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Ghrelin stimulates synaptic formation in cultured cortical networks in a dose-dependent manner

Irina I. Stoyanova *, Joost le Feber, Wim L.C. Rutten

Neurotechnology Group, Biomedical Signals and Systems, Faculty of Electrical Engineering, Mathematics and Computer Sciences, Institute for Biomedical Engineering and Technical Medicine MIRA, University of Twente, Enschede, The Netherlands

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ABSTRACT

Ghrelin was initially related to appetite stimulation and growth hormone secretion. However, it also has a neuroprotective effect in neurodegenerative diseases and regulates cognitive function. The cellular basis of these processes is related to synaptic efficacy and plasticity. Previous studies indicated that ghrelin has an excitatory effect on neuronal activity, and stimulates synaptic plasticity *in vivo*. Plasticity in the adult brain occurs in many different ways, including changes in synapse morphology and number. Therefore, we used *in vitro* neuronal cultures to investigate how ghrelin affects synaptogenesis. We used dissociated cortical cultures of newborn rats, chronically treated with different doses of ghrelin (0.5, 1, 1.5 and 2 μM). After one-, two-, three- or four weeks cultures were immunostained for the presynaptic marker synaptophysin. In parallel, additional groups of non-treated cultures were immunostained for detection of ghrelin receptor (GHSR1). During development, GHSR1was increasingly expressed in all type of neurons, as well as the synaptophysin. Synaptic density depended on ghrelin concentration, and was much higher than in controls in all age groups. In conclusion, ghrelin leads to earlier network formation in dissociated cortical networks and an increase in number of synapses. The effect is probably mediated by GHSR1. These findings suggest that ghrelin may provide a novel therapeutic strategy for the treatment of disorders related to synaptic impairment.

1. Introduction

Ghrelin is a 28-amino acid acylated peptide gastric hormone and a neuropeptide, initially identified in the stomach and related to the appetite stimulation and growth hormone (GH) secretion [1]. The transmitter is an endogenous ligand of the orphan G-coupled protein receptor — the growth hormone secretagogue receptor (GHSR1, the only known receptor for ghrelin), which is strongly expressed in the brain, mainly in the hypothalamus, pituitary gland, hippocampus, and some brain stem nuclei [2,3]. Although limited, there is some data about the presence of GHSR1 in the brain cortex of rat, mouse [4], lemur [5], and human [6]. This distribution also suggests that ghrelin has broader functions than the control of GH secretion and food intake. Indeed, it has been demonstrated that ghrelin has a neuroprotective effect in stroke, in ischemia [7] and in Alzheimer's disease [8] and improves functional recovery after a moderate spinal cord injury [9].

Soon after ghrelin's discovery, ghrelinergic neurons were detected in the central nervous system — in the hypothalamic arcuate [10], in paraventricular and supraoptic nucleus, and in the ependymal layer of the third ventricle [11,12]. Some of the projections of these neurons have been traced to the dorsal vagal complex, thus involving ghrelin in the regulation of the brainstem functions [13]. Additionally, ghrelinergic neurons were demonstrated in the rat cortex *in vivo* and *in vitro* [14,15]. Ghrelin-synthesizing neurons were found in the pyramidal layer V of the sensory-motor cortex, in the cingulate gyrus [13] (Hou et al, 2006), and in the primary sensory cortex in adult rats [15]. On the one hand, a striking reduction of mRNA levels for ghrelin, as well as for the enzyme responsible for its acylation, ghrelin-O-acyltransferase, has been revealed in the temporal gyrus of patients with Alzheimer's disease (AD), thereby suggesting that an impairment of the ghrelin system may contribute to the cognitive deficit in this pathology [6]. Unger et al. [16] reported a decline in ghrelin excretion in the preclinical stage of patients with Parkinson's disease, which could make ghrelin a suitable biomarker for this disorder. On the other hand, behavioral experiments with ghrelin infusions in rats significantly facilitated the maze test performances [17,18], thus relating ghrelin to higher brain functions such as cognition and memory performance. Additionally, it has been shown that ghrelin plays an important role in stress, anxiety, depression, sleep and wakefulness. [19,20]. The cellular basis of these processes is related to synaptic efficacy and plasticity [21].

Synaptic plasticity enables connectivity changes in neuronal networks to meet the requirements of the environment. In the developing brain synaptic plasticity extends into maturity and can arise in response to different stimuli and learning new behaviors [22]. Plasticity in the adult brain occurs in many different ways, including changes in synapse

* Corresponding author at: Institute for Biomedical Engineering and Technical Medicine MIRA, BSS, ZH 226, University of Twente, P. O. Box 217, Enschede 7500 AE, The Netherlands. Tel.: +31 53 489 2760; fax: +31 53 489 2287.
E-mail address: stoyanovai@yahoo.co.uk (I.I. Stoyanova).

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morphology and number [23,24]. It has been previously shown that ghrelin takes part in these changes [25] but it is still unclear how ghrelin affects synaptic formation and the time course of its effect. It is very difficult to answer these questions with *in vivo* experiments therefore this research will focus on the effect of ghrelin on network development *in vitro* using cultured cortical networks. We designed experiments with two groups of cultures: one incubated in plain medium, and the other in medium chronically supplemented with ghrelin. To quantitatively evaluate the alterations in network formation we applied immunostaining for detection of the synaptic marker synaptophysin, which is the major integral membrane protein in pre-synaptic vesicles [26], used as a marker for synaptic formation during development [27]. To determine if the effect of ghrelin is dose dependent, we applied four different concentrations. Additionally, another set of cultures was immunostained for demonstration of GHSR1 development.

2. Materials and methods

2.1. Dissociated cell cultures

This research involving animals was conducted according to Dutch law (as stated in "Wet op de dierproeven"), and approved by the Utrecht Animal Use Committee (DEC). Our study required living cells, and therefore the use of donor animals bred in the animal facility of our department. We introduced neurons from the brains of donor newborn Wistar rats into cultures, and performed research on these cultures. To obtain enough cells approximately five pups (from the same mother) were needed per plating. This approach ensured a minimum number of donor animals while obtaining sufficient experimental preparations.

Rat pups were anesthetized with isoflurane and decapitated. The brains were removed and placed in RPMI-medium. The meninges were removed; the cortices were dissociated and collected in chemically defined R12 culture medium [28], commercially available as Neurobasal-A medium (Invitrogen, Carlsbad, CA, USA), with addition of B-27 Supplement (Invitrogen, 10 ml/500 ml medium) and trypsin for further chemical dissociation. After the trypsin treatment, 150 μl of soybean trypsin inhibitor and 125 μl of DNAse I (20.000 units, Life Technology) were added, followed by mechanical dissociation of the neurons. The suspension was centrifuged at 1200 rpm for 5 min. For immunostaining the pellet was plated on glass cover slips at a density of approximately 3000 cells/mm^2. The cover slips were pre-coated with 20 mg/ml poly-ethylene-imine (Fluka, Buchs, Switzerland) for enhancement of the cell adhesion. Cells were allowed to attach for 2 h at 37 °C and 5% CO_2 in air and kept in 600 μl R12 medium optimized with 50 ng/ml nerve growth factor (Invitrogen, Carlsbad, CA). Medium was serum-free to suppress glial cell proliferation and keep their concentration lower than 5% [29]. Cells were kept either in standard medium, cultures referred to as controls (*ctrl*) or with additional ghrelin (*ghr*) (Abcam, Cambridge, UK) under standard conditions of 37 °C and 5% CO_2 in air. Ghrelin concentrations used in other studies varied between 0.1 and 2 μM [30,31], therefore we decided to determine if the effect of ghrelin is dose dependent and used four experimental groups incubated in medium supplemented with 0.5, 1, 1.5 and 2 μM ghrelin, respectively. The medium was renewed every 2 days (300 μl were removed and replaced with the same amount of fresh medium). Cultures, chronically treated with ghrelin, as well as the controls, were fixed in 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS), pH 7.4 after 7, 14, 21 or 28 days *in vitro* (DIV), and processed for immuno-detection of synaptophysin.

2.2. Immunohistochemistry

We used dissociated cells from fourteen plating procedures from different rats (262 cultures in total) plated on coverslips for synaptophysin demonstration. These cultures were divided into two main conditioning categories *ctrl* and *ghr*, and *ghr* treated cultures were further subdivided into four subgroups based on the ghrelin concentration — 0.5, 1, 1.5 and 2 μM. After one-, two-, three- or four-week incubation, cultures were fixed in 4% paraformaldehyde in 0.1 M PBS, pH 7.4, and processed immunocytochemically with the ABC (avidin-biotin-horseradish peroxidase) method [32] for detection of synaptophysin. Briefly, 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 mouse anti-synaptophysin IgG (Abcam, Cambridge, UK, dilution 1:1000) lasted for 20 h at room temperature, and was followed by 2 h incubation with biotinilated donkey anti-mouse IgG (1:500; Jackson ImmunoResearch, West) and 1 h ABC complex (1:500; Vector Labs, Burlingame, CA, USA) application. 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). The same method was applied for detection of GHSR1, using rabbit anti-ghrelin receptor type 1 (Chemicon/Millipor, Billerica, MA, USA, dilution 1:100) as a primary antibody, biotinilated goat anti-rabbit IgG (1:500; Jackson ImmunoResearch, West) as a secondary antibody, and ABC complex (1:500; Vector Labs, Burlingame, CA, USA). Negative controls included incubation after antigen-antibody preabsorption with the native antigen, at 4 °C for 24 h, or replacement of the primary antibody with non-immune serum at the same concentration.

2.3. Data analysis and photomicrograph production

After staining microscope images were generated through 40× and 60× objectives (Nikon) and the cultures were photographed with a Nikon DS-*Fi*1 digital camera linked to a Nikon Eclipse 50*i* microscope. All digital images were matched for brightness in Adobe Photoshop 7.0. For quantitative analysis of synaptic marker expression we counted the number of granules of the reaction product after synaptophysin staining. We used Nikon NIS-Elements software and obtained estimates of the mean densities and standard deviations. First we qualitatively graded the overall density of immunostaining of neurons into three categories: high, medium and low, following the procedure described by Ljungdahl et al. [33]. Then we calculated the granule density under a high magnification at four different neurons from each category, obtained from all 10 to 24 analyzed specimens per condition (*ctrl* or *ghr*), per age (1, 2, 3 and 4 weeks). To obtain values that were not biased by differences in cell density across the cultures, we restricted this analysis to the area of the perikarya and the initial part of the arbotizations. The analysis of neurons from all three categories obviously yielded relatively high standard deviations in the average density per condition, per age, thus preventing overestimation of statistical significance of differences between average densities of different groups. Two-way ANOVA was applied to assess the statistical significance of density differences. Known sources of variation are ghrelin concentration and culture age. All data are presented as mean ± SD unless stated otherwise. A p-value smaller than 0.05 was considered statistically significant.

3. Results

3.1. Specificity of the immunostaining

Specificity of the immunoreaction was tested with two methods: Preincubation of the antiserum with the native protein totally abolished the immunoreaction. No labeling was observed also when the antiserum was replaced by non-immune serum at the same concentration. The immunoreactivity was readily discernible at the light microscopic level by the presence of a dark-gray immunoreactive product. Neuronal