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Expression of the ionotropic glutamate receptors on neuronostatin neurons in the periventricular nucleus of the hypothalamus

Running title: The ionotropic glutamate receptors and neuronostatin neurons

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Abstract

Background: Neuronostatin, a newly identified peptide, is accepted as an anorexigenic peptide since it suppresses food intake when given intracerebroventricularly. Although the effect mechanisms of neuronostatin have been shown in different studies, there are no reports in the literature describing the mechanisms controlling neuronostatin neurons. In this study, we aimed to determine the presence of the ionotropic glutamate receptor subunits (iGluRs) in neuronostatin neurons in the periventricular nucleus of the hypothalamus.

Materials and methods: The presence of glutamate receptors in neuronostatin neurons was investigated by dual immunohistochemistry. Immunohistochemistry was performed on 40 μm thick coronal brain sections with antibodies against AMPA (GluA1-4), kainate (GluK1/2/3, and GluK5), and NMDA (GluN1 and GluN2A) receptor subunits.

Results: The results showed that the neuronostatin neurons expressed most of the NMDA and non-NMDA receptor subunits. The neuronostatin neurons in the anterior hypothalamic periventricular nucleus were particularly immunopositive for GluA1, GluA4, GluK1/2/3,

GluK5 and GluN1 antibodies. No expression was observed for GluA2, GluA3 and GluN2A antibodies.

Conclusions: For the first time in the literature, our study demonstrated that the neuronostatin neurons express glutamate receptor subunits which may form homomeric or heteromeric functional receptor complexes. Taken together, these results suggest that multiple subunits of iGluRs are responsible for glutamate transmission on neuronostatin neurons in the anterior hypothalamic periventricular nucleus.

Key words: neuronostatin, glutamate, NMDA, kainate, AMPA

INTRODUCTION

Glutamate is the major excitatory amino acid neurotransmitter in the mammalian central nervous system (1, 2). Glutamate mediated neurotransmission occurs via metabotropic and ionotropic glutamate receptors (13). Ionotropic glutamate receptors are classified according to their agonists: N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoazolpropionic acid (AMPA) and 2-carboxy-3-carboxymethyl-4-isopropenylpyrrolidine (kainate) receptors (13, 16, 31). NMDA receptors are composed of subunits named GluN1, GluN2A-D and GluN3A-B (3, 14). These receptors have a critical function in excitatory synaptic transmission, plasticity and neurotoxicity (4, 19, 21-23). NMDA receptors can participate in very different processes because they have different characteristics from AMPA and kainate receptors. In addition to glutamate, an agonist, glycine or D-serine, is required for NMDA activation. AMPA receptors are composed of four subunits named GluA1-4 (19). Kainate receptors consist of five subunits named GluK1-5 (17). While GluK1-3 form functional homomeric receptors, GluK4 and GluK5 only form functional receptors when combined with one of the other subunits (GluK1-3), which generates kainic acid receptors with varying kinetics and agonist affinities (9, 16). The other receptors, kainate receptors have presynaptic and postsynaptic localizations usually on the same neuron (12).

Neuronostatin is a newly identified anorexigenic peptide encoded by the somatostatin gene (28). Immunohistochemical studies have shown that neuronostatin positive neurons are localized in the anterior hypothalamic periventricular nucleus and suprachiasmatic nucleus, while neuronostatin immunoreactive axon terminations are

localized in the arcuate nucleus with median eminence. There are fewer and less densely-marked neuronostatin-expressing cells in the polymorphic layer of the dentate gyrus and motor cortex, amygdala and cerebellum (5). These areas where neuronostatin neurons are localized in the hypothalamus play a role in the control of food intake (28). Also, neuronostatin has regulatory effects on energy consumption (28), cardiovascular system (11), and digestive system (27). In the literature, experimental studies investigating central regulators (such as glutamate) involved in the control of neuronostatin neurons have not been found.

To better understand glutamatergic function in the neuronostatin neurons localized in the anterior hypothalamic periventricular nucleus,it is necessary to determine the localization of various iGluR sub-units in neuronostatin neurons. In the present study, we examined the cellular localization of protein expression of AMPA (GluA1, GluA2, GluA3, and GluA4), kainate (GluK1/2/3 and GluK5), and NMDA (GluN1 and GluN2A) receptor subunits in the neuronostatin neurons in the periventricular nucleus of the hypothalamus by immunofluorescence.

MATERIALS AND METHODS

Animals

All animal experiments were carried out under the National Institute of Health Guide for the Care and Use of Laboratory Animals and approved by the Experimental Ethical Committee of Bursa Uludag University. 60-day-old male Sprague-Dawley rats (200-250 g) (n=10) were used in this study. The rats were maintained at the Bursa Uludag University Experimental Animals Breeding and Research Center and were housed two per cage in a temperature-controlled environment (21°C) with a 12:12-hour light/dark cycle. The animals were allowed to access food and water ad libitum. All the experiments were carried out between 9:00 am and 11:00 am.

Tissue preparation

The animals were deeply anesthetized and fixed by trans-cardiac perfusion with 4% paraformaldehyde in phosphate buffer, pH 7.4 (300 mL per animal). Brains and brainstems were carefully removed and post-fixed overnight in the same fixative. Forty-micrometer-thick coronal serial sections throughout the brains were cut with a vibratome and collected

into Tris-HCl buffer (0.05 M, pH 7.6). The sections were kept in the cryoprotectant solution at -20°C until use.

Immunohistochemistry

Tris-HCl buffer was used for all washing steps. Blocking buffer (10% normal horse serum, 0.2% triton X-100, and 0.1% sodium azide in Tris-HCl buffer) was used for incubations to prevent non-specific binding and to dilute the antibodies. All incubation steps were carried out on an orbital shaker with appropriate agitation.

Free-floating sections were equilibrated to room temperature and washed 3 times in Tris-HCl buffer to remove cryoprotectant and blocked in blocking buffer for 2 hours. Following the washing step, tissues were incubated in preheated Antigen Retrieval (AR) solution (final solution temperature 73-75°C) for 30 minutes. 50 mM trisodium citrate buffer (pH 6, for neuronostatin, GluK1/2/3 and GluA4) or 1 mM EDTA solution (pH 8, for GluN1 and GluN2A) was used in the AR process. The sections were washed three times**.** Tissues were treated with 3% H_2O_2 to quench endogenous peroxidase, washed three times and blocked with 10% normal horse serum for 2 hours. The sections were incubated in rabbit anti-neuronostatin (1/4000 dilution, H-060-50, Phoenix Pharmaceuticals, Inc., USA) and mouse anti-GluA1 (1/500 dilution, AM60040PU-N, Acris, USA), mouse anti-GluA2 (1/500 dilution, MAB397, Millipore, USA), mouse anti-GluA3 (1/250 dilution, MAB5416, Millipore, USA), goat anti-GluA4 (1/250 dilution, LS-B3606, LifeSpan BioSciences, Inc., USA), mouse anti-GluK1/2/3 IgM (1/500 dilution, MAB379, Chemican Int., USA), goat anti-GluK5 (1/250 dilution, sc-8915, Santa Cruz Biotechnology, Inc., USA), mouse anti-GluN1 (1/500 dilution, 556308, BD Pharmingen, USA), and mouse anti-GluN2A (1/3000 dilution, H-060-50, Millipore, USA). The washed sections were processed with donkey anti-goat IgG-Alexa Fluor (1/500 dilution), donkey anti-mouse IgG Alexa-Fluor (1/500 dilution), donkey anti-mouse IgM-biotin-conjugate (1/200 dilution), donkey anti-rabbit IgG-Alexa-Fluor 488 (1/500 dilution) and streptavidin-TR (1/100 dilution) for 2 hours. After washes, the sections were mounted on glass slides, dried and coverslipped with Prolong gold antifade medium.

Neuronostatin antibodies used in this study have been used in many reports in the literature as well as in our previous studies (6, 30). The antibody specificity of ionotropic

glutamate receptors in the rat red nucleus was shown in our previous studies in the literature (20).

Analysis

Sections were analyzed and photographed with Olympus BX-50 photomicroscope attached to a CCD camera (Olympus DP71, CCD color camera, 1.5 million pixels, Olympus Corporation, Japan). Sections between the coordinates determined according to the rat brain atlas (bregma-0.24 mm to -3.60 mm for periventricular nucleus) were used for single and double immunohistochemical labeling (24). Cross-sections were taken at 5 different levels at the same coordinate and an equal distance for each animal. Immunofluorescent staining intensities of neuronostatin neurons co-localized with Kainic acid, AMPA and NMDA receptor subunits in the anterior hypothalamic periventricular nucleus were graded by the following scale: '+' was used for a small number of double immunoreactive neuronostatin neurons, '++' for a moderate number of double immunoreactive neuronostatin neurons, and '+++' for a high number of double immunoreactive neuronostatin neurons.

RESULTS

The results showed that iGluRs subunits were expressed in neuronostatin neurons localized in the anterior hypothalamic periventricular nucleus. Specific staining was localized in neuronal perikarya. The number of stained neuronostatin neurons changed depending on the type of iGluRs subunits. The neuronostatin neurons in the anterior hypothalamic periventricular nucleus were particularly immunopositive for GluA1, GluA4, GluK1/2/3, GluK5 and GluN1 antibodies. No expression was observed for GluA2, GluA3 and GluN2A antibodies. The highest expression was detected for GluN1-positive neuronostatin neurons.

Neuronostatin protein positivity was visualized by green reaction product with a fluorochrome (Alexa 488) (Fig. 1A) and glutamate receptor subunit protein positivity was labeled by red fluorochromes (Alexa 594 or streptavidin-conjugated Texas-Red) (Fig. 1B) in the cytoplasm. In digitally overlapped images, neurons expressing both proteins were visualized in yellow (Fig. 1C).

The expression of kainic acid receptor subunits in neuronostatin neurons (GluK1-3 and GluK5)

Dual immunofluorescence studies showed that GluK1, GluK2, GluK3 and GluK5 positive neuronostatin neurons were expressed in the anterior hypothalamic periventricular nucleus. However, a small number of GluK1, GluK2, GluK3 (Fig. 2A) and GluK5-positive neuronostatin neurons (Fig. 2B) was observed in the anterior hypothalamic periventricular nucleus (Table 1).

The expression of AMPA receptor subunits in neuronostatin neurons (GluA1-4)

GluA1 (Fig. 3A) and GluA4 subunit protein (Fig. 3B) from the AMPA receptor family were co-expressed in neuronostatin neurons. However, GluA2 and GluA3 subunit proteins were not expressed in neuronostatin neurons.

GluA1, GluA4 and neuronostatin neurons were expressed in the anterior hypothalamic periventricular nucleus. A moderate number of neuronostatin neurons expressing GluA1 and GluA4 was detected in the anterior hypothalamic periventricular nucleus (Table 1).

The expression of NMDA receptor subunits in neuronostatin neurons (GluN1 and GluN2A)

GluN1-positive neuronostatin neurons were observed in the anterior hypothalamic periventricular nucleus (Fig. 3C). However, no expression of GluN2A subunit protein was detected in neuronostatin neurons. The number of GluN1-positive neuronostatin neurons varied from moderate to high (Table 1).

DISCUSSION

The present study showed that both NMDA and non-NMDA glutamate receptor proteins were synthesized by neuronostatin neurons. Our results indicated that neuronostatin neurons express iGluRs subunits in varying quantities and intensities. To the best of our knowledge, this is the first descriptive study on the differential expression pattern of iGluRs in the neuronostatin neurons localized in the anterior hypothalamic periventricular nucleus. GluA1, GluA4, GluK1/2/3, GluK5 and GluN1 receptor proteins were expressed in neuronostatin neurons, but no immunoreactivity indicating the presence of GluA2, GluA3 and GluN2A proteins was detected.

Localization of ionotropic glutamate receptors in the anterior hypothalamic periventricular nucleus

Studies showing the distribution at mRNA and protein levels of glutamate receptors in the central nervous system and hypothalamus are available in the literature (7, 8, 18, 25, 26, 29). In studies on immunohistochemistry and in-situ hybridization in the anterior hypothalamic periventricular nucleus, GluA1 and GluA2 proteins from the AMPA receptor family were moderately high in density; GluA3 and GluA4 have been reported to be expressed at low intensity (18, 29). In another in-situ hybridization study, the presence at the mRNA level of kainate receptor subunits was determined in the anterior hypothalamic periventricular nucleus and it was shown that GluK5 expression was high and that other subunits (GluK1, GluK2 and GluK3) were expressed at low-medium density (7). In the same study, mRNA signals of NMDA receptor subunits were also determined and the most intense signal was shown to belong to GluN2A. mRNA signals of GluN1, GluN2B and GluN2D subunits were reported to be moderate, and at low density for GluN2C (7). Results of the present study show that some NMDA and non-NMDA glutamate receptor signals belong to neuronostatin neurons in the anterior hypothalamic periventricular nucleus.

Kainic acid receptor subunits in neuronostatin neurons (GluK1-3 and GluK5)

Glutamate receptor subunits combine to form functional receptor complexes. These receptor complexes may be homomeric or heteromeric. Homomeric ion channels are formed by combining the same subunits, heteromeric functional ion channels are formed by combining different subunits (10, 15, 19). The present study demonstrated that neuronostatin neurons express receptor subunit proteins of the kainate receptor family. The present study suggests two ideas about the kainate receptor proteins expressed in neuronostatin neurons: 1) The GluK5 subunit cannot form a stand-alone channel; it can form a functional heteromeric kainate receptor complex combined with one of the GluK1, GluK2 or GluK3 subunits; 2) GluK1, GluK2 or GluK3, which are low-affinity kainate receptor subunits, may form functional homomeric kainate receptor complexes.

AMPA receptor subunits in neuronostatin neurons (GluA1-4)

AMPA receptor complexes may form homomeric ion channels but are mostly present in the form of heteromers (10, 19). GluA1 and GluA4 subunits, which were determined to be expressed in neuronostatin neurons, were thought to form functional cation channels either individually or together. The results of the present study showed that the activating effect of glutamate on neuronostatin neurons was mediated by AMPA-selective ionotropic glutamate receptors along with kainate receptors.

NMDA receptor subunits in neuronostatin neurons (GluN1 ve GluN2A)

NMDA receptor subunits can only form functional receptor complexes of heteromeric structure (23). The essential subunit of NMDA receptor complexes is GluN1 (15). The present study showed that GluN1, a NMDA receptor subunit protein, was expressed in neuronostatin neurons.

Although GluN2A immunoreactivity was not determined in neuronostatin neurons, they were still thought to carry functional NMDA-selective glutamate receptors. This is because 1) Expression of GluN1, the indispensable subunit of NMDA receptor complexes, was positive in neuronostatin neurons: 2) The GluN1 subunit forms functional receptor channels with other types of GluN2. In the present study, the expressions of GluN2B, GluN2C and GluN2D subunits in neuronostatin neurons were not investigated. Even so, the GluN2B, C and D receptor types are expressed in the anterior hypothalamic periventricular nucleus. The results suggest that neuronostatin neurons localized in the anterior hypothalamic periventricular nucleus may also express these receptor types and therefore may be regulated by NMDA receptors.

For the first time in the literature, our study demonstrated that the neuronostatin neurons express glutamate receptor subunits which may form homomeric or heteromeric functional receptor complexes.

CONCLUSIONS

As conclusion, glutamate plays an effective role in regulating the functions of neurons synthesizing neuronostatin and glutamate shows its effectiveness through NMDA and non-NMDA receptors.

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Table I. The distribution of the staining intensity of ionotropic glutamate receptor subunits

Neuronostatin neurons	GluK1/2/3	GluK5	GluA1	GluA2	GluA3	GluA4	GluN1	GluN2A
	+	+	++	-	-	++	++/+++	-

in neuronostatin neurons localized in the anterior hypothalamic periventricular nucleus.

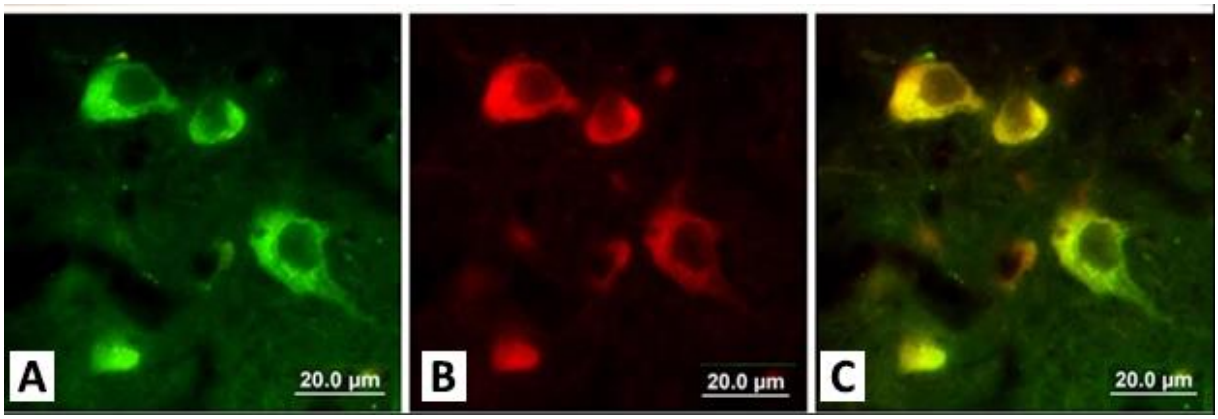

Figure 1. Evaluation of dual immunohistochemical markings. A: Neurons expressing the peptide labeled with green fluorochrome. B: Neurons expressing glutamate receptor subunit protein labeled with red fluorochrome. C: Neurons expressing the glutamate receptor subunit protein together with the peptide are monitored in yellow.

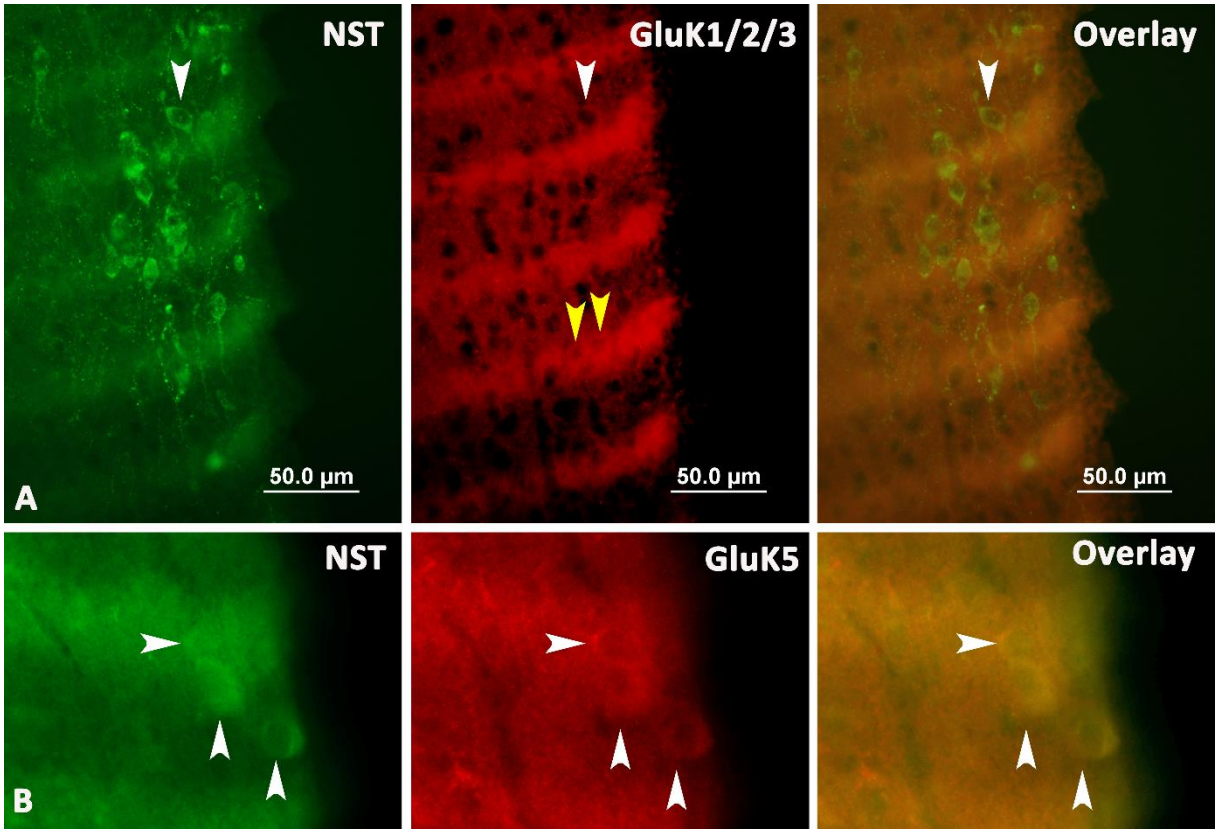

Figure 2. Immunofluorescence image of GluK1/2/3-positive neuronostatin neurons in the anterior hypothalamic periventricular nucleus (A). Immunofluorescence image of GluK5-positive neuronostatin neurons in the anterior hypothalamic periventricular nucleus (B). Neurons co-expressing both proteins (white arrowhead), only GluK1/2/3-positive neurons (yellow arrowhead).

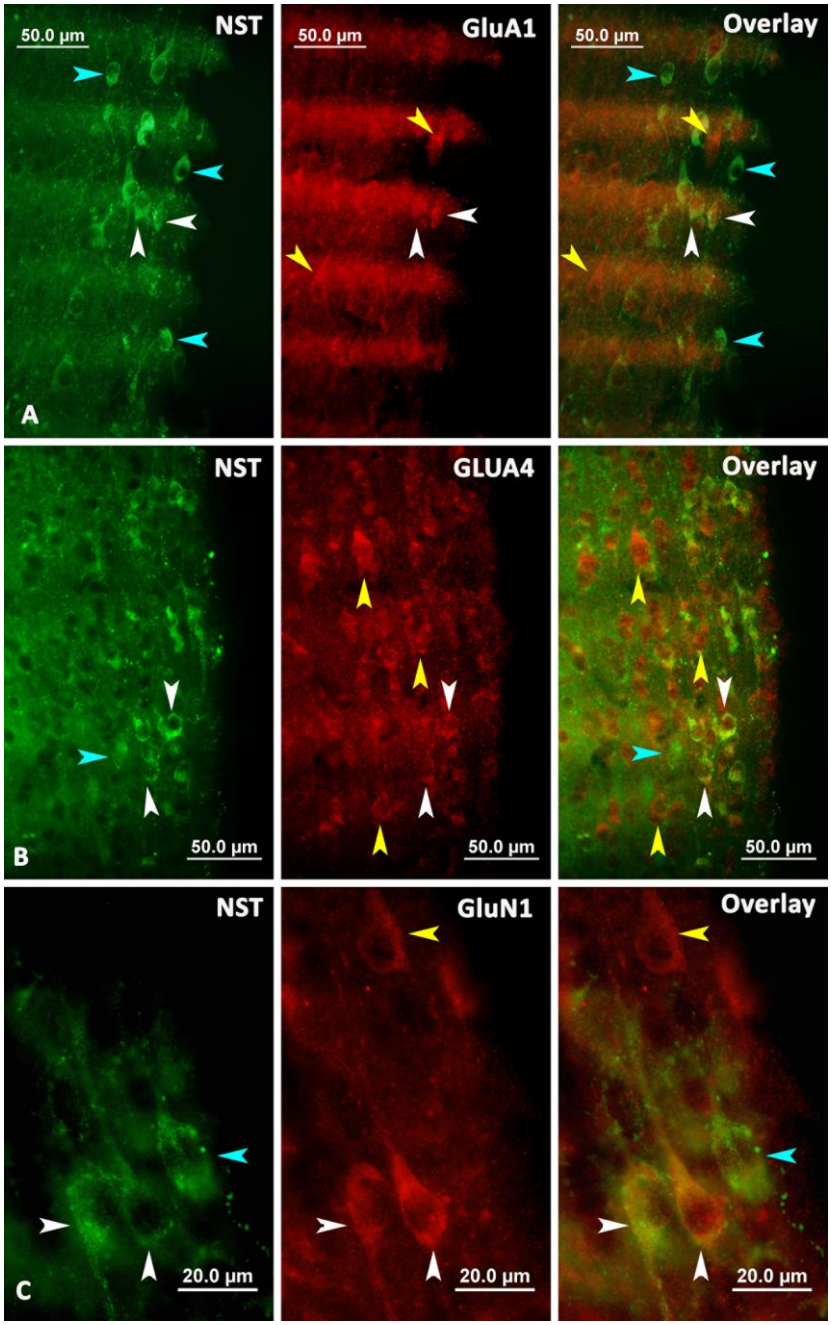

Figure 3. Immunofluorescence image of GluA1-positive neuronostatin neurons in the anterior hypothalamic periventricular nucleus (A). Immunofluorescence image of GluA4-positive neuronostatin neurons in the anterior hypothalamic periventricular nucleus (B).Immunofluorescence image of GluN1-positive neuronostatin neurons in the anterior hypothalamic periventricular nucleus (C). Neurons co-expressing both proteins (white arrowhead), only GluA1-positive neurons, only GluA4-positive neurons, only GluN1-positive neurons (yellow arrowhead) and only neuronostatin-immunoreactive neurons (blue arrowhead).