Proceedings of the 4th International
IEEE EMBS Conference on Neural Engineering
Antalya, Turkey, April 29 - May 2, 2009

Ghrelin expression in dissociated cultures of the rat neocortex

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Abstract— **Ghrelin is a hormone, initially described as a gastric peptide stimulating appetite and growth hormone secretion, which also has an important role in the regulation of many other processes including higher brain functions. Ghrelin has been described in situ in different part of the brain, but so far there are no data about it in cell cultures. Therefore, we aimed this study to investigate the developmental pattern of ghrelin in dissociated cortical neuronal cultures. We applied the ABC immunocytochemical method for detection of ghrelin in dissociated cortical neurons from newborn rats, incubated for one day-, one week-, or two week old cultures. Our results clearly show that at the early stages of development of the network, majority of the neurons are ghrelin-positive, and their number decreases during the culturing period. As ghrelin is omnipresent in the early stages, when the neuronal differentiation and network formation take place, it may also influence the early development of synaptic formation and cell-to-cell interactions, both very important for the network functions like learning and memory.**

Keywords-dissociated cortical neurons, ghrelin development, learning, memory

I. Introduction

GHRELIN is a hormone, initially described as a gastric peptide stimulating appetite and growth hormone secretion. Since ghrelin was discovered in 1999 [1], data have accumulated suggesting that it also plays an important role in regulation of the cardiovascular and immune systems [2]. The wide distribution of the peptide in different tissues suggests that ghrelin plays an important role not only in the appetite regulation and food intake [3], but also could be involved in the control of cellular differentiation, proliferation and apoptosis [4]. Recently, ghrelin-immunoreactive (IR) neurons have been demonstrated in the arcuate nucleus of the rat brain [5], in the hypothalamus, in the sensory-motor cortical area and in the cingular gyrus [6, 7]. The presence of ghreling and its binding sites in the cerebral cortex links the metabolic control of ghrelin with higher brain functions like learning and memory processes [8]. Additionally, electrophysiological studies revealed that ghrelin promotes long-term potentials (LTP) generation in hippocampal slice preparations and when administrated peripherally, rapidly induces the assembly and disassembly of synaptic membrane proteins that modulate synapse formation [9]. As a matter of fact, the number of spine synapse of hippocampal neurons and LTP have a positive correlation with spatial memory and learning [10], thus ghrelin could stimulate these processes.

The mechanism of ghrelin function has been associated with an increase of intracellular Ca^{2+} concentration through voltage-gated L- and T-type channels. As it is well known, Ca^{2+} is an important intracellular second messenger regulating many neuronal functions ranging from secretion to cellular survival. Applying a selective blocker of those channels, Zhang and collaborators [4] demonstrated that ghrelin increases neuronal mitotic activity, and thus stimulates neurogenesis at certain parts of the brain. Ghrelin and its receptor display a developmentally-related pattern of expression, related to the gestational age in the fetal tissues, and in adult animal tissues, which undergo marked development in a short period of time like the placenta [11]. It seems that ghrelin system is linked to the proliferative activity of germ and somatic cells.

Despite the rapidly increasing amount of information about ghrelin *in situ*, so far there are no enough data about it in culturing conditions. Therefore, and because of its importance for the learning and memory formation we underwent our study in order to investigate ghrelin expression in dissociated cortical neurons of the rat neocortex.

II. Methods

A. Dissociated cell cultures

Cortical neurons were obtained from newborn Wistar rats. Under sterile conditions, the whole brain was removed and placed in a Petri dish with a RPMI-medium supplemented with extra glucose to a final concentration of 6.5 mg/ml. The meninges of the cortices were removed, and the striatum and the hippocampus were

Manuscript received March 2, 2009.
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prepared free. The remaining cortices were collected in a tube with chemically defined R12 culture medium and trypsin for chemical dissociation. After removal of trypsin, 150 µl of soybean trypsin inhibitor and 125 ml of DNAse I (20.000 units, Life Technology) were added. It was followed by mechanical dissociation of the neurons into the solution, and the suspension was centrifuged at 1200 rpm for 5 minutes. The supernatant was removed and the pallet of neurons was resuspended. The obtained neurons were plated and cultured on glass cover slips pre-coated with 20mg/ml poly-ethylene-imine (Fluka, Buchs, Switserland) for enhancement of the cell adhesion. Cells were kept in serum-free R12 medium under standard conditions of 37°C and 5% CO2 in air. A cell concentration approximately 3000 cells/mm^2 was used in all experiments. After one day, one week, or two weeks incubation, cultures were fixed in 4% paraformaldehyde in 0.1 M PBS, pH 7.4, and processed for immunohistochemical detection of ghrelin.

B. Immunocytochemistry

The immunohistochemical staining procedure was performed according to the ABC (avidin-biotin-horseradish peroxidase) method. Briefly, a hydrogen peroxide (0.3% in absolute methanol for 30 min) was used to inactivate endogenous peroxidase. Appropriate washes in PBS followed this and subsequent treatments. Incubation in primary antibody (goat anti-ghrelin, 1:500, Everest Biotech, Oxfordshire, UK) was for 20 h at room temperature (RT) and was followed by 2 h biotinilated donkey anti-goat IgG (1:500; Jackson ImmunoResearch, West Grove, PA, USA). The sections were then incubated for 1 h in ABC complex (1:500; Vector Labs, Burlingame, CA, USA). Following rinsing, peroxidase activity was visualized using 2.4% SG substrate kit for peroxidase (Vector) in PBS for 5 min, at room temperature. Finally, the cultures were dehydrated in a graded series of alcohols, cleared in xylene, and coverslipped with Entellan (Merck, Darmstadt, Germany).

Negative controls included sections that were incubated in the absence of primary antibody or in a presence of non-immune normal serum in the same dilution as the primary antibody. Semi-qualitative estimates of ghrelin expression in the dissociated cortical neurons were made subjectively and the following four-point intensity scale was used: (-) background intensity; (+) low intensity; (++) moderate intensity; (+++) high intensity. After immunostaining, the cultures were photographed with AxioCam MRC digital camera linked to a Zeiss Axioplan 2 research microscope. All digital images were matched for brightness and contrast in Adobe Photoshop 7.0 software.

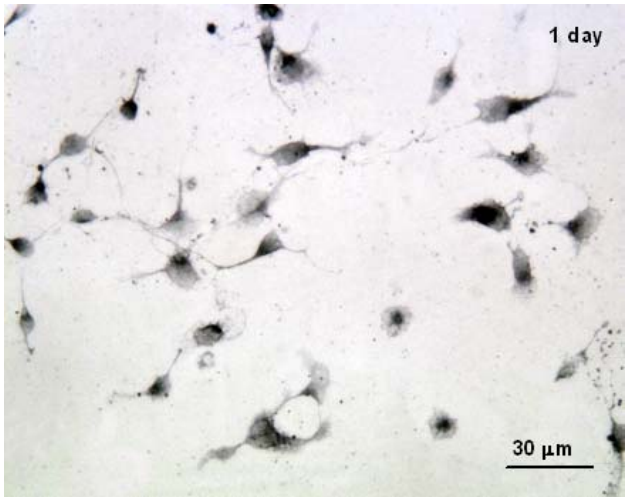

Figure 1. Light micrograph of a culture, incubated for one day. The neurons are underdeveloped and almost all them exhibit ghrelin-IR as dark-gray granules, which are not dispersed in the entire cytoplasm. Scale bar 30 µm.

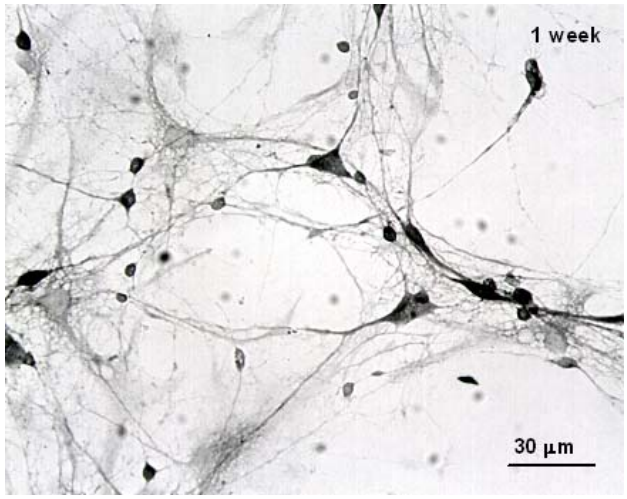

Figure 2. Ghrelin-expression in one-week-old culture. Immunoreactivity varies from negative, mainly in the large-sized perkarya, to highly intensive in the medium- to small-sized neurons of both stellate- and spindle-shaped types. Scale bar 30 µm.

III. RESULTS

No immunoreactivity for ghrelin was detected in the cultures when the primary or secondary antibody was replaced with normal serum (not shown). The immunoreactivity was readily discernible at the light microscopic level by the presence of a dark-gray immunoreactive product. Neuronal structures were considered to be immunopositive when their staining was clearly stronger than that in the background.

Five cortical cultures for each incubating period were used for demonstration of ghrelin. Immunocytochemical labeling revealed thet 86.4% of the neurons in one-day-

old cultures were ghrelin-IR. The reaction product was not evenly distributed throughout the cytoplasm, sometimes concentrated in a restricted aria of the perikaryon (Fig. 1).

The size of the neuronal cell bodies was smaller compared to that in one-week and two-weeks incubated cultures, and the arborizations were shorter and thinner. In cultures grown for 1 week, a lower numbers of neurons were ghrelin-IR (Fig. 2) and they represented 77.6% of the entire cell population. The majority of them were small interneurons but some medium- to big-sized cells were also observed. They already had 2-5 primary dendrites with few secondary arborizations. The immunoreactivity was evenly distributed throughout the perykarya, excluding the nucleus. A relatively high density of ghrelin-IR cells was observed in cultures incubated for two weeks – 59.1% of the neurons. Their population consisted of two types (Fig. 3): spindle-shaped neurons with two major neurites arising from opposite poles of the cell body, and multipolar neurons with several major neurites emerging from a stellate-shaped soma. The results of the quantitative analysis of the ghrelin expression are summarized in Table 1. The intensity of the immunostaining varied from (-), mainly in the large-sized perikarya, to (+), (++) and (+++) in the medium- to small-sized neurons of both mpyramidal and bipolar types. Thick non-varicose neuronal fibers expressing ghrelin-reactivity formed a dense network.

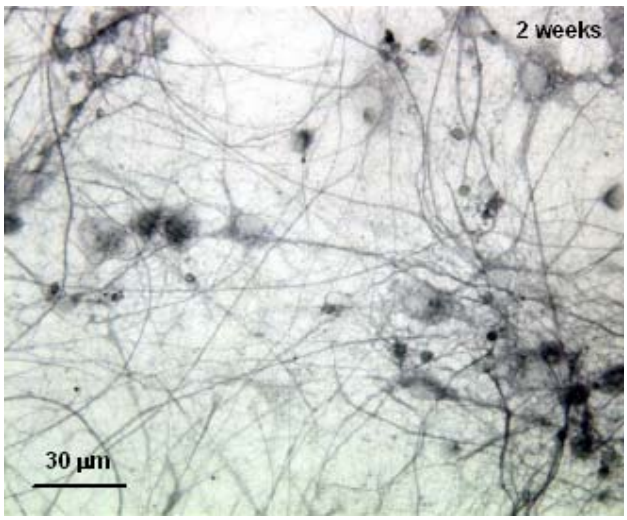

Figure 3. Culture of cortical neurons incubated for two weeks. Cells are well developed and established connectivity. A lower number of cells are ghrelin-positive and they have thick non-varicose arborizations. Scale bar 30 µm.

Table 1. Quantitative analysis of the ghrelin expression in dissociated neuronal cultures at different age.

Age of the neuronal culture	Percentage of ghrelinergic neurons
1day	86.4%
1 week	77.6%
2 weeks	59.1%

IV. Discussion

There are only a few previous studies, which revealed ghrelin-immunoreactivity in the cerebral cortex. Some of them have found that in the cortex, ghrelin is mainly expressed in the processes but not in the somata of the neurons [12]. On contrary, a very recent finding of Hou and collaborators [7] for the first time demonstrated ghrelin-containing cortical neurons, mainly distributed in the sensory-motor area of the frontoparietal cortex and the cingulated gyrus. All these data referred to the ghrelin availability in the brain *in situ*, and so far there are no data about its expression and function in culturing conditions. To the best of our knowledge, the present study demonstrates for the first time ghrelin occurrence in dissociated cortical neurons. Moreover, it clearly shows conditioning- and time-related developmental pattern in the transmitter appearance. In culturing conditions, more neurons are ghrelinergic than in the brain cortex *in situ* (our unpublished data). As early as 24 h after plating the cultures, an initial ghrelin synthesis was observed in most of the cortical neurons. Ghrelin expression was fully developed in one-week-old cultures, but the number of the positive cells was lower compared to the one-day-old cultures. In two-weeks-old cultures there was a tendency for ghrelin expression to be reduced and almost a half of the neuronal perikarya already changed their phenotype into non-ghrelinergic.

As it has been shown in previous experiments of other groups, the first two weeks after plating are crucial for the network formation and survival of the cultures [13]. A rapid synapse formation begins during the first week *in vitro*, and reaches its maximum at day 21, as also do the activity of the network [14]. Hence, we can speculate that the entirely developmentally determined change in the ghrelin expression, as we show in this paper, can serve as a mechanism relevant for the neurite sprouting and cortical network formation during the first weeks *in vitro*. In other words, our finding suggests that in addition to its role as a neurotransmitter, ghrelin may also influence the early development of synaptic formation and cell-to-cell interactions, which are both very important for the network functions like learning and memory.

V. ACKNOWLEDGMENT

The authors would like to thank Prof. Dr. E. Marani for his help with the experimental animals supply. This study is part of the EU research project NEURoVERS-it. It was supported by grant MRTN-CT-2005-019247, the University of Twente, The Netherlands, and Trakia University, Stara Zagora, Bulgaria.

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