NEURAL REGULATION AND DYNAMICS OF PROLACTIN SECRETION IN THE RAT

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Neural Regulation and Dynamics of Prolactin Secretion in the rat

Neurale Regulatie en Dynamiek
van de Prolactine Secretie in de rat

Proefschrift
ter verkrijging van de graad van
doctor in de landbouw- en milieuwetenschappen,
op gezag van de rector magnificus,
dr. H. C. van der Plas,
in het openbaar te verdedigen
op maandag 18 juni 1990
des namiddags te vier uur in de aula
van de Landbouwuniversiteit te Wageningen

Stellingen.

1. Alle thans bekende golven van prolactinesecretie in cyclische, (schijn)drachtige en lacterende ratten staan onder controle van verschillende neurale reguleringsmechanismen.
   Dit proefschrift.

2. Er bestaat een sexeverschil in de neurale regulatie van de prolactinesecretie bij de rat.
   Dit proefschrift.

3. Er is één gemeenschappelijk neuraal centrum, het mediaal preoptisch gebied, dat alle thans bekende golven van prolactinesecretie in de rat controleert.
   Dit proefschrift.

4. Uit het feit dat het bestaan van een autoregulatiemechanisme voor de prolactinesecretie niet kon worden aangetoond, mag niet worden geconcludeerd dat dit fenomeen bij de rat niet zal voorkomen.

5. Het feit dat bepaalde onderzoeksmethodieken stressvrij zouden zijn voor de proefdieren, houdt niet in dat dit ook geldt voor de experimentatoren.

6. Een geëmancipeerde vrouw ervaart positieve discriminatie negatief.

7. Met vaarbelasting vaart de overheid wel.

8. Het bemesten van alle akkerbouwland volgens de normen van de volkstuinder lost het mestoverschotprobleem in de Nederlandse landbouw op.
   Van der Meer, H.G., P.C. Meeuwissen: Emissie van stikstof uit landbouwgronden in relatie tot bemesting en bedrijfsvoering. Landschap 6: 19-32, 1989.

9. Als een bepaalde stressindicator aangeeft dat er geen stress optreedt, betekent dit niet dat er geen stress is.
   Wiersma,J., J.Kastelijn: Stress en welzijn: niet altijd logisch vast te stellen. In: Kwaliteiten in de dierlijke productie, Zodiac-symposium, Wageningen, 1988.

10. Het zenuwstelsel in de darmen van zoogdieren en de cerebrale ganglia van insecten zijn te beschouwen als hersenen.

11. Met betrekking tot de huidige milieuproblematiek zou het Friese spreekwoord: "It is de boer al like folle oft de ko skyt of de bolle" geactualiseerd moeten worden door het woord "like" hierin te vervangen door "to".

Stellingen behorende bij het proefschrift: "Neural regulation and dynamics of prolactin secretion in the rat" door J. Wiersma.

Wageningen, 18 juni 1990

Veel wordt bewezen wat in de grond niet waar is, en
Veel is eeuwig waar, ofschoon 't bewijs niet daar is.

P. de Genestet

Aan mijn ouders,
Aan Taeke, Nynke en Jitty

VOORWOORD

Dit proefschrift is tot stand gekomen dank zij de hulp van velen. Een dankwoord is op zijn plaats aan allen die speciaal tot de totstandkoming hebben bijgedragen.

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CONTENTS

INTRODUCTION

Prolactin (Prl) is one of the trophic hormones that are released from the hypophysis. The hormone acts on several reproductive processes in both males and females, but its effects vary from species to species. The spectrum of actions of Prl differs markedly from that of the other gonadotrophic hormones: luteinizing hormone (LH) and follicle-stimulating hormone (FSH). These latter two hormones only bind to specific cells within gonadal tissue and appear to have the same functions in all mammalian species. Prl, in contrast, not only binds to reproductive tissues, but to other tissues as well, and may also play a role in a variety of processes involved in homeostasis, and responses to stress. In fact, the evolutionary history of Prl is not as a reproductive hormone, but as a regulator of salt and water balance.

It has been difficult to determine exactly what the actions of Prl are. The only clearly defined role of Prl in mammalian reproduction common to all species is its involvement in the regulation of milk production during lactation. The effects of Prl on the male reproductive tract are only now beginning to be understood. The importance of Prl in regulating ovarian function, except in rodents, is not at all clear. In humans, except for lactation, it is not known whether Prl is physiologically important in the functioning of the testis, the regulation of the menstrual cycle, or in breast growth and development. The observation, however, in animals as well as humans, that hyperprolactinemia is usually associated with a suppression of reproductive function indicates that Prl does play an important role. A more precise characterization of the physiological role of Prl awaits further investigation.

The regulation of Prl secretion is exerted mainly via the hypothalamus, and this appears to involve the actions of a Prl-inhibiting factor (PIF) and a possible Prl-releasing factor (PRF). Release of these hypothalamic regulatory factors is under control of both hormonal and neural influences, that is, by the feedback actions of Prl itself and its target cell hormones, and by stimuli carried via afferent neural circuits. Despite numerous investigations, the precise mechanisms of the hormonal and neural control are still far from understood. There are, in fact, serious limitations in any attempt to define the neural events related to a particular neuroendocrine phenomenon. Hormonal secretion is not the primary result of one single trigger stimulus, but, instead, is due to a complex feedback interaction of neural circuits controlling various aspects of hormonal secretion, such as its induction, timing, duration and magnitude. As for Prl, it is not yet possible to define precisely which aspect of the secretion is likely to be mediated by a particular neural circuit. Only with regard to the mechanisms for hypothalamic control there is a general consensus now that a system of tuberoinfundibular dopaminergic neurosecretory (TIDA) neurons represents the "final common pathway" for the inhibitory control of Prl secretion. To date, only biochemical studies have been employed successfully in estimating the activity of these TIDA neurons; electrophysiological studies were unsuccessful. So, as yet, it has still to be established that the TIDA neurons indeed reflect the peptidergic system that regulates Prl secretion. Therefore, specifically electro-physiological data are needed, to elucidate the causal

relationship between electrical activity of TIDA neurons, dopamine release and Prl secretion. A complicating and limiting factor is that hormonal measurements can only be performed at discrete time intervals, which interferes with a more complete characterization of the neuroendocrine control of Prl secretion.

The aim of this study was to contribute to a better understanding of the basic mechanisms involved in the neuroendocrine control of Prl secretion. Throughout the study the rat was used as experimental animal, since in this species the role of Prl in reproductive function is most clearly defined: in cycling rats the surges of Prl secretion induce luteolysis in non-functional corpora lutea, in pregnant rats, however, Prl acts as the initial luteotrophic factor, and in lactating rats Prl stimulates growth and secretory activity of the mammary glands. As such, the female rat displays unique patterns of Prl secretion, dependent upon the reproductive state. Prl secretion in cycling rats is characterised by a surge during the afternoon and early night of proestrus and a smaller surge on the afternoon of estrus. Pregnant and pseudopregnant rats secrete a surge of Prl twice daily for 10 to 12 days, a nocturnal surge in the early morning hours and a diurnal surge in the late afternoon. Lactating rats, in response to suckling, release considerable amounts of Prl, independent of the time of the day. These well-defined endocrine conditions were used as experimental model for the study of the neural control of Prl secretion. Experimentation was usually performed with freely moving individual rats. The neural regulation was studied on the basis of the actual secretion patterns of Prl, the responses to stressful stimuli, and the effects of hormonal and electrical stimulation of the brain. Most of the experiments were performed on the basis of the following hypothesis:

> The various surges of Prl secretion appearing in cycling rats, (pseudo)pregnant rats and lactating rats, respectively, are controlled by different neural regulatory mechanisms

The aim of this study was to find the answer to several questions:

1) Is it possible to demonstrate the existence of different neural regulatory mechanisms for each of the surges of Prl secretion on the basis of a detailed picture of individual release patterns?
2) Is it possible to demonstrate the existence of different neural regulatory mechanisms for each of the surges of Prl secretion on the basis of differences in the Prl responses to stressful stimuli?
3) Is an autofeedback mechanism involved in the regulation of Prl secretion?
4) Is the regulation of Prl secretion different between the sexes?
5) Are the various surges of Prl secretion in cycling, (pseudo)-pregnant and lactating rats finally controlled by one common neural centre?

These questions will be answered in this thesis, which is based on seven already published papers, successively making up the chapters 1 to 7.

The study starts with an investigation of circulating Prl levels in individual undisturbed cycling and pseudopregnant rats (chapter 1). Therefore, a chronic blood sampling/ transfusion procedure was introduced. This procedure permits high-frequency sampling for

several hours at a maximum rate of one blood sample per minute. As such, individual Prl secretory patterns were determined in detail in order to assess the dynamics of Prl secretion and to investigate whether the actual secretory patterns varied with the physiological state (question 1). Since the usual practices of animal handling, surgery, anaesthesia and blood sampling easily produce stress, these aspects are considered in more detail in chapters 2 and 3. The studies presented herein deal with several methodological problems: 1) does cannulation interfere with normal hormone secretion, 2) how long does surgery affect hormone secretion, 3) how much blood loss can be tolerated by the animals, 4) can high frequency blood sampling/transfusion be performed without introducing stress, and finally 5) can blood transfusion be performed with either fresh or preserved donor blood.

The next step was to investigate the existence of different neural regulatory mechanisms for each of the surges of Prl secretion, on the basis of differences in the responses to stress (question 2). Therefore, in chapter 4, secretion patterns of Prl were determined at hourly intervals from 07.00-22.00 h in cycling and pseudopregnant rats. Based on observations from chapter 1, different regulatory mechanisms for Prl secretion were hypothesized for three time periods: a nocturnal (07.00-11.00 h), a prediurnal (14.00-17.00 h), and a diurnal (19.00-22.00 h) period. The hypothesis was tested by investigating the effects of surgery and red light upon Prl secretion.

One of the factors controlling Prl secretion may be an autoregulatory mechanism (question 3). This possibility is investigated in chapter 5, by studying the effect of intracerebroventricular infusion of Prl upon endogenous Prl secretion. The study was restricted to proestrous rats.

The following phase in the study was to identify more precisely which brain areas controlled the secretion of Prl, and in which way. One approach to this problem is to implant electrodes chronically into "suspected" brain areas and investigate the effect of electrical stimulation upon the secretion of Prl under various reproductive states. Particularly the medial preoptic area (MPOA) is an interesting area since it shows sexual dimorphism. As part of the hypothalamus it is concerned functionally with several parameters of homeostasis, (sexual) behavior and endocrine function. Another interesting area is the median eminence (ME) which contains the terminals of the TIDA neurons. The ME is considered to be the source of PIF and perhaps PRF. In order to study sexual differences in the regulation of Prl secretion (question 4) male and cycling females were stimulated electrically to investigate 1) whether the MPOA had a sexually differentiated function in the regulation of Prl secretion and 2) whether electrophysiological evidence could be found for a sexual dimorphism in PIF or PRF activity in the ME (chapter 6). This chapter also deals with the conditions for stressfree experimentation. Finally the effect of electrical stimulation of the MPOA upon Prl secretion was investigated during the appearance of all presently known surges of Prl secretion in cycling, pregnant and lactating rats (chapter 7). This investigation would provide the answer to question 5 whether the various modes of Prl secretion are finally controlled by one common neural centre.

CHAPTER 1

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A Detailed Characterization of Prolactin Secretion Patterns during Daylight in Individual Cycling and Pseudopregnant Rats

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Key Words. Prolactin · Estrous cycle · Pseudopregnancy · Pulsatile secretion · Disappearance rate

Abstract. In order to characterize plasma prolactin (Prl) patterns during the estrous cycle and pseudopregnancy (PSP), I collected sequential blood samples between 8.00 and 19.00 h at 1-hour, 1.5-min or 1-min intervals from conscious, unrestrained female rats. During the afternoon of proestrus and estrus a single Prl surge was observed, while during PSP two daily Prl surges occurred, one nocturnal and one diurnal. During PSP the secretion of Prl occurred occasionally in substantial bursts from baseline levels. This contrasted with the proestrus afternoon surge when plasma Prl levels were constantly elevated due to a more or less continuous release of fluctuating amounts of Prl. The differences in timing and secretion patterns of the three surges suggest a separate neural regulation. The diurnal surge on day 0 of PSP may be composed of two different surges which also may have a separate neural regulation: one peak occurs at 14.00–16.00 h, and is comparable in timing to the Prl surge during the afternoon of estrus; a second surge starts at 17.00 h, and is comparable in time of onset to the diurnal surge on the other days of PSP. Plasma Prl always appeared to increase in an unpredictable manner, discontinuously by means of several bursts, with maximum increments of about 600 ng/ml/min. The shortest half-time values, as calculated from the disappearance of Prl from the circulation, were about 2.2 min. The individual patterns show that Prl release must be the consequence of a very dynamic neural regulatory process.

In the rat the secretion patterns of prolactin (Prl), LH, FSH, estrogen and progesterone during the different states of reproduction have been well described. During the estrous cycle, plasma levels of Prl, LH and FSH are low, except on the afternoon of proestrus when typical proestrus surges are observed [4, 5, 9, 31, 34]. Some investigators have found a second surge of Prl secretion of smaller amplitude and shorter duration on the afternoon of estrus [4, 5, 9, 27], whereas others did not [31, 34]. In pseudopregnant (PSP) rats serum LH and FSH levels remain low, but for Prl two daily surges of secretion are observed, one nocturnal and one diurnal [1, 10, 13, 31, 32]. The two Prl surges during PSP are differently responsive to steroids [11] and stress: a variety of noxious procedures temporarily suppresses the diurnal PSP surge [10, 26]; however, the Prl proestrus afternoon surge, which is comparable in magnitude and timing to the diurnal PSP surge, seems to be less susceptible to stress [27]. These differences between the three Prl surges suggest that at least three distinct neural regulatory mechanisms for Prl release may exist [10]. This study sought to distinguish between the three Prl surges on the basis of a detailed picture of the individual release patterns.

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Materials and Methods

Adult F1 hybrid female rats derived from two inbred strains of female Wistar (R-Amsterdam) and male Sprague-Dawley (U-Amsterdam) rats were used. They were housed in an artificially lighted room with the lights on from 06.00 to 20.00 h, at a temperature of 21–25 °C, and had free access to rat chow and water. At the time of the experiments they were 6–9 months old and weighed 180–240 g.

A silastic tube was inserted into the right external jugular vein of ether-anesthetized rats so that the tip was located near the entrance of the right atrium [see 24]. The cannula was connected to a curved stainless steel tube, which was attached to the skull with screws and acrylic cement. The cannula was filled with saline containing 40% polyvinylpyrrolidone and 500 IU heparin per milliliter. To prevent backflow it was closed with a polyethylene plug.

After surgery the animals were housed individually in transparent plastic cages, where they remained throughout experimentation. They were handled daily for at least 2 weeks to accustom them to the experimental procedure. Vaginal smears were taken 5–7

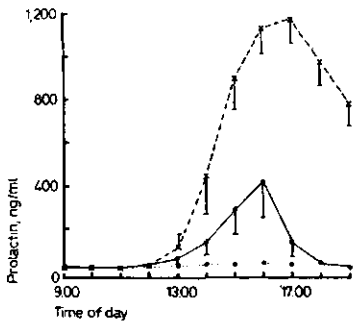

Fig. 1. Mean plasma concentrations (± SEM) of Prl during the estrous cycle of the rat. Measurements were made at 1-hour intervals from 9.00 to 19.00 h. Plasma levels during proestrus (×, n = 9) and estrus (●, n = 18) are first significantly elevated at 14.00 h. ○ = Diestrus (n = 18).

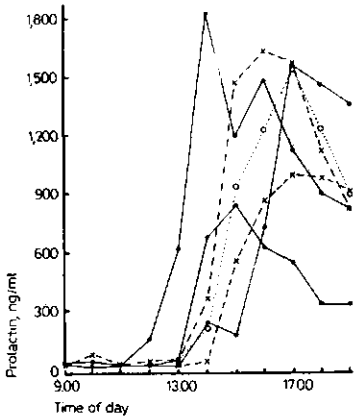

Fig. 2. Plasma Prl secretion patterns from 9.00 to 19.00 h in 6 individual rats serially bled at 1-hour intervals on proestrus, with a 4-day (· · · · ·), 5-day (- - - - -) or 6-day (———) cycle. Note the difference in time of onset of the proestrus surge and peak levels between the animals.

times a week between 7.00 and 11.00 h. Most females had 5-day cycles, although 15% had 6-day cycles and a few 4-day cycles were also observed. Pseudopregnancy was induced by electrical vaginal/cervical stimulation in the afternoon of proestrus. The following day was designated as day 0 of pseudopregnancy.

In a first series of experiments blood samples (200 µl) were collected at 1-hour intervals from unrestrained animals, during the various days of the estrous cycle from 9.00 to 19.00 h, and during pseudopregnancy from 8.00 to 19.00 h. Thus, by the end of the day a total amount of 2.2 or 2.4 ml of blood was withdrawn. This way of blood sampling has been reported not to affect prolactin concentration [24]. After the last sample was taken, the total amount of blood removed was restored with blood from F1 donor-rats. This allowed us to use the animals repeatedly.

In a second series of experiments Prl secretion patterns were determined more in detail by collecting sequential blood samples of 200 µl at 1- or 1.5-min intervals. After every five samples blood volume was restored by injection of 1 ml of blood from cannulated castrated male F1 donor rats. The whole blood sampling/transfusion procedure took less than 40 s. In control experiments this blood sampling/transfusion technique appeared not to affect normal Prl secretion. For example, no correlation was found between the time of injection of 1 ml of blood and the occurrence of a rise or decrease in Prl release. The injected blood usually had a Prl concentration of < 20 ng/ml, although in a few cases the levels were higher, up to 160 ng/ml. However, because of dilution in the blood and interstitial space, even this highest concentration made a negligible contribution to the endogenous Prl levels.

Blood samples were diluted with 100 µl of heparinized phosphate-buffered saline (pH = 7.5). Plasma samples were stored at −20 °C until analyzed by means of a homologous double-antibody radioimmunoassay (RIA) for rat Prl as developed by *Kwa* et al. [20, 21]. Rat prolactin RP-1 (obtained from the NIAMDD Rat Pituitary Hormone Distribution Program) was the standard used (biological potency 11 IU/mg). In some experiments plasma samples were analyzed in triplicate (n = 375). From these data the reliability of the RIA could be estimated. Compared to the average Prl value, 71% of the Prl values had a deviation < 10%; 91% had a deviation < 15%. Half-times ($t_{½}$) for the disappearance of Prl from the blood were calculated according to the formula $Y_t = Y_0 e^{-t/t_{½}}$. Results, where appropriate, are expressed as means ± SEM. Of the plasma Prl levels the logarithm was taken and consequently within rats d_t was calculated as follows: $d_t = {}^{10}\log c_t - {}^{10}\log c_0$, where c_t and c_0 are Prl concentrations at time t and time 0, respectively. An increase in Prl concentration at time t was tested by a deviation of d from zero, using Student's t test.

Results

Plasma Prl Levels at 1-hour Intervals during the Estrous Cycle

Figure 1 shows the average Prl patterns during proestrus (n = 9), estrus (n = 18) and diestrus (n = 18).

Proestrus levels were first significantly increased at 13.00 h ($p<0.02$ vs. 10.00 h). Peak values of 1,100 ng/ml appeared at 16.00 and 17.00 h. At 19.00 h Prl levels were still very high (> 700 ng/ml). In the individual animals the onset of the proestrus afternoon surge occurred between 12.00 and 15.00 h. The shape of the individual proestrus patterns and the maximum Prl levels varied considerably (fig. 2).

Estrus levels showed a first significant increase at 13.00 h ($p<0.02$ vs. 10.00 h), peaked at 16.00 h, but in contrast to the proestrus pattern returned to baseline values by 18.00 h (fig. 1). During the afternoon of estrus individual Prl levels fluctuated erratically and all 18 animals had one or more

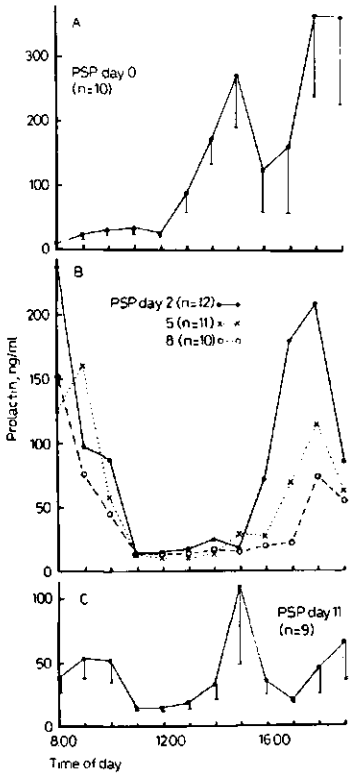

Fig. 3. Mean plasma concentration (± SEM) of Prl on days 0 **(A)**, 2, 5, 8 **(B)** and 11 **(C)** of pseudopregnancy at 1-hour intervals from 8.00 to 19.00 h. Standard errors are not shown on the means of days 2, 5 and 8. Except for day 0, plasma levels on the afternoon are not significantly increased before 16.00 h (see text).

episodes with elevated Prl levels > 50 ng/ml, normally not exceeding 400 ng/ml, but 3 animals had peak levels of about 1,800 ng/ml.

Diestrus levels were low and showed minor fluctuations (fig. 1). Afternoon values between 13.00 and 18.00 h were slightly, but significantly higher ($p<0.05$) compared to morning values (at 10.00 h: 15.3 ± 2.4 ng/ml). In 11% of the observations, elevated levels (40–179 ng/ml) were found throughout the day, slightly more frequently between 12.00 and 16.00 h.

Plasma Prl Levels at 1-Hour Intervals during PSP

In our RU rats pseudopregnancy terminated at day 11 (n = 2), day 12 (n = 14), day 13 (n = 16) or day 14 (n = 3). Figure 3A shows the summarized data (n = 10) for plasma Prl concentration on day 0 of PSP. There was no indication of the falling phase of a nocturnal surge. From 10.00 h throughout the observation period, in the individual rats erratically elevated Prl levels were observed, and, while some animals had greatly elevated leveles, others had basal levels. At 15.00 h, for example, all 10 animals had elevated Prl levels (44–912 ng/ml), while at 16.00, 17.00, 18.00 and 19.00 h the number of animals with Prl levels > 40 ng/ml were 6, 4, 8 and 6, respectively. Compared to the Prl level at 10.00 h (32.2 ± 9.2 ng/ml) afternoon levels from 14.00 h were significantly higher ($p<0.05$), except at 17.00 h.

The Prl patterns for days 2, 5 and 8 are plotted in figure 3B and the Prl pattern for day 11 is presented in figure 3C. The falling phase of the *nocturnal surge* is clearly observed. At 10.00 h Prl levels were still significantly elevated ($p<0.05$) compared to 11.00 h, at which time the nocturnal surge has ended. As for the *diurnal surge*, on day 2, compared to the averaged data of 11.00–13.00 h, the first significant increase was found at 17.00 h ($p<0.002$), on day 5 at 16.00 h ($p<0.02$), on day 8 at 17.00 h ($p<0.01$) and on day 11 at 19.00 h ($p<0.02$). Also before the onset of the diurnal surge, in the individual animals elevated Prl levels were sometimes seen; in the time period 14.00–16.00 h 17% of the blood samples had Prl concentrations of > 40 ng/ml. After 16.00 h, during the diurnal surge, Prl levels were not elevated constantly and towards termination of PSP, baseline levels were observed much more frequently; for example, in the time period 18.00–19.00 h the percentage of Prl levels < 40 ng/ml on days 0, 2, 5, 8 and 11 were 30, 50, 27, 60 and 66%, respectively. So the diurnal surge of PSP represents an average Prl pattern, but in individual animals episodes with baseline as well as increased Prl levels are observed. The same observation was made for the falling phase of the nocturnal surge, although baseline levels were seen less frequently.

Plasma Prl Patterns at Short Time Intervals

In order to investigate more in detail the patterns of Prl secretion during the afternoon of proestrus, blood samples were collected at 1-min or 1.5-min intervals in 14 rats. In several rats, one or more short Prl bursts were observed before the onset of the proestrus afternoon surge (fig. 4A). The disappearance rate ($t_{1/2}$) of plasma Prl was calculated from the decrease between two sample points. In the first 3 min after a burst (fig. 4A) the $t_{1/2}$ values were very short, about 2.2 min, which indicates that immediately after a burst for a few minutes no Prl was released, but after that, as can be seen from the falling phase of these bursts, variable amounts of Prl were released. Once the Prl proestrus surge had started, Prl levels increased in an unpredictable and highly nonlinear manner (fig. 4B, C, D). The longest linear increase observed was of only 6 min duration (fig. 4B: 12.24–12.30 h). The amounts of Prl released also varied considerably. Maximum plasma Prl increments were 400–600 ng/ml/min (see fig. 4). In order to investigate whether this was the highest possible release, 4 rats were injected i.v. with

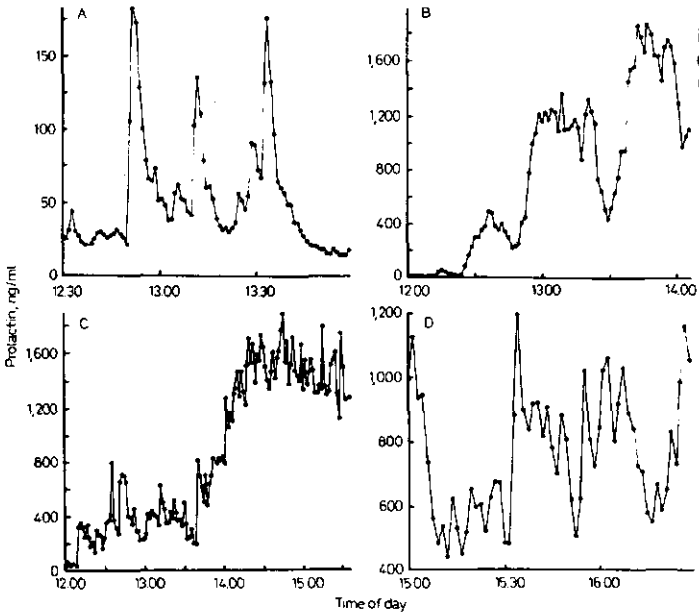

Fig. 4. Plasma Prl secretion patterns of 4 individual rats bled at 1-min **(A)** or 1.5-min **(B, C, D)** intervals on the afternoon of proestrus. Plasma samples of figures **B, D** are analyzed in triplicate and averaged. Note the different scales along the axes.

perphenazine (0.5 mg/100 g body weight), which is known to stimulate Prl release strongly [24]. These injections produced discontinuous release of Prl with increments of up to 700 ng/ml/min (fig. 5).

The t½ values calculated from the falling phase of bursts occurring during the fully established afternoon surge at proestrus had lowest t½ values of about 3 min, but usually the t½ values fluctuated between 4 and 7 min and were often greater. This indicates that during the proestrus afternoon surge there was a more or less continuous release of variable amounts of Prl (see fig. 4B, C, D).

During diestrus, at various times of the day, 1.5-min interval studies of 1.5 h each (n = 12) showed that occasionally very small bursts of Prl were released with peak levels up to 150 ng/ml. 4 rats did not show a burst during the observation period. The same study was performed during estrus in 4 rats. In 1 rat the onset of a burst was seen with a peak level of 800 ng/ml, the other 3 rats had baseline levels during the sampling period.

During PSP, short time interval studies were made in 9 rats. In 1 animal, sequentially sampled at 1-min intervals on day 0 of PSP, Prl levels between 13.30 and 15.30 h were constantly elevated (> 200 mg/ml) (fig. 6). This observation is in accordance with what was found in the 1-hour interval study on day 0 of PSP (fig. 3A), where at 15.00 h the blood samples of all 10 animals had elevated Prl levels as well. Later during PSP, for the falling phase of the nocturnal as well as for the diurnal surge, a different Prl release pattern was observed. Then the release of Prl occurred in short but substantial bursts from baseline levels and with peak levels of up to 800 ng/ml. This is illustrated in figure 7A, C for the nocturnal surge and in figure 7B, D for the diurnal surge. This release pattern is also in agreement with the results of the 1-hour interval study. That study namely showed that during the occurrence of these surges, Prl levels were not elevated constantly, but that, in the individual animals, there were one or more episodes with elevated Prl levels, indicating a burst-like release of Prl. Such a release pattern is confirmed by the short time interval studies (fig. 7). Shortest half-time values were about 2.2 min, but the disappearance of Prl from the blood often occurred more slowly (fig. 7).

Discussion

The present study shows that Prl is released much more dynamically than has hitherto been appreciated. Moreover, the pattern of Prl secretion varies with different physiological states. Adenohypophysial hormone release and particularly Prl release [19] is known to be affected markedly by stress. Even a brief handling or a brief ether stress [24] causes a significant rise in plasma Prl in male rats, and in females the response varies according to the physiological state [27]. Surgery, blood volume reduction and anesthesia also may affect the circulating Prl levels [22, 26]. So for these experiments a blood sampling procedure which does not affect normal Prl secretion [24, 26] was used, while blood vo-

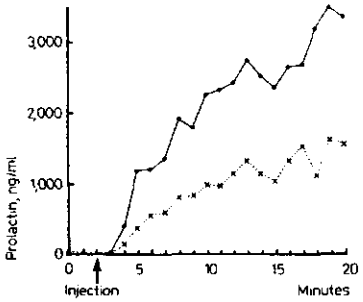

Fig. 5. The effect of i.v. injection of perphenazine (0.5 mg/100 g body weight) on Prl secretion 2 female rats on diestrus (●) and proestrus (×). Time of injection was 10.00 h. A discontinuous release pattern with plasma increments of up to 700 ng/ml/min is observed.

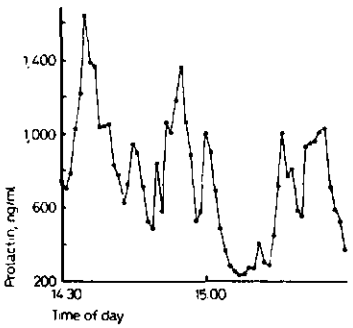

Fig. 6. Plasma Prl secretion pattern of a female rat on day 0 of pseudopregnancy, sequentially sampled at 1-min intervals from 14.30 to 15.30 h. Plasma levels are elevated continuously (> 200 ng/ml).

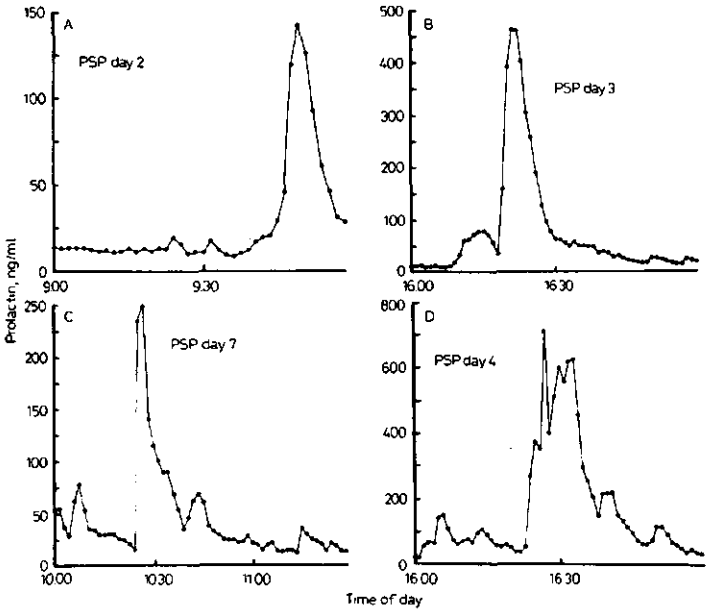

Fig. 7. Plasma Prl secretion patterns of female rats on different days of pseudopregnancy at 1-min (B, C) or 1.5-min (A, D) intervals. The release of Prl occurs in bursts from baseline levels. Note the different scales along the axes.

lume reduction was avoided using a blood transfusion technique. During daylight the plasma Prl levels were followed in individual freely moving rats at 1-hour and at 1-min or 1.5-min intervals in order to characterize the plasma Prl patterns during the estrous cycle and PSP.

Prl secretion patterns during the estrous cycle [4, 5, 9, 31, 34], pseudopregnancy [1, 10, 13, 31, 32] and pregnancy [3, 32] have been well described. There is a general agreement on the appearance of the Prl surge on the afternoon of proestrus as well as on the occurrence of two daily surges of Prl, one nocturnal and one diurnal, during PSP and our study supports this concept (fig. 1, 3).

During the afternoon of estrus both baseline [31, 34] and elevated [4, 5, 9, 27] Prl levels have been reported. In our rat strain there was an afternoon surge of Prl at estrus. The fact that we measured the diurnal surge during PSP, which is known to be suppressed by stress [10, 26], indicates that our blood sampling method does not interfere with normal Prl secretion. So we agree with *Castro-Vazquez and McCann* [5] who argued that Prl release during estrus is not due to stress as *Smith* et al. [31] suggested, but that this surge really takes place.

A circadian variation in the secretion of Prl during diestrus, as observed in the current study, has been reported by

some workers [18]; in other studies it could not be demonstrated [4, 5, 9, 31, 34], possibly because blood sampling has been too infrequent or the number of animals too small. Circadian rhythmicity is a widespread biological phenomenon [for review see ref. 28]. In the male rat a circadian variation in the secretion of Prl has also been reported [for literature see ref. 25]. Although our data in the female rat may indicate that in the afternoon there is an increase in the burst-like release of Prl, it is also possible that an increase in the tonic secretion of Prl [15] may account for the circadian variation in the secretion.

The short time interval studies have shown that in individual rats the release of Prl during the afternoon of proestrus is different from that during PSP. During PSP the secretion of Prl occurs episodically in substantial bursts from baseline levels (fig. 7). A similar release pattern has been reported for other hormones, such as GH in male [36] and female [29] rats and LH in castrated male and female rats [12, 35], and there is evidence for an occasional release of Prl in male [36] and female [29, 36] rats. This secretion pattern contrasts with that during the proestrus afternoon when plasma Prl is elevated constantly due to sustained release of rapidly fluctuating amounts of Prl (fig. 4). Between animals there is a great variability in time of onset and Peak levels (fig. 2, 4). A similar observation has been made for the proestrus surge of LH in intact female rats [2]. However, the rising phase of the LH proestrus surge appeared to be near linear and was reasonably consistent in slope and duration between animals [2], but the rising phase of the Prl surge at proestrus lasted between 1 and 3 h and occurred in a discontinuous and unpredictable manner by rapid pulsatile increases. Such a release pattern of Prl has recently been demonstrated in the male rat [30]. This release pattern is emphasized strongly by the very short half-time for the disappearance of Prl from the blood. The half-time values found in this study are in reasonable agreement with those of some other investigators [14, 18], although shorter than the 7 min reported for male rats after ether [6] or drug [30] treatment. The shortest half-time values of 2.2 min were frequently observed immediately after the occurrence of a burst from baseline level, but in those circumstances Prl may disappear faster than during the proestrus afternoon, when the interstitial space and the receptors are more saturated.

The differences in secretion patterns during various physiological states suggest that there may be different neural regulations for the secretion of Prl. During the afternoon of estrus, in each rat, the plasma Prl level was elevated erratically, indicating that Prl was released occasionally in short bursts, in the same way as during PSP. During estrus this bursting activity peaked at 14.00–15.00 h (fig. 1) when 30 blood samples ($n = 36$) had Prl levels > 40 ng/ml, and the same was true for day 0 of PSP where during the same period this occurred 18 times ($n = 20$) (fig. 3A). This suggests that during this period Prl levels were elevated more or less constantly. However, on the other days of PSP this peak was not observed and since the onset of the diurnal surge did not begin before 16.00 h (fig. 3B), the diurnal surge on day 0 of PSP might be composed of two different peaks, which may have a separate neural regulation, one peak at 14.00–16.00 h comparable to the surge at estrus, and a second starting after 16.00 h. Further support arises from the observation in the one animal sequentially sampled during the occurrence of the first peak on day 0 of PSP (fig. 6), which indeed had a continuously elevated Prl level in contrast with Prl patterns observed after 16.00 h (fig. 7B, D). Furthermore the work of *Beach* et al. [1] indicates that the diurnal surge on day 0 of PSP may be composed of two peaks. However, from the available data it is not possible to draw definite conclusions.

There is much more evidence for a separate neural regulation of the Prl surges during proestrus and PSP. This has first been suggested by *Freeman* et al. [10] on the basis of different responses to stress, and is supported by the present study by the differences in individual afternoon patterns of Prl secretion during proestrus (fig. 4) and PSP (fig. 7) as well as by the differences in time of onset of the proestrus surge (14.00 h, fig. 1), the diurnal PSP surge (16.00 h, fig. 3) and the nocturnal PSP surge. Moreover, the different ways in which steroids [11, 13, 22, 33], several neurotransmitters [7, 23, 26, 33] and various brain areas [8, 16, 17] are involved in the regulation of the three surges strongly support this hypothesis. However, before the brain regions controlling these separate regulatory systems are known, further experiments are necessary. The present study has shown that it is possible to follow rapidly changing levels of Prl secretion on a minute-by-minute basis. Such a dynamic model may prove useful for investigating the neural control of Prl secretion. We hope that electrophysiological recording techniques can be combined with the blood sampling/transfusion technique in future experiments.

Acknowledgments

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CHAPTER 2

A chronic technique for high frequency blood sampling/transfusion in the freely behaving rat which does not affect prolactin and corticosterone secretion

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ABSTRACT

A blood sampling/blood transfusion technique is described for chronically cannulated freely behaving rats. The procedure permits high frequency sampling for several hours at a maximum rate of one blood sample per min. Prolactin and corticosterone were used as indicators of stress.

In male rats the prolactin concentration in blood obtained by rapid decapitation was not significantly different from that obtained through a cannula. A blood volume reduction of 1 or 2 ml did not affect prolactin or corticosterone secretion; however, a reduction of 3 ml or more increased corticosterone secretion but did not consistently increase prolactin secretion. When blood volume reduction was compensated for by blood transfusion, frequent blood sampling did not affect prolactin or corticosterone secretion in dioestrous rats. The surges of prolactin secretion during the afternoon of pro-oestrus and pseudopregnancy were also unaffected by high frequency blood sampling.

It is therefore concluded that the blood sampling/transfusion procedure described does not stress the animals. Its advantages include not only the possibility of following individual hormone profiles, but also the economic and ethical aspects of reducing the number of animals needed for experimentation.
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INTRODUCTION

The need for multiple blood samples from individuals has become clear since it was recognized that many hormones have circadian and other rhythms (Rusac & Zucker, 1979). In fact, hormone secretion appears to be dynamic, since anterior pituitary hormones are secreted episodically rather than continuously, and blood levels of these hormones may fluctuate abruptly (Saunders, Terry, Audet *et al.* 1976; Gallo, 1980; Wiersma, 1981). To assess the dynamics of hormone secretion, frequent sampling of blood is necessary. However, since it is well-known that adenohypophysial hormone secretion can be markedly affected by various stressful stimuli (Stern & Voogt, 1973/74; Krulich, Hefco, Illner & Read, 1974; Borrell, Piva & Martini, 1978; Collu, Taché & Ducharme, 1979; Seggie, 1979; Caligaris & Taleisnik, 1983), the usual practices of animal handling (including anaesthesia) and blood sampling may easily produce stress in the animals and disrupt the endocrine patterns under study.

This paper describes a chronic high frequency blood sampling/transfusion technique which has been in use now for several years in our laboratory. Although a number of chronic blood vessel cannulation techniques have been described for the rat (Steffens, 1969; Neill, 1972; Terkel, 1972; Harms & Ojeda, 1974; Lawson & Gala, 1974; Terkel & Urbach, 1974), it has not been clearly demonstrated that the sampling procedures are stress free. The aim of this study was to investigate whether sequential blood sampling can be performed without stress at rates of up to one sample/min for several hours by compensating for blood volume reduction with blood transfusions.

Since both prolactin and corticosterone secretion are extremely susceptible to stress (Stern & Voogt, 1973/74; Krulich *et al.* 1974; Morishige & Rothchild, 1974; Seggie & Brown, 1975; Riegle & Meites, 1976; Seggie, 1979) we used these hormones as indicators of stress.

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MATERIALS AND METHODS

Animals

Locally bred adult F_1 hybrid male and female rats derived from two inbred strains of female Wistar and male Spraque–Dawley rats were used. They were housed in an artifically lighted room (lights on from 06.00 to 20.00 h) at a controlled temperature (21–26 °C) and humidity (>65%). Rat chow and water were freely available. At the time of experimentation rats were 6–13 months old, the females weighed 180–260 g and the males 340–430 g.

Cannulation technique

Rats were cannulated according to the method of Steffens (1969) with some modifications as described by Mattheij & van Pijkeren (1977). Under ether anaesthesia a Silastic catheter (0·5 × 0·9 mm tubing; Dow Corning, Midland, MI, U.S.A.) was inserted into the right external jugular vein and passed down near the entrance of the atrium. The other end of the cannula was extended subcutaneously to the head where it was connected to a hooked stainless steel tube (0·6 × 0·9 mm) by sliding it a few millimeters over one end. This assembly was then attached to the skull with three 3 mm long M1 stainless steel screws and acrylic cement, so that the free end of the stainless steel tube protuded about 0·6 cm at the surface of the cement. The cannula was filled with 0·9% (w/v) NaCl, containing 500 i.u. heparin/ml (Kabi AB, Stockholm) and 40% polyvinylpyrrolidone (PVP; Merck, Darmstadt, F.R.G.). The external orifice of the steel tube was closed with a small polyethylene plug. After some practice the whole operation could be completed in 20–30 min.

Maintenance

After surgery animals were housed individually in transparent plastic cages, where they remained throughout the experiment. They were handled daily for at least 1 week and accustomed to the various aspects of the experimental situation. The reproductive state of the female rats was followed by taking vaginal smears, and for the appropriate experiments pseudopregnancy was induced by electrical vaginal/cervical stimulation (100 Hz square pulses, 100 μA, 3 min) in the late afternoon of pro-oestrus.

Blood sampling/transfusion procedure

One hour before blood sampling began, a 90 cm long polyethylene sampling cannula (0·75 × 1·43 mm) filled with 0·9% NaCl was connected to the external end of the steel tube. The PVP solution was removed by connecting a syringe (Becton-Dickinson, type SMBDL) to the sampling cannula and drawn up until blood just began to enter the syringe. This syringe was then laid aside and a second syringe filled with 0·9% NaCl containing 50 i.u. heparin/ml was connected to the sampling cannula after a small air bubble had been introduced in order to prevent mixing of blood and saline. A length of about 2·5 cm of the cannula was then filled with the heparinized saline, and a small air bubble was again introduced. A third syringe containing only 0·9% NaCl was connected and this solution was allowed to fill the entire length of the cannula, using the air bubbles as markers. The first air bubble should be close to but not reach the entrance to the heart. The third syringe was disconnected and the sampling cannula closed with a stainless steel pin nail and then clipped to a pulley and counterweight mechanism, holding the cannula vertically above the rat. Under these conditions the animals could move freely.

A blood sample was collected as follows. Using the first syringe, blood was drawn up until it just entered the syringe. Then a polyethylene tube 45 cm long with an inside volume of 200 μl was connected to the sampling cannula and filled with blood using the first syringe. Thereafter, successively, this syringe and the 200 μl cannula (which contained the actual blood sample) were removed and the steps with syringes two and three were repeated to prepare the animal for the next blood sample. The whole procedure lasted about 25s. The 200 μl cannula was cleaned with (tap) water and dried with air before further use.

Blood volume is reduced by repeated sampling. This reduction was avoided by giving the rats blood transfusions at appropriate times, depending upon the experiment. The amount of blood to be transfused was collected from male F_1 donor rats, fitted with a chronic cannula, into a clean heparinized syringe a few seconds before the actual transfusion so that it was still near body temperature. Immediately after the blood sample had been collected from the experimental animal the syringe with the donor blood was connected to the 90 cm sampling cannula and injected slowly into the animal, whereafter the steps with syringes two and three finished the procedure. This whole blood sampling/transfusion procedure took about 40 s.

At the end of the experiment the cannula inside the rat was filled with the PVP solution and closed until the animal was used again.

Experiment 1: Comparisons of different blood sampling techniques

The aim of this experiment was to investigate whether the chronic blood sampling procedure interfered with normal hormone secretion. Blood was collected by rapid decapitation, orbital sinus puncture or the

chronic atrial cannula. Male rats were used in the morning between 10.00 and 12.00 h. In cannulated rats surgery was performed at least 2 weeks beforehand, so the animals had fully recovered and were accustomed to all experimental procedures.

In a first experiment blood was obtained by rapid decapitation ($n = 8$) and the cannula ($n = 10$). In a second experiment blood was collected under light ether anaesthesia by orbital sinus puncture ($n = 23$) and the cannula ($n = 8$). Blood samples were analysed for prolactin concentration.

Experiment 2: Duration of surgical stress

In female rats, pseudopregnancy was induced in the late afternoon of pro-oestrus (15.30–16.00 h). The next day was designated as day 0 of pseudopregnancy. To see how long surgery affected hormone secretion, one group of rats ($n = 15$) was cannulated immediately after the induction of pseudopregnancy (16.00–20.00 h) and a control group ($n = 13$) was cannulated at least 2 weeks before the induction of pseudopregnancy. Blood samples were collected at 07.00 and 15.00 h on day 0 and day 1 of pseudopregnancy and analysed for prolactin and corticosterone concentrations.

Experiment 3: Effect of reduction of blood volume

In this experiment we investigated the acute effect of blood volume reduction on hormone secretion. In rats cannulated at least 2 weeks before, 1 ml blood was collected every 7·5 min until a total amount of 6 ml had been withdrawn. The groups of animals consisted of intact males ($n = 5$), adult castrated males ($n = 10$), dioestrous females ($n = 7$), pro-oestrous females ($n = 10$) and females pseudopregnant for 4–6 days ($n = 6$). Experiments were conducted in the morning between 09.00 and 12.30 h. Blood was analysed for prolactin concentration and also for corticosterone in dioestrous females.

In a separate group of dioestrous females ($n = 7$) blood samples of 200 µl were collected every 2 min for 1 h, so at the end of this experiment blood volume had also been reduced by a total of 6 ml. These blood samples were analysed for prolactin concentration.

Experiment 4: Prolactin secretion under low and high frequency blood sampling

In these series of experiments we investigated whether long-term high frequency blood sampling/transfusion affected normal hormone secretion, particularly: (1) the circadian rhythm of prolactin secretion in dioestrous female rats, (2) the pro-oestrous afternoon surge of prolactin secretion, and (3) the highly stress-susceptible diurnal surge of prolactin secretion in pseudopregnant rats. The experiments were performed with chronically cannulated female rats and in those experiments where blood was sampled sequentially at high rates (<3 min), blood volume was always restored with blood from male donor rats as soon as a total of 1 ml blood had been withdrawn.

In the first experiment in dioestrous day-1 rats, blood samples of 200 µl were collected every 2 min in the morning from 09.30 to 11.00 h and in the afternoon from 14.30 to 16.00 h. In control rats blood samples were only collected at half-hour intervals during these time-periods. Blood was analysed for prolactin and corticosterone concentrations.

In the second experiment the surge of prolactin secretion on the afternoon of pro-oestrus was determined by collecting blood samples at hourly intervals from 12.00 to 17.00 h ($n = 9$). This pattern was compared with that obtained in pro-oestrous rats sampled at time-intervals of 1 min ($n = 3$), 1·5 min ($n = 9$) or 3 min ($n = 4$) for at least 1 h. Some rats were sampled for continuous periods of up to 4 h.

The final subject of investigation was whether chronic cannulation interfered with diurnal prolactin secretion in pseudopregnant rats. We collected blood either by rapid decapitation ($n = 11$) or via the chronic cannula ($n = 11$) from pseudopregnant day-5 rats at 17.00 h. The effect of blood transfusion was also investigated by taking blood samples of 200 µl at intervals of 1 min ($n = 3$) or 2 min ($n = 10$) for 1 or 2 h in the afternoon from 16.00 to 20.00 h.

Radioimmunoassay and data analysis

Blood samples (200 µl) were diluted with 100 µl heparinized phosphate-buffered saline (pH 7·5). Plasma samples were stored at −20 °C until analysed for prolactin or corticosterone concentrations. Plasma prolactin was measured by means of a homologous double-antibody radioimmunoassay as described previously (Wiersma, 1981). Rat prolactin RP-1, kindly provided by the NIADDK, was the standard used (biological activity 11 i.u./mg). The samples were analysed in five or six serial 1:2 dilutions. Plasma corticosterone was measured by radioimmunoassay as described by Van Zon, Eling, Hermsen & Koekkoek (1982). Separation of cross-reacting substances by paper chromatography appeared to be unnecessary, so corticosterone was measured after the extraction step with toluene.

Results are expressed as individual data or as means ± S.E.M. The data were evaluated statistically after a logarithmic transformation by analysis of variance (F-test). A P value <0·05 (two-sided) was considered to be significant.

RESULTS

Comparisons of different blood sampling techniques

Mean prolactin concentrations in blood of decapitated or chronically cannulated male rats were low, being $10{\cdot}1 \pm 1{\cdot}8$ ($n = 8$) and $8{\cdot}7 \pm 1{\cdot}5$ µg/l ($n = 10$) respectively, and were not significantly different. Compared with these two groups, blood obtained under light ether anaesthesia, either by orbital sinus puncture or the cannula had significantly ($P < 0{\cdot}05$) higher concentrations of $39{\cdot}4 \pm 2{\cdot}1$ ($n = 23$) and $23{\cdot}5 \pm 1{\cdot}6$ µg/l ($n = 8$) respectively.

Duration of surgical stress

Figure 1 shows the effect of surgery in the late afternoon of pro-oestrus on mean corticosterone and prolactin concentrations at 07.00 and 15.00 h in pseudopregnant day-0 and day-1 rats. Surgery increased corticosterone secretion in pseudopregnant day-0 rats significantly at 15.00 h, but in pseudopregnant day-1 rats mean corticosterone levels at 07.00 h were significantly lower in the stressed group. Surgery had no significant effect on mean prolactin concentrations.

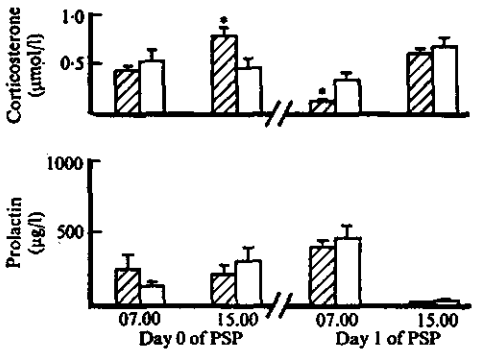

FIGURE 1. Effect of surgery (hatched bars; $n = 15$) in the late afternoon of pro-oestrus on mean corticosterone and prolactin concentration at 07.00 and 15.00 h on day 0 and day 1 of pseudopregnancy (PSP). Control rats (open bars; $n = 13$) were cannulated 2 weeks previously. Vertical lines represent S.E.M. $^*P < 0{\cdot}05$, surgery vs control (F-test).

Effect of reduction of blood volume

The effect of repeated collection of 1 ml blood every 7·5 min on mean prolactin or corticosterone secretion is summarized in Fig. 2. Except for intact male rats, mean prolactin concentration increased in all groups of rats after the blood volume had been reduced by 3 ml. All animals also started to show 'shock-like' behaviour. Despite the apparent state of shock, not all individual animals responded with an increase in prolactin secretion: prolactin values varied considerably, with some animals showing high levels while others showed basal levels. In agreement with this observation was the pattern of prolactin secretion in dioestrous rats where blood samples of 200 µl were collected every 2 min. Some typical secretion patterns are presented in Fig. 3 which demonstrates the variability in the prolactin response to blood volume reduction.

The secretion pattern of corticosterone (Fig. 2) was different from that of prolactin: after a blood volume reduction of 3 ml corticosterone concentration started to increase and remained high in all individual animals.

Prolactin and corticosterone secretion under low and high frequency blood sampling

Mean plasma corticosterone and prolactin concentrations in dioestrous day-1 rats sampled at 30-min intervals or at 2-min intervals are compared in Fig. 4. Mean

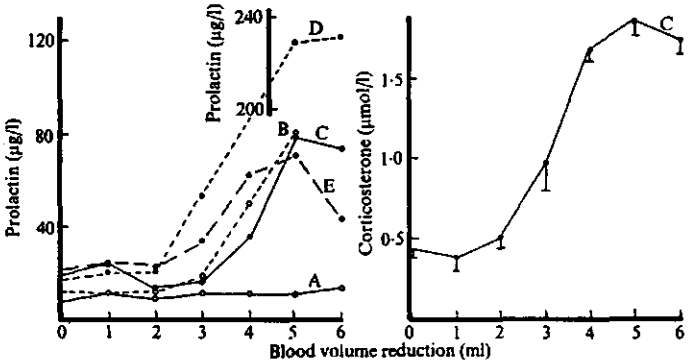

FIGURE 2. Effect of repeated collection of 1 ml blood at 7·5-min intervals on mean prolactin and corticosterone secretion in A, intact male rat ($n = 5$); B, adult castrated males ($n = 10$); C, dioestrous females ($n = 7$); D, pro-oestrous females ($n = 10$); E, pseudopregnant females ($n = 6$).

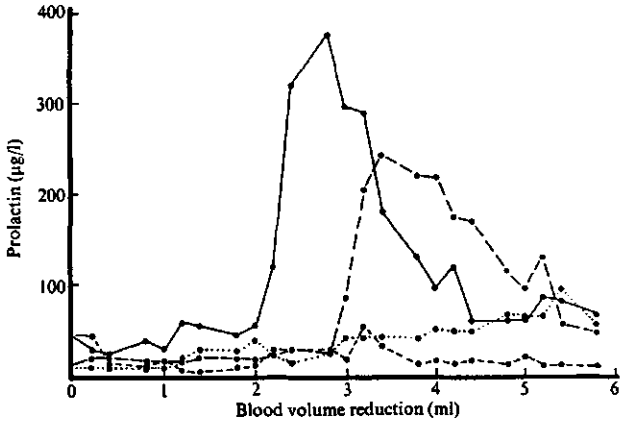

FIGURE 3. Prolactin secretion in individual dioestrous rats. Blood samples of 200 μl were collected at 2-min intervals, without replacement of blood. Due to increased blood volume reduction some rats responded with an increase in prolactin secretion while others did not.

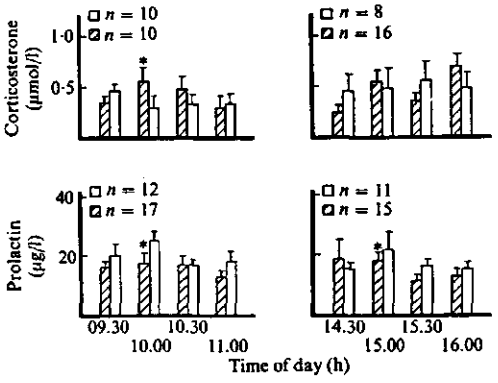

FIGURE 4. Mean corticosterone and prolactin concentration at 30-min intervals in dioestrous day-1 rats. The effect of high frequency blood sampling/transfusion (2-min intervals for 1·5 h; hatched bars) was studied. Control rats (open bars) were sampled at 30-min intervals. Vertical lines represent S.E.M. $*P < 0{\cdot}05$ compared with control rats (F-test).

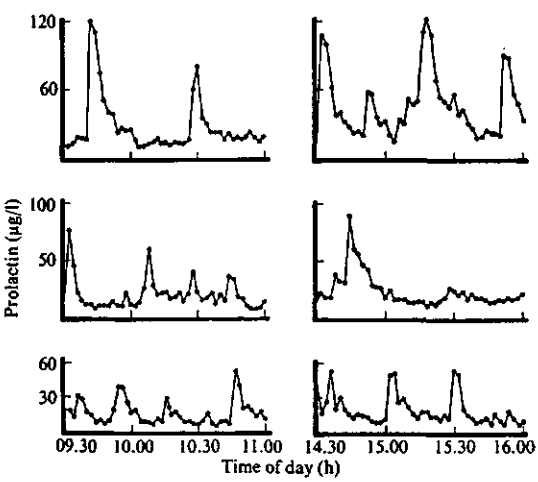

FIGURE 5. Typical individual prolactin secretion patterns in dioestrous day-1 rats at 2-min intervals. Note that prolactin is secreted in short small bursts.

corticosterone concentrations were only significantly different from each other at 10.00 h, whereas prolactin concentrations differed significantly at 10.00 and 15.30 h. The individual prolactin secretion patterns in the dioestrous rats showed that prolactin release occurred in short bursts from baseline levels of relatively low magnitudes (< 150 μg/l). Some representative data are presented in Fig. 5.

There were no significant differences between mean prolactin concentrations in pro-oestrous rats sampled at 1-h intervals or at 1- to 3-min intervals (Fig. 6). The individual data demonstrated that prolactin secretion during the afternoon of pro-oestrus appeared more or less continuously with rapidly fluctuating amounts (Fig. 7).

In pseudopregnant rats mean prolactin concentrations in the blood of decapitated or cannulated rats were not significantly different (Fig. 8). The individual data from these rats showed baseline as well as raised levels. This observation fitted with the pattern of prolactin secretion in individual pseudopregnant rats sampled for 1 or 2 h at short time-intervals; these data demonstrated that prolactin was released in substantial bursts from near basal levels (Fig. 8).

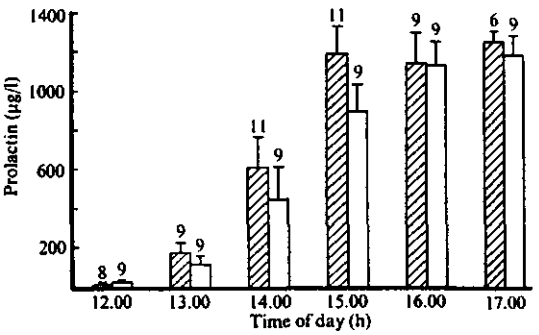

FIGURE 6. Mean prolactin concentration (±S.E.M.) at 1-h intervals (open bars) in pro-oestrous rats (control), compared with that obtained with the high frequency blood sampling/transfusion procedure (1- to 3-min intervals, 1–4 h; hatched bars). There were no significant differences in prolactin concentration between the two groups. Numbers of rats are shown above each bar.

DISCUSSION

Some degree of stress is practically inevitable in animal experimentation. The effect of stress is not limited to activation of the hypothalamo-hypophysial-adrenocortical axis, but involves the other adenohypophysial hormones as well (Krulich *et al.* 1974; Collu *et al.* 1979). Among the various hormones corticosterone and prolactin are very susceptible to stress (Seggie & Brown, 1975; Mattheij & van Pijkeren, 1977; Seggie, 1979). The effect of stress varies with age, sex, time of day and reproductive state. The response of corticosterone to stressful stimuli is always an increase (Stern & Voogt, 1973/74; Smith & Gala, 1977; Seggie, 1979); the effect of acute stressful stimuli on prolactin secretion depends, however, upon the initial level of circulating prolactin, being stimulated when pre-stress levels are low and depressed when pre-stress levels are high (Stern & Voogt, 1973/74; Morishige & Rothchild, 1974; Smith & Gala, 1977; Caligaris & Taleisnik, 1983). Using prolactin and corticosterone as indicators of stress, we investigated: (1) whether cannulation interferes with normal hormone secretion, (2) for how long surgery affects hormone secretion, (3) how much blood loss can be tolerated by the animals, and finally (4) whether high frequency blood sampling can be performed without stressing the animals.

Our data from the different blood sampling techniques indicate that cannulation does not interfere with normal hormone secretion. Mean prolactin concentrations in the blood of decapitated or cannulated rats were not significantly different, whereas bleeding under ether anaesthesia increased prolactin content significantly, all in agreement with results already published (Stern & Voogt, 1973/74; Krulich *et al.* 1974; Riegle & Meites, 1976; Mattheij & Swarts, 1978).

The duration of stress due to surgery is difficult to assess. Surgery can be considered to be a chronic stress and the effects differ from acute stressors (Collu *et al.* 1979). Thus, under chronic stress conditions, hormones may not always be good indicators of stress. Our data show that corticosterone did not indicate the presence of stress 1 or 2 days after surgery consistently

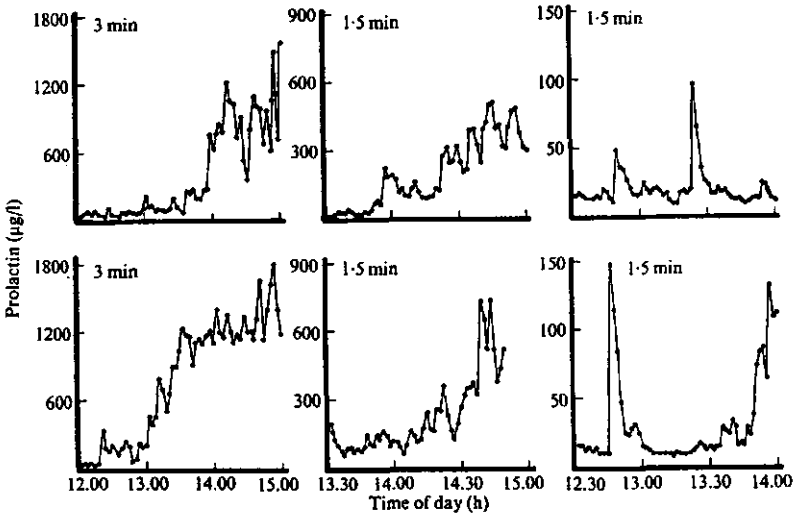

FIGURE 7. Typical prolactin secretion patterns in individual pro-oestrous rats sampled at high frequency at the time-intervals indicated.

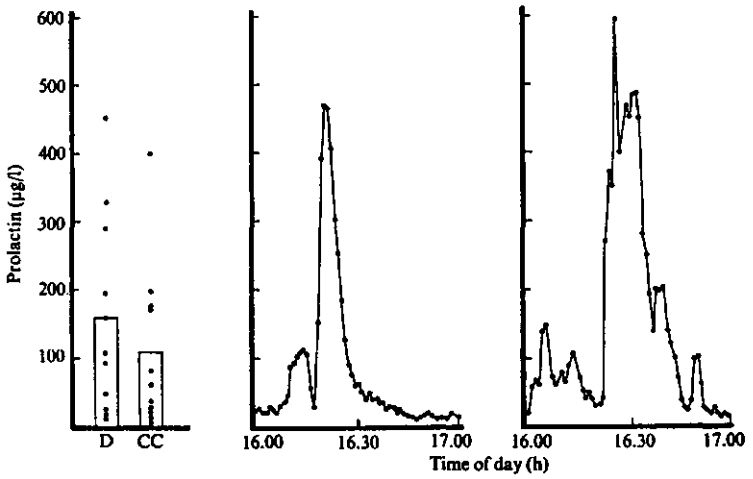

FIGURE 8. Diurnal prolactin secretion in pseudopregnant rats. Blood was collected by decapitation at 17.00 h (D), a chronic cannula at 17.00 h (CC) or under high frequency blood sampling conditions at 1-min intervals. Bars represent the mean. Note that in comparison with pro-oestrous rats, diurnal secretion in pseudopregnant rats occurs in more substantial bursts.

(Fig. 1). Prolactin secretion was not at all affected. Previous results show that the effect of surgery varies from 1 to 5 days (Freeman, Smith, Nazian & Neill, 1974; Lawson & Gala, 1974; Terkel & Urbach, 1974; Hsueh & Voogt, 1975; McLean & Nikitovitch-Winer, 1975; Smith & Neill, 1976). We therefore allowed our animals a post-operative recovery period of at least 1 week to avoid the stress due to surgery.

Blood volume is reduced by repeated sampling and may stress the animals. After a blood volume reduction of 3 ml, corticosterone secretion increased in all individual animals; mean prolactin secretion also increased, although not consistently in all individual animals (Figs 2 and 3). These data demonstrate that a blood volume reduction of 1 or 2 ml is not stressful to the animals, as may also be concluded from other studies (Lawson & Gala, 1974; Mattheij & van Pijkeren, 1977; Chi & Shin, 1978).

We finally investigated whether blood collection/transfusion could be performed free of stress by restoring the blood volume with blood from donor rats as soon as a total of 1 ml blood had been withdrawn. Blood samples of 200 μl were collected sequentially at the highest rates of 1 sample/min for at least 1 h. The blood sampling procedure did not affect corticosterone secretion in dioestrous day-1 rats (Fig. 4). These results strongly suggest that the blood sampling methodology is stress free, since even handling for 5 s may cause a significant increase in corticosterone secretion (Seggie & Brown, 1975; Seggie, 1979). The prolactin data did not indicate the presence of stress either, although mean prolactin concentrations between rats sampled at 30-min and 2-min intervals were significantly different at 10.00 and 15.30 h (Fig. 4). This may be caused, however, by the burst-like secretion of prolactin (Fig. 5); in the group of rats sampled at 2-min intervals mean prolactin concentrations were also significantly different from each other from time to time. If the blood sampling procedure had been stressful the effect would have been consistent and much more pronounced. A circadian rhythm in prolactin secretion could not be established, but this rhythm is very weak, particularly on day 1 of dioestrus (authors, unpublished results; Dunn, Johnson, Castro & Svenson, 1980).

The pro-oestrous afternoon surge of prolactin secretion was not affected by the blood sampling procedure (Fig. 6). The individual patterns observed (Fig. 7) are in agreement with Saunders *et al.* (1976) and Wiersma (1981). Even in rats sampled at high frequency for time-periods of up to 4 h (Fig. 7) the patterns of prolactin secretion were not affected, indicating the absence of stress.

Figure 8 shows data concerning the diurnal surge of prolactin secretion in pseudopregnant rats. It is generally known that stressors inhibit this surge (Freeman *et al.* 1974; Hsueh & Voogt, 1975; McLean & Nikitovitch-Winer, 1975; Smith & Neill, 1976). The data at 17.00 h from decapitated and cannulated rats are compatible with those of individual animals sampled at high frequency and therefore also indicate that the blood sampling procedure is stress free.

On the basis of the data presented herein we conclude that the blood sampling/transfusion technique described is stress free. The technique has now been in use for several years and does not interfere in any way with normal behaviour, cyclicity, (pseudo)-pregnancy, parturition, lactation, etc. Cannulas are usually patent for several months and animals are used repeatedly. The individual hormone profiles presented in Figs 5, 7 and 8 demonstrate the dynamics of hormonal secretion satisfactorily. Knowledge of temporal secretion patterns may be of great value in endocrine studies. The technique is also economic and ethical because it reduces the number of animals needed for experimentation. Our main problem often is obtaining donor blood. At present we are investigating the possibility of using preserved (blood bank) blood instead of freshly collected blood.

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Haematological, immunological and endocrinological aspects of chronic high frequency blood sampling in rats with replacement by fresh or preserved donor blood

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Summary

Haematological, immunological and endocrinological aspects of blood transfusions with either freshly collected or preserved donor blood were investigated in chronically cannulated unrestrained rats. Three anticoagulant preservatives were tested: citrate, citrate-dextrose and citrate-phosphate-dextrose-adenine (CPDA-1). Prolactin was used as an indicator of stress in endocrine studies. The repeated collection of 4 ml blood at 2-week intervals did not affect normal blood composition. Whole blood of rats could be stored in citrate, citrate-dextrose or CPDA-1 for 8, 22 or 35 days, respectively. Blood transfusions with fresh or preserved donor blood of F1 (R × U) rats did not affect normal blood composition nor did it induce immunological responses in F1 rats. Frequent blood sampling for several hours at highest rates of 1 sample/min did not affect prolactin secretion when blood volume reduction was replaced by blood transfusions with fresh donor blood. However, compensation with preserved blood affected prolactin secretion significantly. Blood transfusions did not affect health, behaviour, cyclicity or pseudopregnancy. The application of blood transfusion in chronically cannulated rats appeared to be quite simple. Its advantages are the possibility of following individual secretion patterns of blood-bound substances, the repeated use of animals and the reduction of the number of animals.

Keywords: *Blood sampling*; *Blood storage*; *Blood transfusion*; *Prolactin*; *Rats*

For the rat there are numerous methods for acute and chronic vascular access (see Cocchetto & Bjornsson, 1983). We recently described a chronic technique for high-frequency blood sampling/blood transfusion in the unrestrained rat (Wiersma & Kastelijn, 1985). In that paper we demonstrated that blood could be collected for several hours at a rate up to 1 sample/min without affecting normal hormone secretion. Blood volume reduction was compensated by blood transfusions with freshly collected donor blood. This paper is an extension of that study. We now present data on haematological, immunological and endocrinological aspects of blood transfusions performed with either freshly collected or preserved donor blood. The use of blood transfusions allows repeated use of animals which might be very useful in medical and biological studies.

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Materials and methods

Animals

Most studies were performed with locally bred adult F1 hybrid rats derived from 2 inbred strains of female Wistar (R-Amsterdam) and male Sprague-Dawley (U-Amsterdam) rats. For erythrocyte antigenicity studies 2 other local, random-bred strains were used, the Cpd:WU (Wistar) and the Zucker strain. The animals were housed in an artificially lighted room (lights on from 06.00–20.00 h) at controlled temperature (21–26°C) and humidity (≥ 65%). Food and water were freely available. The rats were used at 6–10 months of age when the females weighed about 225 g and the males about 375 g.

Cannulation technique

Blood was collected either under ether anaesthesia by orbital sinus puncture, or from unrestrained rats by means of a chronic cannula (for details see also Wiersma & Kastelijn, 1985). Rats were cannulated according to the method of Steffens (1969) with some modifications as described by Mattheij & Van Pijkeren (1977). Under ether anaesthesia, a Silastic catheter (0·5 × 0·9 mm tubing; Dow Corning, Midlans, MI, USA) was inserted into the right external jugular vein and passed down near to the atrium. The other end of the cannula was extended subcutaneously to the head where it was connected to a hooked stainless steel tube (0·6 × 0·9 mm) by sliding it a few millimeters over one end. This assembly was then attached to the skull with three 3 mm long M1 stainless steel screws and acrylic

cement, so that the free end of the stainless steel tube protruded about 0·6 cm at the surface of the cement. To prolong the patent lifetime of the cannula, it was filled with 0·9% (w/v) NaCl, containing 500 i.u. heparin/ml (Kabi AB, Stockholm, Sweden) and 40% polyvinylpyrrolidone (PVP; Merck, Darmstadt, FRG). The external orifice of the steel tube was closed with a small polyethylene plug. After some practice the whole operation could be completed in 20–30 min.

After surgery animals were housed individually in transparent plastic cages (l × w × h: 35 × 25 × 34 cm) where they remained throughout the experimental period. The endocrinological studies were performed on rats that were handled daily for at least one week and accustomed to all various aspects of the experimental situation.

Blood sampling procedure

1 h before blood sampling began, a 90 cm long polyethylene sampling cannula (0·75 × 1·43 mm) filled with 0·9% NaCl was connected to the external end of the steel tube. The PVP solution was removed by connecting a syringe (Becton-Dickinson, type SMBDL) to the sampling cannula and drawn up until blood just began to enter the syringe. This syringe was then laid aside and a second syringe filled with 0·9% NaCl containing 50 i.u. heparin/ml was connected to the sampling cannula after a small air bubble had been introduced in order to prevent mixing of blood and saline. A length of about 2·5 cm of the cannula was then filled with the heparinized saline, and a small air bubble was again introduced. A third syringe containing only 0·9% NaCl was connected and this solution was allowed to fill the entire length of the cannula, using the air bubbles as markers. The first air bubble should be close to, but not reaching, the entrance to the heart. The third syringe was disconnected and the sampling cannula closed with a stainless steel pin nail and then clipped to a pulley and counterweight mechanism, holding the cannula vertically above the rat. Under these conditions the animals could move freely.

A blood sample was collected as follows. Using the first syringe, blood was drawn up until it just entered the syringe. Then a polyethylene tube 45 cm long with an internal volume of 200 µl was connected to the sampling cannula and filled with blood using the first syringe. Thereafter, successively, this syringe and the 200 µl cannula (which contained the actual blood sample) were removed and the steps with syringes 2 and 3 were repeated to prepare the animal for the next blood sample. The whole procedure lasted about 25 s. The 200 µl cannula was cleaned with (tap) water and dried with air before further use.

Blood volume is reduced by repeated sampling. This reduction was avoided by giving the rats blood transfusions every time a total of 1 ml blood had been withdrawn. The amount of blood to be transfused was collected into a clean heparinized syringe. Immediately after the blood sample had been collected from the experimental animal the syringe with the donor blood was connected to the 90-cm sampling cannula and injected slowly into the animal, whereafter the steps with syringes 2 and 3 finished the procedure. This whole blood sampling/transfusion procedure took about 40 s.

At the end of the experiment the cannula inside the rat was filled with the PVP solution and closed until the animal was used again.

Occurrence of antibodies in rat blood

The immune response against erythrocyte antigens was investigated in 3 groups of rats: (1) F1 (n=6); (2) Cpd:WU (n=6) and (3) F1 (n=10). These rats were immunized with whole blood from Zucker, F1 and F1 rats, respectively. In the first 2 groups 1 ml blood was injected i.p. 4 times at 2-week intervals. The immune response was followed weekly for 8 weeks. The F1 rats of the third group had chronic cannulae which had been used for at least 2 months in endocrine studies, during which time they had received 10 to 15 blood transfusions of at least 1 ml of freshly collected whole blood from F1 donor rats. 10 F1 rats were tested against 10 donor F1 rats.

The occurrence of haemolysis and/or agglutination was tested. Donor blood was collected in a heparinized tube and washed 3 times in 0·9% NaCl and 25 µl of a 2% erythrocyte suspension in 0·9% NaCl was added to 25 µl of serially diluted serum samples, shaken, and incubated for 15 min at 37°C. Haemolysis was tested by adding 25 µl unabsorbed rabbit serum as a source of complement, shaken, incubated for 30 min at 37°C, shaken and incubated for 90 min at 37°C. Agglutination was looked for after adding 25 µl 0·5% bovine serum albumin, shaken, and incubated for 2 h at 37°C.

Blood composition during repeated blood withdrawal

Normal blood composition may be affected by the repeated bleeding of (donor) rats. In this experiment we investigated how much blood loss can be tolerated by the animals. Amounts of 4 ml blood were collected in heparinized tubes from F1 rats by orbital sinus puncture. The rats were bled 6 times at 1-week intervals (n=8) or at 2-week intervals (n=6). The following tests were performed on the donor blood: haematocrit (hct; method of Strumia; Merck, 1970), total haemoglobin (Hb; Boehringer Mannheim test combination, cat. no. 124 729), red blood

cell count (RBC; method of Hang; Merck, 1970), red blood cell indices (MCV, MCH, MCHC), leucocyte count (WBC; Merck, 1970), thrombocyte count (BPC; method of Brecher-Cronkite; Merck, 1970), osmotic resistance (method of Begemann; Merck, 1970) and blood sedimentation (BSE; micro-method of Westergren; Merck, 1970).

Comparison of anticoagulants
Three different anticoagulant-preservative solutions were tested, citrate (C), citrate-dextrose (CD) and citrate-phosphate-dextrose-adenine (CPDA-1; Fenwal Travenol). Stock solutions (sterile) were stored at 4°C and 100 ml of the solution contained, respectively, (C): Sodium Citrate Ph Eur 3·20 g, (CD): Sodium Citrate Ph Eur 2·70 g, Dextrose (anhydrous) B.P. 2·30 g and (CPDA-1): Sodium Citrate Ph Eur 2·63 g, Dextrose (anhydrous) B.P. 2·90 g, Citric Acid Monohydrate Ph Eur 327 mg, Sodium Hydrogen Phosphate B.P. 251 mg, Adenine 27·5 mg. Blood (2 ml aliquots) was collected from cannulated F1 rats in 3 ml glass tubes (Venoject VT® 030P34, Terumo Corp., Shibuya-ku, Tokyo 151, Japan) prefilled with either 0·2 ml citrate or 0·3 ml citrate-dextrose or 0·3 ml CPDA-1 anticoagulant and stored by 4°C. The effects of storage on red blood cells was investigated by performing the following measurements: pH (n=8), plasma haemoglobin (Hb; n=6; Hainline, 1958), red blood cell adenosine triphosphate (ATP; n=6; Boehringer Mannheim test combination Cat. no. 123 897), red blood cell 2,3-diphosphoglycerate (2,3-DPG; n=6; Boehringer Mannheim test combination Cat. no. 148 334). These data were collected on various days from the initial day of storage (day 0) up till 35. Sterility was tested on *Aspergillus nidulans* complete medium.

Blood transfusion: haematological and immunological effects
The antigenic components of preserved blood may differ from that of fresh whole blood. So under conditions where blood transfusions performed with fresh whole blood may not cause any effects, blood transfusions with preserved blood may induce immunological responses or may affect normal blood composition. This was investigated by performing blood transfusions with blood from F1 rats stored in citrate (7 days), citrate-dextrose (21 days) or CPDA-1 (28 days). Each test group consisted of 4 F1 rats. From each rat 2 ml blood was collected 4 times at 1-week intervals, immediately followed by a blood transfusion with 1 ml preserved blood. Changes in the blood composition were tested weekly by measurement of hct, total Hb, RBC, MCV, MHC, MCHC, WBC, BPC. The development of an immunological response was investigated after 4 weeks by haemolysis and agglutination tests.

Blood transfusion: endocrinological effects
In these series of experiments we investigated whether long-term high-frequency blood sampling/blood transfusion could be performed stress free. Prolactin was used as a marker of stress. Chronically cannulated female F1 rats acclimatized for at least 1 week to all experimental procedures were used. Blood samples (200 µl) were collected sequentially, either at 1-h intervals (control) or at 1–3-min intervals for 1–5 h. Blood volume was always restored as soon as a total of 1 ml blood had been withdrawn. The blood transfusions were performed either with 1 ml freshly collected whole blood from cannulated male F1 donor rats, or with 1·15 ml F1 blood stored in CPDA-1 anticoagulant for 4–10 days. The effect of blood sampling/transfusion was investigated on the surges of prolactin secretion occurring in the afternoon of pro-oestrus and the late afternoon of pseudopregnancy (PSP). The reproductive state of the females was followed by taking vaginal smears. Pseudopregnancy was induced in the late afternoon of pro-oestrus by electrical vaginal/cervical stimulation (100 Hz square wave pulses, 100 µA, 3 min). The next day was designated day 0 of PSP. During the experiments the animals could move freely. Plasma samples were diluted 1:1 with heparinized phosphate buffered saline (pH 7·5) and stored at −20°C until analysed for prolactin concentration by means of a homologous double-antibody radioimmunoassay (Wiersma, 1981). Rat prolactin RP-1 (NIADDK) was used as the standard (biological activity 11 i.u./mg).

Data analysis
Results are expressed as individual data or as means ± SD or SEM. The prolactin data were evaluated statistically after a logarithmic transformation by analysis of variance (F-test). A P-value $< 0{\cdot}05$ (two-sided) was considered to be significant.

Results
Occurrence of antibodies in rat blood
Antibodies to red blood cells were detectable after one week in F1 (R × U) rats immunized with whole blood of Zucker rats (total haemolysis). In Cpd:WU rats given F1 blood, a very weak haemolytic reaction was found. F1 rats given F1 blood showed no haemolytic reaction. The agglutination tests were all negative.

Blood composition during repeated blood withdrawal
Normal blood composition was not affected by the

repeated withdrawal of 4 ml blood at 2-week intervals, however, repeated collection of 4 ml blood at 1-week intervals markedly affected blood parameters. The 1-week interval data are presented in Table 1. These data show that hct and RBC values decreased after 2 and 3 weeks, but increased thereafter. In the course of time there was increase in the number of young red blood cells and reticulocytes, which have a greater volume but contain less Hb than older red blood cells. So Hb values steadily declined as did MCHC (hypochromic anaemia). The increase in BSE values and osmotic resistance also demonstrated that blood composition had changed. WBC and BPC values were only slightly affected.

Comparison of anticoagulants

The data on plasma Hb, red cell ATP and 2,3-DPG levels in preserved blood are summarized in Figs 1, 2 and 3. Plasma Hb concentration in blood stored in citrate, citrate-dextrose or CPDA-1 increased progressively after 10, 21 and 24 days, respectively, reflecting an increase in haemolysis. Clinical data on the *in vivo* viability of stored red blood cells indicate that ATP levels should not have fallen below 40% of their pre-storage levels and 2,3-DPG levels not below 3·72 μmol/g Hb (Simon, 1977). In blood stored in citrate or citrate-dextrose the 40% levels for ATP were reached by day 8 and day 22, respectively, and in the CPDA-1 anticoagulant the ATP level by day 35 had fallen to 50·6%. The critical values for 2,3-DPG were reached by day 4 (citrate), day 8 (citrate-dextrose) and day 15 (CPDA-1).

During storage there was a fall in pH values. The initial (day 0) pH values of blood stored in either citrate, citrate-dextrose or CPDA-1 were 7·54, 7·43 and 7·00, respectively. These values slowly declined in, respectively, 10, 18 and 28 days to more or less constant pH values of 7·10, 6·50 and 6·40.

Blood transfusion: haematological and immunological effects

Repeated blood transfusions with blood stored in citrate (7 days), citrate-dextrose (21 days) or CPDA-1 (28 days) did not affect normal blood composition and did not induce immunological responses.

Blood transfusion: endocrinological effects

Patterns of prolactin secretion in pro-oestrous F1 rats were measured from 12.00–17.00 h. Data are presented in Fig. 4. Mean prolactin concentration of rats bled at 1-h intervals (control) was low at 12.00 h (25 μg/l), peak values of 1150 μg/l appeared at 16.00 and 17.00 h. Mean prolactin concentrations in rats sampled at 1–3-min intervals for time periods of 1–5 h and given blood transfusions with freshly collected F1 donor blood were not significantly different from the controls. Only data obtained after at least 1 h of high-frequency blood sampling were used for this comparison. However, when blood volume reduction was compensated by blood transfusions with blood preserved in CPDA-1, prolactin secretion was significantly affected. In this experiment rats (n=9) were bled at 2-min intervals from 15.00–17.00 h. After 1 h of blood sampling mean Prl concentration (at 16.00 h) did not differ significantly from the control value. Thereafter, in the individual animals prolactin values began to decline and at 17.00 h mean concentration was significantly lower than the control value ($P < 0·05$). Some individual data are shown in Fig. 5.

No differences in the secretion patterns of prolactin secretion were found in PSP rats sampled for 2 h at 2-min intervals when given blood transfusions with either freshly collected blood (n=8) or preserved blood (n=8). In these rats the secretion of prolactin occurred in substantial bursts from near basal levels. Some representative patterns are shown in Fig. 6.

It should be mentioned that the injection of fresh whole blood usually did not induce a behavioural response. If the blood was not near body temperature then, immediately following injection, the rats shook their heads. However, the injection of warmed preserved blood often elicited a well pronounced response: the animals became very alert and looked excited for a few minutes.

Discussion

Biological and medical studies in rats often require the collection of blood. In our field of interest, endocrinology, the need for frequent blood sampling from individuals has become clear since it was recognized that hormones are secreted episodically, rather than continuously, and blood levels of hormones may fluctuate abruptly (Saunders, Terry, Audet, Brazeau & Martin, 1976; Gallo, 1980; Wiersma, 1981). Unfortunately, the usual practices of animal handling, surgery, anaesthesia and blood sampling may easily produce stress and hence affect the endocrine patterns under study (Krulich, Hefco, Illner & Read, 1974; Seggie & Brown, 1975; Riegle & Meites, 1976; Borrell, Piva & Martini, 1978). In the present study we investigated whether sequential blood sampling can be performed without stress by making up the blood volume through blood transfusion either with fresh or preserved blood. Since prolactin secretion is extremely susceptible to stress (Krulich *et al.*, 1974; Morishige & Rothchild, 1974; Seggie & Brown, 1975; Riegle & Meites, 1976; Caligaris & Taleisnik, 1983) we used this hormone

Table 1. Effect of repeated withdrawal of 4 ml blood from rats at 1-week intervals on blood parameters

Week	*hct* *(l/l)*	*Hb* *(mmol/l)*	*RBC* *(10^{12}/l)*	*MGV* *(fl)*	*MCH* *(amol)*	*MCHC* *(mmol/l)*	*WBC* *(10^{9}/l)*	*BPC* *(10^{11}/l)*	*BSE* *(mm/h)*	*osm res (% NaCl min.*	*max.)*
0	0·44	8·58	7·61	58·16	1128	19·40	4·3	7·4	0·9	42	34
	(0·01)	(0·24)	(0·28)	(1·07)	(11)	(0·21)	(1·1)	(0·8)	(0·3)	(13)	(11)
1	0·44	8·58	7·53	58·79	1139	19·39	3·8	7·8	0·9	42	34
	(0·01)	(0·14)	(0·28)	(1·98)	(33)	(0·13)	(1·5)	(1·4)	(0·2)	(13)	(11)
2	0·38	7·32	6·82	55·41	1075	19·34	3·8	7·6	1·2	41	32
	(0·01)	(0·56)	(0·55)	(2·03)	(37)	(0·61)	(1·0)	(1·0)	(0·3)	(15)	(10)
3	0·39	7·15	6·43	59·45	1113	18·41	4·3	6·7	1·3	39	32
	(0·03)	(0·27)	(0·27)	(6·91)	(80)	(1·19)	(1·6)	(1·3)	(0·4)	(10)	(13)
4	0·43	6·77	6·90	62·28	985	15·82	4·4	6·7	2·2	39	32
	(0·03)	(0·29)	(0·33)	(1·43)	(86)	(1·51)	(1·3)	(1·5)	(0·7)	(14)	(09)
5	0·45	5·99	7·43	60·04	845	13·46	3·7	6·2	3·1	38	32
	(0·01)	(0·28)	(0·43)	(2·74)	(104)	(0·60)	(1·1)	(1·6)	(1·3)	(09)	(09)

Data are presented as means (upper) ± SD (lower) n=8. hct: haematocrit; Hb: total haemoglobin; RBC: red blood cell count; MCV: mean corpuscular volume; MCH: mean corpuscular haemoglobin; MCHC: mean corpuscular haemoglobin concentration; WBC: leucocyte count; BPC: thrombocyte count; BSE: blood sedimentation; osm res: osmotic resistance.

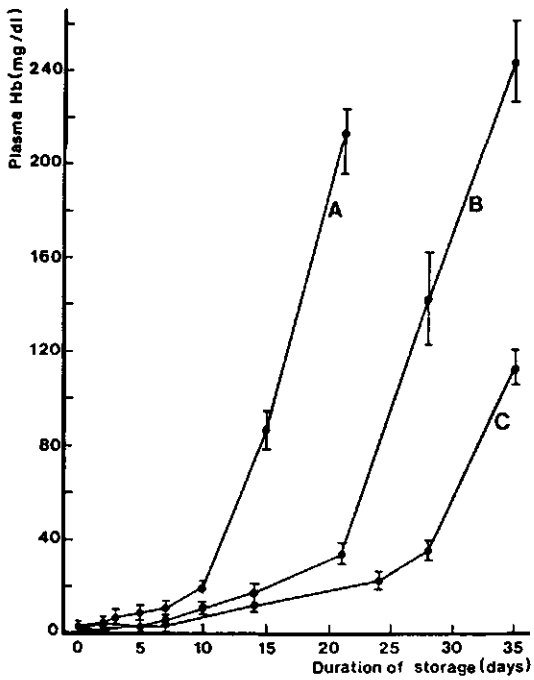


Fig. 1. Change in plasma haemoglobin (Hb) concentration of rat blood during storage in citrate (A), citrate-dextrose (B) or citrate-phosphate-dextrose-adenine (C). Data are presented as means ± SEM (n=6).

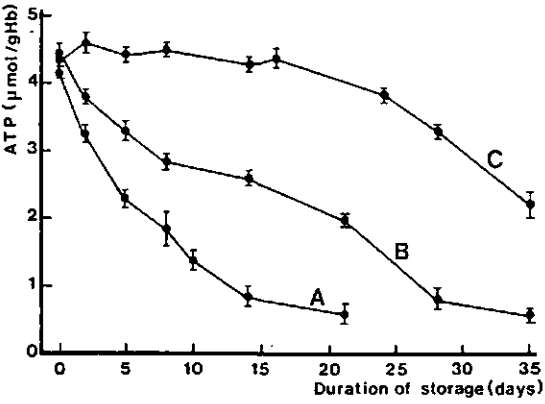


Fig. 2. Change in red blood cell adenosine triphosphate (ATP) level of rat blood during storage in citrate (A), citrate-dextrose (B) or citrate-phosphate-dextrose-adenine (C). Data are presented as means ± SEM (n=6).

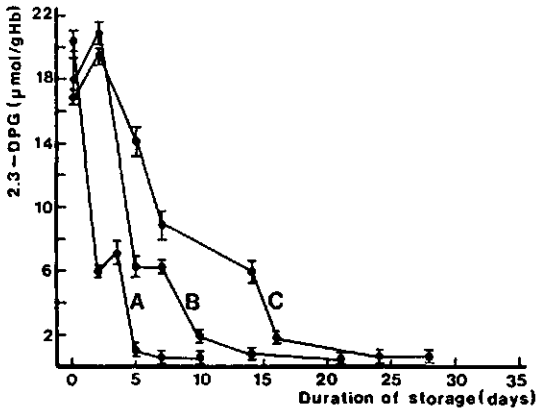


Fig. 3. Change in red blood cell 2,3-diphosphoglycerate (2,3-DPG) level of rat blood during storage in citrate (A), citrate-dextrose (B) or citrate-phosphate-dextrose-adenine (C). Data are presented as means ± SEM (n=6).

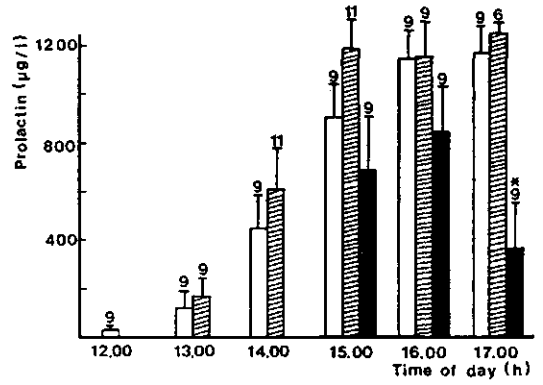
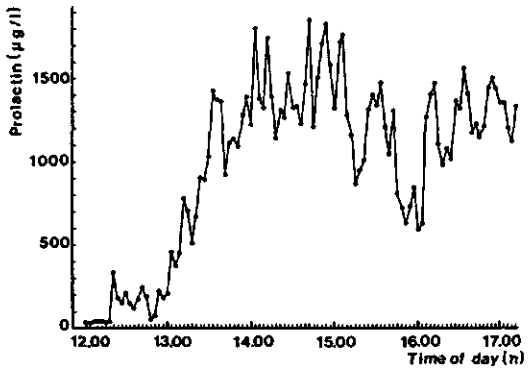


Fig. 4. Mean prolactin concentration (± SEM) at 1-h intervals (open bars) in pro-oestrous rats (control) compared with that obtained during long-term high-frequency blood sampling/transfusion. Blood transfusions with fresh donor blood did not affect prolactin secretion (1–3-min intervals, 1–5 h; hatched bars). Blood transfusions performed with blood preserved in CPDA-1 affected prolactin secretion significantly after 2 h (2-min intervals, 2 h; solid bars; $^{*}P < 0.05$ vs control). Number of rats is shown above each bar.



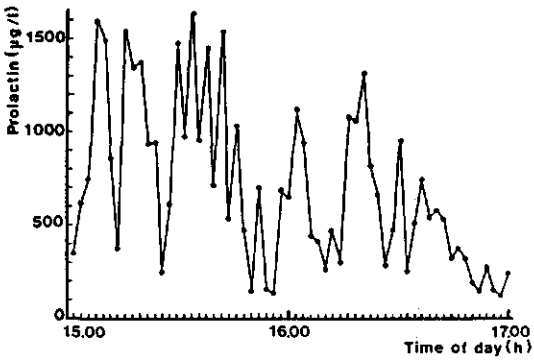
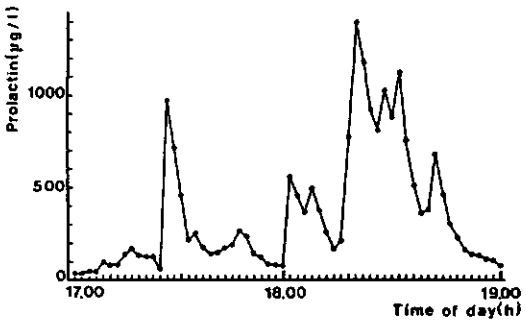


Fig. 5. Representative individual prolactin secretion patterns in pro-oestrous rats. The upper trace shows a pattern of a rat sampled at 3-min intervals for 5 h and given blood transfusions with fresh donor blood. The lower trace presents data of a rat sampled for 2 h at 2-min intervals and transfused with preserved donor blood. Note that prolactin secretion was significantly affected as indicated by the prolactin values < 250 µg/l.



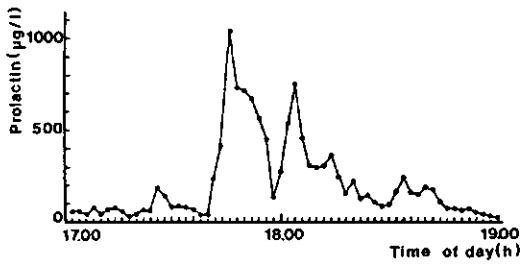


Fig. 6. Individual prolactin secretion patterns of rats pseudopregnant for 5 days, sampled for 2 h at 2-min intervals and given blood transfusions with fresh (upper) or preserved donor blood (lower). Differences in the secretion patterns between the 2 groups could not be found.

as a marker for stress. The haematological and immunological consequences of blood collection, preservation and transfusion were also studied.

The reduction of blood volume by repeated blood sampling can be avoided by giving the rats blood transfusions. However, due to the presence of antibodies to erythrocytes, either present spontaneously or induced immunologically, problems may arise. The immune response to a variety of antigens in the rat is linked to the major histocompatibility complex and may closely resemble that in man (Gill, Cramer & Kunz, 1978). Several serologically detectable antigenic systems have been found in the rat (Palm, 1962; Palm & Black, 1971; Kunz & Gill, 1978; Günther & Štark, 1979). Serologically active antibodies occur spontaneously in normal rat sera, but the titre of these antibodies is usually low in comparison with antisera prepared by immunization (Günther, Elsässer-Beile & Hedrich, 1983). Consequently rats may well accept a single blood transfusion but not when repeated, unless the blood groups systems match. Our data are consistent with this concept. In our F1 (R × U) rats an immunological response developed when given blood of Zucker rats, but not when transfused with blood of F1 rats. For this reason we only used F1 rats in our experiments.

Red blood cell storage in three anticoagulants was

investigated: citrate, citrate-dextrose and citrate-dextrose-phosphate-adenine (CPDA-1). Aliquots of 2 ml blood were stored at 4°C. Blood was usually collected from male F1 donor rats. Amounts of 4 ml were collected at 2-week intervals, since the collection at 1-week intervals induced hypochromic anaemia by 3 weeks (Table 1). In some cases blood was also collected from rats that had to be sacrificed. Clinical data show that blood storage in the 3 anticoagulants is different. Blood collected in sodium citrate can be stored for 5 days and the addition of dextrose extends the storage period up to 15 days (Gibson, Murphy, Scheitlin & Rees, 1956). Further studies on the preservation of human blood show that the addition of phosphates and adenine further increases the storage period to 35 days (Dern, Brewer & Wiorkowski, 1967; Simon, 1977; Zuck *et al.*, 1977; Moore, Peck, Sohmer & Zuck, 1981). Our data concerning the 3 anticoagulant-preservatives (Figs 1, 2, 3) fit well with the clinical data on this subject. Red blood cells of rats, therefore, seem to behave similarly to human cells. Since CPDA-1 has the best red blood cell survival properties we used this solution in our blood transfusion experiments. Based on clinical data, rat red blood cells may survive satisfactorily following 35 days of storage.

After we had assessed that repeated blood transfusions with preserved blood did not affect normal blood composition, nor induced immunological responses, we investigated whether high-frequency blood sampling/blood transfusion affected normal hormone secretion. During the afternoon of pro-oestrus there appears a well-pronounced surge of prolactin secretion (Wiersma, 1981) which is usually not affected by stressors (Riegle & Meites, 1976). High-frequency blood sampling/transfusion did not affect pro-oestrous prolactin secretion when freshly collected blood was used for blood transfusions (Fig. 4). In a previous study, we demonstrated that this blood sampling procedure did not affect corticosterone secretion either (Wiersma & Kastelijn, 1985) which provides further evidence that the procedure is stress free. However, when blood transfusions were performed with blood preserved in CPDA-1, prolactin secretion in pro-oestrous rats was significantly affected (Fig. 4). Mean prolactin concentration was significantly lower after 2 h in rats sampled at 2-min intervals. In rats transfused with preserved blood, prolactin values < 250 µg/l were regularly seen during the time course (Fig. 5), whereas such low values are very exceptional for pro-oestrous rats in the same time period, 15.00–17.00 h (Wiersma, 1981). In rats sampled at 1-h intervals or sampled at short-time intervals and given fresh donor blood, such low values were not seen during that time period (Fig. 5). The prolactin data of pro-oestrous rats, therefore, indicate that blood transfusions with preserved blood affect normal hormone secretion. Surprisingly, diurnal prolactin secretion in PSP rats was not affected. It is generally known that diurnal prolactin secretion in PSP rats is very susceptible to stress and several noxious procedures will block prolactin secretion for 1–5 days (Freeman, Smith, Nazian & Neill, 1974; Hsueh & Voogt, 1975; McLean & Nikitovitch-Winer, 1975; Smith & Neill, 1976). However, in PSP rats during the late afternoon prolactin was still secreted in substantial bursts from near basal levels (Fig. 6), a release pattern in accordance with previous studies (Wiersma, 1981). We have no explanation why prolactin secretion in PSP rats is not affected, in contrast to pro-oestrous rats. It should be realized that stressors do not necessarily need to affect prolactin secretion identically under different physiological conditions. We recently demonstrated that prolactin secretion in the afternoon of pro-oestrus seems to be regulated differently from that in the afternoon of PSP (Wiersma & Kastelijn, in press). However, such data do not really explain why blood transfusions with preserved blood affect prolactin secretion in pro-oestrous rats but not in PSP rats.

The data presented herein demonstrate that blood transfusions between F1 (R × U) rats with fresh or preserved donor blood do not affect normal blood composition or induce immunological responses, or interfere with health, behaviour, cyclicity or pseudopregnancy. However, transfusions with preserved blood may interfere with normal hormone secretion, whereas transfusions with fresh blood do not. The described blood sampling technique has been used now for several years in our laboratory. Cannulae usually remain patent for several months. The technique has not only the scientific advantage of the repeated use of animals but also reduces the number of animals needed for experimentation.

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Hämatologische, immunologische und endokrinologische Aspekte der langfristigen, sehr häufigen Blutentnahme/Bluttransfusion in Ratten unter Anwendung von frisch entnommenem oder konserviertem Spenderblut

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Zusammenfassung

In uneingeschränkten Ratten mit langfristig eingeführten Kanülen wurden die hämatologischen, immunologischen und endokrinologischen Aspekte der Bluttransfusionen mit entweder frisch entnommenem oder konserviertem Spenderblut untersucht. Es wurden 3 antikoagulierende Konservierungsmittel geprüft: Citrat, Citrat-Dextrose und Citrat-Phosphat-Dextrose-Adenin (CPDA-1). Bei endokrinen Untersuchungen wurde Prolactin als ein Indikator für Streß angewendet. Die wiederholte Entnahme von 4 ml Blut, in zweiwöchigen Intervallen, hatte auf die normale Blutzusammensetzung keinen Einfluß. Vollblut von Ratten konnte für eine Dauer von 8, 22 bzw 35 Tagen in Citrat, Citrat-Dextrose oder CPDA-1 aufbewahrt werden. Bluttransfusionen mit frischem oder konserviertem Spenderblut von F1 (R × U) Ratten wirkten sich weder auf die normale Blutzusammensetzung aus noch riefen sie in F1-Ratten immunologische Reaktionen hervor. Die häufige Blutentnahme, über mehrere Stunden hinweg, bei einer Höchstrate von 1 Probe/min hatten auf die Prolactinsekretion keinen Einfluß, wenn die Reduktion in dem Blutvolumen durch Bluttransfusionen mit frischem Spenderblut ersetzt wurde. Die Kompensation mit konserviertem But wirkte sich jedoch in signifikanter Weise auf die Prolactinsekretion aus. Bluttransfusionen hatten keinen Einfluß auf die Gesundheit, das Verhalten, die Zyklusregelmäßigkeit oder Pseudogravidität. Die Verabreichung von Bluttransfusionen an Ratten mit langfristig eingeführten Kanülen schien ganz einfach zu sein. Ihre Vorteile liegen in der Möglichkeit, individuelle Sekretionsabläufe von blutgebundenen Stoffen zu verfolgen, der wiederholten Verwendung der Tiere und der Reduktion in den Tierzahlen.

CHAPTER 4

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Effects of Red Dim Illumination and Surgery on Prolactin Secretion during the Estrous Cycle and Early Pseudopregnancy in the Rat: Different Regulatory Mechanisms for Prolactin Secretion

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Key Words. Prolactin · Estrous cycle · Pseudopregnancy · Surgery · Stress · Circadian rhythm · Red light · Vision · Neural regulation · Rat

Abstract. The effects of surgery and red light on prolactin (Prl) secretion were investigated in cycling and in pseudopregnant (PSP) rats. Secretion patterns of Prl were determined at hourly intervals from 07.00 to 22.00 h. Different regulatory mechanisms for Prl secretion were hypothesized for three time periods: a nocturnal (07.00–11.00 h), a prediurnal (14.00 –17.00 h), and a diurnal (19.00–22.00 h) period. The results demonstrate that red light can affect significantly Prl secretion, in particular nocturnal and prediurnal Prl secretion in estrous, diestrous day 1 and PSP rats. The effect of surgery varied with the time of the day and was dependent upon whether the animals, during the dark period, were maintained under full darkness or constant red dim illumination. In PSP rats the regulation of Prl secretion was different for the three time periods. In PSP day 0 rats there was a prediurnal surge of Prl secretion, comparable in timing and regulation to a prediurnal surge in estrous rats. This prediurnal surge was not evident on the other days of PSP. The regulation of nocturnal as well as diurnal Prl secretion was similar in PSP rats, but differed from cycling rats. The afternoon surge of Prl secretion on proestrus lasted the full afternoon and was basically one surge, distinct from all other surges. In diestrous rats Prl levels were low but showed a circadian variation. In summary, the effects of red light and surgery on Prl secretion varied with the physiological state and with the time of the day, indicating that the regulation of Prl secretion is complex and multimodal in nature.

Pseudoprengnancy (PSP) in the rat is characterized by the secretion of two daily surges of prolactin (Prl): a nocturnal surge (N) occurring in the early morning, and a diurnal surge (D) occurring in the late afternoon [1, 2, 12, 14, 17, 23, 31, 36]. In cycling rats the occurrence of surges of Prl secretion depends upon the state. During the diestrous day Prl levels are low and show minor fluctuations [4, 5, 10, 19, 27, 31, 36], however, in proestrous rats there is a surge (P) of Prl secretion during the full afternoon [4, 5, 10, 27, 31, 36], and in estrous rats there appears a surge (E) of Prl secretion with the same time of onset as the P surge, but of a much shorter duration [1, 4, 5, 10, 27, 29, 36]. We have previously suggested [36] that the secretion of Prl on the afternoon of day 0 of PSP may consist of two different surges: a surge (D1) comparable to the estrous afternoon surge E, and a second surge (D2) starting thereafter, comparable to the D surges on the other days of PSP. The present study was undertaken to determine the existence of such a D1 and D2 surge, and also whether these surges basically are a representation of the E and D surge, respectively, i.e.: D1 = E, and D2 = D. We also wished to know whether the proestrous afternoon surge P basically was the sum of the E and D surge, i.e. an E surge followed by a D surge: P = E + D. Therefore, based on the concept of the existence of the well-defined N, E and D surges of Prl secretion, we delineated three time periods: a nocturnal (07.00–11.00 h), a prediurnal (14.00–17.00 h) and a diurnal (19.00–22.00 h) period, for which we hypothesized that the secretion of Prl was under control of different regulatory mechanisms. The occurrence and timing of the various surges of Prl secretion is schematically summarized in figure 1, together with the above formulated hypothesis. Differences in the regulatory mechanisms for Prl secretion were determined by investigating the effects of surgery and red light on Prl secretion during the three defined time periods. It is well known that stressful stimuli, like surgery, can affect circulating Prl levels, depending

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Physiological State	Time Period of the Day		
	Nocturnal	Prediurnal	Diurnal
Proestrous		P–surge	
Estrous		E – surge	
PSP day 0	N–surge	D_1–surge	D_2–surge
PSP ≥ day 1	N–surge		D – surge

Fig. 1. Schematic presentation of the timing of the well-established P, E, N and S surges of Prl secretion and the hypothesized D1 and D2 surges. The present study investigates whether: (1) D1 = E, (2) D2 = D, and (3) P = E + D.

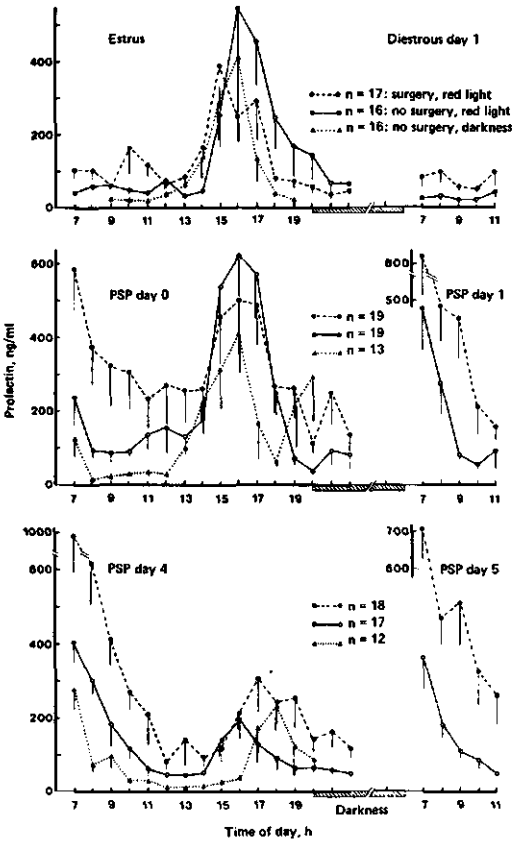

Fig. 2. Mean Prl secretion patterns (± SEM) in estrous and PSP rats maintained under full darkness at night (▲), constant red dim illumination at night (○), and after surgery with constant red dim illumination at night (●). For statistics see table 1.

upon the physiological state and the time of the day [5, 7, 17, 23, 27, 29, 32, 37]. Red light, we had found in a pilot study, also seemed to affect Prl secretion. Our knowledge of the visual capabilities of the rat is fragmentary and it is frequently thought that red light is invisible to rodents [8, 33, 34]. However, there are a few studies that indicate that there may be some perception of red light [3, 6, 21, 22]. It therefore was worthwhile to investigate more systematically the influence of red light on Prl secretion and, in the context of the present study, whether the effect varied with the time of the day.

Materials and Methods

Locally bred adult F_1 hybrid female rats derived from two inbred strains of female Wistar and male Sprague-Dawley rats were used. They were housed individually in a temperature-controlled room (21–26 °C) and had free access to food and water. At the time of experimentation rats were 7–14 months old and weighed 180–280 g. The room was artificially lighted (Philips TL 40W/34) from 06.00 to 20.00 h, with the light intensity in the cages of 290–550 lx. In some experiments blood samples were collected in the night period. In that case, a constant illumination of red dim light (Philips TL 40W/15) was introduced from 19.30 to 06.30 h, from at least 1 week before experimentation, so that the intensity in the cages was of 6–22 lx.

Blood sampling was always performed via a chronic atrial catheter, which was extended subcutaneously to the head, where it was attached to the skull with anchor screws and acrylic cement. The cannula was filled with saline containing 40% polyvinylpyrrolidone and 500 IU heparin/ml. To prevent backflow it was closed with a small polyethylene plug. One hour before blood sampling began, a polyethylene sampling cannula was connected to the animals, so that blood sampling could be performed from unrestrained rats. Samples (200 µl) were collected at hourly intervals from 07.00 to 22.00 h. After every five samples blood volume was restored by injection of 1 ml of blood from male F_1 donor rats. In a separate study this blood sampling/transfusion procedure did not affect Prl or corticosterone secretion [*Wiersma and Kastelijn*, submitted].

Vaginal smears were taken 5–7 times a week between 06.00 and 10.00 h. Pseudopregnancy (PSP) was induced by electrical vaginal/cervical stimulation (100 Hz square pulses, 100 µA, 2–3 min) in the late afternoon of proestrus. The following day was designated as day 0 of PSP.

Differences in the regulatory mechanisms for Prl secretion were determined by investigating the effects of surgery and a constant red dim illumination at night on Prl secretion during three different time periods in cycling and PSP rats (see Introduction). The surgery consisted of the implantation of the cannula and was performed in the late afternoon (16.00–20.00 h) or the day preceding experimentation. If appropriate, pseudopregnancy was induced before surgery between 15.30 and 16.00 h. Control groups were cannulated at least 2 weeks before experimentation and handled daily to accustom them to all experimental procedures. Blood sampling in rats exposed to red light was continued up to 22.00 h, how-

ever, in rats maintained under full darkness at night, blood sampling was restricted to the light period of the day.

Blood samples (200 µl) were diluted with 100 µl of heparinized phosphate-buffered saline (pH = 7.5). Plasma samples were stored at -20 °C until analyzed by means of a homologous double-antibody radioimmunoassay (RIA) as developed by *Kwa* et al. [20]. Rat prolactin RP-1, kindly provided by the NIAMDD, was the standard used (biological potency 11 IU/mg). The samples were assayed in 6 serial 1:2 dilutions. The intra-assay coefficient of variation was 8.2%, the inter-assay coefficient 13.4%. Results, where appropriate, are expressed as means ± SEM. The data were evaluated statistically after a logarithmic transformation and probabilities were derived from analysis of variance (F test). Since Prl is cleared quickly from the circulation with a half-time of about 4 min [19, 36], Prl concentration in hourly blood samples in individual rats were considered independent of one another. In cases where statistics were applied to a full time period (nocturnal, prediurnal, diurnal) all data within such a time period were pooled as independent data.

Results

Prl Secretion Patterns during Estrus, PSP Day 0 and PSP Day 4

Mean Prl secretion patterns of rats during estrus, PSP day 0 and PSP day 4 are summarized in figure 2. In this experiment we investigated the effects of red light and red light with surgical stress on nocturnal, prediurnal and diurnal Prl secretion. A 3-way analysis of variance, accounting for the factors physiological state, treatment and time period, revealed that main effects as well as 2-way interactions were highly significant ($p < 0.000$). In a further evaluation, contrasts for all subfactors were calculated. The results of these calculations are summarized in table I. With reference to this table and figure 2, the following observations could be made:

Contrasts between Different Time Periods of the Day

In estrous rats, maintained under complete darkness at night, the appearance of a prediurnal surge E of Prl secretion was evident ($p < 0.000$). In PSP day 0 rats, under darkness, a prediurnal surge of Prl secretion, comparable to that on estrus, was also evident. This surge seemed to terminate by 18.00 h, when mean Prl concentration was relatively low: 59 ± 16 ng/ml, whereafter the onset of a diurnal surge of Prl secretion could be seen. The appearance of two distinct surges (D1 and D2) became even more marked by noting that at 17.00 h all animals had Prl concentrations of smaller than 179 ng/ml, except for 1 animal with an extremely high concentration of 1,312 ng/ml (fig. 3). By deleting this value, mean Prl concentration at 17.00 h would be 69 ± 13 ng/ml instead of 165 ± 93 ng/ml. Statistical analysis revealed that prediurnal (D1) and diurnal (D2) Prl levels in PSP day 0 rats in darkness were significantly different from each other ($p < 0.032$). Red light and/or surgery affected prediurnal and diurnal secretion differently in PSP day 0 rats (fig. 2), so under both these conditions prediurnal and diurnal Prl levels were significantly different ($p < 0.000$). These data provide good evidence that the D1 and D2 surges on day 0 of PSP are distinct surges.

In contrast to PSP day 0, in PSP day 4 rats in darkness, there appeared no prediurnal surge of Prl secretion, Prl levels were near basal and were significantly lower as compared to the nocturnal (N surge) and diurnal (D surge) Prl levels ($p < 0.012$ and $p < 0.002$, respectively). Red light or surgery had differential effects on prediurnal and diurnal Prl secretion in PSP day 4 rats. Red light increased prediurnal Prl secretion significantly ($p < 0.000$) so that Prl secretion was now significantly higher as compared to diurnal secretion ($p < 0.017$). Surgery just increased diurnal Prl secretion in PSP day 4 rats significantly ($p < 0.000$), so that prediurnal and diurnal Prl levels were no longer significantly different from each other ($p < 0.481$). These data indicate the presence of different regulatory mechanisms for prediurnal and diurnal Prl secretion on day 4 of PSP.

Contrasts between Different Physiological States

The declining phase of the nocturnal surges of Prl secretion in PSP rats was most pronounced in the rats exposed to red light and/or surgery, and this secretion pattern differed from the flat patterns in estrous and diestrous rats. Nocturnal secretion patterns in PSP rats looked quite similar, however, statistical analysis revealed that nocturnal Prl levels between day 0 and day 4 of PSP were significantly different in all three conditions studied: darkness ($p < 0.014$), red light ($p < 0.008$) and surgery ($p < 0.002$).

The prediurnal surge E on estrus looked also very similar to the prediurnal surge D1 on PSP day 0, however, Prl levels between estrus and PSP day 0 were significantly different, under darkness ($p < 0.013$), red light ($p < 0.004$) as well as surgery ($p < 0.019$). It should be noted that during this time period in the individual animals Prl values varied considerably and basal as well as elevated levels were regularly seen (fig. 3), so a mean presentation of the data (fig. 2) may not be well representative.

Such observations on nocturnal secretion in PSP day 0 and PSP day 4 rats, or prediurnal secretion in estrous and PSP day 0 rats, respectively, demonstrate that Prl secretion of similarly timed surges (which may have a same regulatory mechanisms) occurs at different levels.

There was never a significant difference in diurnal Prl levels between day 0 and day 4 of PSP, neither under darkness ($p < 0.995$) nor under red light ($p < 0.344$) or surgery ($p < 0.800$) conditions. Diurnal secretion patterns of these two groups of rats were quite similar in shape, as is illustrated in figure 4 by presenting the data of figure 2 in an appropriate form. Red light or surgery had opposite effects on diurnal Prl secretion in estrous rats, as compared to PSP

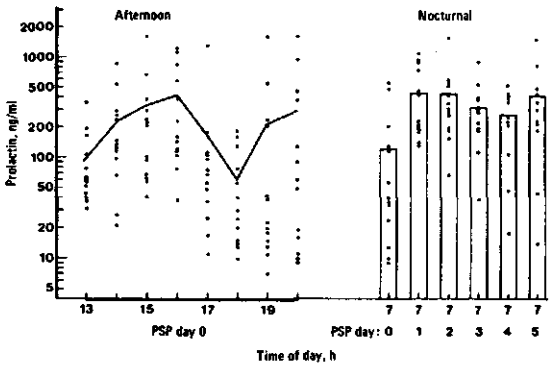

Fig. 3. Distribution of the individual Prl values underlying the afternoon and nocturnal surges of Prl secretion in PSP rats, maintained under full darkness at night.

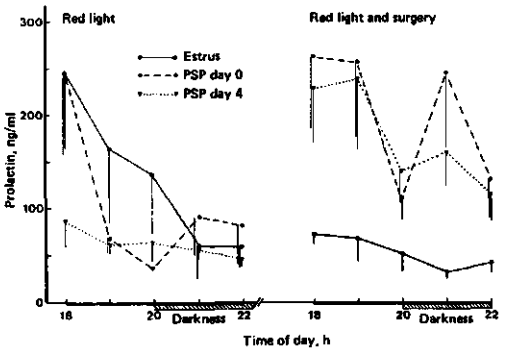

Fig. 4. Mean Prl secretion patterns (± SEM) in estrous and PSP rats, exposed to red light or to red light and surgery. Note the similarity in secretion patterns in PSP rats during the diurnal period (19.00–22.00 h) and the difference with the pattern in estrous rats.

Table I. Probability contrast for Prl secretion patterns presented in figure 2, accounting for the factors physiological state, time of the day and treatment. The physiological state counts three subfactors: estrus (estr), PSP day 0 (day 0) and PSP day 4 (day 4). The time of the day is subdivided into three time periods: a nocturnal (noct), a prediurnal (pred) and a diurnal (diur) time period. The animals were exposed to three different treatments: full darkness at night (drk), red dim illumination at night (rdl) or rdl together with surgery (sur)

	Time of day								
	darkness			red light			red light and surgery		
	noct/pred	pred/diur	diur/noct	noct/pred	pred/diur	diur/noct	noct/pred	pred/diur	diur/noct
Estrus	0.000	0.000	0.668	0.000	0.000	0.664	0.000	0.000	0.000
PSP day 0	0.000	0.032	0.021	0.000	0.000	0.000	0.410	0.000	0.000
PSP day 4	0.012	0.002	0.286	0.004	0.017	0.000	0.000	0.481	0.000
	Physiological State								
	nocturnal			prediurnal			diurnal		
	estr/day 0	day 0/day4	day 4/estr	estr/day 0	day 0/day 4	day 4/estr	estr/day 0	day 0/day 4	day 4/estr
Darkness	0.061	0.014	0.000	0.013	0.000	0.000	0.001	0.995	0.000
Red light	0.000	0.008	0.000	0.004	0.000	0.001	0.578	0.344	0.186
Red light and surgery	0.000	0.002	0.000	0.019	0.000	0.041	0.000	0.800	0.000
	Treatment								
	Estrus			PSP day 0			PSP day 4		
	drk/rdl	rdl/sur	sur/drk	drk/rdl	rdl/sur	sur/drk	drk/rdl	rdl/sur	sur/drk
Nocturnal	0.000	0.002	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Prediurnal	0.025	0.800	0.036	0.008	0.305	0.081	0.000	0.199	0.000
Diurnal	0.001	0.699	0.001	0.157	0.000	0.292	0.140	0.000	0.108

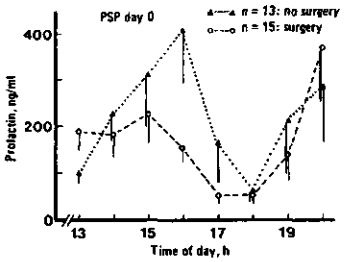

Fig. 5. The effect of surgery in the late afternoon of proestus on mean (± SEM) Prl secretion in PSP rats, maintained under full darkness at night. Surgery reduces prediurnal secretion on day 0 of PSP significantly ($p < 0.037$), but does not eliminate the biphasic secretion pattern.

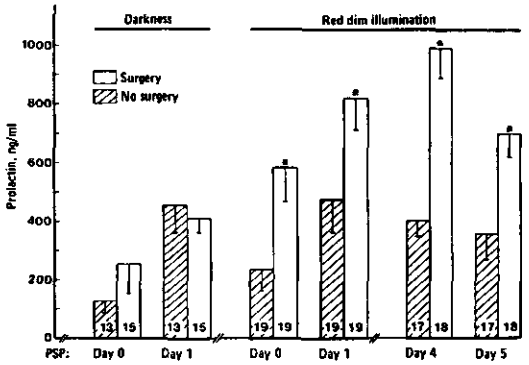

Fig. 6. Effect of surgery on mean (± SEM) nocturnal Prl concentration at 07.00 h in PSP rats maintained at night either under full darkness or under constant red dim illumination. Surgery was performed in the late afternoon before experimentation, i.e. on proestrus or on day 3 of PSP, and the effect was followed for 2 days. The effect of surgery is significant under red dim illumination (*$p < 0.025$) and lasts at least 2 days.

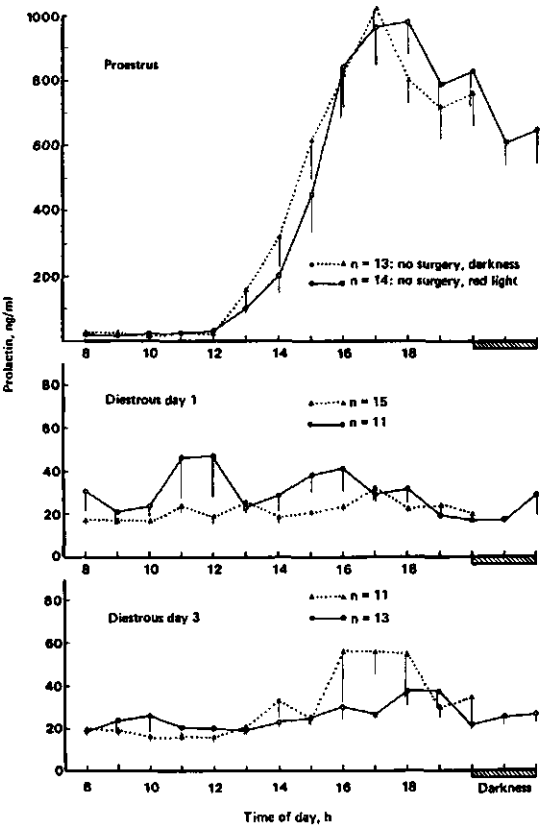

Fig. 7. Hourly Prl secretion patterns (mean ± SEM) in cycling proestrous and diestrous rats maintained at night under full darkness or constant red dim illumination. Red light only affects nocturnal and prediurnal secretion on diestrous day 1 significantly ($p < 0.05$).

rats, and diurnal Prl levels in estrous rats were therefore significantly different from both groups of PSP rats under darkness ($p < 0.001$, $p < 0.000$) or surgery ($p < 0.000$, $p < 0.000$) conditions, but not in the red light situation ($p < 0.578$, $p < 0.186$). These results indicate that diurnal Prl secretion in estrous rats is differently regulated from those of PSP rats, whereas diurnal secretion on day 0 of PSP (D2 surge) and day 4 of PSP (D surge) may have a similar regulation.

Effects of Treatment on the Surges of Prl Secretion

Red light, as compared to darkness, produced in all three physiological states a significant increase in Prl secretion during the nocturnal time period ($p < 0.000$). A further increase was observed when surgical stress was introduced ($p < 0.002$). The effect of the surgery under red dim light conditions lasted more than 36 h: as figure 2 shows, nocturnal Prl secretion was still significantly ($p < 0.000$) increased 36 h or more after the surgery.

Red light, as compared to darkness, significantly increased prediurnal Prl secretion in estrous ($p < 0.025$), PSP day 0 ($p < 0.008$) and PSP day 4 ($p < 0.000$) rats. Surgery, as compared to red light, had no significant effects ($p < 0.800$, $p < 0.199$, respectively). Surgery, as compared to darkness, hat a highly significant effect on Prl secretion in PSP day 4 rats ($p < 0.000$); in estrous and PSP day 0 rats the effect was less pronounced ($p < 0.036$ and $p < 0.081$, respectively). These results demonstrate that prediurnal Prl secretion in estrous, PSP day 0 and PSP day 4 rats react essentially alike to red light or surgery, however, in estrous

Table II. Probability contrast table of Prl secretion patterns presented in figure 7, accounting for the factors physiological state: diestrous day 1 and day 3, and time of the day: nocturnal (noct), prediurnal (pred) and diurnal (diur), under darkness or red dim illumination conditions

Time of day	Diestrous day 1			Diestrous day 3		
	noct/pred	pred/diur	diur/noct	noct/pred	pred/diur	diur/noct
Darkness	0.003	0.538	0.095	0.000	0.760	0.001
Red light	0.073	0.000	0.193	0.005	0.887	0.022

and PSP day 0 rats, in darkness, there appears a well-established surge (E and D1) of Prl secretion, which is absent on day 4 of PSP.

Red light, as compared to darkness, significantly affected diurnal Prl secretion in estrous rats ($p < 0.000$), but not diurnal secretion in PSP day 0 ($p < 0.157$) and PSP day 4 ($p < 0.140$) rats. Surgery, as compared to red light, produced no significant effect on diurnal Prl secretion in estrous rats ($p < 0.699$), but did so in PSP day 0 ($p < 0.000$) and PSP day 4 ($p < 0.000$) rats. Surgery as compared to darkness significantly affected diurnal Prl secretion in estrous rats ($p < 0.001$) but not diurnal Prl secretion in PSP day 0 ($p < 0.292$) and PSP day 4 ($p < 0.108$) rats. These data show that red light or surgery affect diurnal Prl secretion on PSP day 0 and PSP day 4 alike, and the effects are opposite to that on diurnal PSP secretion on estrus.

Throughout experimentation it was a general observation that Prl values varied considerably, particularly in PSP rats where in the individual animals from time to time near-basal as well as elevated levels were occasionally seen, indicative for a burst-like secretion pattern. The erratic distribution of the data is illustrated in figure 3. It was also noted that the length of PSP (13.6 days) was not affected by red light.

Effect of Surgery on Prl Secretion in PSP Rats under Darkness Conditions

In this experiment we investigated the effect of surgery in the late afternoon of proestrus on Prl secretion in PSP day 0 rats maintained under full darkness at night. Figure 5 presents the data. In both experimental groups the occurrence of 2 distinct surges of Prl secretion could be seen. Statistical analysis revealed that surgery produced differential effects on Prl secretion in the two periods: it reduced prediurnal Prl secretion significantly ($p < 0.037$), but it did not affect diurnal secretion ($p < 0.362$).

The effect of surgery in the late afternoon of proestrus on nocturnal Prl secretion in PSP rats was studied at 07.00 h. Results are summarized in figure 6. In rats in darkness, nocturnal Prl secretion was not affected by the surgery. As already mentioned (fig. 2), under red light conditions, surgery produced a significant increase in Prl secretion, either on PSP day 0 as well as on PSP day 4, and the effect of the surgery lasted at least 2 days. For reason of comparisons these data have also been added to figure 6.

Prl Secretion Patterns during Proestrus, Diestrous Day 1 and Diestrous Day 4

The effect of red dim illumination was investigated in proestrous and diestrous day 1 and day 3 rats. Mean Prl secretion patterns are shown in figure 7. In proestrous rats morning values of Prl were low, but a very pronounced surge of Prl secretion was seen in the afternoon. This secretion pattern was not affected by red light: significant differences could not be found, neither on an hourly basis, nor on a basis of longer time periods. The proestrous afternoon surge behaved as one surge: once the surge had started Prl values always remained highly elevated ($>> 250$ ng/ml). This pattern of secretion was in contrast to that in PSP rats where during this time period highly elevated as well as basal levels could be seen (fig. 3).

Prl levels in diestrous rats were low throughout the observation period, although there was a circadian variation (table II). Red light significantly affected Prl secretion in diestrous day 1 rats during the nocturnal ($p < 0.039$) and prediurnal ($p < 0.004$) time period; in diestrous day 3 rats no effect was found.

Discussion

Regulatory Mechanisms for Prl Secretion

Since the early description of *Freeman* et al. [12] that pseudopregnant rats release Prl in two daily surges, one nocturnal (N) and one diurnal (D), there is a general agreement on the occurrence of these surges [1, 2, 14, 17, 23, 31, 36], and this study reconfirms such a release pattern (fig. 2). Moreover, the present study demonstrates the occurrence of also a prediurnal surge (D1) of Prl secretion on day 0 of PSP, which preceeds the diurnal surge (D2) of Prl secretion. The N, D1 and D2 surges on day 0 of PSP behave differently under various experimental conditions (fig. 2, 5, 6, table I). In rats maintained under full darkness at night, surgery only affects prediurnal Prl secretion (fig. 5, 6), but

in animals exposed to red light, surgery increases nocturnal as well as diurnal secretion (fig. 2, 6). Red light on its own has remarkable effects on Prl secretion by enhancing nocturnal and prediurnal secretion (fig. 2). So the N, D1 and D2 surges respond differently to surgery and/or red light. Moreover, there is always a significant difference between nocturnal, prediurnal and diurnal Prl levels in all three conditions studied, except for nocturnal and prediurnal Prl levels in animals subjected to surgery (table I). These data together indicate the existence of different regulatory mechanisms for nocturnal, prediurnal and diurnal Prl secretion on day 0 of PSP.

The finding of the present work, that Prl levels are elevated throughout the full afternoon of day 0 of PSP, under both stress-free and stressful conditions (fig. 2, 5), has been a constant observation [5, 11, 31, 36]. Several workers have also oberved the prediurnal surge E of Prl secretion in the afternoon of estrus [1, 4, 10, 27, 29, 36]. It, however, has never been recognized that the elevated prediurnal Prl levels on day 0 of PSP in fact may be a reflection of the E surge. The available data demonstrate that in rats in darkness, the E surge and the corresponding Prl surge D1 on day 0 of PSP are both quite comparable in timing and magnitude [5] (fig. 2) and contrast with the low levels of Prl secretion on day 4 of PSP during this time period (fig. 2). The E and D1 surges behave similarly under different experimental conditions: surgery under darkness conditions attenuates the E surge [27, 29] as well as the D1 surge [11] (fig. 5); red light potentiates the two surges similarly (fig. 2), whereas red light together with surgery does not affect both these surges (fig. 2). This all indicates that the E and D1 surge must be basically one and the same surge.

It should be noted that red light or surgery affect prediurnal Prl secretion in PSP day 4 rats comparable to that in estrous and PSP day 0 rats: red light produces a significant increase, surgery has no further effect. However, prediurnal Prl levels on day 4 of PSP are much lower as compared to estrus and PSP day 0 (fig. 2). On the other hand, red light has no such profound effect on prediurnal Prl secretion in cycling diestrous rats (fig. 7). Therefore, the regulatory mechanism(s) for prediurnal Prl secretion in PSP day 4 rats may not be fundamentally different from those of estrous and PSP day 0 rats. The main difference, under usual light/darkness conditions, is the appearance of a well-defined E or D1 surge in the latter group. We have no explanation for this phenomenon, the data just indicate that in a state of estrus prediurnal Prl secretion comes to full expression.

Diurnal Prl secretion patterns in PSP day 0 and PSP day 4 rats are remarkably similar (fig. 4), and as table I illustrates, diurnal Prl levels between PSP day 0 and PSP day 4 are never significantly different, since diurnal Prl secretion responds in the same way to surgery and red light. Therefore, the diurnal surges D2 and D of Prl secretion in PSP rats have a similar regulation. This regulation is clearly different from diurnal secretion in estrous rats, where red light or surgery produces opposite effects as compared to PSP rats (fig. 2). It should be mentioned that the D2 and D surge behave differently in one respect. As figure 5 shows, surgery does not block the D2 surge, which is surprising since it is well documented that several noxious procedures would completely block the D surge for at least 1 day [7, 11, 12, 17, 23, 32, 37], with only one exception [7]. However, our observations concerning the D2 surge is supported by other investigators who also found that under stressful conditions Prl levels in the late afternoon of day 0 of PSP [11] and pregnant [29] rats remain elevated. An explanation for this difference between the D2 and D surge is not available.

Another point that should be mentioned is the choice of the prediurnal time period from 14.00 to 17.00 h and the diurnal time period from 19.00 to 22.00 h. Considering figure 2, under darkness conditions, this seems a good choice as far as it concerns the E, D1 and D2 surges, however, diurnal Prl secretion on day 4 of PSP starts at 17.00 h, peaks at 18.00 h and continues at 19.00 and 20.00 h. On the other hand, under red light conditions, the D1 surge seems to terminate by 19.00 h, whereas diurnal Prl secretion in PSP rats is quite similar after 19.00 h. In rats exposed to red light and surgery, the E surge terminates by 18.00 h, whereas diurnal Prl secretion in PSP rats is quite similar after 18.00 h. To avoid interactions due to termination and onset of the two surges it therefore seems a good choice to define the prediurnal time period from 14.00 to 17.00 h, and the diurnal time period from 19.00 to 22.00 h. This choice fits well with the available data in literature [1, 4, 5, 10, 12, 13, 25, 31].

It is well established that stress has little effect on nocturnal Prl secretion in PSP rats [11, 12, 17, 23, 37]. Our data are consistent with this concept when the animals are maintained under full darkness during the night (fig. 6). Red light, however, enhances nocturnal secretion markedly and after surgery Prl levels increase even more (fig. 2, 6). Such an effect of surgery and/or red light on the N surges was unexpected, however, the above procedures affect nocturnal Prl secretion on day 0 and day 4 of PSP essentially alike, indicating that nocturnal Prl secretion is regulated similarly from the very first day of PSP. It should be noted that we only studied the terminating phase of the nocturnal surge, so results may not be true for the full nocturnal period.

The data show that the regulation of Prl secretion is a complex phenomenon: red light and/or surgery can affect Prl secretion differently and the effect depends upon the physiological state as well as the time of the day. These effects were used to demonstrate different regulatory mechanisms for Prl secretion, and we could demonstrate the existence of a distinct regulatory mechanism for each of the three hypothesized time periods: nocturnal, prediurnal, and diurnal, in PSP rats. The different effects of red light and/or surgery within a particular time period can be ex-

plained by their differential effects on the same regulatory mechanism since it is hard to imagine a whole different set of mechanisms for the regulation of Prl secretion for each artificial intervention one does to the animal. It therefore is likely that for each of the three time periods, basically one regulatory mechanism controls the secretion of Prl. Before a regulatory mechanisms becomes effective, the conditions for its induction, timing and expression must be fulfilled. After the induction of PSP, either by cervical stimulation or other means [2, 15, 16, 26, 38], nocturnal and diurnal surges of Prl secretion occur daily, its timing entrained by photoperiod [12, 24, 28] and the expression modulated by ovarian steroids [12–15, 18, 25, 35, 37]. Red dim illumination certainly does not interfere with the induction mechanism(s), since it neither blocks surges of Prl secretion, nor does it affect the duration of PSP. The timing of the surges is also not affected by red light, so it therefore is likely that red dim light interferes with the expression of the surges. The proestrous afternoon surge is not at all affected by red dim illumination and behaves as one surge (fig. 7). This clearly indicates that this surge has a distinct regulation from those of PSP and estrous rats, which is also confirmed by the individual secretion patterns: Prl secretion in the afternoon of PSP rats appears more or less burst-like [36] (fig. 3), whereas in proestrous rats, once the surge has started, Prl levels remain constantly and highly elevated [36].

In summary, the present study demonstrates that Prl secretion is a rather complex process and multimodal in nature. The results also illustrate that the time of day and physiological state are important factors when mechanisms involved in the release of Prl induced by stressful stimuli are studied.

Red Dim Illumination

Rodents possess a rod-dominated retina with rhodopsin as the visual pigment [8, 30, 34]. This pigment absorbs all forms of visible light, although it has a reduced sensitivity to red light. This means that red light may appear as darkness to these animals, as was confirmed by behavioural studies in mice [33]. During experimentation we had no indication that the applied lighting schedule had any effect: the animals exhibited their usual nocturnal behaviour. Most of the daytime they were asleep and only a few hours before the onset of 'darkness' their behavioural activity increased. The animals had a normal smearing pattern. Cycle length and duration of PSP was also not affected. So the effect of red light on Prl secretion was fully unexpected.

In fact, surprisingly little is known about the visual capacities of rats [9]. Behavioural studies indicate that the rat possesses at least three different spectral mechanisms, a scotopic and two photopic mechanisms [3]. The latter provides a potential substrate for colour vision in the rat. The presence of cones in the rat retina has been confirmed anatomically [21] and physiologically [6]: electroretinogram studies indicate that there may be even three cone mechanisms. Most studies concerning physiological effects of light have been on entrainment to circadian rhythms [24, 28, 30, 31], however, systematic studies of the effects of colours are generally not available. In a study in the rat, red light was not very effective in entraining the body temperature, as compared to other spectra [22]. It nevertheless demonstrates perception of red light. The present study has shown that red light can produce significant effects on the endocrine system, and although the mechanism is not understood, the effects were very useful in this investigation. The results demonstrate the perception of red light, however, one may not conclude to colour vision in rats, since we even do not know whether the effect was mediated by the scotopic or photopic mechanism.

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CHAPTER 5

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No Evidence for an Autoregulatory Mechanism for the Proestrous Afternoon Surge of Prolactin Secretion in Rats

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Key Words. Prolactin · Autoregulation · Estrous cycle · Rats

Abstract. The temporal effects of intracerebroventricular (icv) infusion of prolactin (PRL) on endogenous PRL secretion was investigated in intact, freely moving, cycling rats. An icv infusion of 10 ng or 100 ng rat-PRL/min from 13.00 to 17.00 h during the proestrous afternoon did not affect endogenous PRL secretion; infusion from 09.00 to 13.00 h showed a tendency to delay the time of onset of the PRL proestrous afternoon surge. The results do not indicate the involvement of an autoregulatory mechanism in the expression or the termination of the proestrous afternoon surge of PRL secretion.

Introduction

There are several lines of evidence that prolactin (PRL) may control its own secretion. High levels of circulating PRL, introduced by pituitary transplants, PRL-secreting tumors or daily injections of PRL decrease the weight and PRL function of the hosts' pituitary [1–3]. One possible site of action of PRL is on the pituitary itself. However, results from studies on incubated pituitaries and PRL-secreting tumor cells are conflicting, some groups of workers demonstrating autoregulation [4, 5], while others do not [3, 6, 7]. Transplanted pituitaries, which secrete abundant amounts of PRL [2, 8–10] do not inhibit their own secretion. In view of these results it is thought that the site of action of PRL is, at least for the main part, on the central nervous system. Several studies support this concept. Implants of PRL in the median eminence depress the serum PRL concentration [2]. PRL has been shown to be able to enter the brain, either via the cerebrospinal fluid [11, 12] or by a retrograde hypophysial blood transport [13, 14], sufficient to exert short-loop feedback effects. A feedback mechanism is also suggested by the presence of binding sites for PRL in the brain, particularly in those areas likely to be involved in the regulation of PRL secretion [15–17]. That PRL indeed can affect neural function was

shown by electrophysiological [18–20] and behavioural [21] studies and also pharmacologically by the PRL-induced increase in dopaminergic activity in the median eminence [9, 10, 22, 23] and pituitary stalk blood [24]. Little information, however, is available whether increased levels of PRL inhibit endogenous PRL secretion. In a few studies the existence of a short-loop inhibitory feedback could be demonstrated [9, 25, 26]. In the present study we investigated whether an autofeedback mechanism is involved in the regulation of the proestrous afternoon surge of PRL secretion in the rat.

Materials and Methods

Animals

Locally bred F_1 hybrid female rats derived from two highly inbred strains of female Wistar and male Sprague-Dawley rats were used. They were housed under controlled illumination (light 06.00–20.00 h) and temperature (21–26 °C). Rat chow and water were freely available. They were used for experimentation at 5–9 months of age, weighing about 225 g. Blood was collected from freely moving, unrestrained animals via a chronic atrial silastic catheter which was inserted while the animal was anaesthetized with ether. The cannula was extended subcutaneously to the head where it was attached to the skull with anchor screws and dental cement. It was filled with saline containing 40% polyvinylpyrrolidone and 500 IU heparin/ml. In rats which received intracerebroventricular (icv) injections, a stainless steel guide cannula was also implanted, stereotaxically under nembutal anaesthesia. The tip was located just inside the right lateral ventricle (coordinates according to *De Groot* [27]: A = +5.5; L = +1.2; V = +2.6). Correct placement was assessed as described by *Paakkari* [28]. After surgery the animals were housed individually in transparent plastic cages, where they remained throughout experimentation. They were handled daily for at least 2 weeks to accustom them to the experimental procedures. Estrous cycles were followed by taking vaginal smears. 1 h before experimentation a polyethylene sampling cannula was connected to the animals, such that blood sampling could be performed without disturbing the animals. Samples of 200 µl were collected. After every 5 samples blood volume was restored by injection of 1 ml of blood from male F_1 donor rats. This blood sampling/blood transfusion technique does not affect PRL or corticosterone secretion [submitted].

Autoregulation of PRL Secretion

In this experiment we studied the effect of a constant icv infusion of PRL for several hours. Rat-PRL (NIAMDD PRL-RP-B-2, activity 20 IU/mg) was dissolved in physiological saline, at a concentration of 0.1 mg/ml or 1 mg/ml. The infusion apparatus was set at a constant rate of 0.1 µl/min, so the amount of PRL infused was 10 ng/min or 100 ng/min, respectively. Assuming a first order disappearance ratio for PRL in the cerebrospinal fluid (CSF), then the change in the PRL concentration in the CSF can be estimated by:

$$\frac{dP_{csf}}{dt} = \frac{1}{V}\left[-\left(\frac{V}{t_{½}/\ln 2} + a + b\right)P_{csf} + aP_{inf}\right]$$

where P_{csf} = concentration of PRL in CSF, V = volume of CSF, $t_{½}$ = exponential half-life of the disappearance of PRL from the CSF, a = infusion rate of PRL solvent, b = rate of CSF outflow, and P_{inf} = amount of PRL infused. Inserting V = 0.8 ml, a = 0.1 µl/min, $t_{½}$ = 4 min [29], then for P_{inf} = 0.1 mg/ml or 1 mg/ml, steady state levels of 71.1 ng/ml or 711 ng/ml, respectively, will be obtained. The first concentration is within a physiological range, the latter is extremely high, since there appears to be an upper limit to the CSF PRL concentration [12]. The $t_{½}$ value of 4 min for CSF PRL is an estimated value, derived from the $t_{½}$ value found in the blood circulation [30], an actual value is unknown. 1 h before infusion was started an infusion cannula was connected to the animals, such that they could move freely around. Autoregulation of PRL was studied in proestrous rats; PRL was infused in the morning from 09.00 to 13.00 h or in the afternoon from 14.00 to 17.00 h. Control rats received physiological saline.

RIA and Data Analysis

Blood samples (200 µl) were diluted with 100 µl of heparinized phosphate-buffered saline (pH = 7.5). Plasma samples were stored at −20 °C until analyzed by means of a homologous double-antibody radioimmunoassay as described previously [30]. Rat prolactin

RP-1, kindly provided by the NIAMDD, was the standard used (biological potency 11 IU/mg). The samples were assayed in 5 of 6 serial 1:2 dilutions. Results are presented as means ± SEM. The data were evaluated statistically after a logarithmic transformation and probabilities were derived from analysis of variance (t test).

Results

The pattern of PRL secretion was followed in proestrous rats from 09.00 until 19.00 h at 1-hour intervals. The icv infusion of physiological saline (0.1 µl/min) from 09.00 till 13.00 h (n = 8) or from 14.00 till 17.00 h (n = 8) had no effect on the occurrence of the proestrous afternoon surge of PRL secretion as compared to noninfused controls (n = 8). In figure 1 the patterns of PRL secretion in rats infused from 14.00 to 17.00 h with 10 ng or 100 ng rat-PRL/min are compared with the pattern of the control group. Infusion with 100 ng/min did not affect PRL secretion. In rats receiving 10 ng/min, the mean PRL concentration at 17.00 h was significantly lower as compared to the control ($p < 0.05$). The average PRL patterns did not demonstrate a feedback effect of PRL, so we investigated the effect of a long-term infusion from 09.00 to 13.00 h before the onset of the proestrous surge of PRL secretion. The results are presented in figure 2. As can be seen, the afternoon surge of PRL secretion still occurred and was not significantly affected as compared to the control. Short-term effects were also investigated by collecting blood samples at 10-min intervals for 1 h immediately after the onset of the infusion. Neither in the morning-infused groups, nor in the afternoon-infused groups was an effect on PRL secretion found.

Discussion

PRL is involved in a variety of physiological processes, but there are no specific target organ hormones that inhibit PRL secretion. This makes the regulation of PRL secretion a complex phenomenon. In rats the most obvious function of PRL is in reproduction. Particularly the surges of PRL secretion occurring during proestrus, estrus and (pseudo)pregnancy are well documented [30], however, despite numerous investigations the underlying regulatory mechanisms are still poorly understood.

At present it is not known which factors are responsible for the actual secretion pattern and the termination of the proestrous afternoon surge of PRL secretion. One of these factors may be an autoregulatory mechanism. In the Introduction we summarized several lines of evidence for such an autoregulatory mechanism, likely acting via tuberoinfundibular dopaminergic (TIDA) neurons [1, 30–32]. Indeed, at times of elevated PRL secretion, such as during proestrus, dopamine levels in portal blood are low, whereas it is elevated during estrus and diestrus, when plasma PRL levels in the rat are low [31, 32]. In case autoregulation would be involved in the actual secretion of PRL during the proestrous afternoon, one would expect either a burst-like release pattern, the result of a phasic feedback mechanism, or a suppressed release pattern, the result of a tonic feedback mechanism. However, PRL icv infused from 13.00 to 17.00 h, on top of the endogenously elevated levels, did not affect endogenous PRL secretion (fig. 1). Studies on the effects of exogenous PRL on endogenous PRL [9, 25, 26] or dopamine [9, 19, 22, 33] activity all show a certain latency of at least several hours. Such a latency may

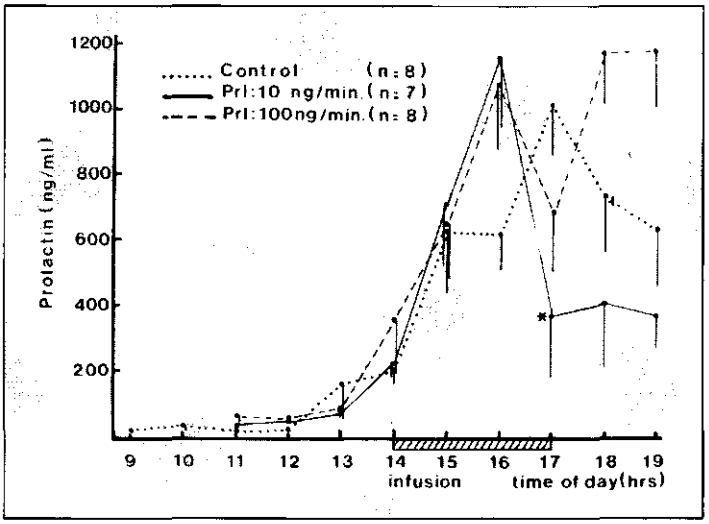

Fig. 1. Effect of intracerebroventricular infusion of PRL from 13.00 to 17.00 h on endogenous PRL secretion in proestrous rats. Blood was collected at hourly intervals. Data are presented as means ± SEM. *$p < 0.05$ as compared to the control.

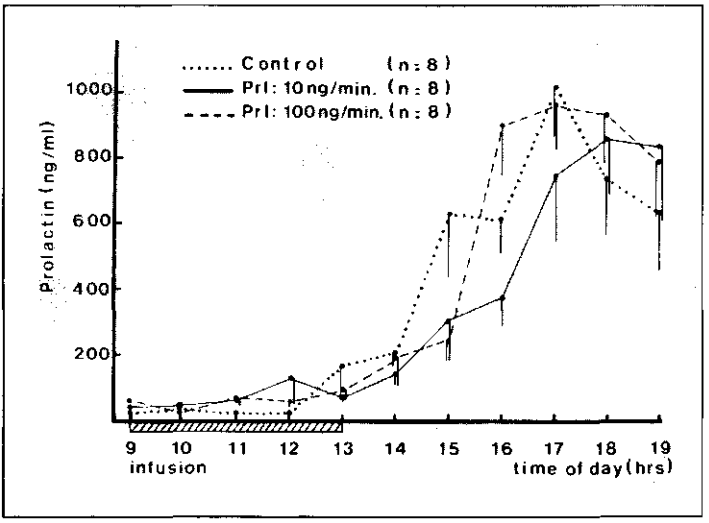

Fig. 2. Effect of intracerebroventricular infusion of PRL from 09.00 to 13.00 h on endogenous PRL secretion in proestrous rats. Blood was collected at hourly intervals. Data presented as means ± SEM.

suggest that the termination of the proestrous surge of PRL secretion may be controlled by an autoregulatory mechanism. To study this possibility we infused rats in the morning from 09.00 till 13.00 h to see whether it would prevent the occurrence of the afternoon surge. However, the only effect noted was a slight delay in time of onset of the proestrous surge (fig. 2). Under physiological conditions the proestrous surge lasts about 8 h and terminates gradually [34], so an autoregulatory mechanism may need the exposure to elevated PRL for at least 8 h. Therefore, PRL secretion in the morning-infused groups of rats might have been terminated after another 4 h of elevated PRL levels

caused by endogenously secreted PRL. However, our results (fig. 2) still show peak levels at 19.00 h, not indicative for any feedback effect. It, therefore, is likely that the proestrous surge of PRL secretion is not terminated by an autoregulatory mechanism, and the observed dopamine rhythm [31] may be considered an intrinsic rhythm. A similar conclusion has been drawn for the dopamine rhythms and the surges of PRL secretion in pregnant rats [26].

Not all effects of PRL have been reported to appear after a latency period of several hours. Iontophoretically applied PRL affected the single unit activity within seconds [18, 20]. Recently, evidence has been presented for the existence of separate types of TIDA neurones: a 'phasic' group that reacts rapidly to an intravenous injection of PRL and a 'tonic' group responding to PRL treatment only following a latency period of 12–16 h [35]. Interesting in this respect is that the surges of PRL secretion in pseudopregnant rats have a different neural regulation from those of proestrous rats [submitted], and that PRL secretion in pseudopregnant rats appears to be more or less burst-like [30]. It is quite possible that the burst-like pattern of PRL secretion in pseudopregnant rats is due to an autoregulatory mechanism, regulated by the 'phasic' tuberoinfundibular dopaminergic component and experiments are in progress to investigate this possibility.

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CHAPTER 6

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Electrophysiological Evidence for a Sex Difference in Neural Regulation of Prolactin Secretion in Rats

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Key Words. Prolactin · Estrous cycle · Pseudopregnancy · Stress · Sex difference · Neural regulation · Brain stimulation · Rat · Electrodes

Abstract. Prolactin (PRL) secretion patterns were determined in freely moving male and female rats chronically fitted with electrodes in the sexually dimorphic component of the medial preoptic area (MPOA) and the median eminence (ME), together with a chronic atrial blood sampling catheter. Electrodes made of stainless steel or platinum-iridium were implanted bilaterally. Female rats with 4 electrodes in the ME exhibited an attenuated surge of PRL secretion on proestrus (injured group), rats with 2 electrodes in the ME had a normal proestrous surge (intact group). Basal PRL levels were not different between the two groups. Stimulation (100-Hz voltage pulses, 0.2 ms width, 10 s on/5 s off, total duration 25 min) did not produce lesions. Evidence is presented that experimentation was performed without stress. Electrical stimulation applied to the MPOA increased PRL secretion in males, decreased PRL secretion in proestrous females and was without effect in conscious or anesthetized diestrous females. Electrical stimulation applied to different locations of the ME was without effect in male and diestrous female rats; it reduced PRL secretion in proestrous females. These data demonstrate that the MPOA has a sexually differentiated function in the regulation of PRL secretion. The data do not provide evidence for a sexual dimorphism in prolactin-inhibiting factor and prolactin-releasing factor activity in the ME.

Prolactin (PRL) in the normal male rat is released in a tonic, acyclic pattern [28, 31], whereas in normal females PRL release is cyclic and varies with the reproductive state [31, 37, 42]. This sexual difference in the regulation of PRL secretion is neural in origin [31]. There is a variety of morphological differences within the brain that might account for this sexual difference. At least the medial preoptic area (MPOA) shows a sexual dimorphism with respect to nuclear volume, neuronal size, synaptic organization and dendritic branching patterns [16, 35]. There is also electrophysiological evidence for a sexual dimorphism [11], and lesion studies of the MPOA show sexual differences in the PRL response [17].

Neurons belonging to the preoptico-infundibular and tubero-infundibular system seem to be involved in the regulation of PRL secretion. These neurons have endings in the median eminence (ME) [11, 32] where they secrete dopamine (DA) a potent PRL-inhibiting factor (PIF) into the portal vessels [3, 15, 19, 30]. While DA inhibits PRL release by releasing PIF or acting itself as PIF, serotonin (5-HT) has a stimulating effect on PRL release. There is accumulating evidence that this stimulatory effect may be mediated through the release of a PRL-releasing factor (PRF) [7]. Sexual differences in the effect of DA and 5-HT on PRL secretion have also been reported, and there is evidence for a more developed PIF system in females and PRF system in males [2, 3, 7, 26, 30].

In order to study sexual differences in the regulation of PRL secretion we investigated the effect of electrical stimulation of the MPOA and the ME in conscious male and female rats. Electrical stimulation of the MPOA in both male and female anesthetized rats has been reported to stimulate [8, 9, 12, 43], inhibit [21–23] or fail to affect [14, 20, 21] PRL secretion, whereas ME stimulation in general was without effect [6, 13, 20, 21].

However, all these data are difficult to compare because of differences in experimental protocol. We, therefore, stimulated males and females under comparable conditions and investigated: (1) whether the MPOA had a sexually dif-

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ferentiated function in the regulation of PRL secretion and (2) whether we could find electrophysiological evidence for a sexual dimorphism in PIF or PRF activity in the ME. Part of this work was previously reported in abstract form [39].

Materials and Methods

Animals

Experiments were carried out upon locally bred adult F1 hybrid rats derived from two inbred strains of female Wistar (R-Amsterdam) and male Sprague-Dawley (U-Amsterdam) rats. The animals were maintained under conditions of controlled lighting (lights on 06.00–20.00 h), temperature (21–26 °C) and humidity (>65%). Food and water were freely available. At the time of experimentation the rats were 8–17 months old, the females weighed 190–250 g and the males 350–500 g. The reproductive state of the females was followed by vaginal smears.

Electrode Implantation and Brain Stimulation

Surgery was performed under pentobarbital anesthesia (40 mg/kg body weight i.p.; Nembutal, Abbott). In some cases the anesthetic period was extended by supplying ether together with an injection of atropine (1 mg/kg body weight i.m.; ACF Holland). The De Groot atlas [10] was used as a guide for the placement of electrodes. The stereotaxic apparatus used was a slightly modified version of that developed by Minderhoud [29]. This instrument enabled us to implant electrodes separately in any structure within the brain from any angle. The electrodes were inserted through holes drilled through the frontal and parietal bones of the skull on both sides and parallel to the midline at lateral distances of about 3 mm. After the electrodes had been implanted and secured to the skull with a rapidly hardening glue (methyl-2-cyano-acrylate) the plug (Amphenol, series 221) for the external cable contact was placed between the electrodes and connected to the electrodes by spot-welding [38]. The complete electrode assembly was then imbedded in acrylic dental cement and fastened to the skull with anchor screws.

Electrodes were made of stainless steel (type 304) or platinum-iridium (70/30) wire of 0.2 mm diameter and insulated with epoxylite (EPR-4, Clark Electromedical Instruments) except for 0.4 mm at the tip. The electrodes were implanted bilaterally into the sexually dimorphic component of the MPOA; [coordinates: A = 7.5, H = -1.5, L = 0.2; see ref. 16, p. 110] and at two locations into the ME (coordinates: A = 5.5, H = -4.25, L = 0.2 and A = 6.2, H = -4.0, L = 0.2, respectively). To improve stereotaxic accuracy, the electrodes aimed for the ME were lowered till they touched the basisphenoid and then withdrawn 0.1 mm.

Rats were stimulated bilaterally with monophasic rectangular 100-Hz voltage pulse trains (0.2 ms width; 10 s on/5 s off; total duration 25 min). Voltage and current were monitored continuously. The experiments were performed with freely moving rats and from the onset of stimulation the intensity was slowly increased to a maximum of 4 V. Stimulation of the MPOA produced increased locomotor activity and the stimulation was usually set at a lower intensity of 2–3 V.

At the end of experimentation the brains were fixed by cardiac perfusion with an aqueous solution containing 43% ethanol, 2% acetic acid, 4% formalin and 3% potassium ferrocyanide. A DC current (30 μA, 60 s) was passed through the stainless steel electrodes. The electrode positions were verified by microscopic examination of serial sections stained in hematoxylin-eosin by the presence of the Prussian Blue spot (stainless steel) or electrode track (PtIr).

Table I. The occurrence of impaired PRL function and/or PSP in relation to the number of electrodes implanted into the ME, electrode material, surgery, or electrical stimulation of the MPOA or ME

Number of electrodes in ME	Injured[a] PRL function	PSP after surgery	PSP after stimulation	
			MPOA	ME
2 stainless steel	3/12[b]	4/14	1/19	2/13
4 stainless steel	10/10	11/14	0/12	2/14
4 PtIr	8/8	0/8	3/4	12/33

[a] Injured as defined in Materials and Methods.
[b] Three out of 12 rats.

Blood Sampling

The rats were fitted with a chronic silastic (0.5 × 0.9 mm, Dow Corning, USA) atrial catheter as described previously [40, 41]. The free end for the external catheter connection was slit a few millimeters over one end of a hooked stainless steel tube (0.6 × 0.9 mm). This tube was integrated in the Amphenol plug for the electrical cable connection. The catheter was filled with 0.9% NaCl, containing 500 IU heparin/ml (Kabi AB, Stockholm) and 40% polyvinylpyrrolidone (PVP, Merck, Germany) and closed with a small polyethylene plug.

The rats were housed singly in transparent plastic cages and could move freely. They were allowed a postoperative recovery period of at least one week, during which time they were accustomed to all experimental procedures. Blood samples of 200 μl were collected at -10, 0, 5, 15, 25, 30, 40, 50, 75 and 90 min. The blood volume reduction was compensated by blood transfusions with freshly collected donor blood every time a total of 1 ml blood had been withdrawn. This blood sampling procedure appeared to be free of stress [40, 41].

Experiments

The aim of this study was to investigate the existence of a sex difference in the neural regulation of PRL secretion. In a first series of experiments rats were fitted chronically with a total of 6 stainless steel electrodes, bilaterally implanted into the MPOA and at the two locations into the ME. However, the females responded with pseudopregnancies (PSP) and impaired PRL function (see Results). We therefore decided to investigate whether these effects were caused (1) by the number of electrodes, by implanting less electrodes, or (2) by the electrode material, by implanting PtIr electrodes instead of stainless steel electrodes. It appeared that rats implanted with a total of 2 electrodes into the ME behaved normally (see Results). We finally fitted rats with two pairs of stainless steel electrodes, one pair into the MPOA and one pair into the ME. The rats were stimulated electrically for 25 min and blood samples were

collected sequentially during 90 min. Unstimulated rats served as controls. Males were stimulated in the morning at 11.00 h, females on proestrus at 10.00 h and 15.00 h and on diestrous day 1 at 11.00 h. Stimulation was also performed in anesthetized diestrous rats.

Radioimmunoassay and Data Analysis

Blood samples (200 µl) were diluted with 100 µl heparinized phosphate-buffered saline (pH 7.5). Plasma samples were stored at −20 °C until analyzed for PRL concentration by means of a homologous double-antibody radioimmunoassay. The samples were analyzed in 5 or 6 serial 1:2 dilutions. Rat PRL RP-1, kindly provided by the NIADDK was the standard, with a biological activity of 11 IU/mg.

The results are expressed as individual data or as means ± SEM. The data were evaluated statistically after a logarithmic transformation using the Student t test. A value of $p < 0.05$ was considered significant.

Results

Effect of Number of Electrodes and Electrode Material on PRL Function

Experimentation with conscious rats, chronically fitted with electrodes and a catheter, was not without problems; the rats developed PSP after surgery and/or electrical stimulation, or showed impaired PRL function. The results are summarized in figure 1 and table I. Based on the magnitude of the proestrous afternoon surge of PRL secretion in female rats, the rats could be classified into 2 groups, *intact* or *injured*, depending upon whether the individual PRL concentrations at 16.00 h were higher or lower than 400 ng/ml, respectively. This classification is justified by realizing that in our rat strain proestrous PRL concentrations smaller than 400 ng/ml at 16.00 h are extremely exceptional. The mean PRL concentration in intact rats was significantly higher ($p < 0.005$) when compared with injured rats: 758 ± 94 and 148 ± 32 ng/ml, respectively (fig. 1). All rats with 4 electrodes in the ME, either made of stainless steel or PtIr, had an impaired PRL function, 3 out of 12 rats with 2 electrodes in the ME could be classified as injured (table I). There were no significant differences between the mean PRL concentrations in intact and injured rats at times when PRL levels are basal, as on proestrus at 10.00 h or on diestrous day 1 at 11.00 h (fig. 1). The data of figure 1 also show that the mean PRL concentrations in comparable groups of rats were not significantly different before or after the rats had been stimulated electrically.

The implantation of 4 steel electrodes into the ME induced PSP in 11 out of 14 rats, however, none of the 8 rats fitted with 4 PtIr electrodes responded with PSP after surgery (table I). In rats fitted with PtIr electrodes PSP was induced more often after stimulation as compared to rats with steel electrodes. Rats with 2 steel electrodes in the ME behaved normally most of the time: surgery evoked PSP in 4 out of 14 rats, 3 out of 12 rats had impaired PRL function, and stimulation rarely induced PSP (2 out of 13).

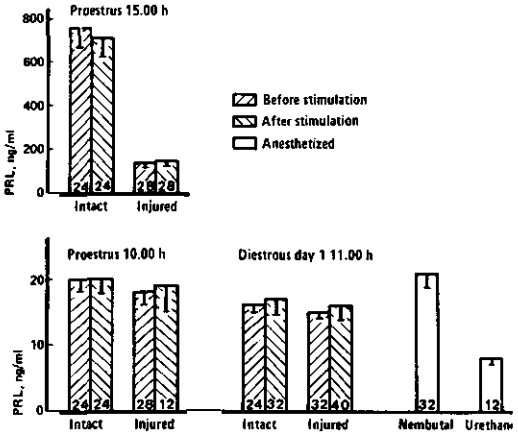

Fig. 1. Mean PRL concentrations in intact and injured proestrous and diestrous day 1 rats. Pre- and poststimulation PRL levels are not significantly different in comparable groups. Nembutal increases ($p < 0.05$) and urethane decreases ($p < 0.005$) the mean PRL concentrations in diestrous rats significantly. The number at the base of each bar represents the number of rats in the respective experiment.

Effect of Electrical Stimulation on PRL Secretion in Intact Rats

These experiments were performed with rats implanted bilaterally with steel electrodes into the MPOA and the ME. Only data of female rats that could be classified as intact were used, males could not be classified. The data are presented in figures 2 and 3. Electrical stimulation of the ME had no effect on PRL secretion in male rats, in proestrous females PRL levels were significantly lower after 25 min of stimulation as compared to the controls ($p < 0.05$). MPOA stimulation produced a significant increase in PRL secretion in males ($p < 0.05$), but a highly significant decrease in proestrous females ($p < 0.005$). In proestrous females the effect of MPOA stimulation was more pronounced and of a longer duration compared with ME stimulation.

Electrical stimulation of the ME ($n = 3$) or MPOA ($n = 8$) in intact diestrous day 1 rats had no significant effect on PRL secretion. The only effect noted was that during stimulation the PRL values varied less, indicating that stimulation had some inhibitory effect on PRL secretion. Some representative individual PRL secretion patterns are presented in figure 3.

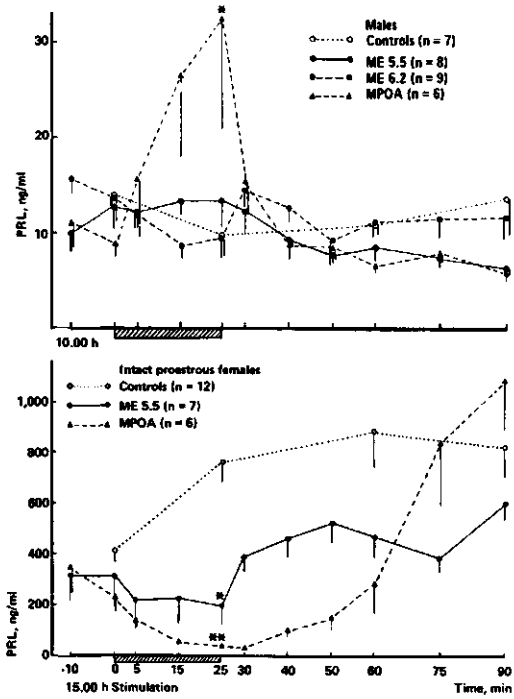

Fig. 2. The effect of electrical stimulation of the ME or the MPOA on mean PRL secretion patterns (-SEM) in conscious male and proestrous female rats. MPOA stimulation significantly increases PRL secretion in males and decreases it in females as compared to sham-stimulated controls. In male rats ME stimulation is without effect, however, in females it reduces PRL secretion significantly. Electrodes were made of stainless steel. (* = $p < 0.05$; ** = $p < 0.005$.)

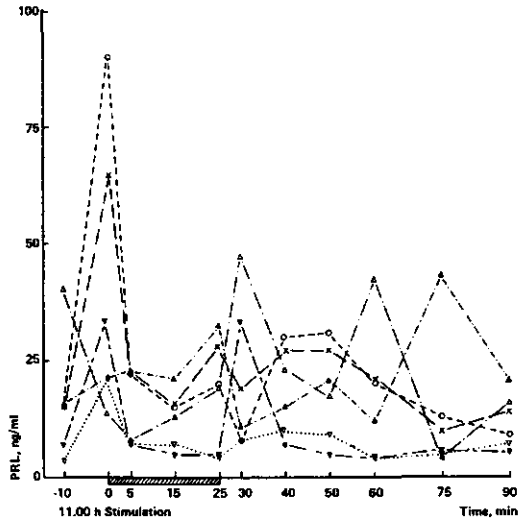

Fig. 3. Time course of individual PRL secretion patterns in intact diestrous day 1 rats receiving 25 min of electrical stimulation of the MPOA with stainless steel electrodes.

Effect of Electrical Stimulation on PRL Secretion in Injured Rats

Female rats classified as injured were used for these experiments. Most rats had 4 electrodes in the ME and 2 in the MPOA, either made of steel or PtIr. The effects of electrical stimulation were quite comparable to those of intact rats. MPOA stimulation ($n = 4$) in proestrous females produced a significant ($p < 0.05$) decrease in PRL secretion after 25 min of stimulation as compared to the unstimulated controls ($n = 18$). Stimulation of the ME had also a significant inhibitory effect on PRL secretion in proestrous rats. This effect was more pronounced in rats implanted with PtIr electrodes ($p < 0.005$) than in rats with steel electrodes ($p < 0.05$). Data on ME stimulation are shown in figure 4.

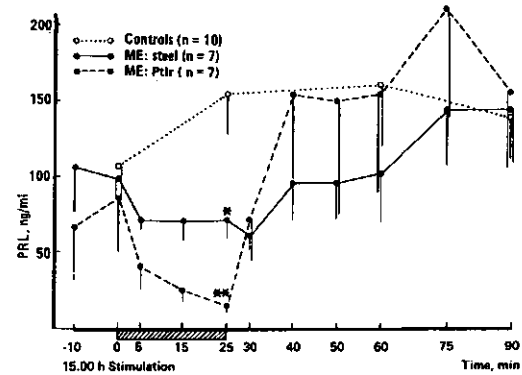

Fig. 4. Time course of mean PRL concentrations in injured proestrous rats receiving 25 min of electrical stimulation of the ME with stainless steel or PtIr electrodes. Stimulation decreased PRL concentrations significantly as compared to the sham-stimulated controls. (* = $p < 0.05$; ** = $p < 0.005$).

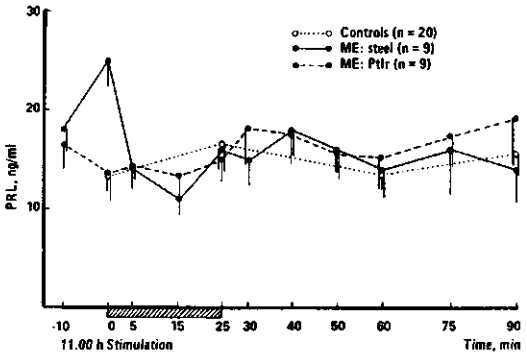

Fig. 5. Time course of mean PRL concentrations in injured diestrous day 1 rats electrically stimulated during 25 min in the ME at location A 5.5 with stainless steel or PtIr electrodes. Similar results were obtained at ME location A 6.2.

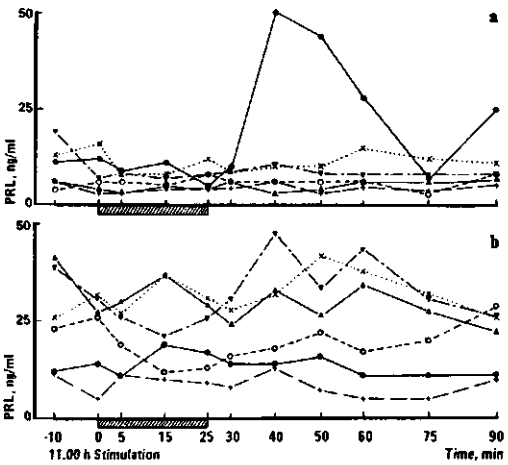

Fig. 6. Individual PRL secretion patterns in intact urethane- (a) or nembutal-anesthetized (b) diestrous day 1 rats receiving stimulation of the ME during 25 min. In the nembutal group the PRL levels are higher than in the urethane group (see also fig. 1). Electrodes used were made of stainless steel.

Electrical stimulation of the MPOA with either steel (n = 5) or PtIr (n = 3) electrodes, or at both locations on the ME with steel (n = 7) or PtIr (n = 7) electrodes had no significant effects on PRL secretion in injured diestrous day 1 rats. As in intact rats (see fig. 3) there was a tendency that individual PRL values varied less during stimulation. The mean PRL secretion patterns in ME-stimulated rats are presented in figure 5.

Effect of Electrical Stimulation on PRL Secretion in Anesthetized Rats

Intact diestrous day 1 rats were anesthetized twice at termination of experimentation, the first time with Nembutal (40 mg/kg body weight i.p.) and the next cycle with urethane (1.5 g/kg body weight i.p.). The effect of ME stimulation on PRL secretion was investigated. Mean basal PRL levels under Nembutal anesthesia were significantly higher ($p < 0.05$) and under urethane anesthesia significantly lower ($p < 0.005$) as compared to conscious intact diestrous day 1 rats (fig. 1). Electrical stimulation of the ME did not at all affect PRL secretion (fig. 6).

Electrical Stimulation and Behavioral Response

In both males and females electrical stimulation of the ME did not induce a behavioral response. Stimulation was performed at 4 V, causing an initial current of about 275 μA and a steady-state current of about 130 μA, as the electrical circuitry behaved capacitive. Stimulation of the MPOA elicited a well-pronounced response that was alike in males and females. The animals responded with increased locomotion and explorative behavior. When the stimulus intensity was too high the rats tried to escape from their cage. This escape behavior was avoided by stimulating rats at a lower intensity, which varied for individual animals between 1 and 4 V.

Discussion

Stimulation of the preoptic area of anesthetized male or female rats may stimulate [8, 9, 12, 43], inhibit [21–23] or fail to affect [14, 20, 21] PRL secretion. However, in most of these studies cited, differential experimental protocols were applied with respect to the anesthesia, the stimulation parameters, the electrode material and/or the precise electrode localizations, which may, at least in part, explain the discrepancies. We, therefore, stimulated the sexually dimorphic component of the MPOA in male and female rats under comparable conditions. Our data (fig. 2) show that this stimulation evokes a fast, short-lived increase in PRL secretion in male rats, but a sustained inhibition of the proestrous surge of PRL secretion in female rats, indicating the existence of a sex difference in the neural regulation of PRL secretion. A similar conclusion was drawn by Gunnet and

Freeman [17] on a basis of lesion experiments in the MPOA. The same authors also found that electrical stimulation of the MPOA in conscious female rats suppressed PRL secretion [18], which is in agreement with our observations in conscious female rats (fig. 2).

As also found by others [24], electrical stimulation of the MPOA elicited a similar stereotype behavior in both sexes: the animals exhibited increased locomotor activity and looked quite alert. The overall behavior, however, was quite unnatural, and we realized that the stimulation might stress the animals. The general effect of acute stressful stimuli on PRL secretion depends upon the initial level of circulating PRL, being excitatory when prestress levels are low and inhibitory when prestress levels are high [4, 25, 33]. In our experimental design (fig. 2) the circulating PRL levels were low in the male and high in the female. The observed increase in PRL secretion in males and the decrease in females during stimulation can also be explained as an indirect effect of stress.

For this reason we investigated the effect of MPOA stimulation in conscious as well as anesthetized diestrous females, where circulating PRL levels are low and comparable to that in males. The data show that electrical stimulation of the MPOA does not affect PRL secretion in both conscious (fig. 3) and anesthetized (fig. 6) diestrous females. Therefore, we may conclude that MPOA stimulation does not introduce stress in the animals. So the observed increase in PRL secretion in conscious male rats (fig. 2), which was also found in anesthetized male rats [8, 9], is a direct effect of the stimulation, and we thus may conclude that the MPOA contains sexually differentiated neural elements involved in the regulation of PRL secretion.

In male as well as female rats, the basal level of circulating PRL is maintained by the tonic secretion of PIF [4, 30, 31]. However, an increase in PRL secretion may be either the result of an inhibition of PIF secretion or the release of PRF [3, 7, 27, 31]. There is evidence that dopaminergic (PIF) control of PRL is greater in females than in males. However, in male rats there is evidence for a more developed PRF release mechanism [2, 3, 7, 26, 30]. One way to explain the differential effects of MPOA stimulation on PRL secretion in male and female rats is that this stimulation evokes the secretion of PRF in male rats and the secretion of PIF in female rats. Since there is evidence for regional differences in PIF and PRF activity in the ME [27], we stimulated the ME at two locations to investigate the presence of PIF or PRF. Our data show that ME stimulation at either location does neither affect PRL secretion in males nor in diestrous females. However, it attenuated PRL secretion in proestrous females (fig. 2, 3, 5). These data and those of others [6, 13, 20, 21] do not provide evidence for the existence of a PRF. So on the basis of the data yet available we can not explain the sexual difference in the neural regulation of PRL secretion in terms of PRF and PIF.

Preferentially, animal experimentation should be performed under stress-free conditions. Although the usual practices of animal handling, surgery, anesthesia and blood sampling may easily induce stress, we recently reported that blood sampling/transfusion under chronic conditions with freely moving rats can be performed without stress [40, 41]. In the present study we added a new element, the chronic implantation of metal electrodes into the hypothalamus. This implantation, however, appears to interfere with cyclicity and/or PRL function (table I, fig. 1): rats develop PSP after surgery and/or electrical stimulation, or respond with impaired PRL release. The induction of PSP is not surprising, since it is well known that lesions in the MPOA or electrical stimulation of the dorso- and ventromedial hypothalamus may induce PSP in female rats [1, 17]. The electrodes aimed for the ME pierced the latter area and may have stimulated neural elements involved in the initiation of PSP. However, surgery induced PSP only in the steel electrode-group, whereas stimulation did so more often in the PtIr group (table I). Since we stimulated electrochemically metal ions must have been deposited. Many studies have shown that iron cations, in contrast to other cations, can affect neuronal hypothalamic activity for prolonged periods of time [5, 36]. This may partly explain the differential effects of steel and PtIr electrodes on the induction of PSP. The direct effects of electrical stimulation on PRL secretion are, however, essentially alike with both types of electrodes: stimulation inhibits PRL secretion in proestrous rats, but is without effect during diestrus (fig. 4, 5).

The observed impaired PRL function (table I) may be surprising, since it is well known that PRL is essential for the induction of PSP [34]. However, sufficient amounts of PRL must have been secreted for the induction of PSP. Rats with impaired PRL function usually had 4 electrodes in the ME (table I). Basal PRL levels in these injured rats were not different from those of intact rats (fig. 1). This indicates that the tonic inhibitory control mechanism for PRL secretion [3, 27, 31] has not been affected. The electrodes also have not produced lesions in the ME since this would have increased basal PRL levels [3, 31]. Therefore, it is likely that the electrodes have damaged neurons involved in the phasic release mechanism. Since the data do not indicate the presence of PRF (fig, 2, 5, 6), interneurons inhibiting PIF-secreting neurons may have been injured. Indeed, Dyer and Saphier [12] provided evidence for the existence of such neurons arising from the preoptic area. The attenuated afternoon surge of PRL secretion in injured proestrous rats (fig. 1, 4) can be explained by the injury of such neurons, which results in a less effective inhibition of PIF-secreting neurons. It should be realized that impaired PRL function was only caused by the surgery, not by electrical stimulation. The data of figure 1 show that pre- and poststimulation levels of PRL are not different. Moreover, in case electrical stimulation should have produced lesions in the

MPOA or the ME, this would have resulted in repetitive pseudopregnancies [17, 18] or increased basel PRL levels [3, 31], respectively, which did not happen either.

In summary, the data presented herein show that brain stimulation and blood sampling/transfusion in freely moving rats can be performed without stress. Surgery, i.e., the implantation of electrodes, but not electrical stimulation, may damage neural tissue. The data also show that the neural regulation of PRL secretion is sexually differentiated.

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CHAPTER 7

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Electrophysiological Evidence for a Key Control Function of the Medial Preoptic Area in the Regulation of Prolactin Secretion in Cycling, Pregnant and Lactating Rats

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Key Words. Prolactin · Estrous cycle · Pregnancy · Lactation · Neural regulation · Brain stimulation · MPOA

Abstract. Female rats were chronically fitted with stainless steel electrodes in the medial preoptic area (MPOA), together with a chronic atrial blood sampling/blood transfusion catheter. The surgery did not affect cyclicity, pregnancy or lactation. Twenty-five minutes of bilateral electrical stimulation (100-Hz voltage pulses, 0.2 ms width, 10 s on/5 s off) of the MPOA significantly reduced the magnitude of the proestrous and estrous afternoon levels of prolactin (Prl) in cycling rats, the nocturnal and diurnal levels of Prl in pregnant rats as well as Prl secretion during suckling in lactating rats. These data demonstrate that the MPO exerts dominant inhibitory control over all known surges of Prl secretion in female rats and may be considered as 'antisurge key control center' for Prl secretion. A functional role of the sexually dimorphic nucleus in the control of Prl secretion is suggested.

In mammals prolactin (Prl) is considered to be primarily a hormone concerned with reproduction, including the growth of the mammary gland and the secretion of milk. As such, the female rat displays unique patterns of Prl secretion, dependent upon the reproductive state. Prl secretion in cycling rats is characterised by a surge during the afternoon and early night of proestrus and a surge on the afternoon of estrus [3, 6, 27, 31, 32, 43–45]. Pregnant rats secrete twice daily a surge of Prl for approximately 10 days, a nocturnal surge in the early morning hours and a diurnal surge in the late afternoon [4, 35, 40]. Lactating rats release considerable amounts of Prl, independent of the time of the day [21, 32, 36]. These surges of Prl secretion in cycling, pregnant and lactating rats are controlled by quite different neural regulatory mechanisms [32, 45] as is indicated by the differences in their induction [4, 21, 25, 35, 37, 40], timing [9, 21, 25, 28], expression [13, 21, 25, 43] and responses to stress [5, 31, 45]. It, therefore, may not be surprising that several brain areas have been shown to be involved in the regulation of Prl secretion [1, 12, 16–20, 27, 37, 38, 41], particularly the medial preoptic area (MPOA). We recently demonstrated that the MPOA has an inhibitory role in the regulation of Prl secretion in the proestrous female [41]. An intriguing question is whether the MPOA has the same inhibitory role in the control of surges of Prl secretion in estrous, pregnant and lactating rats. If so, then the MPOA controls all known modes of Prl secretion in female rats and serves a key control function, and there may be one 'final common neural pathway' for the release of the hormone. The presence of preoptic-tuberoinfundibular projections may form the structural basis for such a common final pathway [10, 30, 37].

To answer these questions we investigated the effect of electrical stimulation of the MPOA on Prl secretion in freely moving rats during the appearance of the surges of the hormone in cycling, pregnant and lactating rats.

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Materials and Methods

Animals

Female F1 hybrid rats derived from two inbred strains of female Wistar and male Sprague-Dawley were maintained under controlled temperature (21–26 °C), humidity (> 65%) and light (light on from 06.00 to 20.00 h, Philips TL 40 W/34, 290–550 lx). All rats had ad libitum access to food (Complete Laboratory Diet, Hope Farms, The Netherlands) and water. The rats were kept individually in transparent plastic cages (length × width × height: 35 × 25 × 34 cm). Vaginal smears were taken daily. Each rat to be mated was placed with a fertile male at 17.00 h proestrus. Successful mating was determined by the presence of sperm in the vaginal smear the following morning, which was designated as day 0 of pregnancy. Pregnancy was confirmed later by the persistence of leukocytic cytology for 6 days, and by the occurrence of delivery. The day of delivery was designated as day 0 of lactation. Litter size was adjusted to 6 pups. At the time of experimentation females were 8–15 months old and weighed 190–310 g.

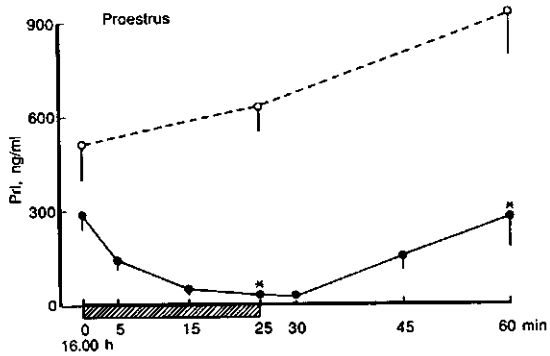

Fig. 1. The effect of 25 min of electrical stimulation of the MPOA on mean Prl secretion patterns (= ± SEM) in conscious proestrous female rats. (*p < 0.05). O = Control (n = 13); ● = stimulation (n = 6).

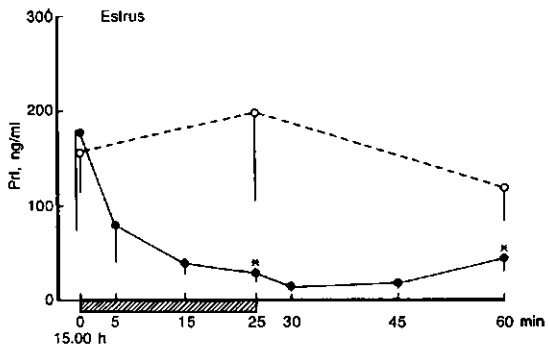

Fig. 2. The effect of 25 min of electrical stimulation of the MPOA on mean Prl secretion pattern (± SEM) in conscious estrous rats (*p < 0.05). O = Control (n = 8); ● = stimulation (n = 9).

Surgery

Rats were fitted chronically with stimulation electrodes and a blood sampling catheter as described previously [41, 42, 44, 45]. Electrodes were made of stainless steel (type 304) wire of 0.2 mm diameter and insulated with epoxylite (EPR-4, Clark Electromedical Instruments, UK) except for 0.4 mm at the tip. These electrodes were implanted stereotaxically under pentobarbital anaesthesia (40 mg/kg body weight i.p.) bilaterally into the MPOA (coordinates: A = 7.5, H = −1.5, L = ± 0.2) using the De Groot atlas [7] as a guide for the placement. A chronic Silastic (0.5 × 0.9 mm, Dow Corning, USA) catheter was inserted into the right external jugular vein and passed down near the entrance of the atrium. The other end was extended subcutaneously to the head, where it was integrated in the plug (Amphenol series 221) for the electrical cable connection. The complete electrode/catheter assembly was imbedded in acrylic dental cement and fastened to the skull. Rats were allowed a post operative recovery period of at least 1 week, during which time they were accustomed to all aspects of the experimental situation.

Experimentation

To investigate the Prl regulatory function of the MPOA, freely moving female rats received bilateral electrical stimulation with monophasic rectangular 100-Hz voltage pulse trains (0.2 width, 10 s on/5 s off) over a period of 25 min. Voltage and current were monitored continuously. From the onset of stimulation (t = 0) the intensity was increased slowly within 1 min to a maximum of 4 V, causing an initial current of about 400 µA and a steady-state current of about 250 µA, as the electrical circuitry behaved capacitively. Stimulation was performed at times of expected surges of Prl secretion, i.e. in cycling rats on proestrus at 16.00 h and on estrus at 17.00 h; in pregnant rats on day 3–8 of pregnancy at 6.30 and 17.00 h, and in lactating rats on day 2–7 of lactation at 15.00 h. Control animals received sham electrical stimulation. In cycling and pregnant rats, blood samples of 200 µl were collected serially at t = 0, 5, 15, 25, 30, and 60 min. In the lactating rat blood samples were also collected at t = −20 and t = −5 min. On the day of experimentation the lactating mothers were separated from their pups for 6–8 h. At t = −20 min the first blood sample was collected after mother and litter were reunited. It took 10.2 ± 1.4 min (n = 18) before all pups were suckling. Five minutes later a second blood sample was collected (t = −5 min). Blood volume reduction was compensated for by blood transfusions with freshly collected donor blood every time a total of 1 ml of blood had been withdrawn. This experimental approach appeared to be free of stress [41, 42, 44, 45].

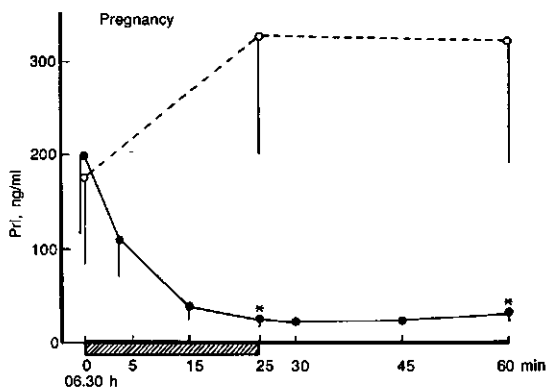

Fig. 3. The effect of electrical stimulation of the MPOA on nocturnal Prl secretion (mean ± SEM) in conscious pregnant rats during day 3–8 of pregnancy (*p < 0.05). O = Control (n = 11); ● = stimulation (n = 11).

Radioimmunoassay and Data Analysis

Blood samples (200 µl) were diluted with 100 µl heparinized phosphate-buffered saline (pH 7.5). Plasma samples were stored at −20 °C until analyzed for Prl concentration by means of a homologous double-antibody radioimmunoassay. The samples were analyzed in 5 or 6 serial 1:2 dilutions. Rat prolactin RP-1, kindly provided by the NIADDK was the standard, biological activity 11 IU/mg.

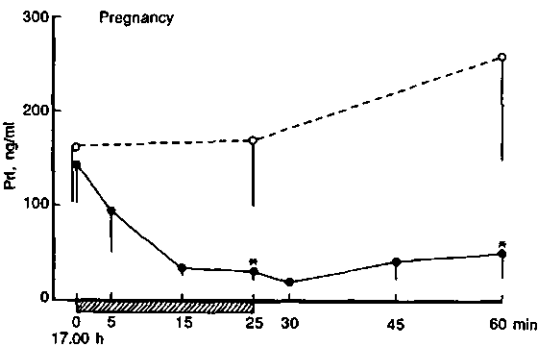

Fig. 4. The effect of electrical stimulation of the MPOA on diurnal Prl secretion (mean ± SEM) in conscious pregnant rats during day 3–8 of pregnancy (*$p < 0.05$). O = Control ($n = 11$); ● = stimulation ($n = 11$).

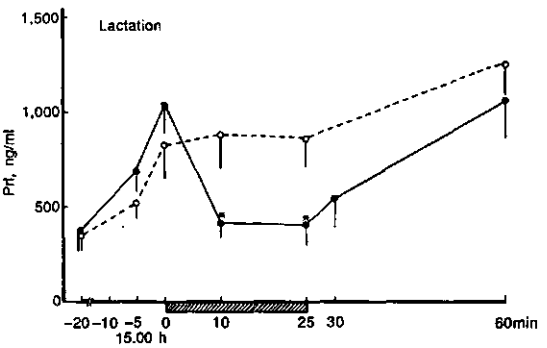

Fig. 5. The effect of 25 min of electrical stimulation of the MPOA on mean Prl secretion patterns (± SEM) in lactating rats, being separated from their pups for 6–8 h and allowed to suckle 10 min before stimulation was startet. At t = –20 min pups were reunited with their mother, at t = –10 min all pups were suckling. Rats were in day 2–7 of lactation (*$p < 0.05$). O = Control ($n = 19$); ● = stimulation ($n = 20$).

Results are presented as means ± SEM. The data were evaluated statistically after a logarithmic transformation using analysis of variance (F test). A value of $p < 0.05$ was considered significant.

Results

Electrical Stimulation and Prl Secretion

Twenty-five minutes of bilateral electrical stimulation of the MPOA significantly ($p < 0.05$) reduced the elevated proestrous and estrous afternoon levels of Prl in cycling rats for at least 60 min (fig. 1, 2). A similar observation was made for nocturnal and diurnal Prl secretion in pregnant rats (fig. 3, 4). In lacting rats, being separated from their pups for 6–8 h, plasma Prl levels rose quickly after reunion and the first 10 min of suckling and remained high during the next 60 min (fig. 5). Electrical stimulation significantly ($p < 0.05$) reduced Prl secretion during the stimulation period (fig. 5).

Electrical Stimulation and Behavioral Response

MPOA stimulation elicited increased locomotion and explorative behavior. For a few rats the stimulation intensity of 4 V was too high and rats tried to escape from their cage. This escape behavior was avoided by stimulating at a lower intensity of 3 V. MPOA stimulation, however, did not appear to interfere with nursing behavior in the lactating rat. For instance, starting at t = –10 min the time that all 6 pups suckled, control as well as stimulated rats spent the same amount of time suckling, 67.3 ± 10.0 min ($n = 8$) versus 60.9 ± 7.7 min ($n = 8$).

Histology

In 8 rats electrode positions were verified by microscopic examination of serial sections stained in hematoxylin-eosin by the presence of the Prussian blue spot after having passed a DC current of 30 μA for 60 s. The electrode positions appeared to be quite accurate, coordinates according De Groot [7]: A = 7.4 ± 0.053; H = –1.5 ± 0.077; L = ± 0.067 (mean ± SEM, $n = 8$).

Discussion

Prl is a multi target hormone and serves more than one function. The surges of Prl secretion in cycling rats induce luteolysis in nonfunctional corpora lutea [11, 47], in pregnant rats, however, Prl acts as the initial luteotrophic factor [11, 33, 34], whereas in lactating rats Prl stimulates development and secretory activity of the mammary gland [11, 33]. These actions of Prl are quite different and may have evolved at different times in evolutionary history. It therefore may not be surprising that several brain areas have been shown to be involved in the regulation of Prl secretion, and that the surges of Prl secretion in cycling, pregnant and lactating rats are controlled by quite different regulatory mechanisms (see Introduction).

From these data one can imagine the existence of separate sets of neural pathways for the release of each of the surges of Prl secretion, perhaps even associated with the release of a specific form of Prl. However, although there is more than one form of Prl, the secretion of a particular form does not seem to be associated with a particular physiological state [2, 26], which makes it conceivable that the surges of Prl secretion share a common final release mechanism. Indeed, it is widely believed that a system of tuberoinfundibular-dopaminergic (TIDA) neurons tonically inhibit the secretion of Prl from the adenohypophysis [8, 23, 30, 37] and under several reproductive states Prl secretion correlates reasonably with TIDA activity and por-

tal dopamine concentration [8, 23]. Therefore the various modes of Prl secretion may be controlled finally by one common neural center from which may originate one final common neural pathway to the TIDA neurons, for the release of the hormone. A likely candidate for such a common center is the MPOA. The MPOA has been shown to be the most sensitive area of estrogen action in the brain for the induction of the proestrous afternoon surge [27]. The MPOA has also been demonstrated as a center involved in controlling the nocturnal and diurnal surges of Prl secretion released in pseudopregnant rats [12, 15, 17], and there is evidence that the neural pathway for the suckling-induced release of Prl in lactating rats may pass through the MPOA [38]. Recently, we demonstrated a physiological role for the MPOA in the control of Prl secretion proestrous female rats [41]. The present study extends our previous observations and demonstrates that the MPOA is involved in the regulation of all known surges of Prl secretion in cycling, pregnant and lactating rats by consistently suppressing Prl secretion during MPOA stimulation at the times of expected surges (fig. 1–5). It is possible, however, that the neural source for the inhibitory effect on Prl secretion may not be the MPOA itself but, instead, another region whose projections continue through the MPOA, since electrical stimulation activates both perikarya and axons of passage.

The present paper only presents data of MPOA stimulation. Our results, therefore, may be explained as a general effect of brain stimulation and not as a specific regional effect. Gunnet and Moore [16], however, have shown that electrical stimulation of the dorsomedial nucleus of the hypothalamus reduced Prl secretion, whereas stimulation of the adjacent ventromedial nucleus was without effect, being evidence for regional specificity. Further evidence for the localized and specific effect of the electrical stimulus is that stimulation of the medial raphe nucleus reduced Prl secretion in cycling rats, but was without effect in pregnant and lactating rats [46]. In addition, it is generally known that electrical stimulation elicits behavioral responses that are regionally characteristics.

Prl secretion is very susceptible to stress. One therefore may explain our results as a nonspecific effect of stress, since the general effect of stressful stimuli is inhibitory when prestress levels are high [5, 31]. However, in our previous study [41] we have demonstrated that the applied experimental procedure does not introduce stress in the animals and that the inhibitory effect on Prl secretion is a direct effect of the stimulation. Also the relatively high concentrations of Prl in the samples collected at –20 min in lactating rats (fig. 5), which should have shown basal levels after 6–8 h of separation from the pups may not be ascribed to stress. This Prl release may be caused by exteroceptive stimuli from the pups in the experimental room, for instance by ultrasonic vocalization [22, 36].

Our data concerning MPOA stimulation (fig. 1–5) is confirmed by others. Electrical stimulation of the MPOA in anesthetized proestrous rats inhibited Prl secretion [19, 20] comparable to our results in conscious proestrous rats (fig. 1). In conscious pseudopregnant rats MPOA stimulation suppressed nocturnal and diurnal Prl secretion [15], similarly as we found in pregnant rats (fig. 3, 4). MPOA stimulation data on estrous and lactating rats are not available. The stimulation data fit well with numerous other studies where on the basis of lesion [12, 17, 20, 29], transection [20] or pharmacological [27] studies it was concluded that the MPOA contains essential neural elements for the control of Prl secretion under various reproductive states.

The location of our electrode tips was very close to (and perhaps in) the sexually dimorphic nucleus (SDN-POA) [14, 39] within the MPOA. Although we do not know the spread of the electrical current from the electrodes and, therefore, the size of the area that was stimulated, it is quite well possible that we have stimulated neural elements of the SDN-POA. Interestingly, electrical stimulation increases Prl secretion in male rats [41], but inhibits Prl secretion in female rats [41, present study], demonstrating a sexually differentiated function of the MPOA in the regulation of Prl secretion. It is tempting to speculate whether this function is governed by the SDN-MPOA. Lesion studies in male rats also suggest a role of the SDN-MPOA in the control of Prl secretion [29]. However, more data are needed to elucidate the precise role of the SDN-POA in the control of Prl secretion.

The data yet available do not answer the question of whether there is one final common pathway originating from the MPOA that directly projects on to TIDA neurons. Brain transection and lesion studies indicate that the neural pathway concerned with the proestrous surge is a relatively diffuse one [20]. Furthermore, outside the MPOA there are also 'surge centers' located in the hypothalamus, involved in the control of surges of Prl secretion [16–18, 37]. Lesion studies suggest that the surges of Prl secretion in pseudopregnant rats may be controlled by two distinct MPOA apparatuses, one inhibitory to the nocturnal surge and the other stimulatory to the diurnal surge [12, 15, 17]. Nevertheless, MPOA stimulation in conscious rats suppressed both nocturnal and diurnal Prl secretion in pseudopregnant [15] as well as pregnant (fig. 3, 4) rats. The picture is quite complex and at present we do not know the course of the preoptic-tuberal route(s), nor the functional relationships between the 'MPOA antisurge center' and 'hypothalamic surge centers'. During Prl secretion TIDA activity is reduced [8, 23], which may be caused by the suppressive action of the hypothalamic surge centers. One possibility is that the MPOA removes this inhibition of the hypothalamic surge centers by a direct effect on these centers, or by a presynaptic inhibition at the level of their projections at the TIDA neurons. Another possibility is that the MPOA neurons directly activate a subset of TIDA neurons, thus causing inhibition of Prl secretion. Although the literature is in favor of a Prl-inhibiting factor for the control of Prl secretion, recent data have shown that the posterior pituitary appears to contain a Prl-releasing factor [24]. Another equally likely possibility therefore is that MPOA stimulation inhibits the release of Prl-stimulating factor. However, this issue still re-

mains unresolved. Nevertheless, the role of the MPOA is clear: it exerts dominant inhibitory control over all known surges of Prl secretion in female rats and thus may be considered as an 'antisurge key control center' for Prl secretion.

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SUMMARY

The subject of this thesis was an investigation of the neural regulation and dynamics of prolactin (Prl) secretion. Experimentation was performed with freely behaving undisturbed male and female rats, chronically fitted with an atrial blood sampling catheter. In some studies rats were also equipped with a chronic intracerebroventricular cannula, or with chronic metal electrodes bilaterally implanted in the medial preoptic area (MPOA) or the median eminence (ME). Stress was always carefully avoided. The animals, therefore, had a post-operative recovery period of at least one week, during which time they were handled daily and fully accustomed to the experimental situation. During experimentation blood samples were collected between 06.00 and 22.00 h. Blood volume reduction was compensated for with blood transfusions.

The study starts with a thorough evaluation of circulating Prl levels in cycling and pseudopregnant (PSP) rats (chapter 1). One-hour interval studies show that diestrous Prl levels were low, about 15 ng/ml, and showed minor fluctuations. During the afternoon and early night of proestrus a single Prl surge was observed with a peak level of 1100 ng/ml at 17.00 h. On the afternoon of estrus there was also a single surge which was of a smaller magnitude and duration, with a peak level of 400 ng/ml at 16.00 h. In PSP rats two daily Prl surges were released during successively 11 days, one nocturnal and one diurnal. During the course of PSP these surges gradually declined in magnitude. Short-time sampling interval studies show that Prl secretion during PSP occurred occasionally in substantial bursts from baseline levels, whereas during the afternoon of proestrus plasma Prl was elevated constantly due to a more or less continuous release of Prl. Such a difference in actual secretion patterns indicates a separate neural regulation.

These data were obtained in rats chronically fitted with a blood sampling/transfusion catheter. Since Prl secretion is extremely susceptible to stress, it was necessary to investigate whether the applied blood sampling/transfusion procedure was free of stress. It appeared that frequent blood sampling for several hours at rates of up to 1 sample/min did not affect normal Prl secretion when blood volume reduction was compensated for with blood transfusion of fresh donor blood (chapter 2). However, compensation with preserved blood affected prolactin secretion significantly (chapter 3). In all later studies, therefore, blood transfusions were performed with freshly collected donor blood. The application of high frequency blood sampling permits the assessment of the dynamics of Prl secretion satisfactorily. The short-time interval studies presented in chapter 1, 2 and 3 show that during a surge plasma Prl always increased in an unpredictable manner, discontinuously, by means of several bursts, with maximum increments of about 600 ng/ml/min. The shortest half-time values, as calculated from the disappearance of Prl from the circulation, were about 2.2 min. The individual release patterns indicate that Prl release must be the consequence of a very dynamic neural regulatory process.

In chapter 4 the effects of red light and/or surgery upon Prl secretion were studied in cycling and PSP rats. Nocturnal (07.00-11.00 h), prediurnal (14.00-17.00 h) and diurnal (19.00-22.00 h) Prl

secretion was differently affected by these "treatments", and the effect was dependent upon the physiological state. The data together demonstrate the existence of different regulatory mechanisms for each of the surges of Prl secretion: the proestrous and estrous surge in cycling rats, and the nocturnal and diurnal surge in PSP rats. Moreover, in PSP day 0 rats, on the first day of pseudopregnancy, the occurrence of a prediurnal surge, preceeding the diurnal surge, was evident, which in fact was a reflection of the estrous afternoon surge in cycling rats.

In the last three chapters the involvement of the brain in Prl regulation was explored by studying the effects of hormonal and electrical stimulation upon Prl secretion. Since Prl has no specific target organ, an autofeedback control mechanism was hypothesized. Therefore, the effect of intracerebroventricular infusion of Prl on endogenous Prl secretion was investigated. However, as far as the proestrous surge of Prl is concerned, there is no evidence for the existence of an autoregulatory mechanism, neither in the expression, nor in the termination of the surge (chapter 5).

In chapter 6 the role of two brain areas in the control of Prl was investigated: the MPOA and the ME. The MPOA shows sexual dimorphism and is concerned functionally with several parameters of homeostasis, (sexual) behavior and endocrine function. The ME contains the terminals of the tuberoinfundibular dopaminergic (TIDA) neurons. Electrical stimulation experiments show that the MPOA is involved in the control of Prl secretion and that this control is different in males and females: electrical stimulation produced an increase in Prl secretion in the male, but reduced Prl secretion in the proestrous female. ME stimulation data do not provide evidence that this sexually differentiated function of the MPOA could be contributed to a sexual dimorphism in prolactin-inhibiting factor or prolactin-releasing factor activity.

The data of chapter 7 show that the MPOA is involved in the control of all presently known surges of Prl secretion in cycling, pregnant and lactating rats: electrical stimulation consistently suppressed Prl secretion at the times of expected surges. So the MPOA may be considered as an "anti-surge key-control" centre for Prl secretion in female rats. Whether the MPOA exerts its control via one final common neural pathway represented by the tuberoinfundibular dopaminergic system, remains to be determined.

In summary: Prl is a multi-target and multi-functional hormone. Based on the differences in actual secretion patterns of Prl and the differential effects of stress on Prl secretion it is concluded that all yet known surges of Prl secretion in cycling, (pseudo)pregnant and lactating rats are controlled by different neural regulatory mechanisms. In proestrous rats there is no evidence for an autoregulatory mechanism. Release of Prl is the consequence of a very dynamic neural regulatory process. The release of Prl is finally controlled by one common neural centre, the MPOA, which control is sexually differentiated: stimulatory in the male and inhibitory in the female.

SAMENVATTING

Dit proefschrift beschrijft een onderzoek naar de neurale regulatie en de secretie van het hormoon prolactine (Prl). De verschillende experimenten zijn uitgevoerd met vrij rondlopende niet verontruste mannelijke en vrouwelijke ratten, die voorzien waren van een permanente bloedafname catheter. In sommige studies waren de dieren daarnaast nog voorzien van een intracerebroventriculaire canule, of van micro-metaal electrodes, bilateraal geïmplanteerd in het mediaal preoptisch gebied (MPOA) of de eminentia mediana (ME). Stress werd altijd zorgvuldig vermeden. Na de operatieve ingreep werd de dieren minstens een week hersteltijd gegeven. Gedurende die tijd werden ze dagelijks gewend en volledig vertrouwd gemaakt met de experimentele situatie. Tijdens de experimenten werden bloedmonsters verzameld tussen 06.00 en 22.00 uur. Bloedafname werd steeds gecompenseerd via bloedtransfusies.

De studie begint met een grondige evaluatie van de Prl spiegels in het bloed in cyclische en schijn-drachtige (PSP) ratten (Hfdst. 1). De 1-uur interval studies laten zien dat de Prl niveaus tijdens de dioestrus laag zijn, ongeveer 15 ng/ml, en nauwelijks fluctueren. Op de dag van de pro-oestrus wordt tijdens de middag en avond een enkelvoudige Prl golf waargenomen, met een piek van 1100 ng/ml om 17.00 uur. Tijdens de middag van de oestrus treedt eveneens een enkelvoudige Prl golf op met een veel kleinere amplitude en duur, met een piek van 400 ng/ml om 16.00 uur. In PSP ratten wordt gedurende 11 opeenvolgende dagen tweemaal daags een Prl golf waargenomen, een nocturnale en een diurnale, die in de loop van de tijd steeds kleiner worden. Studies waarbij met zeer korte tijdsintervallen (1 à 3 min) de Prl secretie werd gevolgd laten zien dat tijdens PSP de afgifte van Prl nogal onregelmatig plaatsvindt in behoorlijk grote bursts vanuit het basisniveau, terwijl tijdens de middag van de pro-oestrus de Prl spiegel constant verhoogd is als gevolg van een min of meer continue secretie van Prl. Een dergelijk verschil in de feitelijke secretie patronen wijst op een gescheiden neurale regulering.

Bovenstaande metingen werden gedaan aan ratten die permanent voorzien waren van een bloedafname/bloedtransfusie catheter. Aangezien Prl secretie uiterst gevoelig is voor stress, was het noodzakelijk te onderzoeken of de toegepaste bloedafname/transfusie techniek vrij was van stress. Het bleek dat herhaald bloed afnemen gedurende verscheidene uren met frequenties tot 1 monster/min de normale Prl afgifte niet beïnvloedde zolang de bloedafname gecompenseerd werd via bloedtransfusies met vers donor bloed (Hfdst. 2). Compensatie daarentegen, met gepreserveerd donor bloed beïnvloedde de Prl secretie echter significant (Hfdst. 3). In alle verdere studies zijn bloedtransfusies daarom steeds uitgevoerd met vers donor bloed. De toepassing van hoog-frequente bloedafname geeft de dynamiek in de Prl secretie voldoende duidelijk te zien. De 1-min interval studies in Hfdst. 1, 2 en 3 laten zien dat tijdens het optreden van een Prl golf, de stijging in de Prl spiegels altijd op een niet te voorspellen wijze geschiedt, discontinu door middel van verscheidene bursts, met maximale incrementen van ongeveer 600 ng/ml/min. De kortste half-waarde tijden, zoals die te berekenen zijn uit de verdwijningssnelheid van Prl uit het bloed, bedragen ongeveer 2.2 min. De individuele afgiftepatronen geven aan dat de secretie van Prl het

gevolg moet zijn van een bijzonder dynamisch neuraal regulatieproces.

In Hoofdstuk 4 worden de effecten van rood licht en/of chirurgie bestudeerd op de Prl secretie in cyclische en PSP ratten. De nocturnale (07.00-11.00 uur), de prediurnale (14.00-17.00 uur) en de diurnale (19.00-22.00 uur) Prl secretie werden alle op verschillende manieren beïnvloed door deze "behandelingen" en het effect varieerde met het fysiologische stadium. De gegevens tezamen tonen het bestaan aan van verschillende regulatie mechanismen voor iedere afzonderlijke Prl golf: de pro-oestrische en oestrische golf in cyclische ratten, en de nocturnale en diurnale golf in PSP ratten. Daarnaast werd op de eerste dag van de schijndracht (PSP dag 0) nog een prediurnale Prl golf waargenomen, voorafgaande aan de diurnale golf. Deze prediurnale golf is in feite een reflectie van de oestrische golf zoals die gevonden wordt in cyclische ratten.

In de laatste drie hoofdstukken wordt de betrokkenheid van de hersenen onderzocht bij de regulatie van de Prl secretie door het effect te bestuderen van hormonale en electrische stimulatie op de Prl afgifte. Aangezien Prl geen specifiek doelorgaan heeft, werd het bestaan van een autofeedback controle mechanisme gehypothetiseerd. Hiertoe werd het effect van een intracerebroventriculair infuus van Prl onderzocht op de eigen Prl afgifte. Voor zover het de pro-oestrische Prl golf betreft zijn er evenwel geen aanwijzingen voor het bestaan van een autoregulatie mechanisme, noch t.a.v. de expressie, noch t.a.v. het beëindigen van de Prl golf.

In hoofdstuk 6 wordt de rol van twee hersengebieden bij de controle van de Prl secretie onderzocht: het MPOA en het ME. Het MPOA vertoont sexueel dimorfisme en is functioneel betrokken bij verscheidene homeostase parameters, (sexueel) gedrag en endocrine processen. Het ME-gebied bevat de terminals van de tuberoinfundibulaire dopaminerge (TIDA) neuronen. Electrische stimulatie experimenten laten zien dat het MPOA betrokken is bij de controle van de Prl secretie en dat deze controle verschillend is bij mannelijke en vrouwelijke ratten: electrische stimulatie leidt tot een verhoging van de Prl afgifte in mannetjes, maar tot een verlaging in pro-oestrische vrouwtjes. ME-stimulatie gegevens geven geen aanwijzingen dat dit sexeverschil in de functie van het MPOA toegeschreven kan worden aan een sexueel dimorfisme in prolactine inhibiting factor of prolactine releasing factor activiteit.

De resultaten uit hoofdstuk 7 maken duidelijk dat het MPOA betrokken is bij de controle van alle op dit moment bekende golven van Prl secretie die kunnen worden waargenomen in cyclische, drachtige en lacterende ratten: electrische stimulatie onderdrukt de Prl secretie in alle gevallen gedurende de tijdstippen dat deze golven optreden. Het MPOA mag dus beschouwd worden als het "inhibitoire sleutelcontrole" centrum voor Prl secretie in vrouwelijke ratten. Het moet evenwel nog vastgesteld worden of het MPOA deze controle uitoefent via één gemeenschappelijke neurale eindweg, gerepresenteerd door de tuberoinfundibulaire neuronen.

<u>Samengevat</u> Prl is een hormoon met meerdere doelorganen en meerdere functies. Vanwege de verschillen in feitelijke secretiepatronen, en de verschillende effecten van stress op de Prl afgifte kan gesteld

worden dat alle thans bekende golven van Prl secretie in cyclische, (schijn) drachtige en lacterende ratten gecontroleerd worden door verschillende neurale reguleringsmechanismen. In pro-oestrische ratten zijn geen aanwijzingen gevonden voor het bestaan van een autoregulatie mechanisme. De secretie van Prl is het gevolg van een bijzonder dynamisch neuraal regulatieproces. De afgifte van Prl wordt uiteindelijk gecontroleerd door een gemeenschappelijk neuraal centrum, het MPOA. Deze controle is sexueel gedifferentieerd: stimulatoir in de man en inhibitoir in de vrouw.

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Curriculum Vitae

De schrijver van dit proefschrift werd op 24 januari 1947 te Roordahuizum, gemeente Idaarderadeel, geboren. Hij behaalde in 1965 het diploma HBS-B aan de rijks-hogere-burgerschool te Leeuwarden. In datzelfde jaar begon hij zijn studie Electrotechniek aan de Technische Hogeschool Twente te Enschede. In januari 1970 behaalde hij zijn Baccalaureaat (cum laude) en in januari 1973 zijn ingenieursdiploma, met als afstudeervakken bio-informatica, meet- en regeltechniek en wiskunde. Gedurende de laatste 4 jaren van de studie werkte hij 1.5 dag/week als research assistent, eerst bij de leerstoel Digitale Techniek, later bij de leerstoel Bio-Informatica. In november 1972 werd in dienst getreden bij de Landbouwhogeschool, bij de sectie Algemene en Vergelijkende Animale Fysiologie te Wageningen. Hier verrichtte hij het onderzoek dat leidde tot dit proefschrift. In het kader van dit onderzoek werd in 1980 gedurende een half jaar in Engeland doorgebracht in Cambridge en Bristol. De schrijver verzorgt tevens onderwijs, met name voor de studierichtingen Biologie, Voeding van de Mens, Zoötechniek en Huishoudwetenschappen.