Exp Brain Res (2000) 133:532–537
DOI 10.1007/s002210000425

RESEARCH ARTICLE

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AMPA receptors colocalize with neuropeptide-Y- and galanin-containing, but not with dopamine, neurons of the female rat arcuate nucleus: a semiquantitative immunohistochemical colocalization study

Received: 15 November 1999 / Accepted: 13 March 2000 / Published online: 21 June 2000

Abstract It is well established that excitatory amino acid (EAA) neurotransmission is an essential component in the regulation of the gonadotropin-releasing hormone (GnRH) delivery system. However, the morphological interconnection of these systems is not fully understood. The objective of the present study was to determine whether or not alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors – as indicators of aspartate/glutamatergic innervation – are present in the major neuronal populations, such as the neuropeptide-Y- (NPY), galanin- (GAL) and tyrosine-hydroxylase- (TH) containing neurons of the arcuate nucleus (AN) of the female rat. Colocalization experiments using the "mirror" technique demonstrated that: (1) AN neurons containing GluR1 are also immunoreactive (IR) for GluR2/3; (2) 38.32% of AMPA-IR cells contain NPY and 31.72% of AMPA-containing neurons are also IR for GAL; in turn, 79.41% of NPY- and 56.19% of GAL-containing neurons are IR for AMPA receptors; none of the neurons are IR for both AMPA receptors and TH. These data suggest that an excitatory aspartate/glutamatergic input is implicated in the regulation of the examined neuropeptide-containing AN neurons but not in that of TH-IR cells of the same area.

Key words Arcuate nucleus · GluR · Neuropeptides · Immunostaining · Colocalization · Female rat

Introduction

It is well established that medial preoptic area (MPO) gonadotropin hormone-releasing hormone (GnRH)-producing neurons are the principal regulators of pituitary gonadotropin (Gn) release. However, these cells are not direct targets of ovarian steroids (Shivers et al. 1983). Thus, the feedback mechanism regulating baseline luteinizing hormone (LH) levels during diestrus and the midcycle LH surge has been suggested to involve other estrogen-sensitive neurons. It has been demonstrated that beta-endorphin (beta-END) cells of the AN play an important role in the regulation of GnRH release and that the activity of beta-END cells is further regulated by other neuron populations. Therefore, it has been postulated that this is an estrogen-sensitive neuronal circuit within the AN which regulates GnRH release through either excitatory or inhibitory innervation of beta-END neurons that, in turn, act as direct inhibitors of myeloperoxidase (MPO) GnRH cells (Leranth et al. 1988). This regulatory effect is facilitated by galanin- (GAL) and neuropeptide-Y- (NPY) containing neurons (Horvath et al. 1995; Xu et al. 1993), while tyrosine-hydroxylase-immunoreactive (TH-IR) cells that terminate on AN beta-END neurons (Horvath et al. 1992b) (known to be exclusively dopaminergic within the AN) enhance GnRH release.

It is described that the EAAs aspartate/glutamate have a facilitatory influence on pituitary LH release when administered subcutaneously or into the third ventricle (Brann and Mahesh 1992, 1994; Olney et al. 1976; Ondo et al. 1976). However, it is not known which of the neuropeptide-containing neurons of the AN are the targets of the aspartate/glutamatergic afferentation. Several lines of data show that with the exception of a minor subset of GnRH-producing neurons [only about 6% of them contain EAA, specifically *N*-methyl-D-aspartic acid (NMDA) receptors], none of the alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor subunits are present within these cells (Eyigor and Jennes 1996; Urbanski et al. 1996). Therefore, it has been suggested that EAAs exert their effects on GnRH neurons indirectly (Saitoh et al. 1991; Lee et al. 1993).

Aspartate/glutamate-containing axon terminals have a widespread distribution in the AN (van den Pol 1991), where they act on the ionotropic AMPA receptors (Brann

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and Mahesh 1994; van den Pol 1991; Halpain et al. 1984). Because of the high number of AMPA receptor-containing neurons within the AN (Wenthold et al. 1992), it is likely that the AN neurons involved in the regulation of pituitary Gn release are also affected by EAA input. In one of our recent experiments, we found that beta-END cells of the AN contain AMPA receptors (unpublished). On the other hand, approximately one-third of GluR-expressing neurons of the AN were found to contain beta-END. Thus, it was of interest to elucidate which of the non-beta-ENDergic neuropeptide-containing neurons of the AN contain AMPA receptors.

The present immunohistochemical study provides evidence for the colocalization of AMPA receptor subunits GluR1 and GluR2/3 with NPY and GAL, but not with TH, within the mediobasal hypothalamus of the female rat.

Materials and methods

Animals and tissue preparation

Four immunohistochemical colocalization studies were conducted (colocalization of GluR2/3 with GluR1, NPY, GAL and TH) using five normal cycling adult female Sprague-Dawley rats (200–250 g body weight) for each study. Animals were kept under standard laboratory conditions, with tap water and regular rat chow ad libitum in a 12-h light, 12-h dark cycle. Twenty-four hours before sacrifice, under deep ketamine (75 mg/kg, i.m.) anesthesia, animals were fixed in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA) and, using a Hamilton microsyringe (Hamilton Co., Reno, NV), a single injection of colchicine (80 µg in 20 µl saline) was applied into the lateral ventricle to enable perikaryal labeling of NPY and GAL. Rats were killed 24 h later under deep ether anesthesia by transcardial perfusion with 50 ml heparinized saline followed by 250 ml fixative [4% paraformaldehyde, 0.1% glutaraldehyde and 15% saturated picric acid, in 0.1 M phosphate buffer (PB), pH 7.4]. Brains were removed from the skull and a tissue block containing the entire hypothalamus was dissected out and postfixed for 2 h in a similar, but glutaraldehyde-free fixative. After fixation, the tissue blocks were rinsed several times in PB and, in order to recognize the two sides of the slices, they were trimmed asymmetrically with a razor blade. Sixty-micometer-thick coronal vibratome sections were cut from the entire rostrocaudal extent of the AN, and the adjacent sections were arranged in pairs. This was followed by 4×15-min rinses in PB. In order to eliminate unbound aldehydes, sections were incubated for 20 min in 1% sodium borohydride, then washed 6×7 min in PB.

Immunostaining

One section of each pair was immunostained for GluR2/3, whereas their counterparts were single immunostained for either GluR1, NPY, GAL or TH. Sections were incubated overnight at room temperature in primary antisera (rabbit-anti-GluR1, -GluR2/3: 1:500, Chemicon; mouse-anti-TH: 1:5000, Chemicon; rabbit-anti-NPY: 1:20,000; rabbit-anti-GAL: 1:5000, Peninsula Laboratories, San Carlos, CA; all in PB containing 1% normal serum produced in the species of the second antibodies). After several rinses in PB, sections were further incubated in biotinylated goat-anti-rabbit second antibody for GluR1, GluR2/3, NPY and GAL; in biotinylated horse-anti-mouse second antibody for TH (all at a dilution of 1:250 in PB, at room temperature, for 2 h). A subsequent rinse in PB for 3×10 min was followed by an incubation in avidin-biotin-peroxidase, at room temperature, for 2 h (ABC Elite Kit, Vector Labs). After 3×10-min rinses in PB, the immunoreaction was visualized as brown by a diaminobenzidine reaction. Pairs of sections were thoroughly rinsed in PB and mounted with their matching surfaces on the upper side. Sections were then dehydrated through increasing ethanol concentrations and, finally, coverslipped.

Colocalization

The "mirror" technique allows immunostaining for two different cytoplasmic antigens within a cell that is vibratome-cut into two halves. The immunostained material was examined at the light microscopic level. Focusing on the upper surface of each section, at a magnification of ×200, camera lucida drawings were made using a drawing tube attached to an Olympus, BH-2 microscope. Examination of the corresponding areas on the adjacent sections allowed us to determine to what extent the GluR2/3-containing neurons also express GluR1, NPY, GAL and TH, and vice versa. Then, the number of cell bodies immunoreactive for both GluR2/3 and one of the examined antigens were counted and expressed as percentages. This gave us an approximation of the degree of colocalization of the above-mentioned substances with GluR2/3. All the preceding animal procedures were carried out under a protocol approved by the Yale University Animal Care Committee.

Results

GluR immunoreactivity

The immunostaining for GluR1 and GluR2/3 revealed a widespread and mostly homogeneous distribution of IR cells in the AN that corresponded with an earlier description (Wenthold et al. 1992). A majority of the AN neurons were found to contain these AMPA receptor subunits. Although their distribution was mostly homogeneous, it is worthy of note that the subependymal and ventromedial subdivisions of the AN contained relatively fewer IR somata. Also, a distinct non-IR zone surrounded and outlined the AN on its lateral and dorsal side.

NPY immunoreactivity

A distinct perikaryal NPY immunopositivity was observed throughout the AN with the greatest number of cells observed in the central and caudal aspects of the nucleus. Immunoreactive perikarya were spherical or oblong in shape. In coronal sections, several NPY-IR cell bodies were seen across the entire mediolateral extent of the subependymal and internal zones of the median eminence.

GAL immunoreactivity

A large group of small to medium-sized GAL-IR perikarya were seen in the AN. GAL-IR cells were distributed ventrally to the ventromedial hypothalamic nucleus, with more intense immunoreactivity at the ventrolateral aspects of the AN. In more caudal sections IR cells were found ventral to, and extending into, the ventral premamillary nucleus.

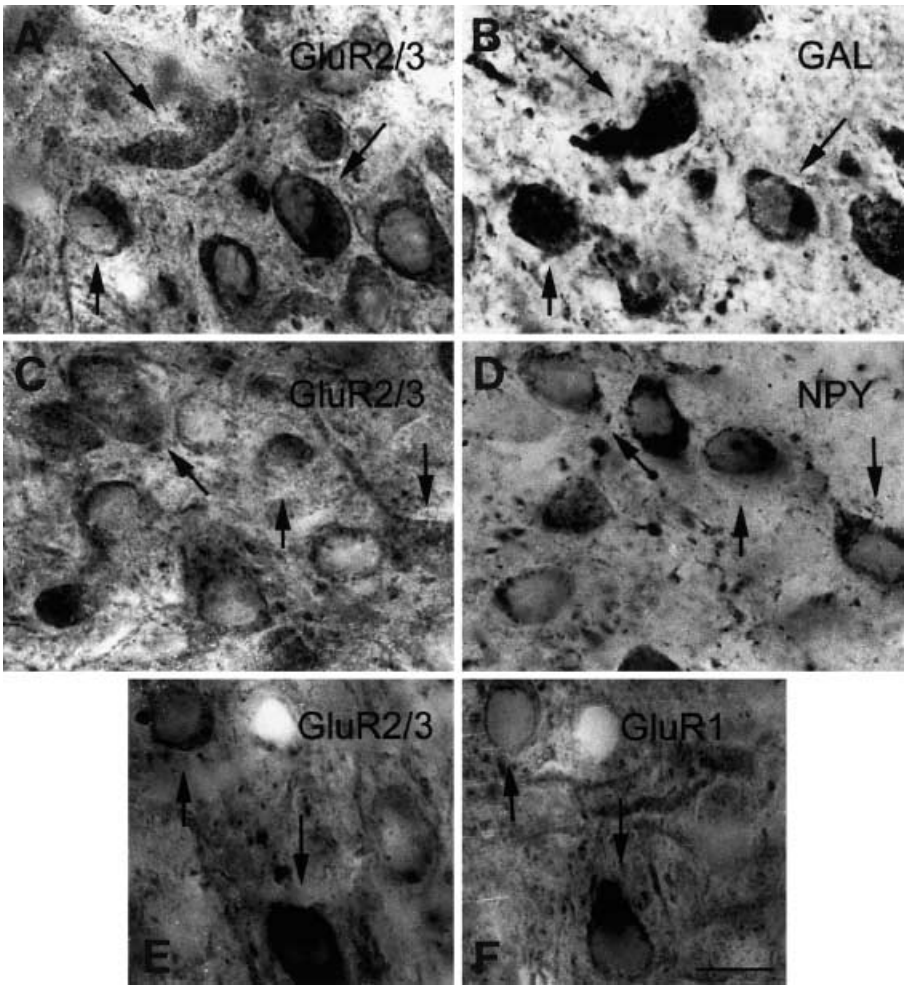

Fig. 1 Colocalization of GluR2/3 with GAL, NPY and GluR1. *Parallel arrows* point to matching cell halves. *Bar* 20 µm

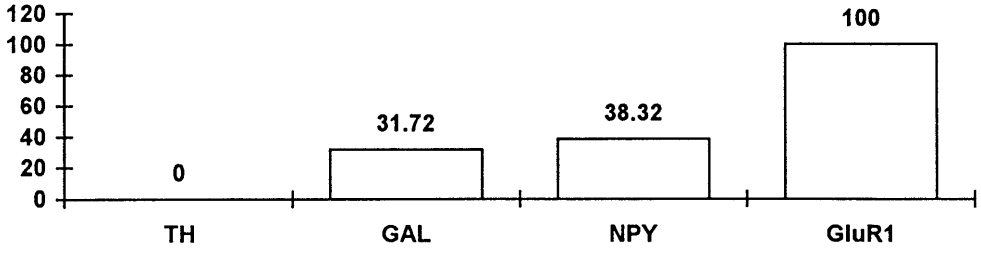

Fig. 2 Numerical calculation of TH, GAL, NPY and GluR1 immunoreactivity in 100 GluR2/3-IR neurons

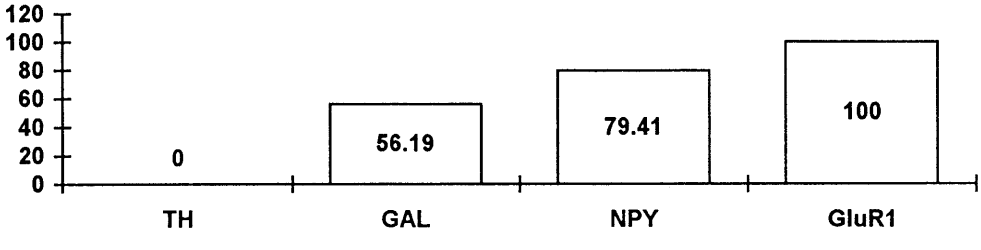

Fig. 3 Numerical calculation of GluR2/3 immunoreactivity in 100 TH, GAL, NPY and GluR1-IR neurons

TH immunoreactivity

The distribution of TH-IR neurons was largely consistent with that previously reported by many authors (Sinkiewitz et al. 1996; Kawano and Daikoku 1987; Palay et al. 1984).

Colocalization of GluR2/3 with GAL, NPY, GluR1 and TH

A total of 4440 GluR2/3-IR cells were examined in the present study (750 for TH, 1860 for GAL, 1080 for NPY, and 750 for GluR1 content). GAL was found in 31.72% and NPY in 38.72% of GluR2/3-IR cells (Fig. 1a–d),

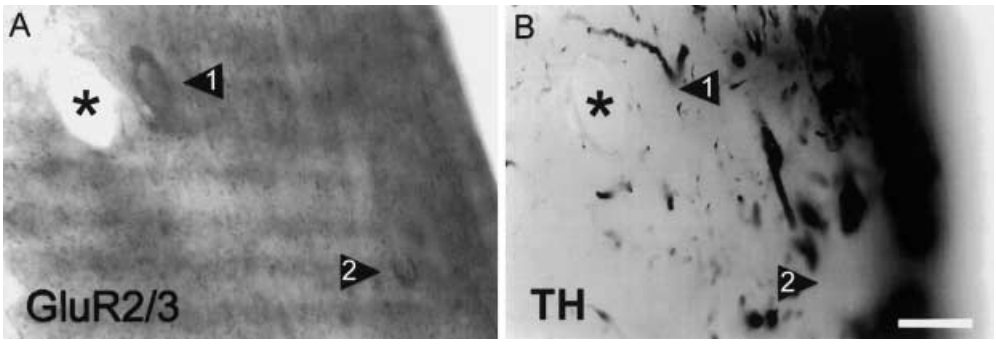

Fig. 4 TH does not colocalize with GluR2/3. *Arrowheads* point to GluR-IR neurons that are not stained for TH. *Asterisks* label the same vessel in adjacent sections. *Bar* 20 µm

whereas all of the examined GluR2/3-IR cells were also IR for GluR1 (Fig. 1e,f). Percentages of colocalizations are illustrated in Fig. 2.

We also determined the rate of GluR2/3 colocalization with 1050 examined GAL- and 510 NPY-IR neurons of the AN. More than half (56.19%) of the GAL and the majority of NPY (79.41%) neurons coexpressed GluR2/3 (Fig. 3). We were not able to find TH immunoreactivity within the GluR2/3-containing neurons (Fig. 4).

Discussion

Anatomical background and functional considerations

Beta-END neurons of the AN are known to be synaptic targets of the cell types that were subjects of the present investigation (Horvath et al. 1992a, 1995), as well as of those acting through GABA neurotransmission (Horvath et al. 1992b; Loose et al. 1991). The anatomical and functional integrity of these neuron populations form a complex circuit, regulating the biphasic GnRH secretion/release of normal cycling female rats via direct inhibitory action of beta-END cells on MPO GnRH neurons. The lack of estrogen and AMPA receptors within GnRH cells further reinforces the importance of this circuit.

The role of AN GAL and NPY neurons in the regulation of GnRH release is well described. Intracerebroventricular application of GAL or NPY readily augmented LH release in ovariectomized, estrogen-primed rats (Sahu et al. 1987). In addition, Horvath et al. (1995) demonstrated that GAL neurons located in the AN terminate on local beta-END cells and exhibit asymmetric membrane specializations. It is also known that NPY neurons of undefined origin – but suggested to be located, at least partially, within the AN – also terminate on beta-END cells (Horvath et al. 1992a), as well as on GAL- (Horvath et al. 1996) and TH-IR (Guy and Pelletier 1988) neurons. Since dopaminergic neurons establish inhibitory synapses on beta-END cells and a substantial number of NPY neurons are known to contain GABA, NPY cells are suggested to have both excitatory and inhibitory effects on beta-END neurons of the AN.

The widespread distribution of aspartate/glutamatergic axon terminals, together with the fact that they terminate on dendrites and cell bodies of AN neurons (van

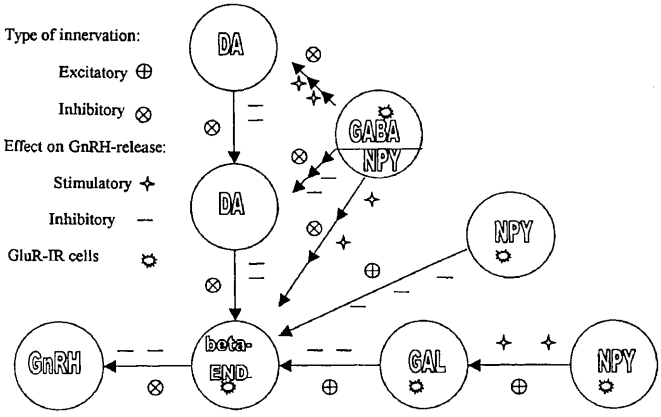

Fig. 5 Schematic illustration of the connectivity between TH-, NPY-, GAL-, GABA- and beta-END neurons involved in the regulation of GnRH cells

den Pol et al. 1990), strongly implies that the EAA innervation of the AN is involved in the regulation of pituitary Gn release. This has been further supported by the physiological finding that glutamate injection into the third ventricle readily increases the plasma LH level, but does not affect the FSH level (Olney et al. 1976), indicating that the site of glutamate action is outside the pituitary.

AMPA receptor content of GAL and NPY neurons

We consider the AMPA receptor content of a cell to be an indicator of aspartate/glutamatergic innervation. GAL and NPY neurons of the AN form excitatory synapses on beta-END neurons, thus facilitating their direct inhibitory effect on MPO GnRH-producing neurons. However, this thinking would lead to the conclusion that since these (GAL, NPY) neurons contain AMPA receptors, their EAA innervation would further strengthen the inhibition of GnRH secretion. This speculation is in contradiction to the finding by Olney et al. (1976) that glutamate effectively increases LH when injected into the third ventricle. The anatomical pattern of connectivity between beta-END neurons and GAL, NPY, GABA and dopaminergic cells (Fig. 5), as well as the extent of the AMPA receptor content of these neurons, may help ex-

plain this paradox. As mentioned above, NPY neurons innervate not only beta-END cells (either directly or indirectly), but they also terminate on dopaminergic neurons that have inhibitory nerve terminals on beta-END cells. In addition, our previous work demonstrated that approximately one-third of the AN NPY neurons are GABAergic (Horvath et al. 1997). This versatility on the part of the NPY neurons could be of great significance since it not only helps to understand the contradictory effects of NPY cells, but implicates that they can both excite and inhibit beta-END neurons and, hence, GnRH release. Our present results showing that the vast majority of AN NPY neurons contain AMPA receptors indicate that a uniform EAA system may upregulate the influence of the examined cell populations on beta-END cells, as well as the function of beta-END cells, themselves, since they also contain AMPA receptors.

A look at the mechanism by which AMPA receptor-containing neurons respond to incoming stimuli has placed further significance on our present findings. Activation of such ionotropic receptors leads directly to the opening of ion channels that are typified by their different permeabilities to Na, K and Ca ions. GluR1 and -3 are known to open Ca channels, allowing an influx of Ca into the cell, whereas GluR2 blocks the Ca permeability of the neuronal plasma membrane. Thus, AMPA receptors substantially affect the dynamic changes of the cytosolic Ca concentration. However, to the best of our knowledge, there are no data regarding the relationship between cytosolic Ca events of AN-neuropeptide-containing cells and GnRH regulation. In the absence of such information, the findings of Garthwaite et al. (1992) could shed some light on the topic. They demonstrated that different cell types of surviving brain slices incubated in Ca-free medium generated whorl-like cytoplasmic inclusion bodies from their rough endoplasmic reticulum, as a response to Ca deprivation, and these inclusions disappeared when Ca was replenished to the incubation medium. These whorl bodies looked similar to those developing in ovariectomized rat and primate AN (Leranth et al. 1985, 1991). The in vitro study of Rizzuto et al. (1998) demonstrated that changes in cytosolic Ca concentration are strongly related to morphological alterations of endoplasmic reticulum, thus confirming the causal connection between intracellular Ca events and the generation of whorl-like inclusion bodies. Along this line, it can be speculated that if the appearance of whorl bodies is based on the lack of cytosolic Ca, then a prolonged lack of AMPA receptor activation might be the cause of the development of hypothalamic whorl bodies. Because the examined AMPA receptor-containing cell types [beta-END (unpublished), GAL and NPY] are known to be estrogen-sensitive and estrogen has been demonstrated to have a stimulatory effect on hypothalamic AMPA expression (Diano et al. 1997), it is possible that the absence of constant levels of estrogen decreases or, for shorter time periods, abolishes the activity of AMPA receptors within these cells.

In the present experiment, we did not observe a colocalization of TH with AMPA receptors. This indicates that the dopaminergic cell population of the AN is not directly influenced by the glutamatergic system.

The exact mechanism by which the aspartate/glutamatergic system affects the AN circuit is not known. The presence of AMPA receptors in both inhibitory and excitatory neurons that are estrogen-sensitive, in conjunction with the idea that estrogen increases AMPA expression, raises the question of whether the aspartate/glutamatergic axon terminals that target AN cells are, in some way, responsible for the negative feedback-controlled baseline regulation of Gn secretion. On the other hand, the lack of AMPA receptors in dopaminergic AN neurons that (unlike the EAA terminals) are known to make symmetric type contacts with their target cells (for example beta-END cells) suggests that these TH-IR neurons may act in direct contrast to the EAA system; thus, they might be involved in the positive feedback-based preovulatory LH surge.

Taken together, the present study provides evidence for the previous proposal that the EAA neurotransmission, at the level of the AN, is highly involved in the complex regulation of GnRH. Our results also show that there are at least two populations of neurons within the AN, one of which (most of the neuropeptide-containing neurons) is under an aspartate/glutamatergic "supervision," while the other (dopaminergic neurons) is not.

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