous cycle, especially the mechanisms which induce oestrous behaviour, the LH and FSH surge and ovulation seem to be vulnerable to stress. The evidence that stress influences reproductive functions is mainly based on studies performed with intact and castrated male rats, and with ovariectomized and ovariectomized, oestrogen-primed female rats, and mainly concern the effect of stress on basal LH secretion. The hormonal status of these animal models differs essentially from those of intact pro-oestrous female rats, and this can of course be of influence on the response to the stressor. However, only a few studies have been performed on the effects of stress on the surge of gonadotropins in the intact cyclic female rat. This thesis focusses on the effect of restraint stress on the surge of gonadotropins and ovulation in the intact cyclic female rat and the underlying mechanisms.

In **Chapter 1** a review is given of stress and reproductive functions. In addition, possible pathways via which stress may influence reproduction are briefly discussed.

Chapter 2 describes experiments on the effects of different periods of restraint stress on preovulatory LH and FSH surge profiles and ovulation in the intact cyclic female rat. In literature, the few studies performed with intact female rats have shown that: 1) the LH surge and subsequent ovulation are suppressed by footshock stress (1), and that ovulation is inhibited by immobilization stress (2,3); 2) the gonadotropin surge can be delayed by one day if on pro-oestrus the surge is blocked (3-5), and 3) a partially suppressed LH surge can induce alterations in the ovulation process, since treatment of rats of which the endogenous LH surge is blocked with a small amount of LH on the morning of pro-oestrus can cause meiotic resumption and induce precocious follicle luteinization (6,7). In the experiments described in chapter 2, groups of pro-oestrous rats were subjected to restraint

which was started 0, 1 or 2 h before the presumed onset of the LH surge and continued during the surge until the beginning of the dark period, thereby covering most of the period during which the surge appears. The presumed onset of the LH surge is approximately 2 h after the middle of the light period (8). Exposure to restraint resulted in a partial or total inhibition of the LH and FSH surge (no significant difference between the three restraint groups). In rats with a partially suppressed LH surge, ovulation occurred or the ovaries contained unaffected graafian follicles on the day following the stress. In restraint rats with a completely blocked LH surge (≤ detectable levels), LH and FSH levels were not elevated the next day, indicating that the surge of gonadotropins had not been delayed by one day. The ovaries of these rats indeed contained unaffected graafian follicles. The results of this study indicate that, during pro-oestrus, restraint stress suppresses (and does not delay) the release of preovulatory gonadotropins in the intact female rat. Partial suppression of the LH surge by restraint induces ovulation or ovulation had not occurred and the graafian follicles were unaffected.

The following chapters are focused on the central mechanisms involved in the inhibition of the pro-oestrous surge of gonadotropins by restraint stress. An interaction of hormones and neuropeptides of the HPA axis (e.g. CRH, AVP and βEND) with those of the HPG axis (e.g. GnRH, LH and FSH) has been suggested. It has been reported that central administration of CRH (the major neuromodulator of the HPA axis) to male or ovariectomized female rats results in a suppression of LH-, but not of FSH-release (9-14). In addition, prolonged administration of CRH can cause downregulation of CRH receptors (15-17) which may alter its effect on the LH and FSH surge. Therefore, **Chapter 3** deals with the effects of central administration of CRH on the pre-ovulatory surge of LH and FSH in the intact female rat. An icv injection (bolus) or infusion (6-h) with CRH resulted in an inhibition of the LH surge and to a lesser degree, the FSH surge. The inhibition of pro-oestrous LH surge levels lasted 3-4 h; subsequently LH increased to control surge levels. A 9-h infusion of CRH which was started 4 h before the presumed onset of the surge, however, did not affect the surge of LH and FSH. This indicates that CRH can inhibit the LH surge (and to a lesser extent the FSH rise) for only 3-4 h and that thereafter adaptive mechanisms are activated that restore LH secretion to surge levels even in the presence of CRH. Inhibition of LH by CRH can occur via activation of the endogenous opioid βEND (11,18-20), and AVP may mediate the CRH-induced βEND release (21). Therefore, we investigated if the inhibitory effect

of CRH on the LH and FSH surge was modified by icv pretreatment with an AVP-antiserum. Pretreatment (icv) with AVP-antiserum did not prevent but, on the contrary, prolonged the inhibitory effect of CRH on the pro-oestrous LH surge. Pretreatment with AVP-antiserum before the start of the 9-h CRH infusion resulted in a suppression of the LH surge. AVP-antiserum alone did not influence pro-oestrous LH surge levels. These results indicate that AVP-antiserum pretreatment potentiates the inhibitory effect of CRH on the pro-oestrous LH surge. Therefore, we suggest that during high central levels of CRH, - as are obtained after icv injection and may occur during stress - AVP pathways become activated that mitigate the suppression of LH release.

In **Chapter 4** experiments are described in which we investigated the role of endogenous CRH and AVP in the inhibitory effect of restraint stress on the pro-oestrous LH surge. Many behavioural and physiological effects observed during stress can be mimicked by central administration of CRH (22), and CRH release is observed during stress (23-25). Additionally, in chapter 3 we showed that central administration of CRH can inhibit the LH surge in the intact cyclic female rat. A possible involvement of endogenous CRH in the restraint-induced inhibition of the LH surge was investigated by icv administration of the CRH antagonist α-helical CRH before application of restraint stress. In pro-oestrous rats, pretreatment with α-helical CRH did not affect the induced suppression, whereas the restraint-induced corticosterone response was partially prevented by both doses of the antagonist. These data suggest that CRH pathways activated during restraint stress do not mediate the inhibitory effect on LH surge levels. As suggested before, high central CRH levels may activate AVP pathways and several studies suggest some role of AVP in the control of LH secretion during stress. Therefore, we pretreated pro-oestrous rats with AVP-antiserum and then subjected them to restraint stress. In control rats, restraint stress partially inhibited the LH surge. Pretreatment (icv) with AVP-antiserum potentiated this inhibition. This result indicates that the residual surge seen in control rats is the result of a stimulatory AVP neuronal pathway activated by restraint stress. In an additional experiment, iv treatment with the GnRH agonist Ovalyse® during restraint resulted in a steep and transient rise of LH levels, indicating that the pituitary was not rendered refractory to GnRH.

During the oestrous cycle opioids tonically inhibit secretion of gonadotropins through inhibition of GnRH release, except for the period during the preovulatory surge of gonadotropins (26-28). Morphine or βEND administration before the onset

of the LH surge can inhibit the spontaneous or steroid-induced LH surge (29-33). Blockade of opioids receptors with the antagonist naloxone before the presumed onset of the LH surge, induces a premature surge (33,34). Several studies have suggested a role for opioids in the effects of stress on reproductive functions (inducing the inhibitory effects on LH secretion) but only a few have used intact female rats (1,2). In **Chapter 5** experiments are described which investigate the role of opioids in the restraint-induced inhibition of the LH surge in the intact cyclic female rat. The aim of our experiments was to distinguish between the putative role of opioids in mediating the inhibitory effect of restraint on the LH surge and their role in the cyclic regulation of LH secretion. Repeated iv injections with the opioid antagonist naloxone during restraint did not affect the restraint-induced suppression of the pro-oestrous LH surge, neither did pretreatment with naloxone or naltrexone, a longer acting opioid antagonist. This indicates that opioids are not critically involved as mediators in the restraint-induced suppression of the LH surge in the intact female rat. Pretreatment with naltrexone did induce a premature rise of LH levels, indicating, in line with results from others, that there is an inhibitory tone of endogenous opioids at that time, and that withdrawal of this inhibition can lead to initiation of an LH surge (34-37).

GABA is a major inhibitory neurotransmitter in the brain, and under non-stress conditions it is involved in the regulation of GnRH release. For instance, it appears to play a role in the negative feedback action of oestrogen on the GnRH release (38-43). Additionally, there is evidence that stress can alter GABAergic activity in the brain (44). Yet, a relationship between the stress effects on reproductive functions and GABAergic activity has not been established. In **Chapter 6** we investigated the role of GABA in the restraint-induced inhibition of the preovulatory LH surge. First, we determined the effect central GABA mechanisms on the pro-oestrous LH secretion. Indeed, icv administration of the $GABA_A$ receptor agonist muscimol as well as the $GABA_B$ receptor agonist baclofen inhibited the LH surge. These data accord with studies obtained by others in ovariectomized and ovariectomized/steroid-primed female rats (45-49). To investigate the conceivable involvement of GABA in the inhibitory effect of restraint on the LH surge, pro-oestrous rats were given an icv injection with the $GABA_A$ receptor antagonist bicuculline, or with the $GABA_B$ receptor antagonist phaclofen, and were subsequently subjected to restraint stress. Neither of these pretreatments, however, prevented the restraint-induced suppression of the LH surge. These data

suggest that activation of GABA receptors does likely not mediate the inhibitory effect of restraint on the LH surge of the intact female rat.

In summary, the experiments performed with intact cyclic female rats described in this thesis yielded the following conclusions;

- restraint stress during pro-oestrus can lead to (partial or complete) suppression of the release of preovulatory gonadotropins. Even when the surge is largely suppressed, the residual LH surge is sufficient to induce ovulation; in case of complete suppression, the graafian follicles remain unaffected and ovulation does not occur.
- exogenous CRH can inhibit the pro-oestrous LH surge (and to a lesser extent the FSH surge), but this effect is only temporary even when prolonged icv infusions with high doses are given ("escape" after 3-4 h). Pretreatment with AVP-antiserum prolongs the inhibitory effect of CRH on LH.
- exogenous CRH can activate a vasopressinergic mechanism that counteracts the inhibitory effects of CRH on the surge, and underlies the "escape".
- during restraint stress CRH pathways are activated that result in a corticosterone response, but appear not to be critically involved in the restraint-induced inhibition of the pro-oestrous LH surge.
- during restraint stress, a vasopressinergic mechanism is activated in the brain, that apparently stimulates LH secretion and can sustain an LH surge.
- endogenous opioids do likely not mediate the effect of restraint. Yet, an inhibitory opioid tone exists (also under non-stress conditions) in the period preceding the LH surge. Withdrawal of this tone can yield a premature surge.
- pharmacological stimulation of GABA receptors in the brain can inhibit the LH surge in the intact cyclic female rat via $GABA_A$ and $GABA_B$ receptors. GABA receptors, however, appear not to be involved in the inhibitory effect of restraint stress on the pro-oestrous LH surge of the intact female rat.

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Glossary

α-MSH	- α-melanocyte stimulating hormone
βEND	- β-endorphin
ACTH	- adrenocorticotropic hormone
AHA	- anterior hypothalamic area
ARC	- arcuate nucleus
AVP	- arginine vasopressin
CNS	- central nervous system
CRF	- corticotropin releasing factor
CRH	- *corticotropin releasing hormone*
DBB	- diagonal band of Broca
EOP	- endogenous opioid peptides
FSH	- follicle stimulating hormone
GABA	- gamma aminobutyric acid
GABA-T	- GABA-a-oxoglutarate transaminase
GAD	- L-glutamic acid decarboxylase
GnRH	- gonadotropin releasing hormone
HPA	- hypothalamus-pituitary-adrenal
HPG	- hypothalamic-pituitary-gonadal
icv	- intracerebroventricular
LH	- luteinizing hormone
LUF	- luteinized unruptured follicle
ME	- median eminence
MPN	- medial preoptic nucleus
MPOA	- medial preoptic area
OVX	- ovariectomized
OXT	- oxytocin
POMC	- pro-opiomelanocortin
PVN	- paraventricular nucleus
SCN	- suprachiasmatic nucleus
SON	- supraoptic nucleus

Samenvatting

Inleiding

Het doel van het in dit proefschrift beschreven onderzoek was een beter inzicht te krijgen in het effect van stress op de hypothalamus-hypofyse-gonaden as in de intacte cyclische rat.

Hoofdstuk 1 geeft een korte beschrijving van stress en de regulatie van de oestrische cyclus in de rat. Het geeft ook een kort overzicht van mechanismen die mogelijk betrokken zijn bij de door stress geïnduceerde remming van voortplantingsfuncties. De hoofdstukken 2 t/m 6 bechrijven de studies naar het effect van stress op de hypothalamus-hypofyse-gonaden as in de intacte cyclische rat.

Stress

Ondanks een steeds weer veranderend extern milieu kan een individu zijn interne milieu binnen bepaalde grenzen handhaven (homeostase). Indien de homeostase wordt bedreigd of verstoord, reageert het individu met een gedrags- en fysiologische verdedigingsrespons, de stressrespons. Deze heeft tot doel de bedreiging/verstoring weg te nemen en het biologisch evenwicht te herstellen.

Of een stimulus de homeostase bedreigt of verstoort, en, zo ja, hoe moet worden gereageerd, wordt in de hersenen beoordeeld. Het resultaat van dat beoordelingsproces hangt niet alleen af van de eigenschappen van de stimulus (bv intensiteit, duur, frequentie). Ook karakteristieken van het individu, zoals genetisch of tijdens de ontwikkeling vastgelegde eigenschappen, opgedane ervaringen en vaardigheden, en de actuele psycho-fysiologische staat van het individu, spelen daarbij een belangrijke rol. Ook de organisatie, coördinatie en evaluatie van de gedrags-fysiologische stressrespons gebeurt door de hersenen.

De gedragsrespons tijdens stress vormt meestal de feitelijke verdediging tegen de stressprikkel en is gericht op beëindiging van de stresssituatie (adaptatie). De fysiologische veranderingen, teweeggebracht via het autonome zenuwstelsel en het hormonale systeem, maken die gedragsrespons mogelijk door mobilisatie en redistributie van energie. Een snelle autonome activatie zorgt voor een onmiddellijke, verhoogde beschikbaarheid van energie. Relatief trage, hormonale mechanismen zijn met name van belang voor het beschikbaar maken van energie op de langere termijn. Daarbij speelt activering van de hypothalamus-hypofyse-bijnier as (HHB-as) een centrale rol.

De fysiologische stressrespons kan worden gezien als een herijking van de

prioriteiten in het individu. Fysiologische processen die energie verbruiken (anabole processen) alsmede onder stressomstandigheden een lage prioriteit hebben, zoals groei, immuniteit, herstel, vertering maar ook reproduktieve activiteit (het onderwerp van dit proefschrift), worden (tijdelijk) geremd. Dit gebeurt ten gunste van processen die een verdedigingsrespons mogelijk moeten maken. De stress-respons heeft dus een prijs die meestal gering is, maar die tijdens langdurige stress kan leiden tot stoornissen en ziekten.

Regulatie van de oestrische cyclus in de rat

De oestrische cyclus van de rat duurt 4 of 5 dagen en bestaat uit verschillende stadia; pro-oestrus, oestrus, metoestrus en dioestrus. Deze stadia zijn goed te volgen omdat onder invloed van geslachtshormonen het epitheel van de vaginawand een cyclus doormaakt. Door het maken van vaginale uitstrijkjes kan men bepalen in welk stadium van de cyclus de rat zich bevindt. De cyclus wordt gereguleerd door hormonen. Het systeem waarbinnen de interactie tussen de verschillende voortplantingshormonen zich afspeelt, wordt aangeduid als de hypothalamus-hypofyse-gonaden as (HHG-as). Het ovarium wordt door de hypofyse-hormonen LH (luteïniserend hormoon) en FSH (follikel stimulerend hormoon) aangezet tot produktie en afgifte van oestrogenen en progesteron. De afgifte van LH en FSH wordt gereguleerd door het GnRH (gonadotropin-releasing hormone) afkomstig uit de hypothalamus.

Tijdens oestrus, metoestrus en dioestrus zorgen de geslachtshormonen via negatieve terugkoppeling voor een remming van de afgifte van zowel GnRH als LH en FSH. Tijdens pro-oestrus leidt een verhoogde concentratie van oestrogenen via positieve terugkoppeling tot een kortstondige verhoogde afgifte van GnRH en vervolgens dus van LH (de LH-piek) en FSH (de FSH-piek). Het verloop van de cyclus wordt beïnvloed door de afwisseling van de licht- en donkerperioden. Het mechanisme dat het tijdstip van de preovulatoire LH- en FSH-piek regelt vertoont een 24-uurs ritme. Het begin van de LH- en FSH-piek valt ongeveer 2 uur na het midden van de lichtperiode waarbij de ratten gehuisvest zijn. De LH-piek induceert hervatting van de meiose van de eicel, ovulatie en luteïnisatie. De FSH-piek zorgt voor de ontwikkeling van een nieuwe set van follikels tot graafse follikels. Tijdens pro-oestrus wordt er naast een verhoging van de oestrogeenproduktie ook meer progesteron geproduceerd, dat vervolgens de afgifte van LH en FSH weer remt (negatieve terugkoppeling).

Stress en voortplanting

Stress kan leiden tot een verstoring van voortplantingsfuncties. Er is daarover al veel onderzoek gedaan. In het algemeen wordt gedacht dat vrouwelijke dieren gevoeliger zijn voor stress dan mannelijke dieren. De ovariële cyclus is immers afhankelijk van een ingewikkeld samenspel van tijdsgebonden neuro-endocrien gereguleerde veranderingen, en verstoring van één enkel proces daarin kan leiden tot verminderde vruchtbaarheid. Met name de mechanismen die het bronstgedrag, en de LH- en FSH-piek reguleren, zijn gevoelig voor stress. De experimentele evidentie daarvoor is echter vooral afkomstig uit onderzoek uitgevoerd met intacte en gecastreerde mannelijke ratten en met vrouwelijke, geovariëctomeerde ratten, die al dan niet met oestrogenen behandeld zijn. In deze diermodellen is met name het effect van stress op de basale LH-afgifte bestudeerd. De hormonale status van deze dieren verschilt echter duidelijk van die van intacte cyclische vrouwelijke ratten. Het is daarom goed mogelijk dat intacte vrouwtjes geheel anders op stress reageren dan geovariëctomeerde. Bij de intacte cyclische rat is er echter nog maar weinig onderzoek gedaan naar de invloed van stress op voortplantingsfuncties.

Onderzoek naar de invloed van stress op voortplantingsfuncties bij het intacte vrouwelijke dier, en de daarbij betrokken mechanismen wordt bemoeilijkt door de complexiteit van de regulatie van de ovariële cyclus. Bovendien veranderen door stress een groot aantal (neurale en hormonale) mechanismen in de hersenen en periferie in activiteit. In de literatuur wordt een verband gesuggereerd tussen de hormonen van de HHB-as die tijdens stress geactiveerd worden en de hormonen van de HHG-as. Centrale toediening van corticotropin-releasing hormone (CRH), vasopressine (AVP) of β-endorphin (βEND) leidt inderdaad tot een remming van de LH-afgifte en blokkering van de ovulatie. Omdat die neuropeptiden/ hormonen een belangrijke rol spelen bij tal van stressresponsen, wordt gedacht dat het effect van stress op de HHG-as ook bij het intacte vrouwelijke dier via deze neuropeptiden/hormonen tot stand komt. Ook tal van andere factoren die tijdens stress vrijkomen kunnen echter betrokken zijn bij de door stress geïnduceerde onderdrukking van voortplantingsfuncties, waaronder bijvoorbeeld gamma amino-butyric acid (GABA), de belangrijkste inhiberende neurotransmitter in de hersenen.

Overzicht van de in dit proefschrift beschreven studies

Hoofdstuk 2 beschrijft het onderzoek waarin het effect bestudeerd wordt van verschillende perioden van restraint-stress op de preovulatoire LH- en FSH-piek en de ovulatie in de intacte cyclische rat. In deze studies verstaan we onder restraint-

stress het opgesloten zitten in een perspex cilinder waardoor de rat in haar bewegingsvrijheid wordt beperkt. In het geringe aantal beschreven studies met intacte vrouwelijke ratten is aangetoond 1) dat de LH-piek en ovulatie onderdrukt worden door voetschok-stress, en dat ovulaties ook geremd kunnen worden door immobilisatie-stress; 2) dat de LH-piek 24 uur uitgesteld kan worden indien tijdens pro-oestrus de piek geblokkeerd is waardoor alsnog - zij het 24 uur later - ovulatie kan optreden; 3) dat een gedeeltelijk onderdrukte LH-piek veranderingen in het ovulatieproces kan bewerkstelligen. In ratten die geïnjecteerd zijn met een kleine hoeveelheid LH in de ochtend van de pro-oestrus, wordt namelijk de meiose hervat. Bovendien wordt dan een vroegtijdige luteïnisatie van de follikels geïnduceerd waardoor de eisprong niet meer kan optreden.

In het onderzoek beschreven in hoofdstuk 2 werden ratten in pro-oestrus onderworpen aan restraint-stress vanaf het tijdstip 0, 1 of 2 uur vóór het verwachte begin van de LH-piek tot vlak voor de donkerperiode. De restraintperiode bestreek daardoor bijna de gehele periode van de LH-piek. Restraint resulteerde in een totale of gedeeltelijke onderdrukking van de LH- en FSH-piek (geen significant verschil tussen de 3 restraintprotocols). In gestresste ratten waarbij geen LH-piek meer detecteerbaar was, waren de LH- en FSH-waarden de volgende dag niet verhoogd. In ratten met een gedeeltelijk onderdrukte LH-piek, bleek het effect van stress op de ovulatie nogal variabel: bij sommige dieren traden er normaal ovulaties op, bij andere was de ovulatie geheel geblokkeerd en bevatte het ovarium de volgende dag onveranderde graafse follikels. De resultaten van deze studie tonen aan dat restraint-stress kan leiden tot een totale onderdrukking van de LH-piek en daardoor tot blokkering van de ovulatie, en dat totale onderdrukking van de LH-piek tijdens pro-oestrus niet leidt tot uitstel van de piek (en dus ovulatie) met 24 uur. Bovendien geven de resultaten aan dat ook na een sterk gereduceerde LH-piek nog ovulatie kan optreden. Het is niet duidelijk of er een relatie bestaat tussen de hoogte van de residuele LH-piek en het ovulatieproces. Een licht verhoogde afgifte van LH gedurende een bepaalde tijd lijkt voldoende te zijn om ovulatie te kunnen induceren.

Hoofdstuk 3 t/m 6 gaan over centrale mechanismen waarvan bekend is dat ze tijdens stress kunnen worden geactiveerd, en die betrokken zouden kunnen zijn bij de door restraint-stress geïnduceerde onderdrukking van de pro-oestrische LH- en FSH-piek. De literatuur suggereert een interactie tussen de hormonen en neuropeptiden van de HHB-as, zoals CRH, AVP en βEND, met die van de HHG-as,

zoals GnRH, LH en FSH. Maar ook andere factoren, in het bijzonder GABA, zouden een rol kunnen spelen.

Het is bekend dat een centrale (icv) injectie met CRH (de belangrijkste modulator van stress-reacties) in mannelijke en geovariëctomeerde vrouwelijke ratten resulteert in een onderdrukking van de LH-, maar niet van de FSH-afgifte. Langdurige icv toediening van CRH kan leiden tot een vermindering van CRH-receptoren wat mogelijk een verandering in het effect van CRH op de LH- en FSH-piek kan veroorzaken. **Hoofdstuk 3** beschrijft het onderzoek waarin het effect bestudeerd wordt van CRH op de preovulatoire LH- en FSH-piek in de intacte cyclische rat. Een icv injectie of een 6-uur durend infuus met CRH (gegeven, respectievelijk gestart, 1 uur voor het begin van de LH-piek) resulteerde in een onderdrukking van de LH-piek en in mindere mate van de FSH-piek. De onderdrukking van LH duurde 3-4 uur, waarna vervolgens het LH-niveau steeg tot het niveau van de controle groep op een vergelijkbaar tijdstip van de surge. Een 9-uur durend infuus met CRH dat 4 uur voor het begin van de LH-piek was gestart, had géén onderdrukkend effect op de LH- en FSH-piek. Dit duidt erop dat CRH de afgifte van LH (en in mindere mate van FSH) maar tijdelijk (3-4 uur) kan onderdrukken, mogelijk doordat er mechanismen geactiveerd worden die er na enige tijd voor zorgen dat de LH-afgifte tot het niveau van de controle wordt hersteld. Volgens literatuurgegevens, zou de onderdrukking van LH door CRH kunnen verlopen via stimulering van de βEND-afgifte; de door CRH geïnduceerde βEND-afgifte zou via AVP tot stand komen. We hebben daarom onderzocht of het remmende effect van CRH op de LH- en FSH-piek kon worden voorkomen door de dieren icv vooraf te behandelen met een AVP-antiserum. Door zo'n voorbehandeling wordt AVP gebonden, en kan het zijn werking niet uitoefenen. We vonden echter dat voorbehandeling met AVP-antiserum leidde tot een verlenging van het onderdrukkende effect van CRH op de LH-afgifte. In AVP-antiserum voorbehandelde dieren leidde een 9-uur durend infuus met CRH, gestart 4 uur voor het verwachte begin van de LH-piek, tot een vrijwel volledig onderdrukte LH-piek. In niet aan stress blootgestelde controle ratten had het AVP-antiserum zelf geen effect op de LH-piek. Deze resultaten wijzen erop dat hoge CRH-concentraties in de hersenen kunnen leiden tot afgifte van AVP in de hersenen, waardoor de door CRH geïnduceerde onderdrukking van de LH-afgifte wordt tegengegaan.

Veel effecten die tijdens stress worden waargenomen, kunnen worden nagebootst door centrale toediening van CRH. In hoofdstuk 3 is reeds aangetoond

dat CRH de LH-piek kan onderdrukken in de intacte cyclische rat. Uit de literatuur is bekend dat gedurende stress de afgifte van CRH en AVP wordt gestimuleerd. In de literatuur wordt een betrokkenheid van beide factoren gesuggereerd in de controle van de LH-afgifte gedurende stress. **Hoofdstuk 4** beschrijft het onderzoek waarin de rol van CRH en AVP onderzocht werd in het onderdrukkende effect van restraint-stress op de pro-oestrische LH-piek. Blokkering van de CRH receptoren door de CRH receptor-antagonist α-helical CRH vóór het begin van de restraintperiode had geen effect. De door stress geïnduceerde corticosteron-respons werd wel gedeeltelijk tegengegaan door de CRH-antagonist. Deze resultaten suggereren dat gedurende restraint-stress CRH-systemen in de hersenen worden geactiveerd, maar dat die niet betrokken zijn bij het remmende effect op de LH-afgifte. Zoals hierboven is vermeld, zouden hoge CRH-concentraties in de hersenen die bv tijdens stress voorkomen, een AVP-systeem kunnen activeren. Aan ratten in pro-oestrus is daarom icv AVP-antiserum toegediend voordat ze aan restraint-stress werden onderworpen. Restraint veroorzaakte een gedeeltelijke onderdrukking van de LH-piek. In ratten die voorbehandeld waren met het AVP-antiserum was deze onderdrukking aanzienlijk sterker. Dit resultaat toont aan dat het residu van de LH-piek dat nog in gestresste controle ratten wordt waargenomen, mogelijk het gevolg is van een door restraint geactiveerd stimulatoir AVP-systeem.

Om uit te sluiten dat door restraint-stress de gevoeligheid van de hypofyse voor GnRH veranderd wordt, is aan ratten in pro-oestrus, tijdens restraint, de GnRH agonist Ovalyse® toegediend. Dit resulteerde in een snelle en kortstondige stijging van het LH-niveau, hetgeen erop wijst dat restraint niet leidt tot ongevoeligheid van de hypofyse voor GnRH. Het is daarom waarschijnlijk, dat het remmende effect van restraint op LH plaatsvindt op het niveau van de hypothalamus.

Endogene opioïden in de hersenen spelen een rol in de regulatie van de oestrische cyclus. Ze vormen een schakel in de negatieve terugkoppeling van de oestrogenen op de GnRH-afgifte. Blokkering van opiaatreceptoren door de opiaatreceptor-antagonist naloxone voor het begin van de LH-piek kan dan ook leiden tot een voortijdige start van de LH-piek. Meerdere studies suggereren een rol voor endogene opioïden in het remmende effect van stress op voortplantingsfuncties. Echter maar in één enkel onderzoek werd gebruik gemaakt van intacte vrouwelijke ratten. In dit onderzoek kon het onderdrukkende effect van voetschok-stress gedeeltelijk worden voorkomen door de dieren vooraf te behandelen met naloxone. In hoeverre hier sprake was van een vervroeging van de LH-piek, of van een

werkelijk antagonisme van door stress geactiveerde endogene opioidsystemen, is onduidelijk. **Hoofdstuk 5** beschrijft het onderzoek waarin de rol van endogene opioïden bestudeerd wordt in de door restraint-stress geïnduceerde onderdrukking van de LH-piek in de intacte cyclische rat. Het doel was onderscheid te maken tussen de rol van endogene opioïden tijdens de cyclische regulatie van de LH-afgifte en de vermeende rol van endogene opioïden tijdens het onderdrukkende effect van restraint-stress op de LH-piek. Herhaalde intraveneuze injecties met naloxone tijdens de restraintperiode hadden geen effect op de door stress geïnduceerde onderdrukking van de LH-piek. Ook een injectie met naloxone of met de langer werkende antagonist naltrexone voorafgaande aan de restraintperiode had geen effect. De voorbehandeling met naltrexone resulteerde echter wel in een voortijdige stijging van het LH-niveau. Uit de resultaten kan worden afgeleid dat endogene opioïden geen rol spelen in de door restraint geïnduceerde onderdrukking van de LH-piek in de intacte cyclische rat. In overeenstemming met de resultaten van onderzoek door anderen is gevonden dat het onderbreken van de remming door opioïden tijdens de periode voorafgaande aan de LH-piek in de intacte rat kan leiden tot een voortijdige LH-piek.

GABA is een belangrijke, inhiberende, neurotransmitter in de hersenen en is betrokken bij de regulatie van GnRH-afgifte. Het lijkt een rol te spelen in de negatieve terugkoppeling van oestrogenen op de GnRH-afgifte. Uit literatuur-gegevens blijkt dat stress de GABAerge activiteit in de hersenen kan beïnvloeden. Tot nu toe is echter een verband tussen het effect van stress op voortplantingsfuncties en een veranderde GABAerge activiteit niet vastgesteld. **Hoofdstuk 6** beschrijft het onderzoek naar de betrokkenheid van GABA bij de door restraint-stress geïnduceerde onderdrukking van de LH-piek in de intacte cyclische rat. In eerste instantie is het effect van centrale activatie van GABA mechanismen op de pro-oestrische LH-afgifte bestudeerd. Zowel de icv geïnjecteerde $GABA_A$ receptor-agonist muscimol als de $GABA_B$ receptor-agonist baclofen waren in staat de LH-piek te onderdrukken. Dit komt overeen met het resultaat van studies waarin gebruik is gemaakt van geovariëctomeerde en geovariëctomeerde/oestrogeen behandelde vrouwelijke ratten. Om een mogelijke rol van GABA vast te stellen in het onderdrukkende effect van restraint op de LH-piek, zijn ratten in pro-oestrus icv geïnjecteerd met de $GABA_A$ receptor-antagonist bicuculline of met de $GABA_B$ receptor-antagonist phaclofen alvorens ze werden onderworpen aan restraint-stress. Geen van beide antagonisten hadden invloed op de door restraint geïnduceerde

remming van de LH-piek. Dit resultaat suggereert dat het onderdrukkende effect van restraint-stress op de LH-piek bij de intacte cyclische rat niet tot stand komt via GABA receptoren.

Conclusie

De resultaten beschreven in dit proefschrift tonen aan dat restraint-stress tijdens pro-oestrus leidt tot een onderdrukking van de preovulatoire LH- en FSH-piek. Een totaal onderdrukte LH-piek gaat gepaard met een totale blokkering van de ovulatie. Een sterk gereduceerde LH-piek is nog in staat om ovulatie te induceren. CRH, opioïden en GABA lijken niet betrokken te zijn bij de door restraint geïnduceerde onderdrukking van de LH-piek in de intacte cyclische rat. Door centrale toediening van CRH, en ook tijdens stress, wordt in ratten in pro-oestrus een AVP-systeem geactiveerd dat een totale onderdrukking van de LH-piek voorkomt. De intacte vrouwelijke rat is daardoor in staat het effect van stress op de HHG-as tijdens pro-oestrus te beperken, zodat het reproduktief vermogen gehandhaafd blijft.

Dankwoord

Dit proefschrift is het eindresultaat van een onderzoek dat eind 1990 is gestart. Een onderzoek dat slechts kon worden uitgevoerd met de hulp van velen.

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Bedankt

Marjolijn

Curriculum vitae

Marjolijn Monique Roozendaal werd geboren op 29 april 1965 te Gouda. In 1983 behaalde zij het Atheneum-β diploma aan het Rijksscholengemeenschap te Gouda. In datzelfde jaar begon zij met haar studie Biologie aan de Rijksuniversiteit Utrecht. Hoofdrichtingen tijdens de doctoraalfase waren Vergelijkende Endocrinologie en Aquatische Toxicologie. Tijdens deze fase heeft zij als studentassistent diverse praktica begeleid en behaalde zij ook de artikel 9 bevoegdheid Proefdierkunde.

In december 1990 werd zij als assistent in opleiding (AIO) bij de vakgroep Fysiologie van Mens en Dier aan de Landbouwuniversiteit te Wageningen. Als AIO verrichte zij het onderzoek dat leidde tot de totstandkoming van dit proefschrift. Het onderzoek is uitgevoerd binnen het kader van de onderzoeksschool "Pathofysiologie van het zenuwstelsel".