Involvement of endogenous opioids in the regulation of LH and testosterone release in the male horse

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To investigate the involvement of endogenous opioids in the regulation of gonadotrophin release in male horses, effects of the opioid antagonist naloxone (0.5 mg kg^{-1} i.v.) on plasma LH and testosterone concentrations and the possible influence of season and of gonadal steroids were investigated. To determine quantitative as well as qualitative changes in gonadotrophin release, LH concentrations were measured by radioimmunoassay and by an in vitro bioassay. Experiments were performed in May, August and December. In stallions, basal LH secretion in May and August was significantly higher than in December (May versus December: P < 0.01; August versus December: P < 0.05); plasma testosterone concentrations were highest in August (August versus May: P < 0.05, August versus December: P < 0.001). The basal bioactive LH concentration and the ratio of bioactive:immunoreactive LH in stallions were highest in May. Therefore, in addition to seasonal changes in quantitative LH secretion, the bioactivity of LH in the circulation also undergoes seasonal variations. Bioactive LH concentrations and the bioactive:immunoreactive ratio in geldings were higher than in stallions. Naloxone caused a significant increase in LH release in stallions in August and December (P < 0.001); no significant increase was found in May (P = 0.06). In geldings, naloxone did not induce any changes in LH secretion; in stallions, a highly significant correlation was observed between basal testosterone concentrations and the LH increment after injection of naloxone (P < 0.001). In August and December, the bioactive:immunoreactive ratio increased significantly (P < 0.05) after injection of naloxone in stallions, indicating a preferential release of LH molecules with high bioactivity. The bioactive:immunoreactive ratio did not change after naloxone injection in May. The naloxone-induced LH release was followed by a significant increase in plasma testosterone concentrations in stallions in August (P < 0.001) and December (P < 0.05). In conclusion, endogenous opioid systems are involved in the regulation of LH and testosterone secretion in stallions. These mechanisms undergo seasonal changes: their activity is increased during winter and decreased during the breeding season. By affecting LH release, endogenous opioids, at least in part, regulate seasonal changes in reproductive activity in the stallion.

Introduction

Endogenous opioid peptides inhibit gonadotrophin release in males and females of different species and an increase in plasma LH concentrations after injection of opioid antagonists is interpreted as evidence of a tonic inhibition of LH secretion by opioid systems. In men (Veldhuis et al., 1984), rams (Lincoln et al., 1987), male hamsters (Chen et al., 1984) and rats (Bruni et al., 1977; Cicero et al., 1979), inhibition of LH release by opioids has been demonstrated. In gonadectomized rats and rams, the opioid antagonist naloxone has no or little effect on plasma LH concentrations, but these effects are reinstated after exogenous testosterone replacement (Cicero et al., 1979; Bhanot and Wilkinson, 1983; Piva et al., 1986; Lincoln et al., 1987). In addition, in female horses (Behrens et al., 1993), as in humans (Quigley and Yen, 1980), sheep (Currie and Rawlings, 1987; Malven and Hudgens, 1987) and pigs (Barb et al., 1986), naloxone causes an increase in plasma LH concentrations during the luteal phase of the oestrous cycle. Opioid effects on gonadotrophin secretion have not yet been investigated in male horses.

An involvement of opioids in the regulation of seasonal changes in reproductive activity has been postulated (Lincoln and Siewannyana, 1989), and it has been shown that LH secretion in mares during the anovulatory season is inhibited by opioids (Aurich et al., 1994). In contrast, in male Syrian hamsters, whose reproductive activity (like that of horses) is linked to increasing daylength, naloxone stimulated the release
of LH only during the long-day period and not during the period of decreased sexual activity (Chen et al., 1984).

In cyclic mares, the biological potency of LH changes with the stage of the oestrous cycle because there are several molecular forms of LH that differ in their half-life and receptor affinity. During oestrus, LH with high bioactivity is secreted (Alexander and Irvine, 1982). In seasonal anovulatory mares, injection of naloxone results in a pronounced increase in plasma LH bioactivity (Aurich et al., 1994); in men, the opioid antagonist naltrexone stimulates a preferential release of LH with high bioactivity (Veldhuis et al., 1983). The effects of opioids or opioid antagonists on the biological potency of gonadotrophins in male animals are not yet known.

In this study, we investigated the influence of the opioid antagonist naloxone on LH and testosterone release in stallions at different times of the year and in long-term castrated geldings. To detect quantitative as well as qualitative changes in gonadotrophin release, LH concentrations were determined by radioimmunoassay and by an in vitro bioassay.

Material and Methods

Animals

All horses used in this study were stallions (n = 20) or geldings (n = 10) of the Hanoverian and Brandenburg breeds, aged between 4 and 23 years [mean (± SD) age: 10.4 ± 5.9] and weighing 590 ± 25 kg. The animals belonged to the stud farm of the State of Niedersachsen at Celle (52.5°N) and the State of Brandenburg at Neustadt/Dosse (53°N). They were kept in loose stalls on straw, and fed oats and hay three times a day; water was freely available. The geldings had been castrated during their first year of life at least 3 years before the experiment.

Experimental procedure

The experiments were performed in August (n = 8 stallions) and December 1992 (n = 7 stallions) and in May 1993 (n = 5 stallions and 10 geldings). Experiments always began between 09:00 and 11:00 h, and during blood sampling the horses remained in their stalls. An indwelling catheter was placed in the left jugular vein 15 min before the first blood sample was taken. Blood for determination of LH and testosterone concentrations was withdrawn at 15 min intervals for 270 min. After 60 min of sampling, 300 mg naloxone–HCl (Sigma Chemical Co., Deisenhofen) per animal (n = 10) or 10 ml saline (n = 11) were injected i.v. via the catheter. Naloxone was freshly dissolved in 10 ml saline and sterile filtered immediately before the injection. The dose of naloxone corresponds closely to 0.5 mg kg⁻¹ bodymass. To determine pituitary responsiveness to GnRH, all animals received 20 µg of the GnRH agonist buserelin (Receptal; Hoechst, Unterscheiβheim) 150 min after naloxone or saline had been injected. Blood was collected into polystyrol tubes containing 25 mg EDTA. Samples were centrifuged immediately for 20 min at 1000 g and plasma was frozen at −20°C until hormone analysis.

Stallions were used as their own controls and were treated with naloxone as well as saline. The interval between these two experiments in the same animal was 1 week, and the order of treatments was randomized, with half of the animals receiving naloxone first and half receiving saline injections first. In contrast to the stallions, geldings were available for one experiment only and were divided into a naloxone and a control group.

Hormone analysis

Steroid hormones. Testosterone was measured by radioimmunoassay after extraction from plasma. Plasma from stallions (50 µl) or geldings (100 µl) was added to 1 ml ethyl acetate. After freezing the tubes at −20°C, the organic phase was decanted into fresh tubes, dried in a vacuum centrifuge and redissolved in 100 µl EDTA buffer: 10 mmol EDTA l⁻¹, 10 mmol Na₂PO₄ l⁻¹ and 1 g lysozyme l⁻¹ (Boehringer Mannheim, Mannheim), pH 7.2. Redissolved sample extracts or standards (Merck, Darmstadt) 3–400 pg ml⁻¹ in 50 µl EDTA buffer, [1,2,6,7-³H]testosterone (Amersham Buchler, Braun¬schweig) at a final dilution of 6500 c.p.m. in 100 µl EDTA buffer, and 100 µl antiserum (HL 35; W. Klingler, Medizinische Universität, Lübeck) at a dilution of 1.500 000 were incubated at 4°C for 18 h. The crossreactivity of the antiserum was 48.8% with 5α-dihydrotestosterone, 2.7% with androstendionone, 2.3% with dihydroandrosterone and < 0.1% with epiantisterone, dihydroepiantisterone, oestrone, oestriadiol, oestriol, cholesterol, cortisol, 3αoxo cholesterol, progesterone and 17β-hydroxyprogesterone. Free and bound ligands were separated by adding 2 mg dextran-coated charcoal (Steranti, St Albans) in 500 µl EDTA buffer. The supernatant was mixed with 1 ml liquid scintillation fluid (Ready Organic, Beckman Instruments, Munich) and radioactivity was measured using a β-counter. The minimal detectable concentration of the testosterone assay was 0.034 nmol l⁻¹⁻¹; the intra-assay and interassay coefficients of variation were 4.5% and 14.7%, respectively. Oestriadiol was determined after extraction from plasma with n-hexane by radioimmunoassay, as described by Behrens et al. (1993). The intra-assay coefficient of variation was 9.5%, the interassay coefficient of variation 11.8% and the minimal detectable concentration 7.3 pmol l⁻¹⁻¹. Oestradiol was measured only in the first sample taken during each experiment.

LH radioimmunoassay. LH was determined using a radioimmunoassay described by Behrens et al. (1993) in which equine LH (UCB, Braine L'Alleu) was used as a standard and that made use of an antibody raised in rabbits against equine LH (UCB). Intra-assay and interassay coefficients of variation of the assay were 5.8% and 18.1%, respectively; the minimal detectable concentration was 0.5 µg l⁻¹⁻¹ and crossreactivity of the antibody with FSH was < 2.8%.

LH bioassay. The LH bioassay measured the LH-induced production of testosterone by murine Leydig cells in vitro. The assay was performed as described by Van Damme et al. (1974) and modified by Aurich et al. (1994) and validated for equine plasma in our laboratory; 1.0 and 2.0 µl of stallion plasma and 0.1 and 0.5 µl of gelding plasma were used. Increasing amounts of plasma from mares in oestrus and dioestrus, as well as from geldings 1 and 2 days after castration, stimulated testosterone.
release in vitro in a manner parallel to the standard curve. Specificity and recovery were tested by comparing testosterone production caused by different equine LH standard preparations [LH assay standard (UCB) and LH standard E98A (H. Papkoff, University of California, San Francisco, CA)], human LH (International reference preparation, WHO International Laboratory for Biological Standards, London), equine FSH standard (E276B, H. Papkoff), and commercially available hCG (Primogonyl: Schering, Berlin) and equine CG (Intergonan: Vemie, Oberdeschleisheim) diluted in plasma. Except for equine FSH, which did not cause detectable changes in testosterone release, the stimulation curves for the tested gonadotrophins were parallel to the standard curve. No losses of standard occurred during the assay procedure. The minimal detectable concentration of the LH bioassay was 0.17 µg l⁻¹, and the intra-assay and interassay coefficients of variation were 7.0% and 20.9%, respectively.

### Statistical analyses

The amount of LH and testosterone released after injection of naloxone or saline was calculated as the area under the curve (µg l⁻¹ min⁻¹) for the time from 0 min to 150 min after treatments. The value of the basal hormone secretion (the mean of the five pretreatment values) was subtracted from each post-treatment value. Statistical comparisons were made using the SPSS/PC + statistics package (Norusis, 1988). The areas under the curves constructed from the naloxone and saline experiments were compared by the paired samples t test in the stallions and one-way analysis of variance in the geldings. Comparisons between stallions tested at different times of the year and between stallions and geldings were made by one-way analysis of variance. The response of LH to buserelin was analysed by the paired samples t test (mean of the four values before versus mean of the four values after buserelin injections). All values given are means ± SEM. For the statistical comparisons, a P value < 0.05 was considered to be significant.

### LH bioactivity and the bioactive:immunoreactive ratio

LH concentrations measured using the in vitro bioassay were always higher than the corresponding values determined by radioimmunoassay, and the ratio of bioactive:immunoreactive LH (B:I ratio) was at least 3. Basal bioactive LH concentrations in geldings were significantly higher than in stallions in August and December (P < 0.05) and the basal B:I ratio in geldings was higher than in stallions irrespective of the time of the year (P < 0.01). In May, when basal LH bioactivity was already high, naloxone did not stimulate any immediate changes in the release of bioactive LH or in the B:I ratio in stallions. The B:I ratio after injection of buserelin was higher than the value before it was injected. In August, the mean bioactive LH concentrations in the stallions increased from 41.2 ± 13.1 µg l⁻¹ immediately before to 83.2 ± 24.8 µg l⁻¹ 15 min after the injection of naloxone (P < 0.05). Buserelin caused a further increase in LH concentrations measured by bioassay as well as in the relative biological potency of LH (P < 0.05). In December, changes in the concentrations of bioactive LH after naloxone injection were not significant, but there was a significant increase in the B:I ratio (P < 0.05; Table 2, Fig. 2). No changes in the relative biological potency of LH after injection of naloxone or buserelin were found in geldings (Table 2, Fig. 3).

### Results

#### LH immunoreactivity

In stallions, there were significant seasonal differences in basal LH concentrations determined by radioimmunoassay. The mean of the five pretreatment values in May (11.6 ± 0.6 µg l⁻¹) and August (10.7 ± 0.4 µg l⁻¹) was signifi-
Fig. 1. Immunoactive LH concentrations in stallions before and after injection of (a) naloxone and (b) saline in May (●, ○), August (▲, △) and December (■, □). All animals were injected with buserelin after 150 min. Values are means ± SEM. Differences between results of naloxone and saline experiments in stallions in May were not significant (P = 0.06), but the LH concentrations were significantly higher in the naloxone experiment compared with the saline experiment in August and December (P < 0.001).

Steroid hormones

In the stallions, there were marked seasonal differences in basal plasma testosterone and oestradiol concentrations. The mean testosterone concentrations before injection of naloxone or saline in August (2.92 ± 0.31 nmol l⁻¹) were significantly higher than in May (2.33 ± 0.45 nmol l⁻¹; P < 0.05) and December (0.93 ± 0.14 nmol l⁻¹; P < 0.001; December versus May: P < 0.05). The basal plasma testosterone concentration in geldings was 0.24 ± 0.10 nmol l⁻¹. Oestradiol concentrations in stallions were significantly higher in May (342.4 ± 24.2 pmol l⁻¹) and August (327.0 ± 17.3 pmol l⁻¹) than in December (272.7 ± 10.6 pmol l⁻¹; P < 0.05). In geldings, the oestradiol concentration in plasma averaged 15.8 ± 1.1 pmol l⁻¹.

Whereas in stallions during May application of naloxone was followed only by a minor, statistically nonsignificant increase in testosterone secretion, the opioid antagonist caused
a marked release of testosterone in August (P < 0.001) and December (P < 0.05) compared with control values (Table 3, Fig. 4). In August, testosterone concentrations increased from 3.16 ± 0.34 nmol l⁻¹ immediately before to 7.00 ± 1.72 nmol l⁻¹ 150 min after naloxone injections; in December the concentration increased from 1.13 ± 0.48 nmol l⁻¹ to 2.81 ± 0.52 nmol l⁻¹. In geldings, plasma testosterone concentrations after the injection of naloxone were not different from corresponding values from control animals.

**Correlation between LH release and testosterone**

A significant correlation was found between the areas under the curve for immunoreactive LH and testosterone (r = 0.3908; P < 0.01) in naloxone-treated stallions, but no correlation could be found between areas under the curve for bioactive LH and testosterone in these animals. There was a highly significant correlation (r = 0.6649; P < 0.001) between basal plasma testosterone concentrations and the increase in LH concentrations after naloxone was injected (i.e. the highest LH value within the first hour after naloxone injection minus the basal LH concentration).

**Discussion**

Basal plasma LH and testosterone as well as oestradiol concentrations in stallions showed marked seasonal variations. Immunoreactive LH concentrations were high in May and low in December, which is in agreement with previous studies (Irvine and Alexander, 1982; Harris et al., 1983). In addition to quantitative changes in LH release, seasonal variations in the relative biological potency of LH in the circulation were also found in this study. The B/I ratio was highest in May, indicating that at the beginning of the breeding season a shift in the LH isohormone profile occurs towards secretion of LH molecules with high bioactivity. The concentration of immunoreactive LH in long-term castrated geldings was in the same range as in stallions. In horses, gonadotrophin secretion increases markedly after castration but returns to normal values within a year (Irvine and Alexander, 1982). In contrast to immunoreactive LH, the B/I ratio for LH was significantly higher in geldings than in stallions, irrespective of the season. Therefore, after castration, changes in the biological potency of LH do not parallel the development of immunoreactive LH release.

Seasonal changes in plasma testosterone concentrations are in agreement with other reports (Berendtson et al., 1974; Harris et al., 1983). Partial differences between variations in immunoreactive LH and testosterone concentrations could be explained by an increase in the number of Leydig cells at the beginning of the breeding season and an increased stimulation of these cells by LH (Johnson and Naeves, 1981).

An involvement of endogenous opioid systems in the regulation of LH secretion has been demonstrated for the first time in stallions, and is in agreement with previous reports from men (Veldhuis et al., 1983), rams (Lincoln et al., 1987), male hamsters (Chen et al., 1984) and rats (Bruni et al., 1977; Cicero et al., 1979). From our studies, it cannot be differentiated whether the regulation of LH release by opioids in the stallion is mediated via hypothalamic or pituitary mechanisms. However, studies in other species have shown that opioids act primarily by regulating GnRH release from the hypothalamus (Cicero et al., 1979; Grossman et al., 1981; Wilkes and Yen, 1982).

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**Table 2.** LH bioactive:immunoreactive ratio (mean ± SEM) in stallions in May, August and December and in geldings in May before and after injections of naloxone and buserelin

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Stallions</th>
<th>Geldings</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>May (n = 5)</td>
<td>August (n = 5)</td>
</tr>
<tr>
<td>(1) 1 h before naloxone</td>
<td>5.8 ± 1.5</td>
<td>3.9 ± 0.9a</td>
</tr>
<tr>
<td>(2) First h after naloxone</td>
<td>6.1 ± 1.4</td>
<td>6.2 ± 1.5b</td>
</tr>
<tr>
<td>(3) Second h after naloxone</td>
<td>6.1 ± 1.4c</td>
<td>5.9 ± 1.4c</td>
</tr>
<tr>
<td>(4) First h after buserelin</td>
<td>7.0 ± 1.4b</td>
<td>7.2 ± 1.5d</td>
</tr>
<tr>
<td>ab P &lt; 0.01</td>
<td>ab: cd P &lt; 0.05</td>
<td>ab P &lt; 0.05</td>
</tr>
</tbody>
</table>

ns: Not significant.

**Table 3.** Testosterone release [area under the curve (nmol l⁻¹ min⁻¹); mean ± SEM] in stallions in May, August and December and in geldings in May after injection of naloxone or saline

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Stallions May (n = 5)</th>
<th>Stallions August (n = 8)</th>
<th>Stallions December (n = 7)</th>
<th>Geldings May (n = 2 × 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naloxone</td>
<td>144.8 ± 38.1</td>
<td>412.6 ± 118.7</td>
<td>178.7 ± 41.5</td>
<td>8.9 ± 6.2</td>
</tr>
<tr>
<td>Saline</td>
<td>93.3 ± 17.5</td>
<td>97.4 ± 73.8</td>
<td>3.4 ± 28.1</td>
<td>NS</td>
</tr>
</tbody>
</table>

ns: Not significant.
and we assume that the same mechanisms occur in the male horse.

The activation of opioid systems inhibiting LH release in stallions undergoes seasonal changes. In May, basal LH concentrations were already high and no significant increase in LH release occurred after naloxone injection, although the difference between the naloxone and control experiment nearly reached statistical significance. In December and

Fig. 2. (a) Bioactive LH concentrations and (b) bioactive:immunoreactive (B:I) ratio in stallions in May (●), August (▲) and December (■) before and after injection of naloxone. Animals were injected with buserelin after 150 min. Values are means ± SEM. For statistical analysis, see Table 2.

Fig. 3. Concentrations of (a) immunoactive LH before and after injection of naloxone (▼) and saline (▼), (b) bioactive LH (□) and bioactive:immunoreactive (B:I) ratio (■) and (c) testosterone after injection of naloxone (▼) and saline (▼) in geldings in May. All animals were injected with buserelin after 150 min. Values are means ± SEM.
Opioids and LH release in stallions

(a) Buserelin (0.02 mg) i.v. Naloxone (300 mg) or saline i.v.
LH (μg l⁻¹)

Time (min)

(b) Naloxone (300 mg) i.v. Buserelin (0.02 mg) i.v.
Bioactive LH (μg l⁻¹)

Time (min)

(c) Naloxone (300 mg) or saline i.v. Buserelin (0.02 mg) i.v.
Testosterone (nmol l⁻¹)

Time (min)
shortly after the end of the breeding season in August, the response of LH to naloxone was more pronounced, indicating a continuous opioid inhibition of GnRH secretion during the nonbreeding season. A partial inactivation of these opioid systems at the beginning of the breeding season could explain why an increased basal release of LH occurs in May.

The effects of opioids on LH release in the stallion, as in male rats (Cicero et al., 1979; Bhanot and Wilkinson, 1983) and men (Veldhuis et al., 1984), are activated only in the presence of gonadal steroids. In geldings, naloxone did not have any effects on the plasma LH concentration. Interactions between gonadal steroids and the activation of opioid systems can also be concluded from the correlation between basal testosterone concentrations and the LH increment after naloxone injection. The absence of an LH reaction to naloxone in geldings and a reduced response in stallions in May are not attributable to an insufficient amount of pituitary gonadotrophin because the
animals released a significant amount of LH after injection of a GnRH agonist.

In contrast to our results in stallions, opioids do not seem to be involved in seasonal changes in LH release in male hamsters: inhibition of LH release by opioids was found only during the breeding season (Chen et al., 1984; Eskes et al., 1984). These differences could be explained by the more pronounced seasonal changes in testicular function in hamsters (Turek et al., 1977; Turek and Campbell, 1979) compared with horses. Therefore, the concentration of gonadal steroids in hamsters during the nonbreeding season might resemble the situation in geldings more than that in intact stallions in our study. Whereas in stallions the naloxone-reversible opioid suppression of LH release was minimal during the height of the breeding season (when basal LH was already high), in mature Soay rams the naloxone-induced increments in LH secretion were maximal in seasons when basal LH and testis size were also at a maximum (Lincoln et al., 1987). This species difference could reflect different reproductive responses to photoperiod in sheep and horses. Whereas reproductive functions are stimulated by decreasing daylength in sheep, they depend on an increase in photoperiod in horses. Although the regulation of LH release by opioids in rams and stallions differs in regard to peak sexual activity, in both species it seems to be activated in autumn and winter. This activation therefore might depend at least partly on similar seasonal or photoperiodical mechanisms in sheep and horses.

In addition to stimulating quantitative LH release, naloxone caused an increase in the relative biological potency of circulating LH in stallions in August and in December but not in May, when basal LH bioactivity was already high. In December, the relative biological potency of LH secreted after injection of naloxone was lower than in August, indicating seasonal changes in the pituitary LH isohormone profile. Although an increased LH bioactivity after injection of an opioid antagonist has also been reported in humans, seasonal changes were not investigated (Veldhuis et al., 1984). Naloxone primarily affects GnRH secretion; therefore, changes in the release of bioactive LH after naloxone injection probably depend on the isohormone profile of releasable LH, and might also be related to circannual changes in the pituitary responsiveness to GnRH and to seasonal differences in the naloxone-induced GnRH signal.

The naloxone-induced LH secretion stimulates testicular testosterone release. It is unlikely that naloxone causes an LH-independent testosterone secretion because the increase in plasma testosterone concentrations was delayed and occurred 30–45 min after LH secretion increased. Injection of exogenous GnRH or hCG also stimulates testosterone release in stallions within approximately 30 min (Lindner, 1961; Cox and Williams, 1975). In May, when LH release was small and nonsignificant, no pronounced increase in testosterone concentrations was observed. The correlation between the magnitudes of the naloxone-induced LH and testosterone release also favours the interpretation of an LH-mediated testosterone secretion. Plasma testosterone concentrations in stallions also tended to increase slightly during control experiments in May and August. This could be explained by diurnal variations in testosterone release with an increase during the morning, as described by Clay et al. (1988).

It can be concluded that endogenous opioids are involved in the regulation of LH release in stallions. Opioid systems do not only influence plasma LH concentrations, but also indirectly affect testosterone release and testicular function.

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