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ACYLATED AND UNACYLATED GHRELIN CONFER NEUROPROTECTION TO MESENCEPHALIC NEURONS

JOHANNA WAGNER,[a] FRANCA VULINOVIĆ,[b] ANNE GRÜNEWALD,[b,c] MARCUS M. UNGER,[a,d] JENS C. MÖLLER,[e] CHRISTINE KLEIN,[b] PATRICK P. MICHEL,[f] VINCENT RIES,[a] WOLFGANG H. OERTEL[a] AND DANIEL ALVAREZ-FISCHER[a,b,g]*

[a] *Department of Neurology, Philipps-University of Marburg, 35043 Marburg, Germany*

[b] *Institute of Neurogenetics, University of Lübeck, 23562 Lübeck, Germany*

[c] *Luxembourg Centre for Systems Biomedicine, Université du Luxembourg, 6, avenue du Swing, L-4367 Belvaux, United Kingdom*

[d] *Department of Neurology, Saarland University, 66421 Homburg, Germany*

[e] *Neurocentro della Svizzera Italiana, Clinical Trials Unit, Ospedale Regionale di Lugano, Via Tesserete 46, 6903 Lugano, Switzerland*

[f] *Sorbonne Universités, UPMC Univ Paris 06 UM75, INSERM U1127, CNRS UMR 7725, Institut du Cerveau et de la Moelle Epinière, F-75013 Paris, France*

[g] *Department of Psychiatry, University of Lübeck, 23562 Lübeck, Germany*

Abstract—The polypeptide ghrelin is an endogenous ligand at the growth hormone secretagogue receptor 1a. To ghrelin multiple functions have been ascribed including promotion of gastrointestinal motility. Postprandial ghrelin levels have been reported to be reduced in patients suffering from Parkinson disease (PD). Experimental studies revealed neuroprotective effects of ghrelin in different PD models. The purpose of the present study was (i) to further elucidate the mechanism underlying the neuroprotective action of ghrelin and (ii) to determine whether these effects occur with both the acylated and the unacylated form. The study was conducted in primary mesencephalic cultures treated with mitochondrial complex I and complex II inhibitors. We show that protective effects of ghrelin against complex I inhibition with MPP^+ were independent of the acylation status of ghrelin, although acylated ghrelin appeared to be more potent. Protection by both forms was also observed when neurons were exposed to the complex II inhibitor 3-NP. Both forms led to higher oxygen consumption rates upon electron transport chain uncoupling, indicating that the two peptides may exert uncoupling effects themselves. We demonstrate that the rescue provided by ghrelin required calcium influx through L-type voltage-gated calcium channels. Whereas the protective effects of acylated ghrelin required receptor binding, effects of the unacylated form remained unaffected by treatment with a ghrelin receptor antagonist. Importantly, inhibition of ghrelin O-acyltransferase failed to reduce the activity of unacylated ghrelin. Overall, our data suggest that both acylated and unacylated ghrelin afford protection to dopamine neurons but through mechanisms that only partially overlap.

Key words: Parkinson disease, ghrelin, ghrelin receptor, mitochondrial respiratory chain, MPP^+, 3-NP.

INTRODUCTION

Ghrelin is a 28 amino acid peptide mainly produced by X/A cells in the stomach and released into the circulation (Kojima et al., 1999; Inui et al., 2004; Unger et al., 2011). Ghrelin functions as an important regulator of growth hormone secretion, energy homeostasis and food intake (Kojima et al., 1999; Andrews et al., 2009; Ejskjaer et al., 2009; Unger et al., 2011; Bayliss and Andrews, 2013; Bayliss et al., 2016a). These effects largely rely on stimulation of the growth hormone secretagogue receptor 1a (GHSR1a), a G protein-coupled receptor, in the hypothalamus and the pituitary gland (Kojima et al., 1999; Inui et al., 2004; Chung et al., 2008; Andrews et al., 2009; Zhang et al., 2012; Bayliss et al., 2016a). Ghrelin exists in two major molecular forms, acylated (AG) and unacylated (UAG). Of both forms, UAG is the most abundant one (van der Lely et al., 2004; Chung et al., 2008; Togliatto et al., 2010, 2014). The acylation is catalyzed by the ghrelin O-acyltransferase (GOAT) and only AG is able to bind to and activate the GHSR1a (Chung et al., 2008; Andrews et al., 2009; Zhang et al., 2012). Traditionally, AG has been regarded as the only active form of ghrelin due to its receptor binding properties (Togliatto et al., 2010). Conversely, both forms have been shown to confer biological effects as they share affinity for common binding sites (Bedendi et al., 2003; Broglio et al., 2004; Toshinai et al., 2006; Gauna et al., 2007; Togliatto et al., 2010, 2014; Heppner et al., 2013; Ku et al., 2016). Yet, it has been reported that UAG additionally

*Correspondence to, D. Alvarez-Fischer: Institute of Neurogenetics and Department of Psychiatry, University of Lübeck, Maria-Goeppert-Str 1, D-23562 Lübeck, Germany.
E-mail address: daniel.alvarez@neuro.uni-luebeck.de (D. Alvarez-Fischer).

Abbreviations: AG, acylated; BBB, blood–brain-barrier; CCCP, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone; GHSR1a, growth hormone secretagogue receptor 1a; GOAT, ghrelin O-acyltransferase; mDA, mesencephalic dopaminergic; NeuN, neuronal nuclei; OCR, oxygen consumption rates; PD, Parkinson disease; UAG, unacylated; VGCC, voltage-gated Ca^{2+} channels.

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causes effects different from those provoked by AG, suggesting two separate molecular pathways (Broglio et al., 2004; Toshinai et al., 2006; Togliatto et al., 2010, 2014; Ku et al., 2016).

Since the ghrelin receptor is expressed in many brain regions including hippocampus and ventral midbrain (Guan et al., 1997; Dong et al., 2009), it is conceivable that a number of studies have reported a responsiveness to AG in many neuron populations outside the hypothalamus, in particular in the midbrain, hindbrain, hippocampus, and spinal cord. These effects range from modulation of membrane excitability to control of neurotransmitter release, neuronal gene expression, and neuronal survival (Ferrini et al., 2009; Bayliss and Andrews, 2013). In particular, it has been shown that AG modulates the activity of mesencephalic dopaminergic (mDA) neurons (Abizaid et al., 2006; Shi et al., 2013; Bayliss et al., 2016b) and protects them from MPTP-induced degeneration both *in vitro* (Dong et al., 2009; Liu et al., 2010) and *in vivo* (Jiang et al., 2008; Andrews et al., 2009; Moon et al., 2009; Bayliss et al., 2016a). More specifically, when injected prior to a fasting period, AG reduced the effects of MPTP in a mouse model of Parkinson disease (PD) (Andrews et al., 2009; Bayliss and Andrews, 2013). This observation is interesting in view of the fact that patients diagnosed with PD already exhibit reduced levels of circulating ghrelin, as well as a reduced postprandial ghrelin response (Unger et al., 2011).

To date, the mechanism by which AG leads to neuroprotection is not fully understood. It has been shown that the mechanism of neuroprotection includes GSHR1a activation (Andrews et al., 2009; Bayliss et al., 2016a). Induction of the diacylglycerol/inositol(1,4,5)trisphosphate pathway, resulting in an increase of cytosolic Ca^{2+} (Mau et al., 1995; Heppner et al., 2013), seems to play a role as well. Moreover, protective effects against MPTP/MPP^+ seem to involve uncoupling protein 2 (UCP2) (Andrews et al., 2009), a mitochondrial protein that operates by buffering mitochondrial ROS production (Andrews et al., 2005a).

The aim of the present *in vitro* study was to investigate whether the protective effects of ghrelin are restricted to its acylated form or whether UAG also confers protective properties. We further aimed to elucidate the underlying mechanism of this action and in particular the possible implication of the mitochondrial respiratory chain. To this end, we treated primary midbrain cultures with complex I and complex II inhibitors and analyzed the oxygen consumption rate of AG- and UAG- treated neurons.

EXPERIMENTAL PROCEDURES

Cell culture and treatment

Primary mesencephalic cells were isolated from embryos at gestational age of 15 days of pregnant Wistar rats (Janvier LABS, Le Genest St Isle, France). The ventral mesencephalon was dissected as previously described (Douhou et al., 2001). The cells were maintained in 500-μl neurobasal medium supplemented with glutamine, glutamate, B27, fetal bovine serum, and penicillin/streptomycine. Cells were treated with 2 μM AraC (Sigma Chemical, Munich, Germany) to block astrocyte growth and 2 μM MK801 (Sigma Chemical) to prevent nonspecific excitotoxic stress (Michel et al., 1997). On average, one well contained 2627 ± 228 TH-positive cells and 34 7,893 ± 21,457 NeuN-positive cells, respectively. Both forms of ghrelin (Bachem, Bubendorf, Switzerland) were dissolved in water and added at indicated concentrations at DIV3 (days *in vitro* 3). All toxins were prepared freshly before treatment. Both, 3-nitroproprionic acid (3-NP, Sigma Chemical) and MPP^+ (1-Methyl-4-phenylpyridinium iodide, Sigma Chemical) were dissolved in water and were added at a concentration of 100 μM (3-NP) and 3 μM (MPP^+), respectively. Toxin treatment was started at DIV4 and lasted for 48 h. Co-treatment of UAG with a GOAT (ghrelin O-acyltransferase) inhibitor, GO-CoA-Tat, was performed at a concentration of 6 μM. The GSHR1a antagonist GHRP-6 was used at a concentration of 0.9 μM. T-type calcium channel blocker flunarizine was used at a concentration of 2.5 μM and L-type calcium channel blocker nicardipine at 3 μM.

Immunocytochemistry and cell counting *in vitro*

Immunocytochemistry was performed as previously described (Douhou et al., 2001) with a monoclonal anti-TH antibody (MAB-5280; Chemicon, Temecula, CA, USA) diluted 1:500 and a secondary antibody (anti-mouse Sigma Chemical, 1:500). Neuronal cells, regardless of their neurotransmitter phenotype, were identified by staining of neuronal nuclei with a monoclonal biotin-conjugated antibody targeted against neuronal nuclei (NeuN) (MAB-377B, Chemicon) diluted 1:100 and a secondary antibody (biotin conjugated, Invitrogen, 1:500 in PBS and 5% horse serum).

Measurement of cell respiration

Respiration was analyzed using Seahorse Biosciences technology on primary mesencephalic cell cultures as described elsewhere (Pesta and Gnaiger, 2012). In brief, a Seahorse 24-well plate was seeded with approximately 380,000 mesencephalic cells/per well. After five days of culture, a third of the wells were treated with 1 nM UAG and 0.1 nM AG, respectively, for 24 h. Respiration and extracellular pH was then measured four times in three successive series. First, we determined basal respiration in the cells. Subsequently, the respiration compensating for the mitochondrial proton leak, maximal respiration and non-mitochondrial respiration were quantified by sequentially adding oligomycin (2 μM), carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (CCCP, 0.5 μM) and potassium cyanide (2 mM) to the media. The oxygen consumption rates (OCR) for each condition were quantified. The data were expressed as % of controls. The results from four independent plates were grouped.

Statistical analysis

The data are expressed as percentage of corresponding control values. Each data point represents the mean ± S.E.M. of at least three independent experiments.

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