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

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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

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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

structures were considered to be immunopositive when their staining was clearly stronger than that in the background.

3.2. GHSR1 immunoreactivity

Immunocytochemical labeling for demonstration of GHSR1 revealed that most of the neurons after one day of culturing expressed some immunoreactivity. The reaction product appeared as small dots unevenly distributed on the neuronal surface (Fig. 1a). Both types of neurons: bipolar 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 were GHSR1-immunoreactive (IR). A fairly higher density of GHSR1-expression was observed in cultures incubated over a period of one, two and three weeks (Fig. 1b–d).

3.3. Effect of ghrelin on synaptophysin expression

Immunostaining for synaptophysin revealed that chronic treatment of the cultures with ghrelin led to considerable changes in synaptic development in a dose-dependent manner. Seven days after plating in ghrelin-supplemented medium, neurons showed some expression of synaptophysin (from 0.25 ± 0.11 granules/μm^2 at dose 0.5 μM to 0.50 ± 0.15 granules/μm^2 at dose 2 μM), while in controls there was less immunoreactivity (0.25 ± 0.12 granules/μm^2) (Fig. 2a,b). Granules of reaction product were located mainly on the cell bodies. Some immunoreactivity was also observed along the neuronal processes. Synaptophysin was expressed in all type of neurons, regardless their type or size. After two weeks of incubation, synaptophysin expression was detected in all cultures (*ghr* and *ctrl*) (Fig. 2c,d), but far more abundant in *ghr* (from 0.42 ± 0.12 (at 0.5 μM) to 0.69 ± 0.10 (at 2 μM) granules/μm^2), than in *ctlr* cultures (0.42 ± 0.12 granules/μm^2). Both the perikarya and the neurites were immunolabeled for synaptophysin. After three weeks (Fig. 2e,f) we found a substantially higher expression of synaptophysin in *ghr* treated neurons (from 0.46 ± 0.15 (at 0.5 μM) to 0.68 ± 0.19 (at 2 μM) granules/μm^2), compared to *ctrl* (0.33 ± 0.09 granules/μm^2). At the end of the fourth week *in vitro* there was a decline in synaptophysin expression with no much difference between the cultures treated with different concentrations of ghrelin (from 0.44 ± 0.13 to 0.47 ± 0.18 granules/μm^2) but it was significantly higher than in the *ctlr* cultures (0.32 ± 0.13 granules/μm^2). Two-way ANOVA showed that the synapse density significantly depended on age ($p < 0.001$) and ghrelin concentration ($p < 0.001$), but not on the interaction of these factors. Densities were always higher in *ghr* than *ctrl*. Results are summarized and illustrated in Fig. 3.

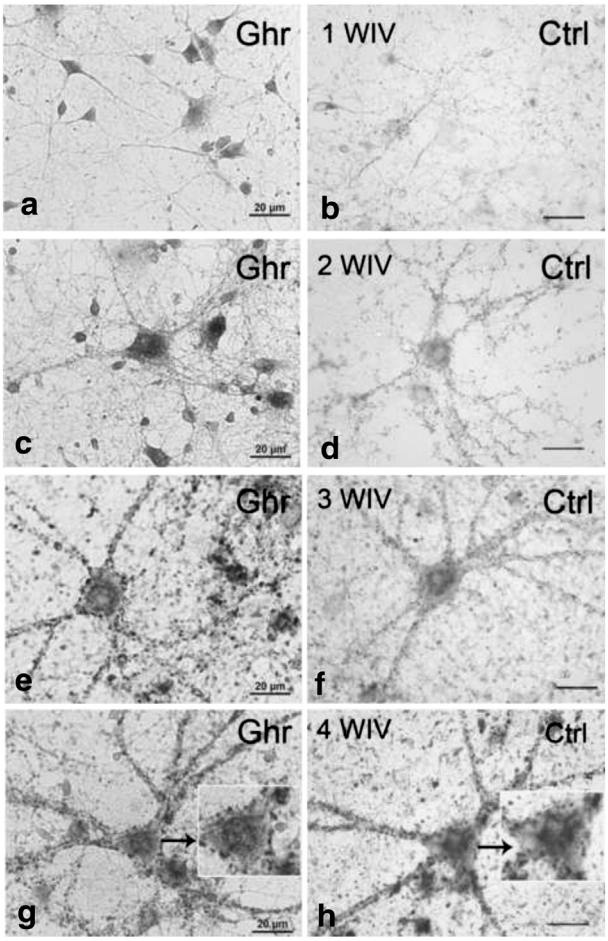

Fig. 2. Example microphotographs of ghrelin pretreated (at concentration 1 μM) (a,c,e,g) and control incubation (b,d,f,h) immunostained for synaptophysin. (a,b) One-week-, (c,d) two-weeks-, (e,f) three-week, and (g, h) four-week-old neuronal cultures. The reaction product appeared as dark-gray dots. Arrows in (g, h) point at panels with closer view of fragments of synaptophysin positive neuronal perikarya. Scale bars: 20 μm.

4. Discussion

In vitro cultured networks maintain many of the cortical cellular properties, while they can be easily manipulated. Therefore, dissociated neuronal cultures provide a suitable model to form an impression of the

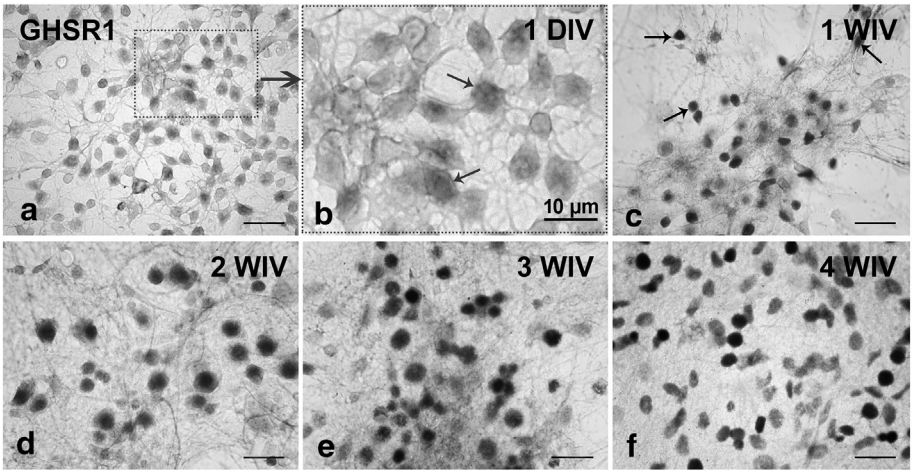

Fig. 1. (a). Immunocytochemical labeling for GHSR1. Already at age of 1 day *in vitro* (DIV) most of the neurons express some immunoreactivity, which appears as small dots unevenly distributed on the neuronal surface. Enlargement of the area in the box is shown in (b). (c–f) A fairly higher density of GHSR1expression was observed in cultures incubated over a period of one, two, three and four weeks *in vitro* (WIV). The arrows in (b) and (c) point at some examples of positive neurons. Scale bars in (a, c–f): 20 μm; scale bar in (b) 10 μm.

possible role of ghrelin in synaptogenesis. Indeed, when ghrelin was chronically applied it had a strong stimulating effect on network formation (accelerated synaptogenesis) in cultured developing cells, and to the best of our knowledge, this study demonstrates it for the first time.

Recent studies reported that ghrelinergic cortical neurons appear very early during development. They were detected in the rat cortex, much more abundant at the earlier postnatal stages (one-week-old rats) than in adult animals [15]. Additionally, ghrelin was found in dissociated cortical neurons with a clear time-related pattern in the transmitter appearance: very early ghrelin expression at a high level (86% of the neuronal population), followed by maturational decrease in the next two weeks of culturing [14]. This qualitatively follows the *in vivo* time course of development of networks, the survival of which requires synaptic consolidation and activation during the first two weeks [29,34].

The paradigm for natural establishment of neural circuits is known to proceed *via* two-stages: 1) early activity-independent wiring to produce a rough map characterized by excessive synaptic connections, when most of the synaptogenesis takes place (between the first and third week), and 2) subsequent, use-dependent pruning to eliminate inappropriate connections and reinforce maintained synapses (the phase of maturation within the fourth and fifth week) [35–38]. Taken together, these previous findings suggest that *in vitro* ghrelin may influence the early synaptic formation, which is very important for network/brain development and functioning. Indeed, the synaptophysin immunostaining clearly illustrates an early expression of a higher density of synapses in ghrelin treated cultures than in control experiments.

Ultrastructural studies of synaptic development in cultured cortical neurons showed a decrease in the synaptic density after 14 DIV [39]. Similarly, in our study we observed a stabilization of synaptic density around that age in the controls, whereas the density of synaptophysin expression gradually and significantly increased up to the end of the third week *in vitro* in ghrelin conditioned cultures. This overshoot appeared proportionally to the ghrelin concentration. During the 4th week *in vitro* the number of synapses in all *ghr* cultures decreased also in a dose-dependent manner — the higher concentration, the bigger decline, and finally leveled off. Ghrelin not only accelerated synaptogenesis but also prolonged the period with excessive connectivity.

Synaptic formation is a complex process which involves a series of events including neuronal fate determination, axon guidance, cell–cell adhesion, local induction of pre- and postsynaptic differentiation, recruitment of neurotransmitter receptors at postsynaptic differentiation and their stabilization at the synaptic junction by proteins packed in electron-dense structures named postsynaptic densities (PSDs) [40,41]. Which parts of this cascade are affected by ghrelin and what are the underlying mechanisms?

In our study we found GHSR1 expression in dissociated cortical neurons already at 1 DIV, which gradually increased during the first two weeks. This is in consent with the finding of Lattuada et al. [42] who reported increasing level of GHSR1 expression in developing hippocampal and cortical neurons, followed by significant reduction in mature neurons at longer times in cultures. Therefore, it is possible that the effect of ghrelin on synaptogenesis in dissociated cortical neurons is mediated *via* GHSR1, as was shown in the ventral thalamic area and substantia nigra of mice and rat [43]. The observation that the receptor, as well as synaptophysin, was expressed in most of the neurons, independently on their type and size, suggests that the synaptogenic effect could be mediated by GHSR1.

Ghrelin binding to GHSR1 activates different intracellular second messengers and triggers different pathways [29,44]. The phospholipase signaling pathway leads to cAMP and protein kinase A activation [29,45], prevention of cytochrome *c* release and subsequent activation of caspase-3, thus inhibiting activation of the apoptotic cascade [46]. When the phosphatidyl inositol 3-kinase (PI3K)/ protein kinase B (Akt) and extracellular-signal-regulated kinase (ERK)1/2 pathways are triggered by ghrelin it results not only in prevention of apoptosis but also induction of cell proliferation [47].

Recent studies on the hippocampus indicate that ghrelin takes part in the synaptic assembling by promoting dendritic spine synapse formation [26]. Additionally it has been shown that ghrelin induces synaptic rearrangement in GHSR1-dependent manner in which excitatory synapses dominated the hypothalamic perikarya [11]. It would be beneficial to investigate further the effect of ghrelin on the postsynaptic density protein 95 (PSD-95) expression, which is anchoring and organizing postsynaptic neurotransmitter receptors [48], known to be expressed only at excitatory synapses [49,50]. Thus, the mechanism regulating the ratio of inhibitory vs. excitatory synapses as a target of ghrelin activity could be determined.

Based on the excitatory nature of the neurotransmitter, it could be speculated that ghrelin affects synaptogenesis by stimulating synaptic activity, which triggers further synthesis and aggregation of neurotransmitter receptors, and synaptic maturation. It is possible that other type of synaptic signals *e.g.*, agrin-like molecules are released in response to electrical activity stimulated by ghrelin [51]. Actually agrin operates a rapid switch from gap junction-mediated electrical communication to synaptic transmission therefore, it is essential in synaptic development and maturation [52]. On the other hand, while it could be a possible mechanism of ghrelin's effect during the second phase of synaptogenesis, when the synaptic activity is essential for the receptor expression and localization [53], it cannot explain the effect on the initial synaptic assembly, when activity is not crucial, as it was shown for the basics of glutamate or GABA synapse formation [40,54]. Similarly, it is equally possible that some other, as yet unidentified transmitter functions are involved, *e.g.* as an inducing agent. At present, we cannot distinguish between these and similar possibilities.

It is also important to mention that during the early development of the central nervous system, neurons form extensive interconnections, and thus create functional networks, which exhibit frequent spontaneous action potential discharges [55]. As it has been previously shown *in vitro*, spontaneous neuronal activity usually emerges toward the end of the first week, when the synaptic contacts become stable enough to enable neurons to trigger one another [56]. Because of the strong mutual influence between neuronal connectivity and activity (structure–function relationship) during early development as, for example, explored *in vitro* at network level [57], further electrophysiological *in vitro* experiments may functionally validate the effects of ghrelin on the network.

In summary, we have demonstrated that when applied to dissociated cortical neurons ghrelin exerted a stimulating effect on synaptogenesis. It accelerated and prolonged synaptic formation in a dose-dependent manner. It affected all neuronal cell types in the cultures, and was probably mediated by GHSR1. These findings suggest that ghrelin may provide a novel therapeutic strategy for the treatment of disorders related to impaired synaptic formation.

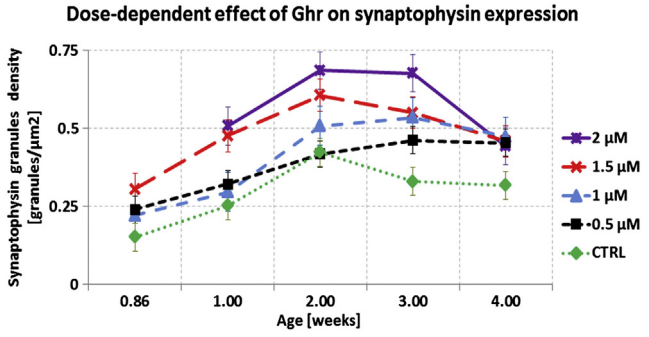

Fig. 3. Quantified synaptophysin expression during culture development. Synaptophysin is quantified from data as in Fig. 2 at different ages under control conditions and after chronic treatment with ghrelin (0.5–2 µM). Expression was significantly higher in ghrelin treated cultures than in control ($p < 0.001$). Error bars indicate SEM and refer to differences between all neurons and across cultures.

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Competing interest

The authors have declared that no competing interests exist.

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