Direct Activation of Gonadotropin-Releasing Hormone Secretion Through Different Receptors to Neuroexcitatory Amino Acids

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Key Words. N-methyl-D,L-aspartate - Kainate - Quinolinic - Quisqualate - Gonadotropin-releasing Hormone - Neuroexcitatory amino acids

Abstract. In order to evaluate the involvement of gonadotropin-releasing hormone (GnRH) in the effects of neuroexcitatory amino acids on luteinizing hormone (LH) secretion, N-methyl-D,L-aspartate (NMDA; 30 ng/kg i.c.v.) was administered to 50-day-old male rats. The in vitro release of GnRH from the hypothalamus showed a maximal increase (4.6-fold) in animals sacrificed 7.5 min after NMDA injection, while serum LH levels rose concomitantly. Incubation of rat hypothalamus in vitro with kainate or NMDA concentrations > 0.1 μM resulted in a dose-related release of GnRH. NMDA being two-fold more potent than kainate. Quisqualate (10 μM) did not affect the release of GnRH. On a molar basis, quinolinic acid (50 μM), a possible endogenous ligand for NMDA receptors, was the most effective in inducing GnRH secretion (34.9 ± 4.9 pg/75 min, mean increment ± SEM, n = 10). The effects of kainate and NMDA were mediated through different types of receptors, since GnRH response to kainate was unchanged in the absence of glucose or in the presence of increased concentrations of Mg²⁺ (2 mM) or Ca²⁺ (5.8 mM). In contrast, the GnRH response to NMDA was reduced by Ca²⁺ (5.8 mM) and abolished in the absence of glucose or in the presence of Mg²⁺ (2 mM). In addition, D,L-α-amino-5-phosphonopentanoic acid (AP5), a competitive antagonist of NMDA receptors, prevented the NMDA-induced release of GnRH. The permissive effect of glucose on GnRH response to NMDA was 2.7-fold more important when using glucose concentrations of 0.01 μM than when concentrations ≥ 100 μM were used. Intermittent incubation with NMDA in vitro (other 7.5-min periods) did not affect the amplitude of GnRH response, while continuous exposure to NMDA resulted in an initial and transient increase in GnRH release followed by a prolonged desensitization period. It is concluded that different neuroexcitatory amino acids acting through distinct receptor types may be involved in the hypothalamic control of LH release by regulating the secretion of GnRH.

In rats and monkeys in vivo administration of agonists of the neuroexcitatory amino acids glutamate and aspartate triggers the release of luteinizing hormone (LH) [14, 17, 21, 27, 30, 37]. These observations may be relevant to the hypothalamic mechanisms thought to be involved in pulsatile secretion of LH and its changes at onset of puberty. Such a hypothesis has been raised in recent studies using agonists and antagonists of neuroexcitatory amino acids in the immature monkey [14, 26] and in the rat [1, 24]. In these experiments, it has been assumed that the study of LH provided an estimate of changes in gonadotropin-releasing hormone (GnRH) secretion. This was based on indirect observations suggesting that the effects of neuroexcitatory amino acids are mediated in the hypothalami [14, 21, 24, 30, 33]. In addition, most studies have been directed towards receptors to N-methyl-D,L-aspartate (NMDA), while in the central nervous system, at least two types of distinct amino acid receptors have been characterized using kainate and NMDA as specific ligands [23]. The aim of this study was to evaluate directly the putative changes in GnRH secretion induced by neuroexcitatory amino acids and to determine if different types of receptors are involved.

Materials and Methods

Rats

Male Wistar rats aged 50 days at the time of the experiment were purchased from Janssen (Beersel, Belgium). They were housed 6 per cage under constant temperature and a light-dark schedule (lights on 06:00-20:00 h) with free access to food and water. For each experiment, a group of 12 rats were killed around 13:00 h. The retrodorsal hypothalamus was rapidly dissected as previously described [6, 7] and in situ incubated in vitro.

Incubation of Hypothalamic Explants

A static in vitro system was used, as previously described [6, 7], in order to study 12 hypothalamic individually, each explant being in 0.5 ml culture medium which was renewed every 7.5-min period. Culture medium was Dulbecco's modified Eagle's medium (Flow Laboratories, McLean, Va., USA) which contained 25 mM glucose, 1.8 mM Ca²⁺, 1.0 mM Mg²⁺, and 0.4 mM glycine. Eagle's minimum essential medium (Flow Laboratories) was also used after being enriched with 5.2 mg/ml glucose and 350 mg/ml in order to obtain a medium similar to Dulbecco's modified Eagle's medium. The absence of glucose. Culture medium contained bacitracin at a final concentration of 20 μM in order to prevent degradation of GnRH by hypotalamic proteases [29].

Radioimmunoassay of GnRH

From the 0.5-ml samples collected every 7.5 min, 0.1 ml was used for analyses. According to a method described previously [5, 6], using the RIK-5 anti-GnRH antiserum generously supplied by Dr. A. Root (St. Petersburg, Fl., USA). Since the sensitivity of the assay varied between 0.5 and 1.0 ng/tube, the detection limit of GnRH release was 5 pg/7.5 min. The standard curves were prepared in Dulbecco's modified Eagle's medium. The absence of nonspecific effects on the binding of radiolabeled GnRH and on the second antibody was checked in the presence of the different substances used.

Radioimmunoassay of LH

Serum concentrations of LH were determined in duplicate by double-antibody radioimmunoassay previously described [5] using NIAMD rat pituitary gonadotropin reagents kindly supplied by the Pituitary Agency of the National Institutes of Health. Results were expressed with reference to the rat standard IRP-1.

Study Protocols

Experiment 1. Intact rats (4-12/series) were injected subcutaneously with NMDA (Sigma, St. Louis, Mo., USA) 30 μg/kg body weight or with saline (controls). One, 3, 5, 7.5, and 10 min after the injection, the rats were sacrificed. Trunk blood was collected, and serum for the radioimmunoassay of LH was obtained after centrifugation. Immediately after decapitation, the hypothalami were dissected and transferred to the incubator where the release of GnRH in culture medium was measured after a 7.5-min incubation period.

Experiment 2. The in vitro release of GnRH from 9 to 12 individual hypothalami was studied in the presence of increasing concentrations (0.001-100 μM) of kainate (Sigma) or NMDA applied for 7.5 min at 15-min intervals. Quisqualate (Sigma), a ligand to receptors distinct from those binding kainate and NMDA [32, 33], and quinolinic (Sigma), a possible endogenous ligand to NMDA receptors [31], were also tested. The results were expressed as increments in GnRH release, i.e., the difference between the levels of GnRH release observed immediately before and after the application of a test substance.

Experiment 3. The effect of kainate 50 μM or NMDA 50 μM on GnRH release in vitro was evaluated under different conditions. Controls were obtained in medium containing Ca²⁺ 1.8 mM, Mg²⁺ 1.0 mM, and glycine 0.4 mM. The effects of kainate and NMDA were studied in the presence of D-600 0.1 μM (Medoxy Verapamil; Knoll, Ludwigshafen, FRG), a calcium channel blocker [4], and in medium enriched with Ca²⁺ 4.0 mM or Mg²⁺ 1 μM in using glycine-free medium. In addition, the effect of NMDA was studied in the presence of equimolar concentrations of AP5 (Tocris, Neuraceptors, Ltd., Bathurst Hiltt, UK), a competitive antagonist of NMDA receptors [16]. In these experiments, the basal level of GnRH release was determined under the same conditions as for NMDA or kainate stimulation.

Experiment 4. The in vitro GnRH release induced by NMDA 50 μM was studied in the absence of glycine or in the presence of increasing glycine concentrations (0.05-10.0 μM).

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Fig. 1. Serum LH concentrations (lower panel) and levels of GnRH release from individual hypothalami in vitro (upper panel) in relation to time after subcutaneous injection of NMDA 30 μg/kg or saline in 50-day-old male rats. The significance of differences is indicated by n values.
Experiment 3. The effects of high frequency or continuous stimulation of GnRH release by NMDA were studied by incubating hypothalami in the presence of NMDA 50 mM applied every other 7.5-min period for 120 min or during continuous exposure to NMDA 50 mM for 75 min.

**Results**

As shown in figure 1, in vivo administration of NMDA 30 mg/kg s.c. induced an increase in the secretion of LH in vivo and in the release of GnRH in vitro. When compared to the data obtained 1 min after NMDA injection or 5 min after saline injection, LH serum concentrations and GnRH release in vitro were significantly increased (p < 0.005) 5 min after NMDA injection. The peak level of GnRH release induced by NMDA was observed 7.5 min after NMDA injection, while the rise in LH serum concentrations occurred up to 10 min after NMDA injection.

Incubation of rat hypothalami in vitro in the presence of increasing concentrations of kainate or NMDA resulted in a dose-related stimulation of GnRH release (fig. 2, left panel). At concentrations of 10, 50 and 100 mM, NMDA was about twice as potent (p < 0.05) as kainate in eliciting the release of GnRH. At a 50-mM concentration, quinolinate, a putative endogenous ligand of NMDA receptors, induced a GnRH increment of 34.9 ± 4.9 (SEM) pg/7.5 min (n = 10) which was 2.2-fold greater than the response to 50 mM NMDA (p < 0.001). In contrast, 10 mM quisqualate did not affect the release of GnRH (increment 1.7 ± 1.0 pg/7.5 min; n = 11).

As shown in the right panel of figure 2, the response of GnRH to kainate or NMDA 50 mM was abolished (p < 0.001) in the presence of D-600, a Ca²⁺ channel blocker. When compared to controls obtained in medium containing 1.8 mM Ca²⁺, 1 mM Mg²⁺, and 0.4 mM glycine, the stimulatory effect of 50 mM kainate was not significantly affected by increased concentrations of Ca²⁺ (up to 5.8 mM) or Mg²⁺ (2 mM) or by using glycine-free culture medium. In contrast, the stimulatory effect of NMDA 50 mM was blunted in Ca²⁺-enriched medium (p < 0.001) and abolished (p < 0.001) in the presence of Mg²⁺ 2 mM or when using glycine-free medium. In addition, equimolar concentrations of APV, a competitive antagonist specific of NMDA receptors, suppressed (p < 0.001) the response of GnRH to NMDA (fig. 3).

While no response to 50 mM NMDA was observed in the absence of glycine (fig. 3), the use of low glycine concentrations (0.01-1 μM) maximized the NMDA-induced release of GnRH which was significantly greater (p < 0.001) than the release seen in the presence of higher glycine concentrations (100-10,000 μM).

As shown in figure 4, the increment in GnRH release induced by NMDA 50 mM occurred repetitively when intermittent NMDA stimulation was performed every other 7.5-min period. The amplitude of GnRH response to NMDA remained similar during eight successive stimulations. In contrast, continuous exposure to NMDA 50 mM for 75 min resulted in an initial increase in GnRH release, immediately followed by a decrease back to the baseline level which was unchanged throughout the entire subsequent period of stimulation.

**Discussion**

In this paper, we provide the first direct evidence that neuroexcitatory amino acids trigger the release of GnRH. Several authors have previously suggested that the hypothalamus is the primary site where neuroexcitatory amino acids could affect the secretion of LH. After neonatal exposure of rodents to excitotoxins such as monosodium glutamate, which destroy the vast majority of perikarya in the arcuate nucleus [22], the response of LH to NMDA administration is abolished [23]. Although not tested at concentrations higher than 10 mM, NMDA does not affect the release of LH from rat and monkey pituitary cultures in vitro [30, 33]. In the rat, pituitary hormone secretion including LH is activated by intrahypothalamic injection of quinolinic acid, a ligand to NMDA receptors [21]. More recently, the LH-releasing effect of NMDA in monkey was shown to be prevented by pretreatment with a GnRH receptor antagonist [14].

In the central nervous system, the receptors to excitatory amino acids have been classified in three groups according to the exogenous agonists which bind selectively to these receptors: NMDA, kainate, and quisqualate [32, 35, 36]. Receptors to NMDA and kainate have been demonstrated in the hypothalamus where the concentration of kainate-binding sites is predominant over NMDA-binding sites [9]. A subtype of NMDA receptors is bound by quinolinic acid which is derived endogenously from tryptophan [31, 32]. Among these ligands, NMDA [27, 37], kainate [27], and
Quinoline [17, 21] have been shown to induce the release of pituitary hormones, particularly LH. Our data indicate that both NMDA receptors, particularly NMDA receptors have been implicated in the mechanisms regulating GnRH secretion, while receptors to quinolinate do not seem to play any role. Quinolinate was not effective at 10 μM, while NMDA was consistently effective at the same concentration. A possible effect of quinolinate at higher concentrations has not been excluded. Our data confirm the observations of Price et al. [27] showing that LH release can be mediated by the stimulation of LH release. After the early report of Price et al. [27], the difference between effects mediated through kainate and NMDA receptors has been elucidated. In fact, a series of mechanisms are selectively involved in the activation of NMDA receptors: noncompetitive antagonism by Mg2+ [3, 12], inhibition by elevated Ca2+ concentrations [20], modulation by glycine which potentiates NMDA effects at low concentrations [18], competitive antagonism by AP5 [16], and desensitization during continuous exposure to NMDA [13]. In this paper, we have shown that the NMDA-induced release of GnRH bear all the characteristic of NMDA receptor mediated effects as evidenced in other systems. We have also shown that kainate-induced release of GnRH is mediated through non-NMDA receptors, since this effect is not affected by factors such as glycine or Mg2+ concentrations. While NMDA and kainate are potent excitatory agonists of receptors to excitatory amino acids, quinolinate, instead, represents a possible endogenous ligand to NMDA receptors [25, 31]. On a molar basis, the GnRH-releasing activity of quinolinate is greater than that of NMDA, although the potency of quinolinate has been originally reported to be 10% of the NMDA potency [31].

A major unresolved issue is the significance of our observations for the release of hypothalamic peptides under physiological conditions. In this respect, questions arise from the concentrations of agonists used which may be much higher than the concentrations of endogenous substrates activating NMDA and kainate receptors. For instance, quinolinate has been found at micromolar concentrations in most brain areas [19], while we have used millimolar concentrations in vitro. Furthermore, Nemerooff et al. [21] have observed a stimulation of LH after an intrahypothalamic injection of a quinolinate amount which was 100 times less than the one we have used in vitro. In contrast, glycine and glutamate have been applied by some investigators to the hypothalamus in vivo [10] or in vitro [15] in order to obtain neuroeffectiv effects. In our experiments, repeated exposure to very high concentrations of NMDA (50 μM) for 2 h failed to alter the response of GnRH. Thus, it may be argued that the neurotoxic effect which characterizes the use of high concentrations of excitatory amino acids [22, 23] did not occur during our experiments. This suggests either that, under our experimen- tional conditions, only a small fraction of the amount of agonist used is related to the receptors or that the agonists used have a low receptor sensitivity for the agonists. The first hypothesis is consistent with the high affinity of many excitatory amino acids to divalent cations [3]. Thus, it is possible that some of the agonists are partially complexed with Ca2+ or Mg2+ and inactivated. The second hypothesis is supported by the recent findings of Ciceri et al. [8]. These authors have found that the LH response of male rats to NMDA was maximal between concentrations of 10 μM and 1 mM, and virtually absent at 60 days. This may explain the require- ment of very high concentrations during our experi- ments in 50-day-old rats. The concentrations of Mg2+ used in our experiments may be an alternative explanation to the high concentrations of NMDA required. In some systems, micromolar concentra- tions of glycine concentrations may be sufficient to elicit the effect, since it represents a possible endogenous ligand to NMDA receptors. [25, 31]. On a molar basis, the GnRH-releasing activity of quinolinate is greater than that of NMDA, although the potency of quinolinate has been originally reported to be 10% of the NMDA potency [31].

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Regulatory Activity and Topological Distribution of Folliculo-Stellate Cells in Rat Anterior Pituitary Cell Aggregates

(With 1 color plate)

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Key Words. Folliculo-stellate cells - Reaggregate cell cultures - Prolactin secretion - Growth hormone secretion - Angiotensin II - Dopamine - Somatostatin

Abstract. An enriched population of cells immunoreactive to antisera against S-100 protein, a marker of folliculo-stel-

late (FS) cells in the rat pituitary, was obtained by separation of dispersed pituitary cells from adult female rats by gradient sedimentation at unit gravity. The effect of FS cells on the stimulation and inhibition of prolactin (PRL) and growth hor-
mone (GH) release was studied by coaggregation experiments of the FS cell-enriched population with respectively a lacto-

troph-enriched and a somatostatin-enriched population from adult female rats. The FS cell population not only attenuated the stimulation of PRL and GH release, but also significantly attenuated the inhibition of PRL release by 10, 30 or 300 nM dopamine (DA), and the inhibition of GH release by 0.1 nM somatostatin (SRIF). The stimulation of FS cells on the angiotensin II (AII) on PRL secretion in the presence of DA was also attenuated by the FS cells. Light microscopic evaluation of immu-
nostained semithin sections showed a meshwork of cytoplasmic extensions of FS cells as well as follicular structures in the aggregates. There was no preferential association of FS cells with certain cell types. The permeability of the aggregates to diffusing molecules was tested at the ultrastructural level by the lanthanum hydroxide tracing technique. Lanthanum traced the intercellular gaps over the entire aggregate irrespective of whether the proportional number of FS cells was high or low, indicating that FS cells do not seal off certain areas in the aggregate by the formation of tight junctions. It is suggested that FS cells attenuate the action not only of stimulatory but also inhibitory secretagogues on hormone-secreting cells in the aggregates, which closely resembles that of the intact pituitary.

Although on histological and ultrastructural grounds many possible functions have been ascribed to the folliculo-

stellate (FS) cells in the anterior pituitary [reviewed in 23], the functional significance of these cells remains unclear. Recently, several laboratories have expressed renewed in-

terest in this matter. Ferrara et al. [15] have demonstrated transport epithelial characteristics of bovine pituitary FS cells, grown in monolayer cultures. FS cells produce basic fibroblast growth factor [16]. Most, if not all [21], pituitary FS cells contain the glic protein S-100 [7, 22]. Ishikawa et al. [20] established an S-100-producing clonal cell line from PRL release under tonic inhibition by DA. All these studies were performed using an experimental design in which an FS cell-enriched population was allowed to coaggregate with populations enriched in lactotrophs or somatotrophs, as described previously [2]. Furthermore, we wanted to vali-
date this in vitro model by studying the topological distribu-
tion of FS cells in the aggregates in order to determine whe-
ther FS cells in these three-dimensional structures express

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