Effect of Ghrelin on the Network Development and Activity in Cultured Cortical Neurons of Newborn Rats

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Ghrelin is a gastric hormone and a neuropeptide, initially related to the appetite stimulation and growth hormone secretion. Ghrelin has also an important role in the regulation of many other processes, including higher brain functions like memory performance, sleep and wakefulness. Previous studies on the ghrelin activities indicate that the transmitter has an excitatory effect on the neuronal activity of hypothalamic slices in vitro by direct synaptic interaction, and controls synaptic plasticity in the hippocampus when applied intravenously. However the effect of ghrelin on developing neuronal networks has not been studied yet. Therefore, we cultured dissociated cortical neurons of newborn rats in a medium containing ghrelin for a period of three weeks and recorded the network activity using multy electrode arrays. In addition, after a cultivation of the cultures for one-, two- and three weeks they were stained immunocytochemically with the ABC method for detection of the synaptic marker Synaptophysin. The control recording experiments included culturing of the neurons in medium without ghrelin. The ghrelin conditioning of the medium led to earlier formation and activation of the networks. The activity pattern we observed at 6 DIV in Ghr cultures normally did not appear before the end of the second week. The immunostaining for synaptophysin revealed that the density of the synapses is much higher in Ghr cultures than in ctrls at all stages of incubation. In conclusion, ghrelin has a strong stimulating effect on functional network formation and earlier collective burst activity. This high level of functional connectivity is a possible consequence of a rapid chemical synaptogenesis, as the immunostaining clearly showed.

1 Introduction

Ghrelin is a gastric hormone and a neuropeptide, initially related to the appetite stimulation and growth hormone (GH) secretion [1]. It is produced mainly in the stomach, but also in the brain by neurons in the hypothalamic arcuate nucleus [2] and paraventricular neurons [3]. Ghrelin is an endogenous ligand of the orphan G-coupled protein receptor – the growth hormone secretagogue receptor (GHS-R), which is greatly expressed in the brain. This distribution suggests that ghrelin has broader functions beyond the control of GH secretion and food intake. Indeed, it has been demonstrated that ghrelin has an important role in the regulation of many other processes, including higher brain functions like memory performance, sleep and wakefulness [4], the cellular basis of which are the synaptic efficacy and plasticity. Previous studies on ghrelin induced activity indicate that the transmitter has an excitatory effect on the neuronal activity of hypothalamic slices in vitro by direct synaptic interaction [5], and controls synaptic plasticity in the hippocampus when applied intravenously [6]. However, the importance of ghrelin for developing neuronal networks *in vitro* has not been investigated yet. Therefore, we performed electrophysiological experiments on cultured neuronal networks coupled to microelectrode arrays (MEAs) combined with immunocytochemistry for synaptic detection.

The pattern of collective electrophysiological activity changes with time during the first three weeks of development widely recognized as a maturation of the network. The natural changes in the activity dynamics comprise two major phases: the first phase is between the first and third week *in vitro*, when the synaptogenesis takes place and the electrophysiological behaviour of the network changes, both in spike and burst activity; and the second phase of maturation (within the fourth and fifth week), characterised by the modulation and shaping of the synaptic connectivity [7,8,9]. The previous work on cultured cortical neurons is mainly connected with the correlation between the age of the culture and the resulting activity pattern, but these are only limited aspects of the complex dynamics in the developing networks *in vivo*, missing the natural input in the developing brain. In this study we focus on the input from the ghrelin system, to see how the network formation and electrophysiological behaviour are influenced.

2 Material and Methods

Dissociated cortical neurons of newborn rats were obtained at post natal day 1 and plated on 60 electrode multi electrode arrays (MEA) (Multi Channel Systems, Reutlingen, Germany) 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 (*ctrl*) or with additional ghrelin (*Ghr*) 2 □M, Abcam, Cambridge, UK) under standard conditions of 37°C and 5% CO2 in air. An initial cell density of approximately 3000 cells/mm^2 was used in all experiments. The neurons were cultured for a period of at least three weeks, and their activity was recorded for at least one hour on different days. For each culture we plotted the mean firing rate as a function of age. To enable comparison across cultures, the individual curves were normalized to their mean values and then averaged. Finally, all data was grouped in bins of 5 days of age (mean±SD) to obtain curves as in Figure 2.

In addition, after one-, two-, and three-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 for detection of the synaptic marker synaptophysin. 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 mouse anti-synaptophysin IgG (Abcam, Cambridge, UK dilution 1:1000) lasted for 20 h at room temperature and was followed by 2 h biotinilated donkey anti-mouse IgG (1:500; Jackson ImmunoResearch, West) and 1 h 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 incubation after antigen-antibody preabsorption with the native antigen, at 4 °C for 24 h. 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.

3 Results

Experimental data presented here were obtained from 8 cultures, coming from different preparations, monitored for at least 3 consecutive weeks. All the cultures exhibited highly variable patterns of spontaneous activity during their temporal maturation. When the cortical networks were incubated under control conditions, at day 7 *in vitro* (DIV) only random spikes were generated (average 1.1 spikes/sec), and there was no clear evidence of collective bursts (Fig. 1). However, the ghrelin conditioning of the medium led to earlier formation and activation of the networks. At young age, the activity of the Ghr cultures was substantially higher then that of the controls, the Ghr cultures exhibitted some activity as early as 3 DIV. The activity pattern we observed at 6 DIV in Ghr cultures normally did not appear before the end of the second week (11-12 DIV) in ctrl cultures (Fig. 1). At that age we recorded on average 8.8 spikes/sec, while in the ctlrs showed no activity before 7 DIV. in Ghr cultures the mean firing rate reached a plateau at day 10-12, which is also considerably earlier than the controls (17-18 DIV) (Fig. 2). The immunostaining for synaptophysin revealed that the density of the synapses is much higher in Ghr cultures than in ctrls at all stages of incubation (Fig. 3, 4).

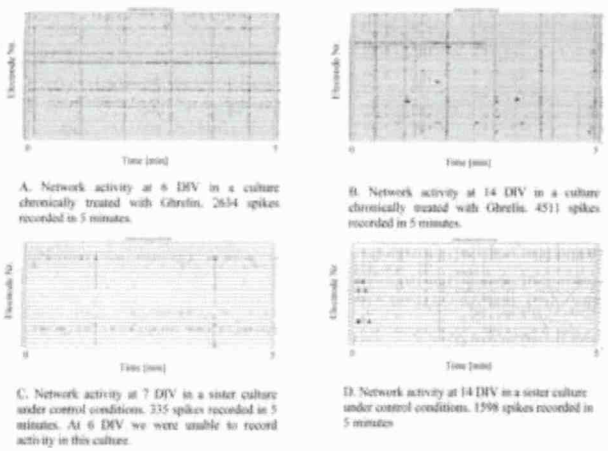

Fig. 1. Raster plots of the neuronal activity recordings in cultures incubated with ghrelin (top panels) and controls (bottom panels).

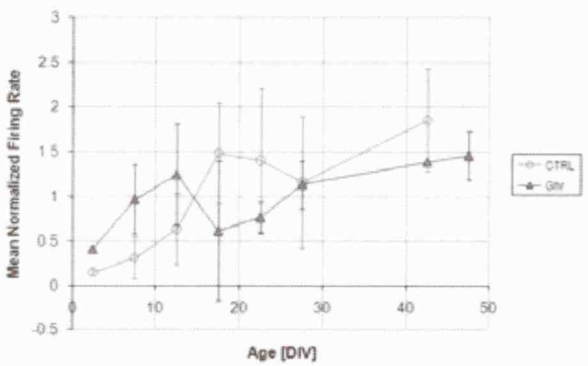

Fig. 2. **Development of mean firing rates (normalized) of cultures incubated with ghrelin (Ghr, N=3) and under control conditions (N=5). All data was normalized and pooled into 5 day bins**

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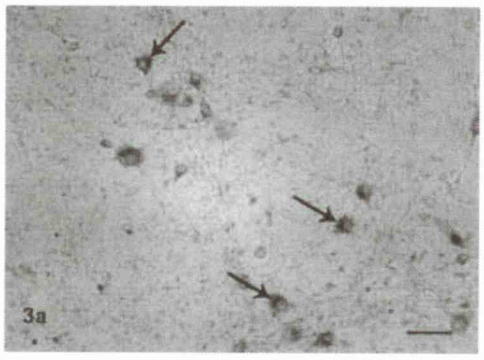
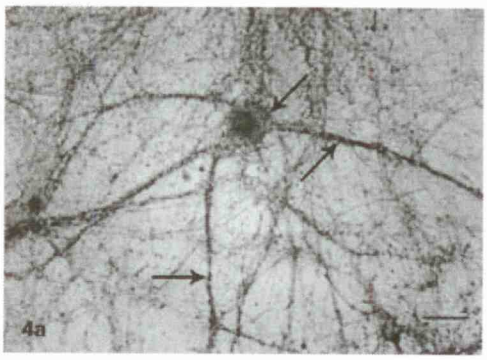


Fig. 3. One-week-old neuronal cultures immunostained for synaptophysin after ghrelin pretreatment (a) and control (b). Synapses appeared as dark-gray dots (arrows). Scale bars 20 μm.



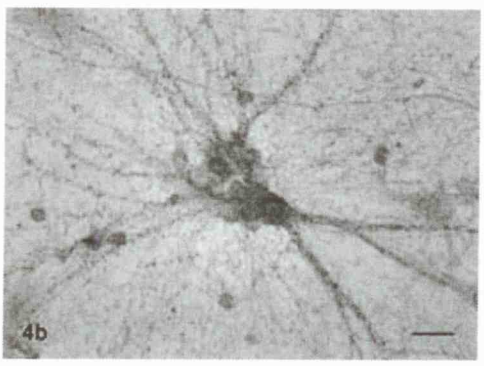


Fig. 4. Immunolabeling for synaptophysin after culturing for three weeks in medium containing ghrelin (a) and a control, incubated in plain medium (b). Arrows point at the synapses on the perikaryon and the neurites. Scale bars 20 μm.

4 Conclusions

Previous studies on *in vitro* networks of cortical neurons, deprived of external stimuli, report the existence of a correlation between the age of the culture and the resulting activity pattern [10], and characterize the main phases of their development [7]. The results from our study provide clear evidence that chronic application of ghrelin has a strong stimulating effect on functional network formation. Ghrelin increases spike generation, and leads to earlier and better expressed collective burst activity. This high level of functional connectivity is a possible consequence of a rapid chemical synaptogenesis [11]. Indeed, immunostaining clearly showed an increased density of synapses observed in ghr cultures compared with the control experiments. Thus, it supports our electrophysiological findings of higher network activity, and clearly indicates that ghrelin also significantly increases synaptogenesis.

This effect of ghrelin on synaptic formation and function could provide a molecular target in developing novel therapeutics for disorders related to neurodegeneration and impaired synaptic plasticity.

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