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Identification of Glutamate Receptor Subtype mRNAs in Gonadotropin-Releasing Hormone Neurons in Rat Brain

Ozhan Eyigor and Lothar Jennes

Department of Anatomy and Neurobiology, University of Kentucky College of Medicine, Lexington, KY

The aims of the present study were to determine:

1. **If glutamate neurites can provide input to gonadotropin-releasing hormone (GnRH) neurons;**
2. **Which glutamate receptor subtype mRNAs are expressed in GnRH neurons; and**
3. **If GnRH neurons synthesize kainate 2 receptor (KA_2) protein.**

Immunohistochemical double stainings for GnRH and glutamate or for GnRH and KA_2-receptor protein were applied to rat brain sections containing the medial septum–diagonal band and preoptic area or the median eminence; in addition, dual *in situ* hybridization studies were carried out with digoxygenin-labeled cRNA probes encoding GnRH in combination with ^{35}S-labeled cRNA probes encoding the glutamate receptor subtypes $GluR_{1-4}$, KA_2, NMDA R_1, or NMDA R_{2A-D}. The results show that GnRH neurons are surrounded by glutaminergic neurites, which form puncta-like close appositions with the GnRH perikarya, and that an extensive overlap exists in the distribution of GnRH-positive axon terminals and glutaminergic neurites in the median eminence. Similarly, KA_2-receptor immunoreactivity is present in the perikarya of many GnRH neurons and in their axon terminals in the median eminence. Dual *in situ* hybridization experiments show that about 32% of all digoxygenin-labeled GnRH neurons also contain KA_2-receptor mRNA, 17% contain NMDA R_{2A} mRNA, 8% contain NMDR R_1, whereas <5% of the GnRH neurons express measurable amounts of $GluR_{1-4}$ or NMDA R_{2B-D} mRNA. The results suggest that glutaminergic neurons innervate the GnRH neuronal system directly through activation of KA_2 receptors on GnRH neurons, whereas the effects of AMPA and NMDA on GnRH release are likely to be exerted indirectly through interneurons.

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Author to whom all correspondence and reprint requests should be addressed: Lothar Jennes, University of Kentucky, College of Medicine, Department of Anatomy and Neurobiology, MS 210 Medical Center, Lexington, KY 40536.

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Introduction

Gonadotropin-releasing hormone (GnRH) is the key neuronal peptide that regulates the activity of the anterior pituitary gonadotropes and is thereby, together with the ovarian steroid hormones, responsible for the maintenance of appropriate estrous or menstrual cycles in mammals. It is clear that the activity of the brain–pituitary–gonadal axis is controlled by complex feedback interactions in which estradiol inhibits GnRH and luteinizing hormone (LH) release from the brain and the pituitary, respectively, during all stages of the cycle, except during proestrus when a change to a positive feedback mode occurs (Freeman, 1988). At present, the mechanisms causing such a change in feedback properties are not known. However, since GnRH neurons do not contain nuclear receptors for the gonadal steroids (Shivers et al., 1983), a direct genomic action of estradiol on the GnRH neurons is not likely to occur. Instead, estradiol is thought to activate select neurotransmitter systems in the central nervous system, which in turn convey the steroid feedback signal to the GnRH neurons and cause a synchronized release of the peptide into the fenestrated capillaries of the median eminence.

Glutamate is the most abundant excitatory neurotransmitter in the brain and plays important roles in diverse processes, such as cognition, memory (Monaghan et al., 1989), and neuroendocrine regulation of the pituitary (Brann and Mahesh, 1994). Glutamate can activate two families of membrane receptors, which have been classified according to their preferential binding of glutamate agonists and antagonists: the metabotropic receptor family, which forms G-protein coupled receptors with seven transmembrane spanning domains, and the ionotropic receptor family, which forms ion-specific channels. The ionotropic family of receptor proteins includes the AMPA-preferring receptors $GluR_1$ through $GluR_4$, the kainate-preferring receptors KA_1, KA_2, and $GluR_{5-7}$, and the NMDA-preferring receptors NMDA R_1 and NMDA R_{2A-D} (Seeburg, 1993; Hollman and Heineman, 1994). All three ionotropic receptor sub-

families appear to be involved in the control of GnRH release either directly or indirectly, since iv, sc, and ip injections of AMPA, kainate, or NMDA cause a rapid increase in circulating LH within 10–15 min, which is mediated by activation of the GnRH neuronal system (for review, *see* Brann, 1995; Cowell, 1995). In order to determine which glutamate receptor subtypes stimulate GnRH neurons directly, we used dual *in situ* hybridization to detect colocalizations of GnRH mRNA and one of the above glutamate receptor subtype mRNAs. Since this procedure does not show where in the GnRH neurons a specific receptor protein is located, we also applied dual immunohistochemistry to determine the anatomical relationships between the KA_2-receptor protein and the GnRH neuronal system.

Results

Immunohistochemistry

In order to determine where in the basal forebrain interactions could occur between the GnRH neuronal system and the glutamate-containing neurites, the areas of the medial septum–diagonal band through the medial preoptic area were analyzed, since GnRH perikarya are restricted to these areas as well as the median eminence, which is the site of termination of most GnRH axons (Silverman, 1988). Immunohistochemistry for GnRH and glutamate revealed that in the septum–diagonal band and preoptic area, numerous glutamate-immunoreactive neurites were present throughout the extent of this region. Many glutamate-containing fibers were observed juxtaposed to GnRH perikarya, and these fibers contained punctae-like swellings at the sites of close appositions to the GnRH-containing cell bodies (Fig. 1A and B). In the median eminence, glutamate immunoreactive fibers were present in all areas that also contained GnRH axons, including the region next to the infundibular sulcus where GnRH axon terminals were most numerous (Fig. 1C and D).

Immunohistochemistry for the KA_2-receptor protein and GnRH showed that many GnRH neurons contained punctae of KA_2-receptor immunoreactivity in their cell bodies, preferentially in the perinuclear region, whereas such aggregations were usually absent in the proximal neurites (Fig. 1E and F). In the median eminence, KA_2-receptor immunoreactivity was most dense in the lateral palisade layer including the region next to the infundibular sulcus. In addition, KA_2-receptor immunoreactivity was present in a slightly lesser density in the medial part of the palisade layer and in a punctate pattern throughout the reticular and fibrous layers. Immunohistochemical dual stainings for KA_2-receptor protein and GnRH showed that many, but not all GnRH preterminal axons in the palisade layer also contained KA_2-receptor immunoreactivity (Fig. 1G–J). Absorption of the GnRH antisera with 10 μg GnRH peptide/mL dilute antiserum, omission of the primary antisera, and tests for possible crossreactivities of the second antibodies resulted in the absence of specific staining.

In Situ *Hybridization*

In situ hybridization using digoxygenin-tagged cRNA probes followed by immunohistochemistry to localize the mRNA encoding GnRH resulted in the labeling of select neurons in the medial septum–diagonal band complex and preoptic area. These neurons were similar in number, location, and morphology when compared to immunohistochemically identified GnRH neurons.

Hybridization with ^{35}S-labeled cRNA probes encoding the $GluR_1$- and the $GluR_2$-receptor subunits resulted in dense labeling of a large number of neurons in the septum–diagonal band and preoptic area, whereas the hybridization signals of cRNA probes of $GluR_3$ and $GluR_4$ were relatively sparse. Dual *in situ* hybridization for GnRH and one of the above receptor subunits showed that no GnRH neurons contained detectable levels of $GluR_1$ or $GluR_4$ subunits, and only <5% of the digoxigenin-labeled GnRH neurons expressed measurable amounts of mRNA encoding $GluR_2$- or $GluR_3$-receptor subunits (Fig. 2A).

KA_2-receptor mRNA-expressing neurons were present in large numbers in the medial septum–diagonal band and preoptic area, and most of these neurons were heavily labeled with the ^{35}S-riboprobe (Fig. 2C). Dual hybridization for GnRH and KA_2-receptor mRNAs revealed an extensive colocalization of the two mRNAs, such that 32% of all digoxygenin-labeled GnRH neurons also expressed the KA_2-receptor mRNA (Fig. 2D and E). These dual-labeled GnRH neurons were preferentially located in the region of the diagonal band and rostral preoptic area that surrounds the organum vasculosum of the lamina terminalis (OVLT), whereas the medial septum was usually devoid of dual-labeled GnRH neurons. However, most GnRH neurons that did not contain measurable amounts of KA_2-receptor mRNA in their cytoplasm were situated next to KA_2 mRNA-positive neurons.

NMDA R_1, NMDA R_{2A}, and NMDA R_{2B} mRNA-containing neurons were similar in number and anatomical distribution compared to the KA_2-expressing neurons in the septum–diagonal band and preoptic area, whereas these regions contained only a few, weakly labeled neurons containing the mRNAs for NMDA R_{2C} and NMDA R_{2D}. Although no colocalizations were observed for GnRH and NMDA R_{2B}, NMDA R_{2C}, and NMDA R_{2D}, about 8% of the GnRH neurons contained the mRNA-encoding NMDA R_1 and 17% of the GnRH neurons contained the mRNA-encoding NMDA R_{2A} (Fig. 2B). The majority of these dual labeled GnRH neurons were located in the diagonal band and preoptic area next to the OVLT. The control experiments, including incubation of the sections with ^{35}S-labeled sense probe, pretreatment with RNase, and coincubation of the labeled antisense probe with a 100-fold excess unlabeled probe, resulted in the absence of specific labeling. The results are summarized in Table 1.

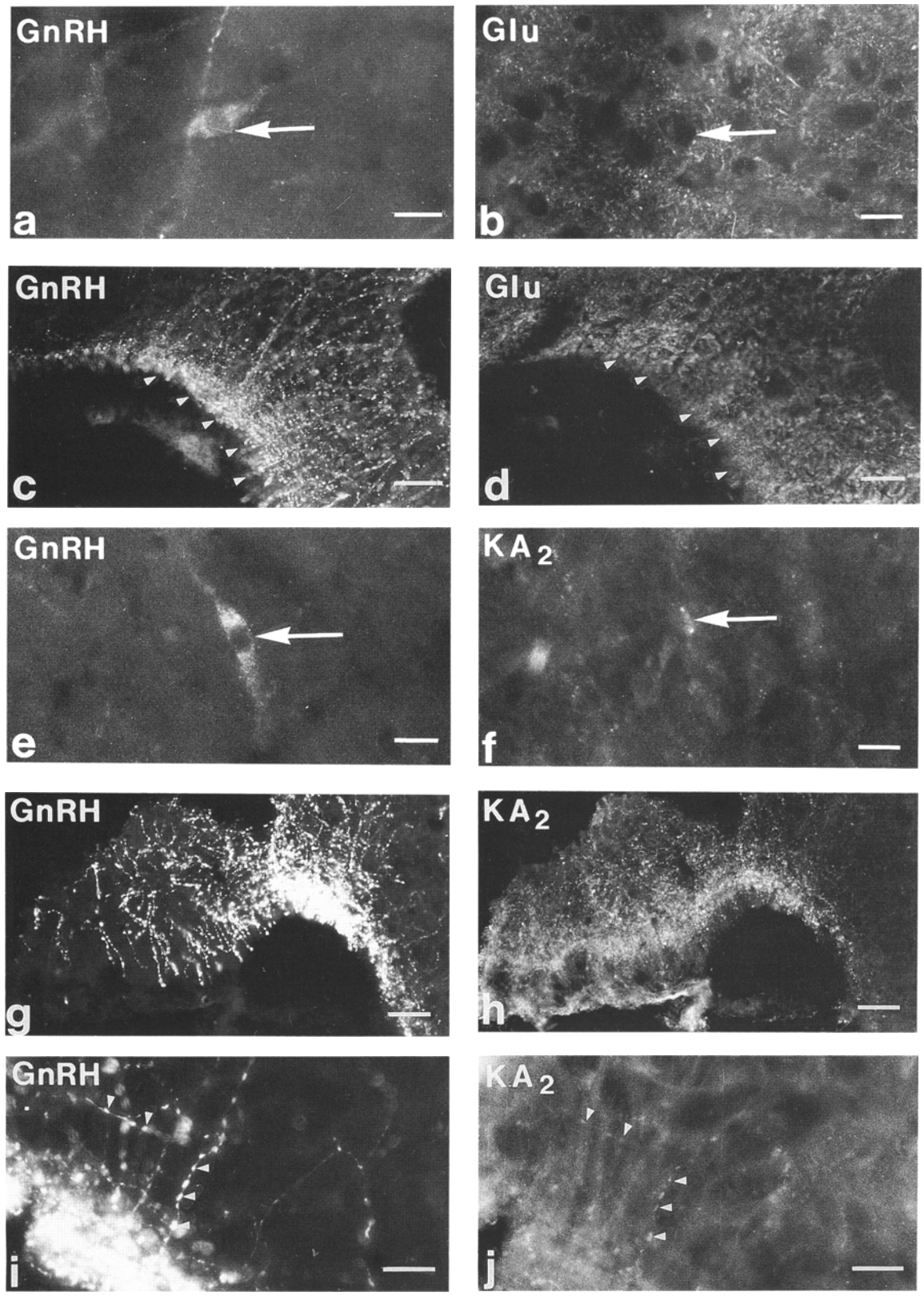
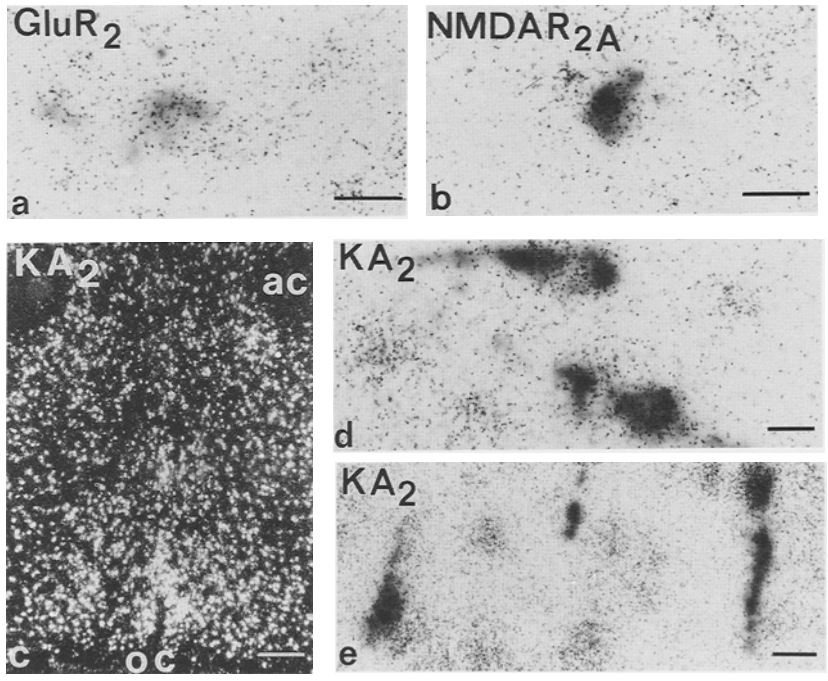


Fig. 1. a–j: Immunohistochemical double stainings for GnRH (a, c, e, g, i) and glutamate (b and d) or KA_2 receptor (f, h, j) showing close appositions of glutamate-containing neurites on a GnRH neuron (a and b, arrows), and extensive overlap in the distribution of GnRH axons and glutamate-containing neurites in the median eminence (c and d, arrowheads). e and f show the presence of KA_2-receptor immunoreactivity (f, arrow) in a GnRH neuron (e, arrow) and extensive overlap in the distribution of GnRH axons (g) and KA_2-receptor immunoreactivity in the median eminence (h). i and j show colocalization of GnRH (i) and KA_2-receptor immunoreactivity (j) in some, but not all preterminal GnRH axons (arrowheads). Bar: a, b, e, f, i, and j: 20 μm; c, d, g, and h: 50 μm.



Figs. 2. a–e: Examples of autoradiograms after *in situ* hybridization with ^{35}S-labeled cRNA probes encoding $GluR_2$ (a), NMDA R_{2A} (b), or KA_2 (c–e) combined with digoxygenin-labeled cRNA probe encoding GnRH (a, b, d, and e) showing colocalizations of GnRH mRNA and one of the glutamate-receptor subtype-specific mRNAs. c gives an overview of the distribution of KA_2 mRNA-containing neurons in the septum–diagonal band and rostral preoptic area. Abbreviations: ac: anterior commissure; oc: optic chiasm. Bar: a, b, and e: 20 μm; c: 250 μm; d: 15 μm.

Discussion

Consistent with data from other neuroanatomical studies, the results of the present study show that glutaminergic neurons are, from an anatomical point of view, in the appropriate locations to provide excitatory inputs to the GnRH neuronal system. This input is likely to be mediated by synaptic specializations at the level of the GnRH perikarya or initial dendrites, as has been described in the arcuate nucleus of the monkey (Goldsmith et al., 1994), or by asynaptic neurotransmission in the median eminence. The results of the present study also suggest that glutamate activates GnRH neurons directly through KA_2 receptors, which are located in the GnRH perikarya in the diagonal band and preoptic area, and preterminal axons in the median eminence. The general location of KA_2-receptor immunoreactivity matches the results of Petralia et al. (1994), who described the presence of immunoreactive KA_2-receptor protein throughout the hypothalamus with a relatively high density in the external layer of the median eminence. These immunohistochemical findings are further supported by in vitro binding or *in situ* hybridization studies showing the presence of KA_2-binding sites (Unnerstall and Wamsley, 1983; Meeker et al., 1994) or receptor mRNA (Herb et al., 1992; Wisden and Seeburg, 1993; Van Den Pol et al., 1994; Petralia and Wenthold, 1996) throughout the hypothalamus with highest densities in the arcuate and ventromedial regions, as well as in the median eminence.

Glutamate has been known for some time to stimulate GnRH-mediated LH release. However, the mechanisms and the rank order of glutamate analog potencies are still unclear. Although some studies have shown that iv administration of NMDA or kainate in vivo and administration of the agonists to hypothalamic slices in vitro cause a rapid, dose-dependent, and reversible increase in GnRH release (Bourguignon et al., 1989a), others could detect such effects only for kainate, but not for NMDA (Donoso et al., 1990; Lopez et al., 1992). On the other hand, pulsatile GnRH release from male rat hypothalamic explants is suppressed by the NMDA receptor channel blocker MK-801, but not by the kainate receptor blocker DQNX (Bourguignon et al., 1989b) suggesting that NMDA receptors are important regulators of coordinated, pulsatile release from deafferented mediobasal hypothalami, whereas kainate receptors may act on other sites, such as the GnRH perikarya, which were not contained in the tissue slices, or stimulation by kainate may require complete intact GnRH neurons to function. Similar to the male rat, peripheral injections of either kainate or NMDA induce a rapid stimulation of GnRH-mediated LH release in the cycling female rat (Abbud and Smith, 1991), whereas administration of MK-801 to adult animals in early proestrus or to pregnant mare serum gonadotropin-primed immature rats blocks the LH surge (Brann and Mahesh, 1991). These results indicate that activation of NMDA receptors by endogenous glutamate is necessary for an LH surge to

Table 1
Colocalization of GnRH mRNA
and Glutamate-Receptor Subtype mRNAs

Glutamate-receptor subtype	Number of GnRH cells counted	Percentage of colocalized cells
Glu R_1	109	0
Glu R_2	90	<5
Glu R_3	107	<5
Glu R_4	30	0
KA_2	484	32
NMDA R_1	196	8
NMDA R_{2A}	92	17
NMDA R_{2B}	102	0
NMDA R_{2C}	78	0
NMDA R_{2D}	66	0

occur. Further support that glutamate is important for the regulation of the GnRH neurons has been provided by studies in which a significant increase in GnRH mRNA was measured after administration of NMDA (Petersen et al., 1991; Gore and Roberts, 1994) or kainate (Gore and Roberts, 1994), which is probably caused by an increase in GnRH mRNA stability and not increased mRNA synthesis (Gore and Roberts, 1994). Similarly, blockade of NMDA receptors by MK-801 causes a reduction in GnRH mRNA content (Seong et al., 1993).

Taken together, these studies suggest that glutamate is important for the regulation of pulsatile basal GnRH-LH release in both male and female animals, as well as in the stimulation of phasic GnRH-LH release during the preovulatory surge. It appears that both NMDA and kainate receptors mediate the excitatory effects of glutamate, but the above physiological studies cannot distinguish between direct and indirect effects of glutamate on the GnRH neurons.

Based on the results of the present study, it is likely that glutamate exerts the stimulatory effects by direct activation of the GnRH neurons through KA_2 receptors. We could show that a large number of GnRH neurons contain KA_2-receptor mRNA and express the receptor protein, which accumulates in the perikaryal region in the septum–preoptic region, as well as in the terminal compartment of the GnRH axons in the median eminence. This pattern of intracellular location of the KA_2-receptor protein suggests that the GnRH neurons receive glutaminergic synaptic input to the perikaryon and, in addition, GnRH release can be regulated in the median eminence through asynaptic activation of KA_2 receptors.

The results of the present study also suggest that the effects of NMDA on the GnRH system are probably mediated by interneurons and not by direct activation of NMDA receptors on GnRH neurons, since the NMDA R_1 subunit, which is required for functioning NMDA receptors (Monyer et al., 1992a), was detected only in a very small number of GnRH neurons. The presence of NMDA R_1-subunit mRNA in only a few GnRH neurons was previously noted in male and female rats under varying endocrine conditions (Abbud and Smith, 1995), as well as in the hamster (Urbanski et al., 1995), and it appears unlikely that GnRH neurons contain the $NMDAR_1$ mRNA only for such a short period of time that it would have been missed in the present as well as in the above-cited studies (Abbud and Smith, 1995; Urbanski et al., 1995). Our data are, however, in contrast to results obtained from immortalized neurons (GT-1) that synthesize GnRH. These cells apparently contain the mRNA encoding the NMDA R_1 subunit as determined by Northern blot analysis (Mahachoklertwattana et al., 1994) and they respond to NMDA, kainate, and AMPA with increased release of GnRH (Mahachoklertwattana et al., 1994; Spergel et al., 1994). Since the NMDA R_1-subunit mRNA is present in only 8% of the GnRH neurons, the biological significance of our finding that the NMDA R_{2A} subunit is expressed in a subset of GnRH neurons is unclear. It is possible that other yet unidentified NMDA receptor subunits can assemble with the NMDA R_{2A} to form a functioning receptor.

Materials and Methods

A total of 30 adult female Sprague-Dawley rats were used for the dual *in situ* hybridization localization of GnRH mRNA and the glutamate-receptor subtype mRNA localization. The animals were ovariectomized for 2 wk ($n = 7$) or ovariectomized for 2 wk followed by an estrogen-progesterone treatment mimicking proestrus conditions and sacrificed at 0800 h ($n = 5$), 1000 h ($n = 4$), 1600 h ($n = 5$), 1800 h ($n = 3$), 2000 h ($n = 3$), and 2200 h ($n = 3$) of the day of the progesterone administration. For immunohistochemistry, 10 adult intact female rats were used at random stages of the estrous cycle. Animals were kept at the animal facility unit of University of Kentucky Medical Center with a light cycle of 14 h lights on and 10 h lights off.

Immunohistochemistry

Animals were perfusion fixed with 4% paraformaldehyde in 0.1 phosphate-buffered saline (PBS) and postfixed overnight in the above fixative containing 30% sucrose. Twenty-five-micrometer thick coronal sections were taken with a freezing sliding microtome. The sections were washed for 30 min at room temperature in Tris-HCl buffer (0.05*M*, pH 7.6) containing 0.1% Na-azide, 4% normal horse serum, and 0.2% Triton X-100 followed by the exposition overnight to a mixture of rabbit anti-GnRH #640 (Jennes) and monoclonal antiglutamate (1:500; kindly provided by A. J. Beitz, Minnesota; Madl et al., 1986) or a mixture of monoclonal mouse anti-GnRH (4H10, Jennes) and rabbit anti-KA_2 (2 μg/mL; kindly provided by R. J. Wenthold, NIH, Bethesda, MD; Petralia and Wenthold, 1996). After a 15-min wash in Tris-HCl buffer, the sections were incubated for 60 min in the mixture of affinity-purified and crossabsorbed FITC-labeled donkey anti-

mouse (1:70, Jackson Lab, West Groove, PA) and Texas red-labeled donkey antirabbit (1:70, Jackson Lab). After two final washes of 15 min each, the sections were mounted and observed with an Olympus BH-2 fluorescence microscope. Pictures were then taken with Kodak (Rochester, NY) Tri-X pan film (ASA 400). Control experiments included absorption of the GnRH antisera with 10 μg synthetic decapeptide, omission of the primary antisera, and tests for possible crossreactivities of the second antibodies.

In Situ *Hybridization*

The animals were decapitated, and the brains rapidly removed and frozen on dry ice. Twelve-micrometer-thick coronal cryostat sections were cut through the medial septum–diagonal band complex to the anterior hypothalamus, collected on Superfrost Plus slides (CMS, Houston, TX), and stored at –80°C until use. The slides were equilibrated to room temperature, fixed for 15 min in 4% paraformaldehyde, followed by two 5-min rinses in PBS (0.1*M*), two 5-min rinses in PBS containing 10 m*M* glycine, and two 5-min PBS rinses. The sections were then acetylated for 10 min in triethanolamine (0.1*M*, pH 8.0) and 0.25% acetic acid anhydride, dehydrated, and air-dried.

A cDNA clone complementary to rat GnRH was generously provided by J. P. Adelman, Vollum Institute, Portland, OR; cDNA clones complementary to glutamate-receptor subunits $GluR_{1-4}$, KA_2, and NMDA R_{1A}, and NMDA R_{2A-D} were generously provided by J. Boulter, Molecular Neurobiology Laboratory at the Salk Institute, San Diego, CA. Plasmids containing the cDNA clone complementary to rat GnRH were linearized with *Hinc*2 and transcribed in vitro in the presence of digoxigenin-11-UTP (Boehringer Mannheim, Mannheim, Germany), whereas other plasmids were linearized with *Eco*R1 (Glu R_1), *Xho*1 ($GluR_4$), *Not*1 ($GluR_{2,3}$, KA_2, and NMDA $R_{1A,2A,2D}$), *Eco*R5 (NMDA R_{2B}), or *Cla*1 (NMDA R_{2C}), and transcribed in vitro in the presence of ^{35}S-UTP and T7 polymerase ($GluR_{1-3}$, KA_2, NMDA $R_{1A,2A,2D}$) and T3 polymerase ($GluR_4$, NMDA $R_{2B,2C}$). The characteristics of these clones have been published: $GluR_1$, EMBL/GenBank accession number (an) X17184 (Hollman et al., 1989); $GluR_2$, an M85035 (Boulter et al., 1990); $GluR_3$, an M85036 (Boulter et al., 1990); $GluR_4$, an M85037 (Bettler et al., 1990); KA_2, an U08258 (Herb et al., 1992); $NMDAR_{1A}$, an U08261 (Moriyoshi et al., 1991); $NMDAR_{2A}$, (Monyer et al., 1992b); $NMDAR_{2B}$, an U11419 (Monyer et al., 1992b); $NMDAR_{2C}$, an U08259 (Monyer et al., 1992b); $NMDAR_{2D}$, an U08260 (Ishii et al., 1993).

Dried sections were hybridized overnight at 60°C with 1.5×10^6 cpm ^{35}S-labeled probe/60 μL/slide and 2 μL/slide digoxigenin-labeled probe, which were diluted in hybridization cocktail (Amresco, Solon, OH) in the presence of 0.15 mg/mL yeast tRNA and 40 m*M* dithiothreitol. After 18 h, sections were rinsed twice in 2X SSC for 15 min each and treated with RNase (10 mg/100 mL) for 30 min at 37°C, followed by two 10-min washes in 0.2X SSC at room temperature. The sections were then kept for 1 h at 60°C in 0.2X SSC, rinsed in 0.2X SSC at room temperature followed by 70 and 95% ethanol, and air-dried.

The dried sections were circled with a PAP-pen and incubated for 3–4 h with 2% normal lamb serum and 0.05% Triton-X-100 in 2X SSC followed by two washes in buffer A (100 m*M* Tris, pH 7.5, and 150 m*M* NaCl). The sections were then exposed overnight to sheep antidigoxigenin antibody (1:1000, Boehringer Mannheim) in buffer A containing 1% NLS and 0.3% T-X-100. After two washes in buffer A for 10 min each, sections were rinsed for 15 min in buffer B (100 m*M* Tris, pH 9.5, 100 m*M* NaCl and 50 m*M* $MgCl_2$) and stained in the dark for 2–4 h in chromagen solution (45 μL NBT, 35 μL X-Phosphate, Boehringer Mannheim, and 10 mg levamisole, Sigma, St. Louis, MO, per 10 mL of buffer B). The reaction was stopped by two 15-min washes in buffer B followed by rapid rinses (5 s each) in 70 and 95% ethanol, and air-dried. Sections were then dipped in parlodion (3%) in isoamyl acetate and coated with Kodak photographic emulsion NTB2, diluted 1:1 with water, followed by a 3–8-wk exposure. Slides were developed in Kodak Developer D19 (for 2 min at 15°C) and fixed in Kodak Rapid Fixer (for 5 min). Specificity controls included incubation with ^{35}S-labeled sense probe, pretreatment with RNase, and coincubation with a 100-fold excess unlabeled antisense probe.

Image Analysis

Each digoxygenin-labeled GNRH neuron was examined for the presence of overlying silver grains under a 40× oil immersion objective. A GnRH neurons were identified as "double-labeled" if the number of silver grains over the neuron was higher than three times background as determined by measurements of the number of silver grains in a similarly sized area over the anterior commissure.

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