Mechanisms mediating the response of GnRH neurones to excitatory amino acids

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Excitatory amino acids, such as glutamate, exert a profound stimulatory effect on the reproductive axis of several mammals. Although glutamate receptor agonists stimulate GnRH secretion, both in vivo and in vitro, it is unclear whether GnRH neurones respond directly to glutamatergic excitation. Immortalized GnRH neurones (GT1 cells) express glutamate receptors when grown in culture and also show enhanced GnRH secretion in response to glutamate receptor agonists. In addition, immunocytochemical evidence at the electron microscope level supports the possibility of a direct interaction between glutamatergic and GnRH neurones. In general, however, double-label histochemical studies (using immunocytochemistry, in situ hybridization, or a combination of these techniques) have not shown significant glutamate receptor gene expression in GnRH neurones of adult animals. It remains to be determined whether a higher degree of glutamate receptor gene expression occurs during development. This general lack, or very low amount, of glutamate receptor gene expression in the GnRH neurones of adults supports the view that excitatory amino acids exert their stimulatory action on the reproductive axis primarily through interneuronal pathways that impinge on the GnRH neurones, rather than by stimulating GnRH release directly.

Acidic amino acids, such as glutamate and aspartate, are generally considered to be the predominant excitatory neurotransmitters within the mammalian central nervous system. They have been implicated in physiological processes such as learning and memory and, because of their excitotoxic potential, in neurodegenerative diseases such as Huntington’s, Parkinson’s and Alzheimer’s diseases, and amyotrophic lateral sclerosis (McDonald and Johnston, 1990; Baron et al., 1995; Kalb, 1995). Because glutamate receptors are most abundant in the hippocampus, cerebral cortex and cerebellum, these areas of the brain were the focus of early radioligand binding studies, while the hypothalamus was largely ignored (Meeker et al., 1994). Nevertheless, it has been known for some time that excitatory amino acids (EAAs) stimulate LH secretion, in rodents and in non-human primates (Ondo et al., 1976; Wilson and Knobil, 1982; Taj et al., 1983; Gay and Plant, 1987). Numerous experiments in vivo have now confirmed the marked stimulatory effects of EAAs on the reproductive axes of several species. For example, in juvenile female rats and also in male rhesus macaques, the pulsatile intravenous administration of N-methyl-D-aspartate (NMDA), an EAA receptor agonist, induces precocious puberty (Urbański and Ojeda, 1987; Plant et al., 1989). In addition, in photoperiodic species such as hamsters, single daily systemic injections of NMDA prevent the regression of the testes that occurs when the animals are transferred from long to short days (Urbański, 1990; Ebling et al., 1995). Support for the idea that endogenous EAAs may be physiologically involved in controlling the mammalian reproductive axis is strengthened by observations that EAA receptor antagonists inhibit the preovulatory LH surge in female rats and significantly delay the onset of puberty (López et al., 1990; Urbański and Ojeda, 1990). In recent years, the number of studies implicating EAAs in the control of reproductive function has increased exponentially (for recent reviews see Brann and Mahesh, 1994; Brann, 1995; Brann et al., 1995; Urbański, 1995; van den Pol et al., 1995). However, it is still unclear exactly where along the hypothalamo–pituitary–gonadal axis EAAs exert their primary stimulatory influence.

**Glutamate receptor subtypes**

Receptors that are activated by EAAs or glutamate are divided into two broad groups, the metabotropic and the ionotropic, the former being G protein-coupled receptors while the latter are ligand-gated ion channels (Hollmann and Heinemann, 1994; Kalb, 1995; Petralia and Wenthold, 1995). It is the ionotropic glutamate receptors that have become the focus of much neuroendocrine research. There are three main classes of ionotropic glutamate receptor and each is named after its principal receptor agonist: NMDA, α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA), and kainate (Table 1).

These receptors are composed of different subunits which are thought to occur in multimeric groups and collectively determine the functional properties of the receptor. The NMDA receptors, for example, are believed to contain an NR1 (also referred to as NMDAR1) subunit and one or more of the following subunits, which together form pentameric complexes: NR2A, NR2B, NR2C or NR2D. In addition, because of possible alternate splicing of the primary RNA transcripts, the presence of numerous variations is possible (Hollmann and Heinemann, 1994). Similarly, AMPA receptors are thought to be multimeric complexes consisting of combinations of subunits GluR1, GluR2, GluR3 and GluR4 (originally referred to as GluRA, GluRB, GluRC and GluRD). As with the NMDA receptors, the specific combination of subunits determines the functional properties of...
the AMPA receptor complex. Although functional kainate receptors are likely to be composed of various combinations of GluR5, GluR6, GluR7, KA1 and KA2 subunits, their structure and biochemical characteristics remain to be elucidated (for review see Hollmann and Heinemann, 1994). In general, the AMPA and kainate receptors underlie fast excitatory postsynaptic potentials and allow the permeation of Na⁺ and K⁺ ions, although combinations of AMPA subunits that do not include GluR2 are also thought to be permeable to Ca²⁺ ions. The NMDA receptors operate over a slower period but are highly permeable to Ca²⁺ ions (Hollmann and Heinemann, 1994; Kalb, 1995; Petralia and Wenthold, 1995). With the recent cloning of cDNAs encoding EAA receptors and the commercial availability of antibodies to many of the subunits, it has become technically feasible to explore the distribution of glutamate receptors within specific neurones, including those that produce GnRH. However, because there is little published information regarding the role of metabotrophic EAA receptors in neuroendocrine function (Brann and Mahesh, 1994; Brann, 1995; Petralia and Wenthold, 1995) this review will focus on recent studies aimed at disclosing the presence of ionotropic glutamate receptors in GnRH neurones.

Table 1. Classification of major ionotropic glutamate receptors

<table>
<thead>
<tr>
<th>Subtype</th>
<th>Subunits</th>
<th>Agonists</th>
<th>Antagonists</th>
</tr>
</thead>
<tbody>
<tr>
<td>NMDA</td>
<td>NR1, NR2A, NR2B, NR2C, NR2D</td>
<td>Glu, NMDA, Gly</td>
<td>D-AP5, MK-801, CPP</td>
</tr>
<tr>
<td>AMPA</td>
<td>GluR1, GluR2, GluR3, GluR4</td>
<td>Glu, AMPA, KA, QA</td>
<td>Quinoxalinediones</td>
</tr>
<tr>
<td>Kainate</td>
<td>GluR5, GluR6, GluR7, KA1, KA2</td>
<td>Glu, KA, QA, DM</td>
<td>Quinoxalinediones</td>
</tr>
</tbody>
</table>

AMPA: α-amino-3-hydroxy-5-methylisoxazole-4-propionate; D-AP5: 2-amino-5-phosphonopentanoic; CPP: 3-(2-carboxypropyl)aziridine-4-carboxylate; DM: domoate; Glu: glutamate; Gly: glycine; KA: kainate; MK-801: (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine hydrogen maleate; NMDA: N-methyl-D-aspartate; QA: quisqualate.


Supra-pituitary site of action for excitatory amino acids

Although there is some evidence to suggest that the anterior pituitary gland may express glutamate receptors, it is questionable whether these receptors function in the same capacity as those present within the hypothalamus (Onondo et al., 1976, 1988; Meeker et al., 1994; Bhat et al., 1995). Most of the published data supports the view that EAs influence reproductive neuroendocrine function by acting primarily at a supra-pituitary level. In vitro studies using rat hypothalamic explants demonstrate that GnRH secretion can be stimulated by EAA receptor agonists and inhibited by specific antagonists (Bourguignon et al., 1989a, b; López et al., 1992). Similarly, EAs stimulate GnRH secretion from immortalized GnRH neurones (that is, GT-1-1 cells; Mahachoklertwattana et al., 1994). In vivo, NMDA has been shown to significantly increase cellular concentrations of mRNA coding for GnRH in male rats (Petersen et al., 1991) and, when infused into the medial preoptic nucleus (in the vicinity of the GnRH neuronal perikarya), it was more effective at stimulating LH release than when infused into the hypothalamus itself (Ondo et al., 1988). In female rats, glutamate release is enhanced in the preoptic area at the time of the preovulatory LH surge and may play a causal role in stimulating GnRH secretion (Jarry et al., 1995). In addition, it has been demonstrated using in vivo push-pull perfusion that NMDA stimulates GnRH secretion in the stalk-medium eminence of pre- and peripubertal female rhesus macaques (Claypool and Terasawa, 1988). Finally, it has been shown in rats and hamsters (Urbański, 1990; Luderer et al., 1995), as well as in rhesus macaques (Fig. 1) (Gay and Plant, 1987; Plant et al., 1989), that the stimulatory effects of NMDA on LH secretion are blocked or significantly attenuated by the concomitant administration of a GnRH receptor antagonist; presumably because the stimulatory effect of NMDA is mediated primarily through enhanced GnRH secretion. Taken together, these findings suggest that the stimulatory influence of EAs on gonadotrophin secretion is exerted primarily on the GnRH neuronal system. Nevertheless, it has not yet been determined whether this excitation influences GnRH secretion directly by acting through receptors located on the GnRH neurones themselves or whether interneurones or glial cells play an intermediary role.

Direct interaction between excitatory amino acids and GnRH neurones

Glutamatergic nerve fibres are abundant in the hypothalamus and preoptic area and most of the neurones in these areas can be depolarized with EAs (van den Pol et al., 1990, 1995). These observations alone warrant the assumption that a direct interaction between glutamatergic neurones and GnRH neurones is likely. More direct evidence in support of this hypothesis is the immunocytochemical demonstration, at the electron microscope level, that GnRH neurones of macaques closely interact with glutamate-immunoreactive neurones of the hypothalamus (Goldsmith et al., 1994; Thind and Goldsmith, 1995). On the basis of these findings, it is possible that GnRH neurones show a high expression of the glutamate receptor gene. Indeed, in situ hybridization studies have already established that mRNA encoding EAA receptors of both the NMDA and non-NMDA subtypes is present in the hypothalamus and in brain areas that contain GnRH neuronal perikarya (Urbański et al., 1994; van den Pol et al., 1994), although it should be emphasized that the expression is considerably lower than that in the hippocampus or cerebral cortex (Fig. 2). However, because GnRH neuronal perikarya are scattered throughout various forebrain regions, rather than being confined to discrete nuclei, it is impossible to establish with single-label histochemistry whether they express EAA receptors. Similarly, because of this characteristic diffuse distribution of GnRH neurones in the forebrain, electrophysiological examinations of whether they can be depolarized by EAs are not practicable (the electrical recordings need to be performed...
blind’, without a priori knowledge of whether the individual neurones being examined are GnRH-immunopositive). One way of overcoming this problem has been to study glutamate gene expression in immortalized GnRH neuronal cell lines, such as GT1 cells (especially the GT1-1 and GT1-7 subtypes). These were produced originally in mice by specifically targeting expression of the oncogene SV40 T-antigen to neurones that express the GnRH promoter (Mellon et al., 1990). Numerous investigators have demonstrated that these cells have the capacity to both synthesize and secrete GnRH and to respond to depolarizing stimuli (for review see Wetsel, 1995). Furthermore, they can be cultured on microscope slides and processed for histochemical staining (Fig. 3a). In addition, they can be grown on a three-dimensional matrix, such as one consisting of collagen-coated beads, and their secretion of GnRH can thus be studied in a dynamic perfusion system (Fig. 3b). By taking advantage of the high density at which GT1 cells can be grown, it has been possible to extract sufficient mRNA from monocultures to perform northern blot analysis using molecular probes specific to glutamate receptors. At least two laboratories have used northern blot analysis to demonstrate the expression of the NR1 receptor subtype in GT1 cells (Mahachoklertwattana et al., 1994; Urbariski et al., 1994). This finding is corroborated by the in situ hybridization and northern blot data shown in Fig. 3c and d, respectively. Studies have also shown that these cells respond to NMDA in vitro by releasing GnRH (Mahachoklertwattana et al., 1994; Spergel et al., 1994). Taken together, these findings

Fig. 1. Plasma LH concentrations in female rhesus macaques, during the luteal phase of their menstrual cycle. Blood samples were collected remotely, at 10 min intervals, through indwelling jugular vein catheters and plasma LH was determined using a mouse Leydig cell bioassay. At time 0, the animals received an intravenous injection of either (a) saline, or (b) and (c) N-methyl-D-aspartate (NMDA); the NMDA was administered in the form of a racemic mixture (20 mg kg\(^{-1}\) body mass). In addition, one of the NMDA-treated groups (c) received an intravenous injection of a GnRH receptor antagonist ([D-Phe\(^2\), Pro\(^3\), D-Phe\(^6\)]LHRH; 0.4 mg kg\(^{-1}\) body mass) at time –20 min. Each point represents the mean of four or five animals and the SEM are shown as vertical lines. Note that in the presence of the GnRH receptor antagonist the plasma LH response to NMDA was significantly attenuated.

Fig. 2. In situ hybridization histochemistry of coronal hamster brain sections using \(^{35}\)S-labelled cRNA probes specific to NR1 and GluR1 receptor subunits. The autoradiographic images were obtained by apposing the hybridized sections to \(\beta\)max Hyperfilm (Amersham Corporation, Arlington Heights, IL). Compared with the hippocampus (hip) or cerebral cortex (ctx), very little hybridization is evident in the medial septum (ms), an area rich in GnRH neuronal perikarya. More hybridization is evident in the median septum (ms), an area rich in GnRH neuronal perikarya. More hybridization is evident in the hypothalamus (hyp) in the vicinity of GnRH neuronal fibres, the most intense hybridization being in the ventromedial and arcuate regions.
are consistent with the hypothesis that GnRH neurones express functional EAA receptors.

**Indirect interaction between excitatory amino acids and GnRH neurones**

Although the evidence obtained from GT1 cells suggests that glutamatergic neurones interact directly with GnRH neurones in vitro, the findings should be treated with caution. First, because the GT1 cells are transformed cells and are typically grown in monoculture, in the absence of glia or normal target tissue, they may not necessarily express the same genes as GnRH neurones in vivo. Second, although GT1-1 and GT1-7 cells can be cultured readily in enormous quantities it should not be overlooked that ultimately they are each descendants of only a single immortalized GnRH neurone, which may or may not be representative of the GnRH neuronal population as a whole. Third, because the GT1 cells were transformed at a very early stage of development, their gene expression and function may not be truly reflective of fully differentiated and mature GnRH neurones in vivo. Mindful of these limitations, workers in several laboratories have attempted to examine EAA receptor gene expression in GnRH neurones that have matured naturally in vivo, by performing double-label histochemistry on brain sections (Munro and Urbanski, 1994; Munro et al., 1994; Ulibarri et al., 1994; Abbud and Smith, 1995; Urbanski et al., 1995; Eyigor and Jennes, 1996). One such approach has been to combine immunocytochemistry for GnRH with in situ hybridization histochemistry for EAA receptors (Fig. 4). Although this double-labelling technique is quite feasible, certain precautions need to be taken to ensure maximum preservation of the mRNA within the tissue. For example, in our laboratory we perfuse-fix brains using 4% (w/v) paraformaldehyde fixative in borate buffer (pH 9.5). Immunocytochemistry is performed first, on free-floating frozen sections, 20–30 µm thick, using diethyl pyrocarbonate-treated water for all of the antibody solutions and buffers. The sections

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**Fig. 3.** Glutamate receptor gene expression in two GnRH-producing mouse cell lines (GT1 cells) (a) Immunocytochemistry performed on cultured GT1-1 neurones showing strong positive GnRH immunostaining (using monoclonal HU4H antibody, the avidin–biotin–peroxidase complex, and 3,3'-diaminobenzidine tetrachloride as the chromogen). Scale bar represents 50 µm. (b) Scanning electron photomicrograph of GT1-7 neurones cultured on collagen-coated beads (approximate diameter: 175 µm; Cytodex, Pharmacia LKB Biotechnology, Piscataway, NJ) showing a dense three-dimensional network. (c) In situ hybridization histochemistry performed on cultured GT1-7 neurones, showing a strong hybridization signal (using a digoxigenin-labelled antisense cRNA probe of approximately 450 bp that is specific to the NR1 receptor subtype). Scale bar represents 50 µm. (d) Northern blot analysis of RNA extracted from GT1-1 and GT1-7 neurones and hybridized with a 450-bp 32P-labelled cRNA probe specific to the NR1 receptor subtype. The arrow indicates an mRNA band of approximately 4.2 kb. Lanes 1 and 3 were loaded with 10 µg of total RNA from GT1-1 and GT1-7 neurones, respectively, while lanes 2 and 4 were loaded with 10 µg of poly(A)+ RNA from GT1-1 and GT1-7 neurones, respectively.
Excitatory amino acids and GnRH neurones

Fig. 4. Examples of double-label histochemistry used to examine the expression of glutamate receptors by GnRH neurones of rhesus macaques. (a – d) In situ hybridization histochemistry for the GluR1 glutamate receptor subunit combined with immunocytochemistry on rhesus macaque brain sections. Bright-field photomicrographs are shown in (a) and (b) and corresponding dark-field photomicrographs are depicted in (c) and (d). Dense silver grain deposition (c) is clear over a small population of GnRH immunopositive neurones found in the extreme lateral portion of the horizontal limb of the diagonal band of Broca (indicated by black arrows in (a)). In contrast, the amount of silver grain deposition (d) over the GnRH immunopositive neurones of the arcuate region (indicated by white arrows) in (b) is similar to the background amount. For the immunocytochemistry, 3,3'-diaminobenzidine tetrachloride was used as the chromogen (brown) and thionin was used as a Nissl counter stain (blue). (e, f) In situ hybridization histochemistry for GnRH combined with immunocytochemistry for GnRH on rhesus macaque brain sections. Bright-field photomicrographs showing dense silver grain deposition over immunopositive (brown) neurones, confirming that both the lateral (e) and arcuate (f) populations produce mRNA coding for GnRH, despite having a different perikaryal morphology. Scale bar represents 50 µm.
are then mounted on RNase-free glass microscope slides and processed for in situ hybridization histochemistry. So far, we have used this double-label histochemical approach to examine several thousand immunopositive GnRH neurones for evidence of GluR1, GluR2, GluR3 or NR1 EAA receptor subunit gene expression, both in male hamsters and in female rhesus macaques. Overall, we have been unable to detect any significant expression, both in male hamsters and in female rhesus macaques. We have used this double-label histochemical approach to examine several thousand immunopositive GnRH neurones for evidence of GluR1, GluR2, GluR3 or NR1 EAA receptor subunit gene expression, both in male hamsters and in female rhesus macaques. Overall, we have been unable to detect any significant expression of RNA encoding EAA receptor in GnRH neurones of any adult animal (Munro et al., 1994; Urbanński et al., 1995), except in a small population of GnRH neurones that lie in the extreme lateral portion of the diagonal band of Broca of the rhesus macaque brain (Fig. 4a, c). Although these GnRH neurones are immunopositive for GnRH and show strong in situ hybridization to a monkey GnRH cRNA probe (Fig. 4e) (Rodrigues et al., 1996), their perikarya do not show the usual fusiform morphology characteristic of mature GnRH neurones (Fig. 4b, d, f); instead, their perikarya are more spherical and resemble those of embryonic GnRH neurones before they begin their migration from their place of origin in the olfactory placode (Schwanzel-Fukuda et al., 1992). A slightly different double-labeling approach was adopted by Abbud and Smith (1995), who used a digoxigenin-labelled cRNA probe to identify GnRH neurones of adult male and female rats, and an 35S-labelled cRNA probe to identify mRNA encoding for NR1 receptor subunit. When compared with non-GnRH neurones in the hypothalamus or cerebral cortex, the NR1 hybridization in GnRH neurones was negligible: fewer than 5% of the GnRH neurones were considered to be double-labelled. Similar results were reported by Eyigor and Jennes (1996) and L. Jennes (personal communication), who used double-label fluorescence immunocytochemistry as well as a combination of immunocytochemistry and in situ hybridization histochemistry to examine the expression of a wide spectrum of NMDA, AMPA and kainate receptor subunits in the GnRH neurones of rats. Few, if any, of the GnRH neurones appeared to express the NR1, NR2A, NR2B, NR2C, NR2D, GluR1, GluR2, GluR3 or GluR4 receptor subunits. The only notable expression was that of the KA2 receptor subunit: 32% of the 484 GnRH neurones examined showed positive colocalization. Other studies have relied on double-label fluorescence immunocytochemistry using commercially available antibodies to specific glutamate receptor subunits (Munro and Urbanński, 1994; Urbanński et al., 1995). Several hundred immunopositive GnRH neurones were examined in adult male hamsters and few, if any, showed GluR1, GluR2/3, GluR4 or NR1 immunopositive staining. The main advantage of using double-label fluorescence immunocytochemistry over combined in situ hybridization histochemical approaches is that it helps to establish the extent to which the mRNA encoding the glutamate receptor is translated; also, from a practical perspective it avoids problems associated with the maintenance of an RNase-free environment. However, trying to colocalize glutamate receptor immunoreactivity within GnRH immunopositive neurones is associated with its own set of problems. First, most of the glutamate receptor immunoreactivity occurs in the periphery of the neurone, in association with the cell membrane, whereas the GnRH immunoreactivity typically occurs within the cell body itself, making visualization of colocalization difficult; this problem can be overcome to some extent by using confocal microscopy (Fig. 5; NR1, GluR1, GluR2/3). Second, because secretory neuropeptides such as GnRH are usually present only within perikarya, axons and terminals, any glutamate receptors that are localized postsynaptically on GnRH neuronal dendrites may escape detection. Of all the recent published studies aimed at disclosing glutamate receptor gene expression in GnRH neurones of adult mammals, only one claims to have observed significant colocalization (Ulibarri et al., 1994). With the use of in situ hybridization histochemistry, 28.9% of immunopositive GnRH neurones were shown to express significant mRNA encoding GluR1 in ovariectomized rats, and the percentage increased to 47.5% after treatment with oestradiol. It is unclear whether the discrepancy between these and the previous data stems from differences in gonadal status of the animals or from differences in the specific glutamate receptor probes that were used. However, it may also be significant that the study in which significant GluR1 gene expression was observed in GnRH

Fig. 5. (a) NR1, (b) GluR1, (c) GluR2/3) Double-label fluorescence confocal microscopy demonstrating a general lack of excitatory amino acid receptors (red) in GnRH neurones (green) of Syrian hamsters; colocalization of both fluorophores (tetramethyl rhodamine and fluorescein isothiocyanate, respectively), had it been present, would have appeared in yellow. The antibodies to NR1 (that is, NMDAR1), GluR1, and GluR2/3 were all rabbit polyclonals and are commercially available (Chemicon International Inc., Temecula, CA). The GnRH antibody was a mouse monoclonal, HU4H, produced in our laboratory. Scale bar represents 50 µm.
Given that so many neurones in the hypothalamus respond to EAAs (van den Pol et al., 1995), it is surprising that GnRH neurones appear to show so little glutamate receptor gene expression, especially when compared with neurones of the hippocampus. One possible reason for this difference is that GnRH neurones, unlike other hypothalamic neurones, originate outside of the brain, in the olfactory placode, and migrate in during embryonic development (Schwanzel-Fukuda et al., 1992). Alternatively, the general lack of glutamate receptor expression in GnRH neurones may reflect sensitivity limitations of the double-labelling techniques used, and low amounts of expression may be disclosed in the future as more sensitive techniques become developed. However, it should be emphasized that without supporting electrophysiological data any positive colocalization of mRNA encoding glutamate receptor or corresponding protein in GnRH neurones does not in itself prove that functional receptors are expressed. For example, cerebellar Purkinje cells clearly express abundant amounts of mRNA encoding both NRI receptor and protein but, in numerous electrophysiological studies, these cells have failed to show strong electrical responses to NMDA and its antagonists (Garyfallou et al., 1996).

If the primary influence of endogenous glutamate on GnRH secretion is indirect then which interneurones play a role? Both stimulatory and inhibitory circuits are known to influence GnRH secretion (Fig. 6). Furthermore, because glutamate is ubiquitous within the mammalian central nervous system, it may exert some degree of influence on all of them. It is likely, therefore, that the overall response of GnRH neurones to EAAs is context dependent, showing either a net increase or decrease depending on the physiological state of the animal and the relative strength of the underlying stimulatory and inhibitory inputs (Fig. 6). In support of this idea are results from rhesus macaques showing that NMDA can markedly stimulate LH secretion in the early follicular and luteal phases of the menstrual cycle, but not in the midfollicular phase. Moreover, in ovariectomized macaques NMDA strongly inhibits LH secretion (Reyes et al., 1990, 1991; Urbatski and Garyfallou, 1995). Exactly which neurotransmitters play a principal role in mediating the effects of EAAs on GnRH secretion is unclear. However, it is well established that catecholaminergic neurones form synapses with GnRH neurones and that noradrenaline can stimulate GnRH secretion. In addition, it has been demonstrated in rodents that NMDA induces expression of the immediate early gene c-fos, an acute marker of neuronal activation, in catecholaminergic neurones of the locus coeruleus (Saitoh et al., 1991), but not in GnRH neurones (Abbud and Smith, 1995; Ebling et al., 1995; Urbatski, 1995). In addition, mRNA encoding NMDA receptor has been demonstrated in this nucleus (Luque et al., 1995); because efferents from the locus...
coeruleus represent one of the major sources of noradrenaline into the hypothalamus, these neurones are anatomically well placed to influence GnRH secretion. Further support that the primary influence of EAs on GnRH secretion may be mediated through noradrenaline comes from the observation that the stimulatory effect of NMDA on LH secretion in rats is blocked by the concomitant administration of prazosin, an α₁-adrenergic receptor antagonist (Suh et al., 1994). The importance of such indirect stimulatory pathways on gonadotrophin secretion in other species is presently unclear; although the locus coeruleus of rhesus macaques also appears to express glutamate receptors (Urbański and Kohama, 1996), the administration of prazosin at doses that obliterate pulsatile LH secretion in ovariectomized animals appears to have little, if any, effect at suppressing NMDA-doses that obliterate pulsatile LH secretion in ovariectomized rhesus macaques also appears to express glutamate receptors (Urbański and Garyfalliou, 1995). The reason for the discrepancy between the responses to NMDA/prazosin in rats and monkeys is unclear but may stem from differences in the anatomical distribution of the GnRH neurones, as well as differences in their morphology. In rats, the GnRH neurones rarely possess more than one or two neurites and their perikarya are generally less fusiform in shape compared with GnRH neurones in monkeys. In rats, the perikarya are located primarily in the preoptic/media1l septum and in the diagonal band of Broca, whereas in monkeys, a large population is also located more caudally in the hypothalamus, especially in the arcuate region, which is itself rich in glutamate receptors. Although the involvement of other stimulatory and inhibitory interneurones in glutamate-mediated GnRH secretion is highly plausible, this remains to be established experimentally.

Conclusions

Excitatory amino acids, such as glutamate and aspartate, are distributed widely throughout the mammalian central nervous system and probably exert some stimulatory influence on most neurones, including those that secrete GnRH. Whether a direct stimulation of GnRH neurones is the primary route by which EAs influence gonadotrophin secretion is not entirely clear. There is evidence to suggest that EAs may be involved in controlling the neural circuits associated with time measurement, especially in photoperiodic seasonal breeding species (Colwell and Menaker, 1995). Some of the long-term stimulatory effects of EAs on the reproductive axis in hamsters, therefore, may be attributed to this relatively indirect pathway (Urbański, 1990, 1995). However, the rapid and short-lived stimulatory effect of EAs on gonadotrophin secretion (Fig. 1) is most likely mediated either directly through the GnRH neurones themselves or through other neurotransmitter circuits closely associated with GnRH secretion (Fig. 6).

Overall, the body of published evidence does not provide strong support for glutamate receptor gene expression in a significant number of GnRH neurones, at least not in adult mammals, suggesting that the stimulatory effects of excitatory amino acids on GnRH secretion are likely to be mediated indirectly. In contrast to GnRH neurones in vivo, immortalized GT1 cells appear to express abundant mRNA encoding the glutamate receptor. One possibility for this discrepancy is that the GT1 cells represent GnRH neurones that were immortalized at a very immature stage of development. It may be significant that the only GnRH neurones of adult rhesus macaques that expressed significant amounts mRNA encoding GluR1 mRNA (Fig. 4a, c) were the ones that had a relatively simple non-fusiform morphology. Moreover, preliminary double-immunocytochemical observations of GnRH neurones in the olfactory placode of fetal macaques (before their developmental migration to the preoptic/medial septal area and hypothalamus) suggest that NMDA and AMPA receptor gene expression is greater than in the adult (H.F. Urbański, S.G. Kohama and O. Ronneklev, unpublished). Similarly, double-immunocytochemical observations made in peripubertal female rats suggest that approximately 36% of the GnRH neurones express the NRI receptor subunit (Gore et al., 1996). Therefore, as with other brain neural circuits (Kalb, 1995), EAs may play an important role in GnRH neuronal migration, elaboration of GnRH neural circuits and activity-dependent GnRH neurone maturation in general. In adults, EAs are clearly capable of exerting profound stimulatory effects on gonadotrophin secretion. So far, however, it has not been convincingly demonstrated that EAs play an important role in directly modulating the pattern of GnRH secretion. However, if interneurones are involved in mediating the response, these also remain to be elucidated.

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Key references are identified by asterisks.


Bourguignon J-P, Gérard A and Franchimont P (1989a) Direct activation of gonadotropin-releasing hormone secretion through different receptors to neurotransmitter amino acids Neuroendocrinology 49 402–408


References

Eigsti O and Jensen L (1996) Identification of glutamate receptor subtype mRNAs in gonadotropin-releasing hormone neurons in rat brain Endocrine 4 133–139

Garyfallou VT, Kohama SG and Urbatski HF (1996) Distribution of NMDA and AMPA receptors in the cerebellar cortex of rhesus macaques Brain Research 716 22–28


Goldsmith PC, Thind KK, Perera AD and Plant TM (1994) Glutamate-immunoactive neurons and their gonadotropin-releasing hormone neuronal interactions in the monkey hypothalamus Endocrinology 134 858–866


Kalb RG (1995) Current excitement about the glutamate receptor family Neuroscience Update 1 60–63


McDonald JW and Johnston MV (1990) Physiological and pathophysiological roles of excitatory amino acids during central nervous system development Brain Research Reviews 15 41–70

Mahachoklertwattana P, Sanchez J, Kaplan SL and Grumbach MM (1994) N-methyl-D-aspartate (NMDA) receptors mediate the release of gonadotropin-releasing hormone (GnRH) by NMDA in a hypothalamic GnRH neuronal cell line (GT1-1) Endocrinology 134 1022–1030

Meeker RB, Glende-Robins LF and Hayward JN (1994) Glutamate receptors in the rat hypothalamus and pituitary Endocrinology 134 621–629


Munro G and Urbatski HF (1994) Double immunofluorescence staining of excitatory amino acid receptors and luteinizing hormone-releasing hormone (LHRH) neurons in the male Syrian hamster Journal of Reproduction and Fertility Abstract Series 14 16

Munro G, Garyfallou VT, Kohama SG and Urbatski HF (1994) Lack of NMDAR1 expression in luteinizing hormone-releasing hormone neurons of the male Syrian hamster Society for Neuroscience Abstracts 20 1739


Ono JD, Wheeler DD and Dom RM (1988) Hypothalamic site of action for N-methyl-D-aspartate (NMDA) on LH secretion Life Sciences 43 2283–2286


Phelan TD, Gay VL, Marshall GR and Aslan M (1989) Puberty in monkeys is triggered by chemical stimulation of the hypothalamus Proceedings of the National Academy of Sciences USA 86 2506–2510


Reyes A, Xia L and Ferin M (1991) Modulation of the effects of N-methyl-D,L-aspartate on luteinizing hormone by the ovarian steroids in the adult rhesus monkey Neuroendocrinology 54 405–411


Ulbrizzi C, Cao Z and Akesson TR (1994) Estrogen regulates GluR1 mRNA in GnRH immunoreactive neurons of the rat Society for Neuroscience Abstracts 29 202

*Urbatski HF (1990) A role for N-methyl-D-aspartate receptors in the control of seasonal breeding Endocrinology 127 2225–2228


Urbatski HF and Garyfallou VT (1995) Stimulation of LH secretion in female rhesus macaques by N-methyl-D-aspartate (NMDA) is not mediated by an adrenergic pathway Biology of Reproduction 52 (Supplement 1) 156


Urbatski HF and Ojeda SR (1987) Activation of luteinizing hormone-releasing hormone release advances the onset of female puberty Neuroendocrinology 46 273–276

Urbatski HF and Ojeda SR (1990) A role for N-methyl-D-aspartate (NMDA) receptors in the control of LH secretion and initiation of female puberty Endocrinology 126 1774–1787


*van den Pol AN, Wuarin J-P and Dudek FE (1990) Glutamate, the dominant excitatory transmitter in neuroendocrine regulation Science 250 1276–1277


Wedekind WC (1993) Immortalization of hypothalamic luteinizing hormone-releasing hormone (LHRH) neurons: a new tool for dissecting the molecular and cellular basis of LHRH physiology Cellular and Molecular Neurobiology 15 43–78