Regulatory Peptides 158 (2009) 86–90

Contents lists available at ScienceDirect

Regulatory Peptides

journal homepage: www.elsevier.com/locate/regpep

# Time-dependent changes in ghrelin-immunoreactivity in dissociated neuronal cultures of the newborn rat neocortex

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ARTICLE INFO

*Article history:*
Received 19 March 2009
Received in revised form 15 May 2009
Accepted 12 June 2009
Available online 21 June 2009

*Keywords:*
Dissociated neuronal cultures
Ghrelin expression
Development
Neuronal networks
Immunocytochemistry
Rat

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 parts of the brain, but so far there has been no data about its expression in cell cultures. Therefore, we aimed in this study to investigate the levels of ghrelin in dissociated cortical neurons at various times in culture. We applied the ABC immunocytochemical method for the detection of ghrelin in one-day-, one-week-, and two-week-old cultures. Our results clearly show that at the early stages after plating the cultures 86.2% (±8.93) of the neurons are ghrelin-positive and their number decreases during the culturing period. As ghrelin is present in the majority of cultured newborn neurons, when the neuronal differentiation and network formation take place, it may also influence the early synaptic formation and cell-to-cell interactions, which are both very important for network functions like learning and memory.



## 1. Introduction

Ghrelin is a hormone, initially described as a gastric peptide stimulating appetite and growth hormone secretion. Since ghrelin was discovered as an endogenous ligand for the growth hormone secretagogue receptor (GHS-R) in 1999 [1], data have accumulated suggesting that it plays an important role not only in the appetite regulation and food intake [2] but also in the gastrointestinal, cardiovascular, reproductive and immune system function [3,4]. The wide distribution of the peptide and its binding sites in different tissues indicate that ghrelin has multiple biological effects besides the growth hormone release.

Ghrelin and its receptor display a developmental pattern of expression, related to the gestational age in fetal tissues, and also in adult animal tissues, which undergo marked development in a short period of time, such as the placenta [5,6]. It seems that the ghrelin system is linked to the proliferative activity of germ and somatic cells. Moreover, it has been shown to have important roles in the control of cellular proliferation, differentiation, and apoptosis [7]. Ghrelin has also been implicated in the increase of neuronal mitotic activity, thus stimulating neurogenesis in certain parts of the brain [8]. Recently, ghrelin-immunoreactive (IR) neurons have been demonstrated in the arcuate nucleus of the rat brain [9]. Some studies have found that in the cerebral cortex, ghrelin is mainly expressed in the processes but not in the somata of the neurons [10]. On the contrary, very recent findings of Hou and collaborators [11] for the first time have demonstrated ghrelin-containing cortical neurons, mainly distributed in the sensory-motor area of the frontoparietal cortex and cingulated gyrus.

Despite the rapidly increasing amount of information about ghrelin *in situ*, so far there are no data about its expression and function in culturing conditions. Dissociated neuronal cultures are often used as a model for neuronal network formation and functional inquiry [12]. Therefore, we undertook our study to investigate the time-related pattern in the appearance of ghrelin in cultured dissociated cortical neurons of the newborn rat neocortex.

## 2. Materials and methods

### 2.1. Dissociated cell cultures

Cortical neurons were obtained from newborn Wistar rats. All housing facilities and procedures used were supervised and approved by the Animal Care and Use Committee of the University of Twente and Trakia University, Bulgaria, and were carried out in accordance with the European Communities Council Directive of 24 November 1986. The animals were anesthetized with ether, decapitated, and subsequently the heads were immersed in 70% ethanol. Under sterile conditions, the whole brain was removed and placed in a Petri dish with a RPMI-medium supplemented and extra glucose to a final concentration of 6.5 mg/ml. The meninges of the cortices were removed, as well as the striatum and the hippocampus. The remaining cortices were collected in a tube with chemically defined R12 culture

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0167-0115/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.regpep.2009.06.008

medium [13] and trypsin for chemical dissociation. After removal of trypsin, 150 µl of soybean trypsin inhibitor and 125 µl of DNAse I (20,000 units, Life Technology) were added. It was followed by mechanical dissociation of the neurons in the solution, and the suspension was centrifuged at 1200 rpm for 5 min. The supernatant was removed and the pellet of neurons was resuspended. The obtained neurons were plated and cultured on glass coverslips which were pre-coated with 20 mg/ml poly-ethylene-imine (Fluka, Buchs, Switzerland) for enhancement of the cell adhesion. The cells were allowed to attach to the coverslips for 2 h under standard conditions of 37 °C and 5% CO2 in air. Cells were kept in serum-free R12 medium, which suppresses glial cell proliferation [13,14]. The medium was optimized for neuronal survival with 50 ng/ml nerve growth factor (Invitrogen, Carlsbad, CA), renewed first after 24 h, and then subsequently every 2 days. A cell concentration of approximately 3000 cells/mm$^2$ was used in all experiments. After a succession of one day, one week, two weeks, three weeks, and four weeks, cultures were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer saline (PBS), pH 7.4 and processed for immunohistochemical detection for ghrelin and for identification of the cellular type by application of specific markers, i.e. anti-Microtubule Associated Protein (MAP2) antibody as a neuronal marker and anti-Glial Fibrillary Acidic Protein (GFAP) antibody, a marker for glial cells respectively.

### 2.2. Animals and tissue preparation

Five one-week-old and five adult Wistar rats (250–350 g b.w.) were used in this study for demonstration of ghrelinergic neurons in the cortex *in situ*. The animals were deeply anesthetized with ether, and transcardially perfused first with 100 ml heparinized cold 0.9% NaCl (1 U heparin/ml saline) and followed by 500 ml 4% paraformaldehyde in 0.1 M PBS, pH 7.4. After perfusion, the brains were removed, blocked and postfixed in the same fixative solution for 5–6 h at 4 °C, and then cryoprotected in 25% sucrose in PBS overnight at 4 °C. The brains were embedded in TissueTek OCT compound (Miles Inc., Elkhart, NI, USA), frozen, and 30 µm thick sections were cut in a cryostat at −20 °C. The sections were separated into five series, according to the method proposed by Guillery and Herrup [15]. After rinsing in 0.1 M PBS each complete series of one-in-five frontal sections, stretching through the entire rostrocaudal dimension of hemispheres was processed for ghrelin immunocytochemistry.

### 2.3. Immunocytochemistry

The immunohistochemical staining procedure was performed according to the ABC (avidin–biotin–horseradish peroxidase) method [16]. Briefly, hydrogen peroxide (0.3% in absolute methanol for 30 min) was used to inactivate endogenous peroxidase. Appropriate washes in PBS followed this and the subsequent treatments. Incubation in primary antibody (goat anti-ghrelin, 1:500, Everest Biotech, Oxfordshire, UK; rabbit anti-MAP2, 1:200 and rabbit anti-GFAP, 1:750, both Abcam, Cambridge, UK) was for 20 h at room temperature (RT) and was followed by 2 h treatment with biotynilated donkey anti-goat IgG or goat anti-rabbit IgG (1:500; Jackson ImmunoResearch, West Grove, PA, USA). The specimens 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 slices and the cultures were dehydrated in a graded series of alcohols, cleared in xylene, and coverslipped with Entellan (Merck, Darmstadt, Germany). Negative controls included specimens that were incubated in the absence of either primary or secondary antibody or in the presence of non-immune normal serum in the same dilution as the respective antibodies.

### 2.4. Data analysis and photomicrograph production

Seven culturing experiments were performed; eight to ten brains of newborn rats were used in each experiment and plated on 120 coverslips. For the assessment of ghrelin expression in the cortical slices, sections from 4.20 to −9.30 mm (from Bregma) were selected according to the Coggeshall and Lekan [17]. Cell counts were performed on randomly chosen slices taken from various rostrocaudal locations within the sensorimotor frontoparietal cortex, where the ghrelin-immunopositive neuronal cell bodies were found. The statistical computations were performed using the StatView package for Windows, v 4.53 (Abacus Concepts Inc., Berkeley, California, USA). All data were presented as the mean ± S.D. (standard deviation). Statistical comparisons between each of the groups were made by Mann–Whitney *U* and Kruskal–Wallis tests. A $p$ value of less than 0.05 was considered statistically significant.

Semi-qualitative estimates of ghrelin expression in the dissociated cortical neurons or brain slices were made subjectively. The following four-point intensity scale was used: (−) background intensity; (+) low intensity; (++) moderate intensity; (+++) high intensity. After immunostaining, images were generated through 10×, 20×, and 40× objectives and the cultures and brain slices were photographed with an AxioCam MRC digital camera linked to a Zeiss Axioplan 2 research microscope. All digital images were matched for brightness in Adobe Photoshop 7.0 software.

## 3. Results

### 3.1. Specificity

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.

### 3.2. Identification of neuronal and glial cells

The application of immunolabeling for the glial marker GFAP indicated that a very low proportion of the cells were glial cells in one-day-old cultures (Fig. 1). After one or more weeks of culturing, the application of GFAP demonstrated that glial cells were already well developed (Fig. 2) and no one of the ghrelin-labeled cells had their morphological characteristics. In contrast, immunostaining of the

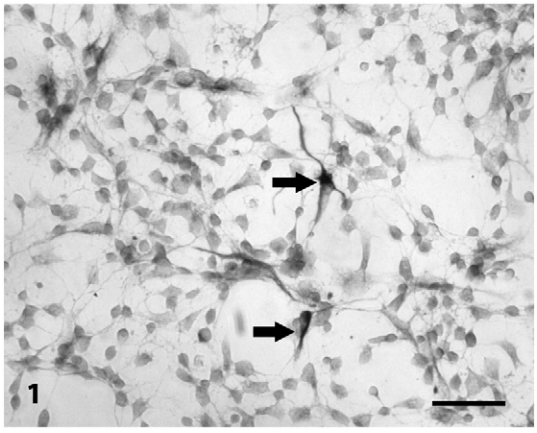


**Fig. 1.** Light micrograph of one-day-old culture. The immunostaining with anti-GFAP antibody indicates a low proportion of the glial cells (arrows). Scale bar 30 µm.

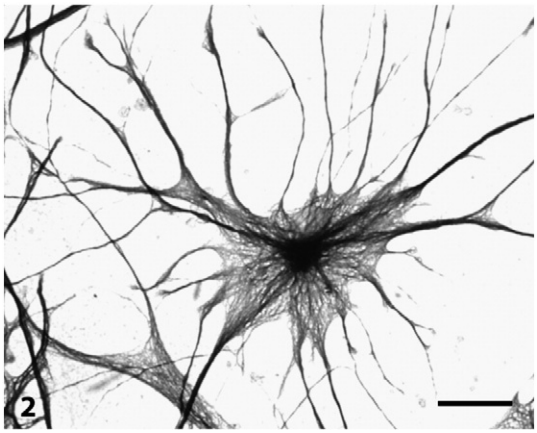


**Fig. 2.** Fully developed glial cell in culture labeled for the glial marker GFAP. Scale bar 30 μm.

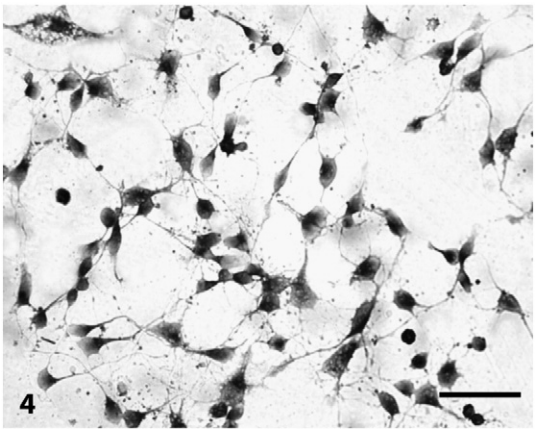


**Fig. 4.** Light micrograph of one-day-old culture processed immunohistochemically for ghrelin detection. The neurons are underdeveloped most of them exhibit ghrelin-IR as dark-gray granules, which are not dispersed in the entire cytoplasm. Scale bar 30 μm.

cultures for the neuronal marker MAP2 (Fig. 3) and for ghrelin, clearly showed identity of the morphology of the labeled cells, i.e. only neuronal cells were ghrelin-positive.

### 3.3. Distribution of ghrelin-immunoreactivity

Immunocytochemical labeling for ghrelin revealed that 86.2% (±8.93) of the neurons in one-day-old cultures were ghrelin-IR. The reaction product was not uniformly distributed throughout the cytoplasm, but was sometimes concentrated in a restricted area of the perikaryon (Fig. 4). The size of the neuronal cell bodies was smaller compared with that in one-week- or two-week-old cultures, and the arborizations were shorter and thinner.

In cultures grown for one week (Fig. 5), a lower numbers of neurons were ghrelin-IR and they represented 76.9% (±26.24) of the entire neuronal population, but Mann–Whitney *U* test showed no significant difference compared with one-day-old cultures ($p = 0.175$). The majority of ghrelinergic neurons in one-week-old cultures were small interneurons, mostly equipped with fine bipolar or tufted dendrites, but some medium- to large-sized cells with basket-like morphology, 2–5 primary dendrites and few secondary arborizations, were also observed. The immunoreactivity was evenly distributed throughout the cell soma, excluding the nucleus. It was also found in the proximal parts of the dendrites and axon.

In two-week-old cultures the proportion of ghrelin-IR neurons was 63.6% (±19.01), which was significantly lower than in one-day- (tied $p = 0.0034$) and one-week-old cultures (tied $p = 0.036$). The neurons were already well developed and the ghrelinergic population consisted of two well distinguishable types of cells: spindle-shaped neurons with two major neurites arising from the opposite poles of the cell body, and multipolar, pyramidal neurons with several major neurites emerging from a stellate-shaped soma. The intensity of immunostaining varied from (−), mainly in the large-sized parikarya, to (+), (++) and (+++) in the medium- to small-sized neurons of both multipolar (pyramidal) and bipolar types (Fig. 6). Thick non-varicose neuronal fibers expressing ghrelin-reactivity formed a dense network (Fig. 7). After growth during three weeks (Fig. 8), the clusters of neurons became very dense and it was hard to count the number of ghrelin-expressing neurons. Nonparametric Kruskal–Wallis testing revealed a significant difference in the ghrelin expression among the three age groups ($p = 0.0073$).

For quantitative comparison of ghrelin expression in dissociated neurons with the brain cortex *in situ*, we used sections of the frontoparietal part of the brain of one-week-old (Fig. 9) and adult rats (Fig. 10). Sections were taken at Bregma 2.70 mm. It was difficult to determine the exact percentage of the immunoreactive cells but the visual comparison of the slices indicated that there are much more ghrelin-positive neurons in one-week-old than in adult brain. We were able to count the ghrelin-IR neurons only in cortical layers 3–5 of

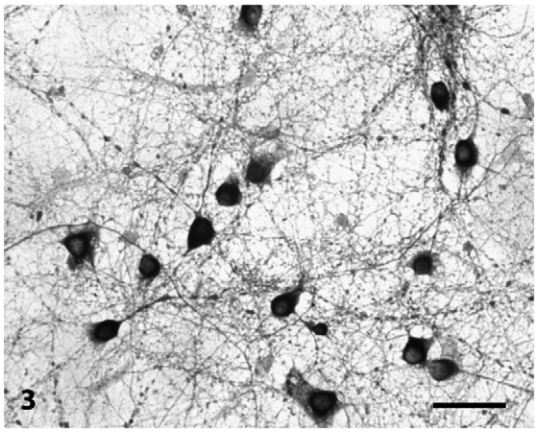


**Fig. 3.** Photograph of two-week-old culture. The immunostaining with neuronal marker anti-MAP2 Ig identifies the neurons and shows their morphology. Scale bar 30 μm.

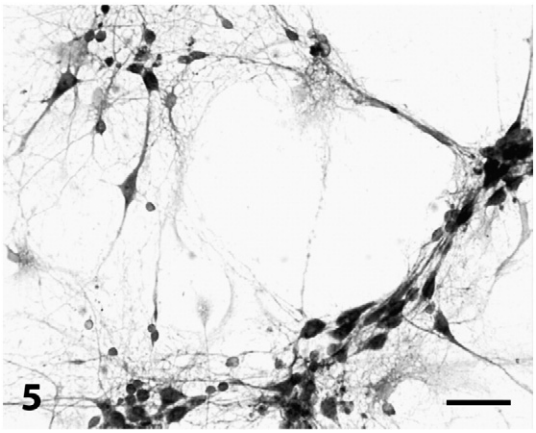


**Fig. 5.** Ghrelin-expression in one-week-old culture. Immunoreactivity varies from negative, mainly in the large-sized perikarya, to highly intensive (+++) in the medium- to small-sized neurons of both stellate- and spindle-shaped types. Scale bar 50 μm.

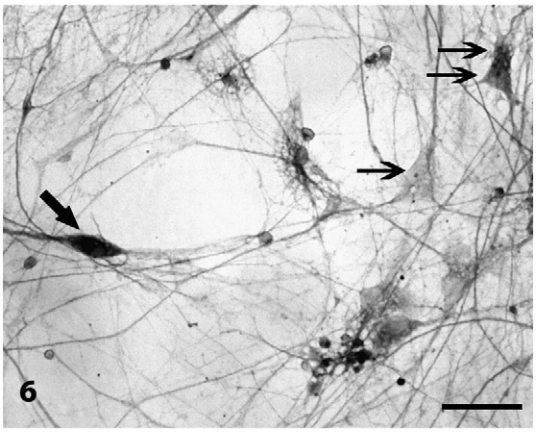


**Fig. 6.** Microphotograph of two-week-old culture of dissociated newborn cortical neurons. Cells and connectivity are well developed. The thick arrow indicates a spindle-shaped neuron exhibiting high intensity (+++) of ghrelin-immunostaining. A multipolar neuron with moderate (++) expression of ghrelin-IR is indicated by a double arrow, and the thin arrow is pointing to a pyramidal perikaryon, which is ghrelin-negative. Scale bar 30 μm.

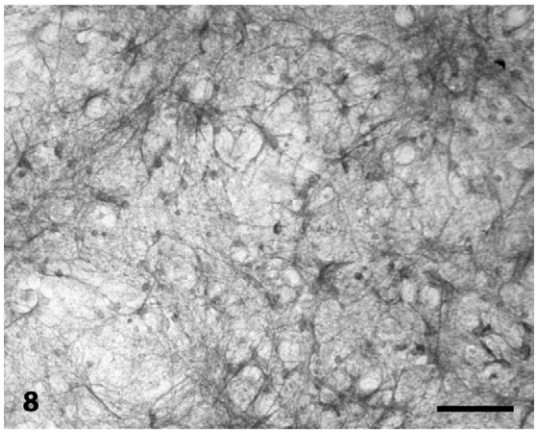


**Fig. 8.** Microphotograph of three-week-old culture immunostained for ghrelin. It shows a dense cluster of neurons, both ghrelin-positive and -negative, and their thick non-varicose processes forming a solid network. Scale bar 40 μm.

adult animals, and their relative percentage was 14.6 (±12.9) which was significantly lower compared with the three groups of cultured neurons (tied $p<0.0001$). The assessment of the immunoreactivity in the cortical ghrelinergic neurons established that 19% of their population demonstrated (+++) staining intensity, 27% were (++), and 54% showed low intensity (+) of ghrelin expression.

## 4. Discussion

The results presented in this paper revealed that dissociated cortical neurons of newborn rats express ghrelin-IR. To the best of our knowledge, the present study demonstrates for the first time ghrelin synthesis in cultured cortical neurons. Moreover, there is a clear manifestation of time-related dynamics in the transmitter appearance. The analysis of our results indicates that as early as 24 h after plating the cultures, an initial ghrelin synthesis appears in the vast majority of the neurons. Ghrelin expression is fully developed in the cytoplasm of single neurons in one-week-old cultures, the number of the positive cells is lower compared with that in the one-day-old cultures but this difference was statistically insignificant ($p=0.1752$). In two-week-old cultures the proportion of ghrelin-IR neurons changes significantly compared with the cultures at the other two early ages ($p=0.0073$). Thereafter, there is a tendency for the number of ghrelin-expressing somata to be reduced, and 36.4% of the neuronal perikarya already change their phenotype into non-ghrelinergic. In the temporal domain, we initially observed a very early ghrelin expression at a high level in dissociated newborn cortical neurons, which shows maturational decrease in two weeks time of culturing.

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. There are two major events, which indeed play a significant role in neuronal development: formation of synaptically connected networks and the emergence of bioelectrical network-wide activity patterns. 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 [18]. This qualitatively mimics the *in vivo* time course of development [19,20]. If the axons do not establish anatomical and functional connections till day 14 *in vitro*, they degenerate [21]. The consolidation of intrinsic connections during early brain development is possible only if there is synchronous network activity, which develops in immature neocortical networks shortly after neurogenesis and disappears before the onset of experience-dependent plasticity [22]. The synchronous network activity triggers collective activation of the silent synapses in the culture [23], a process which starts at day 6 and reaches its maximum at day 12 *in vivo* [21]. Our findings in culture suggest that, *in vivo*, ghrelin may influence synaptic formation and cell-to-cell interactions during development, which are both very important for network

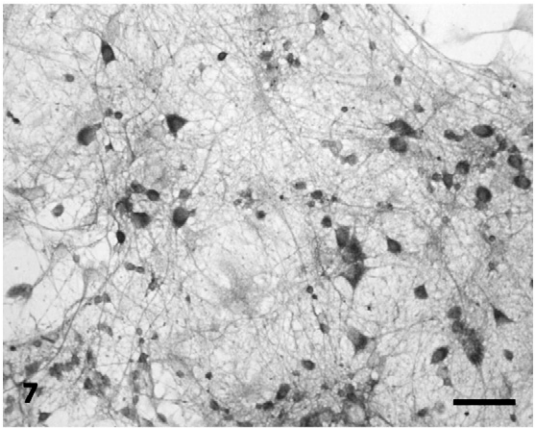


**Fig. 7.** High intensity of ghrelin-immunolabeling of bipolar and multipolar neuronal somata in a two-week-old culture. Thick non-varicose arborizations form a dens network. Scale bar 40 μm.

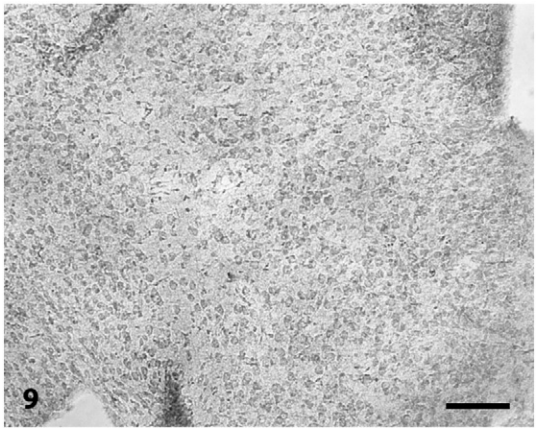


**Fig. 9.** Photograph of the ventral orbital and piriform cortex of one-week-old rat. Most of the neurons express ghrelin-immunoreactivity. Scale bar 100 μm.

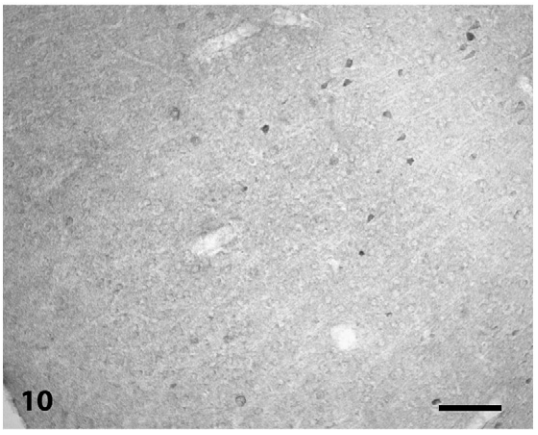


**Fig. 10.** Immunohistochemical labeling for ghrelin in a brain slice of an adult rat, demonstrating ghrelinergic neurons in the primary sensory cortex, jaw region. Immunoreactivity varies from intensive to negative. Scale bar 100 μm.

functions like learning and memory. Further physiological experiments are required to elucidate the exact mechanism of this putative ghrelin function.

## Acknowledgements

The authors would like to thank Dr. Tatyana Vlaykova, Trakia University, for the data statistical analysis. We also thank Prof. Dr. E. Marani for his help with the experimental animals supply, and Dr. J. le Feber for his critical comments on the manuscript. 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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