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Further Studies on Possible Dynorphin Involvement in the Ovulatory Luteinizing Hormone Surge in the Proestrous Rat

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A decrease in inhibitory tone of endogenous opioid peptide on the afternoon of proestrus is one event underlying generation of the ovulatory luteinizing hormone (LH) surge, since premature removal of this inhibitory tone (i.e., disinhibition) results in an early onset of the surge. Our laboratory demonstrated that blockade of κ-opioid receptors in the medial preoptic area (MPOA) advanced the onset of the LH surge on proestrus. Since dynorphin is the endogenous ligand for the κ-opioid receptor, the present studies examined the possible role of dynorphin in this disinhibition response. 1) Neutralization of endogenous dynorphin peptides, by push-pull perfusion of the MPOA with antibodies specific for dynorphin $A_{1–17}$ or $A_{1–8}$ from 1030–1355 h on proestrus, tended to prematurely advance the increase in plasma LH levels normally occurring on this day of the estrous cycle. Although this increase was not statistically significant when compared with controls, plasma LH levels in two antibody-treated rats were sufficiently elevated to cause full ovulation, a response that did not occur in controls. These data suggest that dynorphin $A_{1–17}$ and $A_{1–8}$ might have a role in the MPOA, although a minor one, in suppressing LH secretion early on proestrus. MPOA levels of prodynorphin mRNA decreased at 1700–1800 h on proestrus when plasma LH levels were high, compared with values at 1300–1400 h when plasma LH levels were low. This change did not occur on diestrous d 1 when there was no LH surge. 2) MPOA levels of κ-opioid receptor mRNA did not change on proestrus or diestrous d 1. These results suggest that a reduction in prodynorphin gene expression on the afternoon of proestrus may be one event involved in a possible decrease in dynorphin inhibitory tone on the ovulatory LH surge-generating signal.

Key Words: Dynorphin; luteinizing hormone surge; prodynorphin; κ-opioid receptors; medial preoptic area.

Introduction

A significant decrease in inhibitory opioid peptide tone has been shown to be an important event underlying induction of the luteinizing hormone (LH) surge on the afternoon of proestrus *(1)*. Evidence has supported a role for β-endorphin originating in the hypothalamic arcuate nucleus and acting through μ-opioid receptors in this disinhibition process *(2–6)*. However, we have shown that blockade of κ-opioid receptors in the medial preoptic area (MPOA) early on proestrus advanced the LH surge by 3 h *(7)*. This premature increase in LH secretion indicates that there is a κ-receptor-mediated inhibitory opioid tone in the MPOA suppressing LH release early on proestrus.

Dynorphin is the endogenous ligand for the κ-opioid receptor *(8,9)*. The prodynorphin precursor in the rat hypothalamus is proteolytically cleaved to yield a series of smaller opioid peptides, including dynorphin $A_{1–17}$, dynorphin $A_{1–8}$, dynorphin B, as well as α- and β-neoendorphin *(10–13)*. All five peptides are active at κ-opioid receptors *(14,15)*, and thus each could be involved in suppressing LH secretion early on proestrus. Recently, we reported that while each of these peptides could inhibit the normally occurring afternoon LH surge on proestrus, acting through κ-opioid receptors, dynorphin $A_{1–17}$ and $A_{1–8}$ were much more potent than the others in this regard *(16)*. Therefore, we employed specific antibodies against dynorphin $A_{1–17}$ or $A_{1–8}$ to fulfill the first objective of the present study—to determine whether neutralization of these two dynorphin ligands in the MPOA on the morning of proestrus would result in a premature increase in LH secretion.

Reduction in inhibitory dynorphin tone within the MPOA on the day of proestrus could result from neuronal events occurring pre- or postsynaptically, and it may be involved in timing of the LH surge. Such a reduction could be due to a decrease in the synthesis of dynorphin and/or κ-opioid receptors, eventually resulting in less suppression of LH secretion, and an LH surge. The MPOA contains prodynorphin and κ-opioid receptor mRNA synthesizing cell bodies *(17–19)* as well as dynorphin peptides *(20–24)*. Many reports have demonstrated a direct relationship between the level of prodynorphin mRNA and that of dynorphin peptides *(25–32)*. Thus, the second objective of the present study was to determine whether changes in prodynorphin- or κ-opioid

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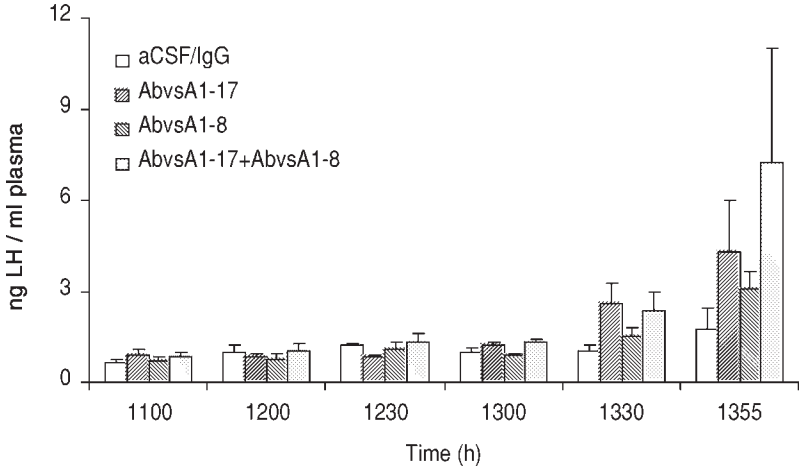

Fig. 1. Mean plasma LH levels in rats treated with aCSF/IgG, antibody vs dynorphin $A_{1–17}$ (AbvsA 1–17), antibody vs dynorphin $A_{1–8}$ (AbvsA 1–8) or both antibodies (AbvsA1–17 + AbvsA1–8), from 1030–1355 h on proestrus.

receptor-mRNA levels in the MPOA occur in association with generation of the ovulatory LH surge on proestrus.

Results

Experiment 1: Push-Pull Perfusion with Antibodies vs Dynorphin Peptides

In control rats perfused in the MPOA with artificial cerebrospinal fluid (aCSF) alone or aCSF containing normal rabbit immunoglobulin G (IgG), there was no change in plasma LH levels between 1100 and 1355 h on proestrus (Fig. 1). None of these rats ovulated the next morning, indicating that pentobarbital injection immediately following the blood sample taken at 1355 h had blocked the normal LH surge. Since there was no difference in plasma LH levels between these two groups at any time, the LH values were combined (i.e., aCSF/IgG) for further comparison with antibody-treated animals.

Plasma LH levels remained stable during the first 2½ h of MPOA perfusion with antibodies vs either dynorphin $A_{1–17}$, $A_{1–8}$, or both peptides, but tended to increase at 1330 and 1355 h, with the highest plasma LH levels occurring in rats treated with both dynorphin antibodies (Fig. 1). Although these increases were not statistically significant, plasma LH levels in two of the antibody-treated rats (one given antibody vs dynorphin $A_{1–17}$, and one given both antibodies) were sufficient to cause full ovulation (18 and 15 ova, respectively), a response that did not occur in aCSF/IgG-treated controls. Examples of changes in plasma LH levels during MPOA perfusion with aCSF, IgG, or antibodies vs dynorphin $A_{1–17}$, dynorphin $A_{1–8}$, or both peptides are given in Fig. 2.

The locations of push-pull cannula tips in antibody-treated rats are shown in Fig. 3. This is the same region where we have previously shown that perfusion with a κ-opioid receptor antagonist resulted in a premature increase in LH secretion early on the afternoon of proestrus *(7)*.

Experiment 2: Prodynorphin- and κ-Opioid Receptor-mRNA Levels

On proestrus, as plasma LH levels increased from 1300–1400 to 1700–1800 h ($p < 0.01$; Fig. 4 left), prodynorphin mRNA levels in the MPOA decreased by 19% ($p < 0.05$). By contrast, this change did not occur on diestrous d 1 when there was no LH surge (Fig. 4 right). Moreover, prodynorphin mRNA levels in the MPOA at both times on diestrous d 1 were very similar to levels measured at 1300–1400 h on proestrus. MPOA levels of κ-opioid receptor mRNA did not change on either proestrus when plasma LH levels increased (Fig. 5 left), or diestrous d 1 when plasma LH levels remained low (Fig. 5 right).

Discussion

A previous report from our laboratory demonstrated that blockade of κ-opioid receptors in the MPOA on the morning of proestrus advanced the LH surge by 3 h, indicating the presence of an inhibitory κ-opioid receptor–mediated tone that acts to suppress LH secretion early on proestrus *(7)*. Dynorphin is the ligand for the κ-opioid receptor *(8,9)*, and the prodynorphin precursor is enzymatically broken down within the hypothalamus and other brain regions into dynorphin $A_{1–17}$, dynorphin $A_{1–8}$, dynorphin B, and α- and β-neoendorphin *(10–13)*. Immunohistochemistry studies have shown that dynorphin $A_{1–17}$- and $A_{1–8}$-positive soma and neuronal fibers are present in the MPOA *(20,21,24)*. In the present study, neutralization of these two dynorphin peptides in the MPOA tended to cause premature increases in LH secretion on the day of proestrus. Although the increases were not statistically significant, in two animals they were sufficient to cause ovulation. It is important to indicate that all antibody-treated rats were injected with pentobarbital between 1355 and 1400 h to block the normal afternoon LH surge. This was done to determine whether any resulting ovulation was due to an antibody-induced increase in LH

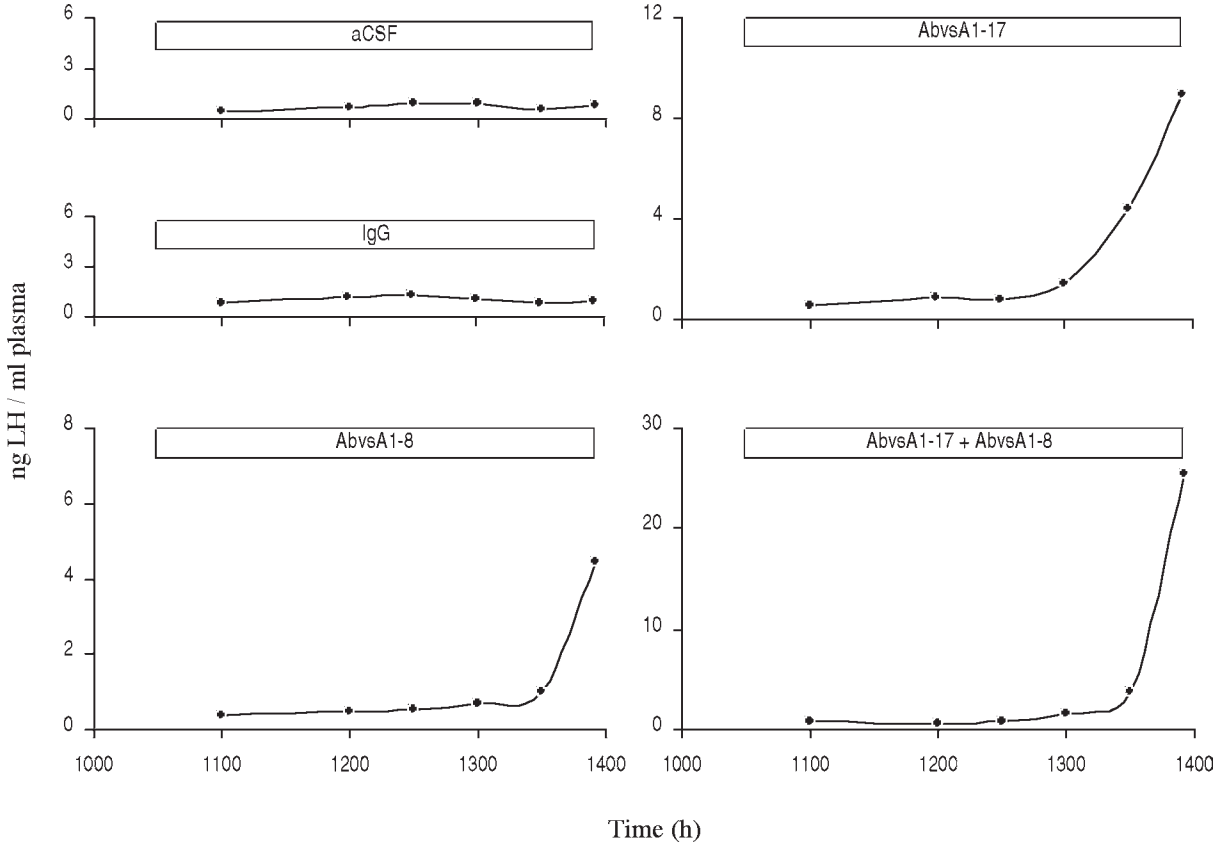

Fig. 2. Examples of LH secretion during push-pull perfusion in the MPOA with aCSF, IgG, antibody vs dynorphin $A_{1–17}$ (AbvsA 1–17), antibody vs dynorphin $A_{1–8}$ (AbvsA 1–8), or both antibodies (AbvsA1–17 + AbvsA1–8), from 1030–1355 h (horizontal bar) on proestrus. The increase in plasma LH levels resulted in ovulation in the two rats shown on the right.

release, and not the normal LH surge. However, pentobarbital will not only block the normal afternoon LH surge, it will also decrease any ongoing LH secretion. Thus, it is possible that had pentobarbital not been given, the antibody-induced LH secretion would have continued, and higher plasma LH levels would have been reached, resulting in more rats ovulating. These data suggest that dynorphin $A_{1–17}$ and $A_{1–8}$ may have a role within the MPOA, although a minor one, in suppressing LH secretion on the morning of proestrus. Moreover, if a reduction in inhibitory dynorphin tone by the afternoon of proestrus is involved in timing of the LH surge, this may be due in part to a decrease in prodynorphin gene expression, as evidenced by a reduction in prodynorphin mRNA levels in the MPOA by the late afternoon of proestrus when plasma LH levels were increased.

Blockade of κ-opioid receptors in the MPOA on proestrus resulted in a premature, marked elevation in plasma LH levels and ovulation in a majority of the rats *(7)*. By contrast, the change in LH release produced by administration of antibodies to neutralize dynorphin $A_{1–17}$ and dynorphin $A_{1–8}$ was of lesser magnitude. One possible explanation for this lesser response is inadequate neutralization of endogenously released dynorphin. In our preliminary experiment (see Materials and Methods), the antibodies were combined with dynorphin peptides prior to intraventricular infusion. The antibodies prevented the ovulation-blocking action of each peptide, and thus were effective in neutralizing exogenously administered peptides. The objective in experiment 1 was to neutralize the action of endogenously released dynorphin peptides by push-pull perfusion of the MPOA with these antibodies. The literature indicates that 5–10% of a perfused compound would be expected to be continuously retained at a central nervous system (CNS) site with the push-pull perfusion technique *(33)*. Based on the reported content of dynorphin peptides within various CNS loci, including the hypothalamus *(10–12,34)*, and the low release rate of the peptides (<1% of the content/h) *(35–38)*, the amount of antibody perfused in the MPOA in our study would be expected to neutralize the endogenous dynorphin peptides. Moreover, in two rats presumed neutralization of dynorphin resulted in ovulation, a response that did not occur in control animals.

Alternatively, the lesser magnitude of the LH increase in response to antibody administration, as opposed to that seen in response to blockade of κ-opioid receptors *(7)*, could be due to the fact that, in addition to the possible involvement of dynorphin $A_{1–17}$ and $A_{1–8}$, other prodynorphin-derived peptides are also released within the MPOA and contribute

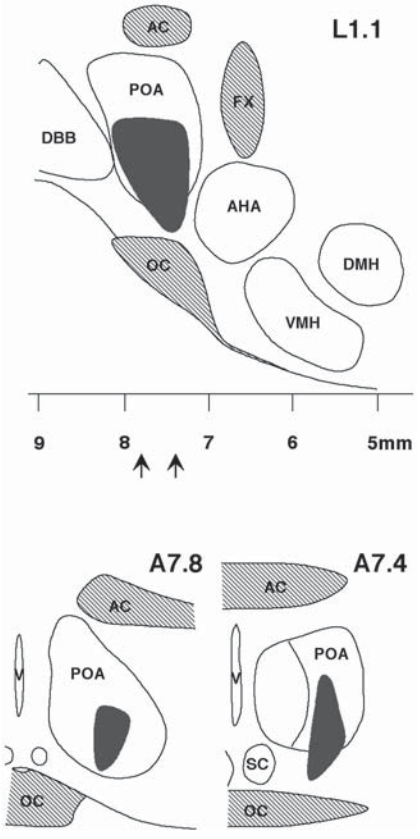

Fig. 3. Sagittal (top) and transverse (bottom) sections through the rat brain indicating location of push-pull cannula tips (shaded area) in rats treated with antibodies specific for dynorphin $A_{1–17}$ and/or dynorphin $A_{1–8}$ in the MPOA. Arrows indicate the level of the transverse sections. Sections are labeled according to the atlas of deGroot *(49)*. AC, anterior commissure; AHA, anterior hypothalamic area; DBB, diagonal band of Broca; DMH, dorsomedial hypothalamic nucleus; FX, fornix; OC, optic chiasm; POA, preoptic area; SC, suprachiasmatic nucleus; V, third ventricle; VMH, ventromedial hypothalamic nucleus.

to this inhibitory dynorphin tone. As a result, neutralization of dynorphin $A_{1–17}$ and $A_{1–8}$ would only partially remove the dynorphin inhibition, resulting in an increase in LH secretion of lesser magnitude than that produced by blockade of κ-opioid receptors, which would block the inhibitory action of all dynorphin peptides.

Regardless of magnitude, the change in plasma LH levels that occurred in response to either blockade of κ-receptors *(7)* or neutralization of dynorphin $A_{1–17}$ and $A_{1–8}$ took about 2½ to 3 h to develop in each case. This delayed LH response to reduction of dynorphin inhibitory tone could be due to the fact that other neuronal events that are involved in generation of the afternoon LH surge, such as an accumulation of neuropeptide Y and gonadotropin-releasing hormone in the median eminence *(39–42)*, do not occur until the early afternoon of proestrus. In support of this explanation, we recently found that an LH surge could be readily induced within ½ h after the onset of κ-opioid receptor blockade on the afternoon of proestrus (unpublished observations).

If a decline in inhibitory dynorphin tone occurs on the day of proestrus, it may be due, at least in part, to a decrease in prodynorphin- but not κ-opioid receptor-gene expression. While κ-opioid receptor mRNA levels did not change on proestrus, prodynorphin mRNA levels in the MPOA were decreased by the late afternoon on proestrus during the LH surge, when compared to values in the early afternoon when plasma LH levels were low. By contrast, this change in prodynorphin mRNA levels did not occur on diestrous d 1 when plasma LH levels remained low throughout the day. Simerly et al. *(43)* also reported that on proestrus a decline of similar magnitude in prodynorphin mRNA levels occurs in the anteroventral periventricular nucleus, which has a high concentration of dynorphin-containing neurons *(44)*, and which was included in the MPOA tissue blocks examined in the present study. Although the decrease in prodynorphin mRNA levels was relatively small in the study of Simerly et al. *(43)* and our own, this may nevertheless be a physiologically important change resulting in a larger decline in dynorphin $A_{1–17}$ and $A_{1–8}$ release within the MPOA. In an analogous manner, a decrease in β-endorphin inhibitory tone has been shown to be involved in the occurrence of the LH surge on proestrus *(2–6)*. While proopiomelanocortin mRNA levels in the hypothalamic arcuate nucleus decreased only by 30% on the afternoon of proestrus *(6)*, a 90% decline in β-endorphin release occurred at the same time *(5)*. Further studies examining dynorphin release in the MPOA on the day of the LH surge are needed.

Prodynorphin- as well as κ-opioid receptor-mRNA-positive neurons are regularly found together in the CNS *(45)*. The MPOA contains prodynorphin, dynorphin peptides, and κ-opioid receptors *(20–24, 46–48)*. Thus, it is possible that there is a local circuit within this brain region in which dynorphin is synthesized, released, and acts on κ-opioid receptors to suppress the onset of the LH surge. The results of the present study suggest that dynorphin $A_{1–17}$ and $A_{1–8}$ may play a minor role within the MPOA in suppressing LH secretion early on proestrus. A reduction in prodynorphin gene expression on the afternoon of proestrus may be one event involved in a possible decrease in dynorphin inhibitory tone on the ovulatory LH surge–generating signal.

Materials and Methods

Animals

Adult female rats in our colony, derived from Charles River Sprague-Dawley CD rats, were maintained on a 14: 10 h light/dark schedule (lights on at 0500 h), and fed rat chow and water. Estrous cyclicity was determined by daily examination of vaginal smears. The experimental procedures were approved by the local University Animal Care and Use Committee.

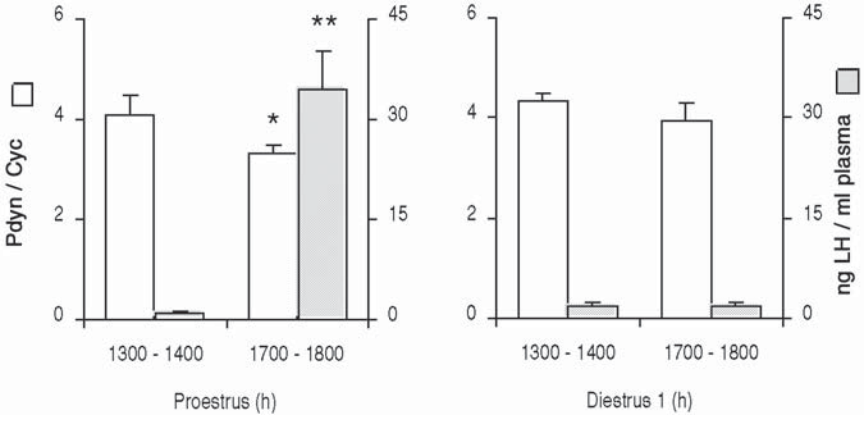

Fig. 4. Prodynorphin mRNA (Pdyn/Cyc; see Materials and Methods for explanation) and plasma LH levels on proestrus (left) and diestrus 1 (right). **p* < 0.05: prodynorphin mRNA levels at 1700–1800 h vs those at 1300–1400 h; ***p* < 0.01: plasma LH levels at 1700–1800 h vs those at 1300–1400 h.

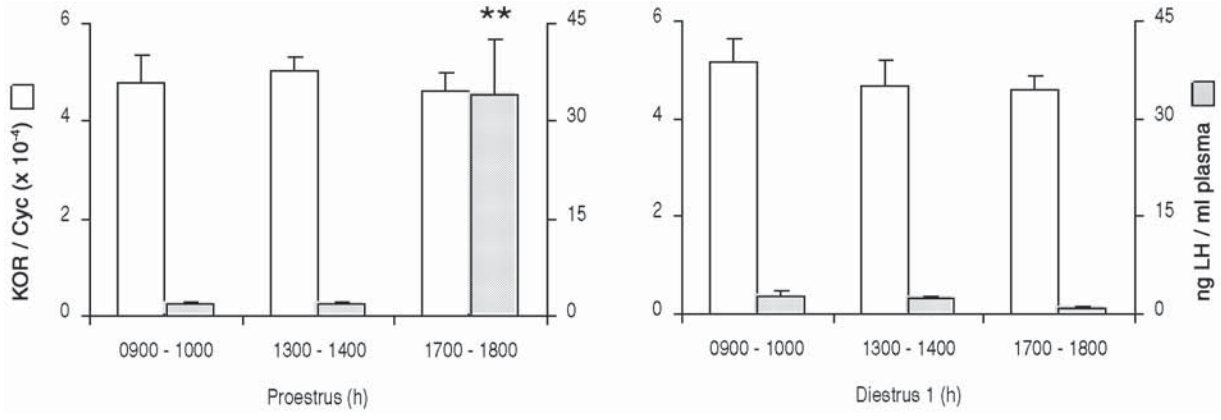

Fig. 5. κ-Opioid receptor mRNA (KOR/Cyc; see Materials and Methods for explanation) and plasma LH levels on proestrus (left) and diestrus 1 (right). ***p* < 0.01: plasma LH levels at 1700–1800 h vs those at 0900–1000 or 1300–1400 h.

Implantation of Push-Pull Cannula

About 2–4 wk prior to the experiment, rats displaying 4-d estrous cycles and weighing 280–320 g were anesthetized with pentobarbital (3.5 mg/100 g of body wt intraperitoneally). A 21-gauge stainless steel guide cannula (Plastics One, Roanoke, VA) was then stereotaxically implanted into the MPOA (coordinates: 7.8 mm anterior to the interaural line, 0.8 mm lateral to the midline, and 6.8 mm below the surface of the brain), according to the atlas of deGroot *(49)*. The cannula was cemented in place, and a 26-gauge stylette was inserted so that its tip was flush with the tip of the guide cannula. Cannula implantation was done on the day of estrus, resulting in minimal disruption of the estrous cycle. Daily vaginal smears were continued, and only rats displaying at least two consecutive 4-d estrous cycles were used in experiments. The inner stylette was removed 4–10 d before the day of push-pull perfusion, and a new stylette was inserted so that its tip extended 1 mm beyond the tip of the guide cannula.

Cannulation of Jugular Vein

Between 0800 and 1000 h on diestrous d 2 (the day before the push-pull perfusion experiment), rats were anesthetized briefly with ether, and a polyethylene (PE50) cannula was inserted into or near the right atrium via the external jugular vein. The next day rats displaying nucleated epithelial smears characteristic of proestrus were used in experiments.

Push-Pull Perfusion

The technique of push-pull perfusion was similar to that previously used in our laboratory *(7)*. On the morning of the experiment, the flow rates of the push and pull peristaltic pumps (Pharmacia, Piscataway, NJ) were carefully balanced and set to deliver 10 μL/min. This was done by setting the push pump to deliver 10 μL/min, and then adjusting the flow rate of the pull pump until a drop of aCSF at the tip of the push-pull cannula system remained unchanged in size for at least 1 h. The inner stylette was then removed, and a push-pull cannula assembly was inserted into the outer cannula.

The tip of the inner cannula extended 0.75 mm beyond the tip of the guide cannula. The push end of the tubing was inserted into a test tube containing the perfusion solution. Constancy of flow rates during the perfusion was monitored gravimetrically by weighing the perfusate.

Specificity of Antibody

Rabbit anti–dynorphin $A_{1–17}$ IgG and anti-dynorphin $A_{1–8}$ IgG were obtained from Peninsula Laboratories (Belmont, CA). These antibodies are highly specific. The antibody against dynorphin $A_{1–17}$ is 100% crossreactive with dynorphin $A_{1–17}$ and 0.43% with dynorphin $A_{1–13}$, and it does not crossreact with dynorphin $A_{1–8}$, dynorphin B, leu-enkephalin, or α- and β-neoendorphin. The antibody against dynorphin $A_{1–8}$ is 100% crossreactive with dynorphin $A_{1–8}$; <0.01% with big dynorphin, dynorphin $A_{1–17}$, and dynorphin $A_{1–13}$; and 0% with dynorphin B, α- and β-neoendorphin, metenkephalin, and leu-enkephalin arg.

Preliminary Experiment

A preliminary experiment was conducted to determine whether the antibodies to be used in experiment 1 were immunologically effective in neutralizing corresponding dynorphin peptides. We have recently shown that although eight of nine rats (90%) infused intraventricularly with aCSF from 1330–1800 h on proestrus ovulated by the next morning, this ovulatory response was blocked by dynorphin, since only one of seven and two of seven rats ovulated when infused with 0.5 ng/h of dynorphin $A_{1–8}$ or 1 ng/h of dynorphin $A_{1–17}$ (14 and 27%, respectively; $p < 0.05$ in each case) *(16)*. In the present preliminary study, antibodies were mixed with dynorphin peptides in aCSF and infused through the third ventricle from 1330–1800 h in proestrous rats (0.5 ng/h of dynorphin $A_{1–8}$ + 3.57 μg/h of anti–dynorphin $A_{1–8}$ IgG, or 1 ng/h of dynorphin $A_{1–17}$ + 3.57 μg/h of anti–dynorphin $A_{1–17}$ IgG). The ovulation-blocking action of each dynorphin peptide was prevented, since four of five (80%) and six of seven (86%) of the rats ovulated in each group, respectively ($p < 0.05$ vs peptides alone). These results demonstrated that the antibody preparations to be used in experiment 1 were effective in inactivating the dynorphin peptides.

Experiment 1

The objective of this experiment was to determine the possible role of endogenous dynorphin $A_{1–17}$ and dynorphin $A_{1–8}$ in suppressing the onset of the ovulatory LH surge. If involved, MPOA administration of antibodies directed against each dynorphin ligand should prematurely remove this suppression and advance the increase in plasma LH levels that occurs on proestrus. Push-pull perfusion of the MPOA with aCSF (140 m*M* NaCl, 4 m*M* KCl, 2.3 m*M* $CaCl_2$, 1 m*M* $MgSO_4$, 1.2 m*M* Na_2HPO_4, 0.3 m*M* NaH_2PO_4, 3.4 m*M* glucose, pH 7.4; $n = 4$), aCSF containing normal rabbit IgG (Peninsula; 6.25 μg/h; $n = 4$), or aCSF containing either rabbit anti–dynorphin $A_{1–17}$ IgG (6.25 μg/h; $n = 5$), rabbit anti–dynorphin $A_{1–8}$ IgG (6.25 μg/h; $n = 5$), or both anti–dynorphin $A_{1–17}$ IgG and anti–dynorphin $A_{1–8}$ IgG (each at 6.25 μg/h; $n = 6$), was done in rats between 1030 and 1355 h on proestrus. Animals were injected with 400 U of heparin following the onset of perfusion. Blood samples (300–400 μL each) were withdrawn through the bleeding cannula with a syringe at 30–60-min intervals starting at 1100 h. An equal volume of saline was given to the animal following each blood sample. Blood samples were centrifuged, and the plasma was collected and stored at –70ºC until assayed for LH by radioimmunoassay (RIA). The animals were injected with pentobarbital (3.5 mg/100 g of body wt intraperitoneally) between 1355 and 1400 h to block the normal afternoon LH surge *(50)*. This was done in order to determine whether any resulting ovulation was due to an antibody-induced increase in LH release.

At the end of the experiment, rats were returned to their quarters overnight. The following morning (estrus) the ovaries were removed, the oviducts were separated from the ovaries, and ovulation was verified by counting the ova with the aid of a low-power microscope. The animals were then anesthetized with pentobarbital and their brains fixed in 10% formalin + 1% $CaCl_2$. Frozen 50-μm serial sections were stained with a Nissl stain using basic fuchsin, and the location of the cannula tip was determined.

Experiment 2

The objective of this experiment was to determine whether prodynorphin- or κ-opioid receptor-mRNA levels in the MPOA change in association with the ovulatory LH surge. Female rats were sacrificed by decapitation, and MPOA tissue blocks and trunk blood were collected at 0900–1000 and/or 1300–1400 h (before the LH surge), and 1700–1800 h (during the LH surge) on proestrus, and during the same time periods on diestrous d 1 when there is no LH surge ($n = 6$–8 per time point). Prodynorphin- and κ-opioid receptor-mRNA levels were determined with quantitative reverse transcription polymerase chain reaction (RT-PCR), a variant of real-time RT-PCR. Cyclophilin mRNA levels were also determined as an internal control to minimize the effect of variations in tissue weights and RNA extraction efficiency on the results. Blood samples were centrifuged, and the plasma was collected and stored at –70ºC until assayed for LH by RIA.

Measurement of Prodynorphin- and κ-Opioid Receptor-mRNA Levels

MPOA Tissue Collection

The MPOA tissue fragments were separated from the remaining brain on a metal plate with ice underneath. Cuts were made anteriorly through the junction of the optic nerves, laterally through the hypothalamic sulci, posteriorly through the caudal border of the anterior commissure, and dorsally at the base of the anterior commissure. The optic chiasm was removed. The MPOA tissue block included the anteroventral periventricular nucleus, which also contains dynorphin

neuronal cell bodies *(44)*. MPOA tissue was weighed and stored at –70ºC until determination of prodynorphin- and κ-opioid receptor-mRNA levels. The average tissue weights did not differ between groups.

Total RNA Extraction

The RNeasy Kit (Qiagen, Germany) was used to obtain total RNA from brain tissue according to the modified manufacturer's protocol with DNase I treatment to eliminate genomic DNA contamination. The average RNA yields did not differ between groups.

Reverse Transcription

mRNA was reverse transcribed to cDNA using murine leukemia virus reverse transcriptase and oligo dT as primer (Perkin-Elmer, Ranchburg, NJ) in a 90 μL vol. The thermal parameters for the reaction were 25ºC for 10 min, 42ºC for 15 min, 99ºC for 5 min, and 5ºC for 5 min. Residual RNA was eliminated with RNase A treatment.

Primer Design

All primers were designed using OLIGO 5.0 primer analysis software (National Biosciences, Hamel, MN). The upstream and downstream primers for prodynorphin are 5'-AGGCTACACGGCACTGACCAA-3' and 5'-AAGTCC TCCTCGTTGAAATGG-3', respectively, which give rise to a 204-bp PCR product. The upstream and downstream primers for the κ-opioid receptor are 5'-AGTCCCCCATCCA GATTTTCC-3' and 5'-ACGGCAATGTAACGGTCCAC-3', respectively, which give rise to a 475-bp PCR product. The upstream and downstream primers for cyclophilin are 5'-ATGGTCAACCCCACCGTGTT-3' and 5'-CGTGTGA AGTCACCACCCT-3', respectively, which give rise to a 206-bp PCR product.

PCR and Sampling

Two 100 μL vol PCR reactions were set up for each cDNA sample. The thermal cycling parameters were an initial denaturation at 95ºC for 5 min, then 28 cycles of denaturation at 95ºC for 30 s, annealing at 66ºC (prodynorphin and cyclophilin) or 60ºC (κ-opioid receptor) for 30 s, and elongation at 72ºC for 45 s. Beginning at cycle 12, 5 μL was collected from each PCR reaction every two cycles and dispensed into a well of a 96-well plate having a transparent bottom (Corning, Corning, NY) that was kept on ice. After all samples were collected, 95 μL of TE buffer was added to each well, followed by 100 μL of Picogreen fluorescence dye at a 1:200 dilution (Molecular Probes, Eugene, OR).

Measurement of Fluorescence

The intensity of fluorescence in each well was measured with an HTS 7000 plus Bio Reader System (Perkin-Elmer, Foster City, CA) at an excitation wavelength of 485 nm and an emission wavelength of 535 nm. A standard curve for each plate was set up to convert the fluorescence reading in each well to DNA content. The specificity of the PCR reactions was verified by gel electrophoresis.

Calculation of Initial cDNA Contents with the Recursive Model

The cDNA contents of prodynorphin, κ-opioid receptor, and cyclophilin at cycle 0 were calculated using the recursive model *(51)*. Briefly, the PCR reaction curve for each cDNA sample was plotted as DNA contents vs reaction cycles and the curve was then logarithmically converted. The amplification efficiency of each PCR reaction was obtained by running linear regression on the converted curve. A PCR product accumulation model was then established using the obtained amplification efficiency to approximate the real reaction curve, and the cDNA concentrations of each gene at cycle 0 were finally extrapolated from the model. The target mRNA levels were finally expressed as the ratio between the target cDNA levels and cyclophilin cDNA levels.

Radioimmunoassay

Plasma samples were analyzed for LH by the ovine:ovine rat LH double-antibody RIA of Niswender et al. *(52)*, as previously described *(53)*. The sensitivity of the assay is 6–8 pg/tube. Inter- and intraassay variations determined at a mean plasma LH level of 3.1 ± 0.2 ng/mL (n = 13) were 16.8 and 15.6%, respectively. LH values (ng/mL of plasma) were expressed in terms of the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) rat LH-RP-3 standard.

Data Analysis

In experiment 1, comparison between plasma LH levels in controls (aCSF/IgG) and antibody-treated groups was done using two-way analysis of variation (ANOVA) with repeated measures. In experiment 2, differences between groups in prodynorphin mRNA levels and plasma LH levels at 1300–1400 and 1700–1800 h were compared using the unpaired student's t-test. Differences between groups in κ-opioid receptor mRNA levels and plasma LH levels were compared using one-way ANOVA followed by the Tukey HSD test. Results were considered significant at $p < 0.05$. All results are expressed as the mean ± SEM.

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