Neuroscience Letters 653 (2017) 126–131

Contents lists available at ScienceDirect

Neuroscience Letters

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

Research article

Hippocampal Ghrelin-positive neurons directly project to arcuate hypothalamic and medial amygdaloid nuclei. Could they modulate food-intake?

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HIGHLIGHTS

- Hippocampal Ghre-containing neurons projecting to the Hypothalamus and Amygdala.
- Double labeled ghrelin neurons were mainly detected in H-CA1.
- Appetite and food intake are controlled by hippocampal Ghre-containing neurons.
- Hippocampus is essential part of a circuit for Ghre-mediated control of food intake.

ARTICLE INFO

Article history:
Received 28 March 2017
Received in revised form 17 May 2017
Accepted 22 May 2017
Available online 25 May 2017

Keywords:
Ghrelin
Hippocampus collateralized neurons
Retrograde tracers
Immunohistochemistry
Food intake
Rat

ABSTRACT

Feeding is a process controlled by a complex of associations between external and internal stimuli. The processes that involve learning and memory seem to exert a strong control over appetite and food intake, which is modulated by a gastrointestinal hormone, Ghrelin (Ghre).

Recent studies claim that Ghre is involved in cognitive and neurobiological mechanisms that underlie the conditioning of eating behaviors. The expression of Ghre increases in anticipation of food intake based on learned behaviors. The hippocampal Ghre-containing neurons neurologically influence the orexigenic hypothalamus and consequently the learned feeding behavior.

The CA1 field of Ammon's horn of the hippocampus (H-CA1) constitutes the most important neural substrate to control both appetitive and ingestive behavior. It also innervates amygdala regions that in turn innervate the hypothalamus. A recent study also implies that Ghre effects on cue-potentiated feeding behavior occur, at the least, via indirect action on the amygdala.

In the present study, we investigate the neural substrates through which endogenous Ghre communicates conditioned appetite and feeding behavior within the CNS. We show the existence of a neural Ghre dependent pathway whereby peripherally-derived Ghre activates H-CA1 neurons, which in turn activate Ghre-expressing hypothalamic and amygdaloid neurons to stimulate appetite and feeding behavior. To highlight this pathway, we use two fluorescent retrograde tracers (Fluoro Gold and Dil) and immunohistochemical detection of Ghre expression in the hippocampus. Triple fluorescent-labeling has determined the presence of H-CA1 Ghre-containing collateralized neurons that project to the hypothalamus and amygdala monosynaptically. We hypothesize that H-Ghre-containing neurons in H-CA1 modulate food-intake behavior through direct pathways to the arcuate hypothalamic nucleus and medial amygdaloid nucleus.

1. Introduction

1.1. Ghrelin

Ghrelin (Ghre) is a gastrointestinal peptide hormone of 28-amino-acid acylated, isolated from the stomach of rat [1,2]. Ghre regulates food intake, body weight, adiposity and glucose metabolism through the activation of orexigenic neural circuits

Abbreviations: BBB, blood brain barrier; GHS-R, Ghrelin receptor; H-CA1-3, CA1 CA2 and CA3 fields of Ammon's horn of hippocampus; PVH, paraventricular nucleus; LH, lateral nucleus; Me, medial amygdaloid nucleus; FG, fluoro Gold; Dil, 1,1′-dioctadecyl 3,3,3′,3′-tetramethyl-indocarbocyanine perchlorate; NTS, nucleus of the solitary tract.

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http://dx.doi.org/10.1016/j.neulet.2017.05.049
0304-3940/

[3–5]. Ghrelin is commonly referred to as a "hunger hormone", but new researches describe it as a signal to the prediction of food intake [6–8]. It is supposed that the Ghre is involved in regulating energy homeostasis because it is able to cross the Blood Brain Barrier (BBB).

Ghre acts within the hypothalamic arcuate nucleus (ARH), directly activating neuropeptide Y neurons, stimulating feeding [9] and gastric motility [3]. In recent years, several researchers have shown that Ghre levels increase in pre-prandial stage and when administered it stimulates food intake in humans and rodents [10,11].

The orexigenic activity of Ghre is very apparent when delivered on several targets in the brain, such as the amygdala [12] and the hippocampus [13].

1.2. Ghrelin and hippocampus

In recent years, many researchers have shown the presence of several receptors for Ghrelin (GHS-R) in some hippocampal fields, in relation to the regulation of feeding behavior. In particular high-density signals were displayed in CA1, CA2 and CA3 fields of Ammon's horn of hippocampus (H-CA1-3) and the dentate gyrus [14]. These signals reach the hippocampus through various Ghre neurons in the brain, including the hypothalamus and amygdala.

Appetite and food intake are controlled by processes of learning and memory [15,16].

Stimuli related to food can affect the feeding behavior through the construction of a complex mnemonic pattern that requires a neural process in the hippocampus [17–19] that regulates the consolidation of food-related declarative memory, thus increasing its plasticity.

Kanoski et al. (2013) have showed that hippocampal neurons are fundamental for the integration of learned factors, with the detection and utilization of food-relevant stimuli that inform us about both external and internal cues. Moreover, the decision to acquire the food is conditioned by cognitive factors like the incentive motivation and/or factors modulating feeding behavior [20].

The internal signals of hunger and satiety involve the hippocampus in the control of feeding behavior [21], through Ghre and other endocrine cues (e.g., leptin, glucagon-like peptide-1, insulin). The receptors for these signals are expressed in hippocampal neurons, which collect information to regulate food intake and body weight even from the periphery [20]. As result of these hormonal signals, hippocampal neurons change their synaptic plasticity and neurogenesis, thus helping to create new memories [22,23]. In particular, the Ghre reaches the CA1 region of the hippocampus causing synaptic changes that promote greater spatial learning and memory [24]. So there is integration between ghrelin, hormones and other cephalic neural substrates that control learning and memory processes related to feeding behavior.

1.3. Ghrelin and hypothalamus

Ghre-immunoreactive neurons are found widely distributed within the hypothalamus. The ARH, is an important structure for the hormonal, metabolic and neuronal signals, reflecting the state of the body's energy; it contains a set of key neurons that co-express high levels of GHS-R and also orexigenic neuropeptides, neuropeptide Y (NPY) and aminobutyric acid (GABA) [14,25]. A feeding behavior adjustment model predicts that primary neurons in the ARH respond to Ghre and in turn modulate the activity of secondary hypothalamic nuclei, such as paraventricular nucleus (PVH) nucleus and the lateral nucleus (LH), in the production of orexigenic peptides to adjust intake energy and maintain body weight [3,25–28].

1.4. Ghrelin and amygdala

Experimental evidence suggests that the amygdala participates in the regulation of memory and learning processes [29,30]. Several electrophysiological and neuroanatomical studies identify the amygdala, the hypothalamus and the hippocampus, as well as a target for Ghre. The amygdala is considered a key target of the CNS for the integration of the acquisition of the food and the emotional reactivity [31,32].

Many authors describe projections from the dorsal hippocampus CA1 to the amygdala [33,34] and from CA2 to the hypothalamus [35] in mediating social and emotional input for memory processing. Other authors show that the synaptic plasticity linked to spatial learning in the CA3 field of the hippocampus is activated by the amygdala [36]. Further extensive data indicate that the amygdaloid complex is reciprocally connected with the hippocampus [37,38], as well as with the hypothalamus [39], this is also demonstrated in the human brain [40]. So it is possible that the hippocampus plays, engaging with amygdala and hypothalamus, a very important role in the regulation of feeding behavior processes. In this study we wanted to verify the existence of a neural pathway direct from the H-CA1-3, both towards the medial hypothalamus (in particular to ARH) and towards the medial amygdaloid nucleus (Me), having assumed that this pathway, involving a collateralized projective arrangement, uses the Ghre as a mediator of feeding behavior learned.

To highlight the presence and the distribution of hippocampal Ghre-containing collateralized neurons that directly project to the hypothalamus and amygdala, two fluorescent retrograde tracers and immunohistochemical technique were used.

2. Materials and methods

2.1. Animals

Experiments were performed on 9 male Sprague-Dawley rats (250–300 g) from Envigo RMS s.r.l. Italy. The animals were housed in standard conditions of temperature (23 ± 1 °C) and humidity with 12 h light/dark cycle and *ad libitum* access to water and food. Upon arrival, animals were allowed to acclimate for at least one week before being used in the experiments. Experiments were carried out in compliance with the Italian law on animal care (D. Lgs. 26/2014) and in accordance with the European Community Council Directive 2010/63/EU. All efforts were made to minimize animals suffering and to reduce the number of animals used.

2.2. Experimental procedures

Two fluorescent tracers were injected into the same rat: Fluoro Gold (FG, Biotium; diluted 6% in saline solution) was monolaterally injected into ARH and Dil (1,1′-dioctadecyl 3,3,3′,3′-tetramethylindocarbocyanine perchlorate, Molecular Probes, Inc., solubilized in 5% N,N-dimethylformamide) was injected into the Me, on the same side, to retrogradely label the hippocampus direct projective neurons.

Rats were placed in a Kopf stereotaxic frame and injected with 0,04 μl of freshly dissolved FG into the ARH nucleus, at the following coordinates (anterior-posterior AP = −3,30 mm; lateral L = 0,2 mm; vertical V = −10,2 mm); freshly dissolved 0,04 μl Dil was injected into the Me at the following coordinates (AP = −3,30 mm; L = 3,0 mm; V = −9,4 mm) [41]. Both tracers were pressure-injected at a rate of 50 nl/min using 1 μl Hamilton microsyringe advanced by an electronic microdrive (David Kopf).

Seven days after the injections, the animals were anaesthetized again and perfused through ascending aorta with 60 ml saline, fol-