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Des-acyl ghrelin attenuates pilocarpine-induced limbic seizures via the ghrelin receptor and not the orexin pathway

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ABSTRACT

Des-acyl ghrelin, widely accepted to work independently of the ghrelin receptor, is increasingly being implicated in a number of biological functions. The involvement of des-acyl ghrelin in epilepsy has only been recently reported. In this study, apart from unravelling the effect of des-acyl ghrelin on seizure thresholds and seizure severity in two models of pilocarpine-induced seizures, we mainly attempted to unravel its anticonvulsant mechanism of action. Since it was found that des-acyl ghrelin administration affected food intake via the orexin pathway, we first determined whether this pathway was responsible for des-acyl ghrelin's seizure-attenuating properties using the dual orexin receptor antagonist almorexant. We noted that, while des-acyl ghrelin showed dose-dependent anticonvulsant effects against focal pilocarpine-evoked seizures in rats, almorexant did not affect seizure severity and did not reverse des-acyl ghrelin's anticonvulsant effect. Subsequently, to investigate whether the ghrelin receptor was implicated in des-acyl ghrelin's anticonvulsant properties, we tested this peptide in ghrelin receptor deficient mice and wild type mice, all infused with pilocarpine intravenously. Unexpectedly, we found that des-acyl ghrelin significantly elevated seizure thresholds in C57Bl/6 and wild type mice but not in ghrelin receptor knockout mice. Taken together, our results indicate the involvement of the ghrelin receptor in the anticonvulsant effects of des-acyl ghrelin on pilocarpine-induced seizures. We also show for the first time that dual antagonism of hippocampal orexin receptors does not affect seizure severity.

1. Introduction

Des-acyl ghrelin (DAG), together with ghrelin and obestatin, form the products of the prepro-ghrelin gene (Chen et al., 2009). Ghrelin has been given the most attention since its discovery, with DAG being initially regarded as a non-functional ghrelin ligand. This stance towards DAG has changed in the recent years after identifying various physiological processes regulated by this peptide (Baldanzi et al., 2002; Bedendi et al., 2003; Lear et al., 2010; Muccioli et al., 2004). Both ghrelin and DAG share an identical amino acid sequence, with the only difference that DAG lacks an *O-n*-octanoylation at serine 3 (Chen et al., 2009). This was believed to render DAG unable to bind with the growth hormone secretagogue receptor (now preferably denoted as ghrelin receptor (IUPHAR/BPS, 2015), and numerous studies have been performed to try to elucidate the receptor on which DAG exerts its function (Bulgarelli et al., 2009; Delhanty et al., 2012, 2013; Pei et al., 2014).

Ghrelin is mostly known for its orexigenic properties, whereas the role of DAG on feeding is controversial with studies claiming an orexigenic, anorexigenic or no effect (Inhoff et al., 2009; Toshinai et al., 2006). One of these studies was that of Toshinai and colleagues, who found a link between the action of DAG on feeding and the orexin pathway (Toshinai et al., 2006). They noted that intracerebroventricular (icv) administration of either ghrelin or DAG significantly induced feeding during both light and dark phases in rats. Contrary to ghrelin, icv administered DAG was able to induce feeding in ghrelin receptor deficient mice but not in orexin-deficient mice. Also pretreatment with antiorexin IgG, but not anti-neuropeptide Y IgG or control serum IgG, completely abolished DAG's food intake. DAG also increased intracellular calcium concentrations in isolated orexin neurons. In the light of these findings, the authors concluded that DAG-induced feeding is mediated by activation of the orexin pathway.

Ghrelin has been repeatedly reported to exert anticonvulsant effects in different models (Aslan et al., 2009; Lee et al., 2010; Obay et al., 2007). Biagini et al. tested the effects of ghrelin and DAG in the pilocarpine- and kainate-induced status epilepticus (SE) models

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by looking at the induction and latency to generalized seizures as well as induction and latency to SE, and mortality (Biagini et al., 2011). They found that while ghrelin failed to display any effect in these two models, DAG presented a trend in the prevention of pilocarpine-induced SE. With regard to kainate-induced SE, DAG was found to significantly delay the onset of SE in rats.

The initial aim of this study was to further characterize DAG's role on limbic seizure severity and to establish whether its anticonvulsant effect was due to the involvement of the orexin pathway. Here the rat intrahippocampal pilocarpine model for limbic seizures was used (Portelli et al., 2009). This model allows researchers to observe the sequential development of partial seizures to generalized seizures, and very rarely results in mortality. Unlike the systemic pilocarpine model for status epilepticus (Curia et al., 2008), the focal pilocarpine model used in this study is more considered as a model for repeated limbic seizures with secondary generalization. For this experiment, we also tested for the first time the effect of the dual orexin receptor antagonist almorexant against limbic seizures.

After noting that DAG's anticonvulsant effect did not involve the orexin pathway, we decided to rule out the involvement of the ghrelin receptor. To this end, seizure thresholds were determined using the intravenous pilocarpine tail infusion model in C57Bl/6 mice, ghrelin receptor knock-out mice and their wild type littermates. The mouse intravenous pilocarpine tail infusion model also allows the experimental study of the various behavioural stages accompanying seizure phenomena and to determine robustly whether test compounds are capable of altering seizure thresholds (De Bundel et al., 2011).

2. Materials and methods

2.1. Animals

Experimental procedures were performed on male Wistar rats (270–310 g; Charles River Laboratories, France), male C57Bl/6 mice (25–30 g; Charles River Laboratories, France), and male ghrelin receptor wild type (ghrelin receptor$^{+/+}$) and ghrelin receptor knock-out (ghrelin receptor$^{-/-}$) littermate mice (25–35 g). The knock-out mice and their littermates were generated as explained previously (Portelli et al., 2012b). All experiments were carried out in accordance with the national rules on animal experiments and were approved by the Ethics Committee on Animal Experiments of the Vrije Universiteit Brussel, Belgium.

2.2. Ligands, chemicals and reagents

DAG was supplied by PolyPeptide Laboratories (Strasbourg, France). The dual orexin receptor antagonist almorexant was generously provided by Dr F. Jenck (Actelion Pharmaceuticals, Switzerland). Pilocarpine was supplied by Sigma-Aldrich (Bornem, Belgium). All other chemicals were of analytical reagent grade or better and were supplied by Merck (Darmstadt, Germany). Aqueous solutions were made with purified water (Arium pro UV, Sartorius Stedim, Vilvoorde, Belgium) and filtered through a 0.2 μm membrane filter.

2.3. In vivo *microdialysis in rats*

2.3.1. Surgery

Rats received intraperitoneal (ip) injections of a mixture of ketamine and diazepam (start dose of 90.5:4.5 mg/kg) until full-body anaesthesia was achieved. The animal was mounted on a stereotaxic frame for precise intracranial steel guide cannula (CMA/Microdialysis, Solna, Sweden) implantation in the left CA1–CA3 hippocampal area using the coordinates 4.6 mm lateral from midline, 5.6 mm posterior to bregma, and 4.6 mm ventral from dura (Portelli et al., 2009). Ketoprofen (3 mg/kg) was administered subcutaneously for post-operative analgesia at the end of the surgical procedure.

2.3.2. Microdialysis

Immediately after surgery, the hippocampal guide cannula obturator was replaced by a microdialysis probe (CMA/12; 3 mm membrane length; theoretical cut-off 20 kDa; CMA/Microdialysis), which was continuously perfused with modified Ringer's solution (in mM: NaCl, 147; $CaCl_2$, 2.3; and KCl, 4) at 2 μL/min. All experiments started the next morning with a 2 h perfusion of modified Ringer's solution alone ('baseline' conditions), and followed at random one of the protocols depicted in the upper panel of Figs. 1a and 2a. Different doses of DAG and almorexant were tested, as depicted in the lower panel of Figs. 1a and 2a. Each collection period was of 20 min duration. DAG concentrations were chosen based on ghrelin experiments performed in the same model (Portelli et al., 2012b). The concentration of almorexant to fully block the hippocampal orexin receptors was determined by taking into account the pKi values (orexin 1 receptor (OX1R) = 7.8 nM; orexin 2 receptor (OX2R) = 8.0 nM) towards the orexin receptors as well as microdialysis probe recovery.

2.3.3. Seizure severity assessment

Following pilocarpine perfusion, typical seizure-related behavioural changes were rated on a Seizure Severity Score (SSS) that is based on Racine's scale (Racine, 1972). This scale was previously optimized and validated with electrocorticographical monitoring to take into account the typical behavioural changes associated with pilocarpine-induced motor seizures (Meurs et al., 2008), and consists of 6 different stages: (0) normal, non-epileptic activity; (1) mouth and facial movements, hyperactivity, excessive grooming, sniffing, scratching, wet-dog shakes; (2) head nodding, staring, tremor; (3) forelimb clonus, forelimb extension; (4) rearing, salivating, tonic–clonic activity; and (5) falling. The total seizure severity score (TSSS) for each animal is calculated as the sum of the highest SSS attained in each of the seven collection periods following the start of pilocarpine administration (total time: 140 min).

2.4. In vivo *mice experiments*

C57Bl/6, ghrelin receptor$^{+/+}$ and ghrelin receptor$^{-/-}$ mice were used to determine seizure thresholds, based on the pilocarpine-induced stereotyped seizure behaviour (De Bundel et al., 2011). The threshold for the different phases of pilocarpine-induced seizure activity was determined by infusing a pilocarpine (Sigma Chemical Company, St. Louis, MO, USA) solution (24 mg/mL) through a 29 G needle, attached to polyethylene tubing (Smiths, Keene, USA) and inserted into the tail vein of the animals at a constant rate of 150 μL/min (De Bundel et al., 2011) using a Hamilton syringe mounted to an infusion pump (CMA, Microdialysis, Solna, Sweden). The animal was allowed to move freely in a cage made of Plexiglas. In order to prevent peripheral cholinergic symptoms, all mice were administered methylscopolamine (1 mg/kg, subcutaneously) 30 min prior to pilocarpine infusion. Mice were also administered (according to body weight) DAG (1.8, 3.0 or 5.0 μg/g) or saline via ip administration 30 min prior to pilocarpine tail infusion. The following endpoints were used to determine the seizure threshold: (1) shivering; (2) rearing; (3) clonus with loss of righting reflexes (falling); (4) tonic hindlimb extension (tonus); (5) death. Time was measured from the start of the pilocarpine infusion until the onset of these stages. The seizure thresholds were determined for each animal according to the following equation: dose (mg/kg) = duration of infusion (s) × rate of infusion (mL/min) × drug concentration (mg/mL) × 1000/(60 s × weight of mouse (g)) (De Bundel et al., 2011).