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Ghrelin alters neurite outgrowth and electrophysiological properties of mouse ventrolateral arcuate tyrosine hydroxylase neurons in culture

Shuo Huang, Samantha A. Lee, Karen E. Oswald, Mark Fry*

Department of Biological Sciences, University of Manitoba, Winnipeg, MB, Canada

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ABSTRACT

While the appetite-stimulating hormone ghrelin can act to acutely modulate electrical activity of neurons in the appetite regulating network, it also has a role in regulating neuronal outgrowth, synaptic connectivity and intrinsic electrophysiological properties. In this study, we investigated whether ghrelin may cause alteration in neurite outgrowth and electrophysiological properties of tyrosine hydroxylase (TH) neurons from the ventrolateral arcuate nucleus (VL-ARC), which are thought to contribute to regulation of energy balance. We prepared dissociated neuronal cultures from the VL-ARC of transgenic mice expressing EGFP under control of the tyrosine hydroxylase (TH) promoter, thus allowing visual identification of putative catecholaminergic (TH-EGFP) neurons. After five days of treatment with 100 nM ghrelin, TH-EGFP neurons exhibited significantly more and longer neurites than control treated neurons, and the effects of ghrelin were abolished by 100 μM ghrelin antagonist, D-Lys-GHRP-6. To investigate whether ghrelin altered electrophysiological properties of TH-EGFP neurons, we carried out patch clamp experiments measuring electrophysiological properties. No significant differences were identified for resting membrane potential or spontaneous action potential frequency, however we observed a hyperpolarization of threshold for action potentials and increased input resistance, indicating increased excitability. This increased excitability is consistent with an observed hyperpolarizing shift in the activation of voltage-gated Na^+ currents. These data indicate that the hunger signal ghrelin induces plastic changes in TH-neurons from VL-ARC.

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1. Introduction

The arcuate nucleus of the hypothalamus (ARC) is a key component of the network within the CNS which regulates energy homeostasis. Neurons of the ARC are not static, but exhibit considerable plasticity in response to satiety signals such as ghrelin and leptin. Pinto et al. [1] observed that administration of ghrelin and leptin to mice caused synaptic reorganization in the AGRP/NPY (agouti gene related peptide, neuropeptide Y) and POMC/CART (pro-opiomelanocortin, cocaine-amphetamine regulated transcript) neurons of the ARC. Yang et al. [2] demonstrated that increasing levels of circulating ghrelin caused long-lasting but reversible enhanced glutamate signaling onto AGRP neurons. Recently, Steculorum and colleagues [3] demonstrated that ghrelin signaling plays an organizational role in the ARC during development, blunting axonal growth to downstream targets of the ARC.

Studies examining plasticity within the ARC have typically focused on the AGRP/NPY and POMC/CART neuronal subpopulations (for review see Ref. [4]), however little is known about plasticity of tyrosine hydroxylase (TH) neurons of the ventrolateral ARC (VL-ARC). The VL-ARC neurons are distinct from the well-known dorsomedial TH-neurons which regulate prolactin release [5,6]: indeed a substantial proportion of VL-ARC neurons lack expression of aromatic amino acid decarboxylase, the key enzyme required for converting L-DOPA to dopamine [7]. While the roles of VL-ARC TH-neurons are not well-understood, they are positioned to play a role in regulation of energy balance. TH-neurons of the VL-ARC express the ghrelin receptor [8] and are activated by ghrelin agonists [9]. Destruction of VL-ARC TH-neurons by monosodium glutamate results in obesity [10], while leaving prolactin secretion intact [6]. Moreover, NPY-neurons of the ARC synapse with TH-

* Corresponding author. Rm 469 Duff Roblin Building, Department of Biological Sciences, University of Manitoba, Winnipeg, MB, R3T 2N2, Canada.
E-mail address: mark.fry@umanitoba.ca (M. Fry).

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neurons and activate Y1 receptors [11–13]. Conversely, VL-ARC TH-neurons may also synapse with ARC NPY-neurons [14]. In this study, using dissociated neurons prepared from transgenic mice that express green fluorescent protein (EGFP) under the control of the TH promoter, we explored the possibility that ghrelin causes plastic changes in VL-ARC TH-neurons. We observed that application of ghrelin to VL-ARC neurons resulted in significant increases in the length and number of neurites on TH-EGFP neurons. Ghrelin treatment also caused an increase in excitability of these neurons by hyperpolarizing the threshold to fire action potentials (APs), increasing input resistance, and shifting the activation of voltage gated Na^+ currents to more hyperpolarized potentials. These effects were abolished by the antagonist D-Lys-GHRP-6. These results suggest that ghrelin may modulate the plasticity of TH-neurons within the VL-ARC, resulting in long term changes to the circuits that regulate energy balance.

2. Materials and methods

2.1. Animals and cell culture

All animal protocols were approved by the University of Manitoba Animal Care Committee in compliance with guidelines from the Canadian Council for Animal Care. Five to six week-old male transgenic mice (background C57BL/6, obtained from Dr Kazuto Kobayshi, Fukushima Medical University). These mice expressed EGFP under control of the TH promoter, allowing accurate visual identification of TH-neurons [15] (Fig. 1A).

2.2. Cultures and treatments

Briefly, for each set of dissociated neuronal cultures, a TH-EGFP transgenic mouse was decapitated, the brain removed and placed in ice-cold oxygenated artificial cerebrospinal fluid consisting of (in mM): 87 NaCl, 2.5 KCl, 7 $MgCl_2$, 1.25 NaH_2PO_4, 0.5 $CaCl_2$, 25 $NaHCO_3$, 25 glucose and 75 sucrose. Brain slices containing the ARC were cut to 300 μm and transferred into Hibernate media (Brain Bits, Springfield, IL) containing 1X B27 supplement and 1X glutamax (Invitrogen, Burlington, ON). VL-ARC was dissected from the slices and dissociated using papain as previously described [16]. Dissociated cells were plated at low density (100–300 cells) on untreated 35 mm glass bottom dishes (MatTek Ashland, MA) in Neurobasal media containing B27 and glutamax (Gibco, Burlington, ON). Cultures were incubated for 5 days at 37 °C, 5% CO_2 for morphological measurements, and 4–6 days for patch clamp electrophysiology. Cultures were maintained in either control media (containing supplemented media only); control media with 100 nM ghrelin (Phoenix Peptide, Burlingame, CA); or control media with 100 nM ghrelin and 100 μM of the ghrelin receptor antagonist D-Lys-GHRP-6 (Tocris, Minneapolis, MN). Concentrations of ghrelin used in other studies to examine excitation, synaptogenesis and neurite outgrowth have varied from 0.1 nM to 30 uM [17]. For the present experiments, we used 100 nM ghrelin, as this concentration is near the top of a dose response curve [18] and is close to the 30 nM used by Steculorum et al. [3] in examining neurite outgrowth from ARC explants. Initial experiments indicated that neuronal survival was not influenced by ghrelin or antagonist.

2.3. Image acquisition and morphology analysis

On the fifth day of culture neurons were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) and examined using DIC optics and epifluorescence (Fig. 1B). Digital images were captured with a QImaging EXi Blue camera (Surrey, BC). Starting on the upper left portion of the dish and moving in a zigzag pattern, 12–15 EGFP-expressing neurons per dish were identified and photographed. Soma size, neurite number, neurite length and number of branches were quantified using ImageJ software. Only neurons that were not contacting other neurons or debris were analyzed. The experiment was replicated six times for each treatment group.

2.4. Electrophysiology

At least 20 min before patch clamp analysis, culture media was replaced with external recording solution (ERS) to wash away ghrelin and antagonist in order to eliminate possible acute effects of these agents. Whole-cell patch clamp recordings were carried out on TH-EGFP neurons on days 4–6 of treatment using methods essentially as previously described [16,18]. TH-EGFP neurons were identified using a Zeiss IM-35 inverted microscope equipped with epifluorescence. Data were acquired using a HEKA EPC10 patch clamp amplifier and Patchmaster software (v2.53–2.59; Mahone Bay, Nova Scotia, Canada). During recording, cells were bathed with ERS containing (in mM): 140 NaCl, 4 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 HEPES and 10 glucose, pH 7.3 with NaOH. Electrodes had resistances of 3–6 MΩ when filled with internal recording solution containing (in mM): 130 K^+-gluconate, 10 KCl, 10 HEPES, 5 EGTA, 1 $MgCl_2$, 0.1 $CaCl_2$ and 4 Na_2ATP, pH 7.25 with KOH. Spontaneous APs were recorded in cell-attached configuration, before establishing whole cell configuration. Resting membrane potential (RMP), input resistance and AP properties were recorded in current clamp configuration (data filtered at 2.9 kHz and acquired at 20 kHz). AP threshold (defined as the point where the slope of the voltage trace reached 10 mV/ms) was determined using a 100 ms current ramp from −10 to +10 pA. Voltage clamp data were filtered at 3 kHz and acquired at 40 kHz. Series resistance was less than 20 MΩ and compensated from 70% to 80%.

2.5. Statistical analysis

All statistical tests were carried out using Origin Pro v9.0. Initial exploration of morphological data revealed no significant differences between replicate groups, so all data were pooled. All experiments were analyzed using either ANOVA followed by Fisher post hoc test (for normally distributed data) or non-parametric Kruskal–Wallis ANOVA followed by pair-wise Mann–Whitney tests with Bonferonni corrections (for non-normally distributed data) as appropriate. Data are presented as mean ± SEM.

3. Results and discussion

A striking difference in the morphology of TH-EGFP neurons was observed with addition of 100 nM ghrelin as compared to control-treated cultures and 100 μM D-Lys-GHRP-6 GHSR-treated cultures (Fig. 1). Ghrelin treatment stimulated neurite outgrowth, resulting in more neurites and longer neurite length compared to control and antagonist treatments (Fig. 1C–E). Specifically, ghrelin-treated neurons had a mean of 2.4 ± 0.1 neurites per neuron ($n = 224$) compared to 1.5 ± 0.1 ($n = 388$) for control and 1.3 ± 0.1 for ghrelin plus antagonist treatments ($n = 136$; $p < 0.05$, Kruskal–Wallis ANOVA; Fig. 1E). Neurite length for ghrelin-treated neurons was significantly longer at 70.0 ± 6.9 μm ($n = 224$), compared to 52.7 ± 4.8 μm for control ($n = 388$) and 47.9 ± 7.5 μm for ghrelin plus antagonist treatments ($n = 136$; $p < 0.05$, Kruskal–Wallis ANOVA; Fig. 1D). No significant difference was noted for mean soma size (Fig. 1D) of TH-EGFP neurons in treated cultures.

We next investigated whether ghrelin treatment could also alter

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