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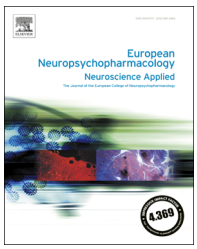

# Ghrelin treatment prevents development of activity based anorexia in mice

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**Abstract**
Stimulation of feeding is necessary for treatment of pathological conditions of chronic malnutrition due to anorexia. Ghrelin, a hunger hormone, is one of the candidate for pharmacological treatments of anorexia, but because of its instability in plasma has limited efficacy. We previously showed that plasmatic IgG protect ghrelin from degradation and that IgG from obese subjects and mice may increase ghrelin's orexigenic effect. In this study we tested if ghrelin alone or combined with IgG may improve feeding in chronically food-restricted mice with or without physical activity-based anorexia (ABA) induced by free access to a running wheel. Mice received a single daily intraperitoneal injection of ghrelin (1 nM) together or not with total IgG (1 nM) from obese *ob/ob* or lean mice before access to food during 8 days of 3 h/day feeding time. We found that both ghrelin and ghrelin combined with IgG from obese, but not lean mice, prevented ABA, however, they were not able to diminish body weight loss. Physical activity was lower during the feeding period and was increased shortly after feeding in mice receiving ghrelin together with IgG from obese mice. In food-restricted mice without ABA, ghrelin treatments did not have significant effects on food intake. Thus, this study supports pharmacological use of ghrelin or ghrelin combined with IgG from obese animals for treatment of anorexia accompanied by elevated physical activity. The utility of combining ghrelin with protective IgG should be further determined in animal models of anorexia with unrestricted access to food.


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## 1. Introduction

Reduced appetite or anorexia may be present in chronic pathological conditions resulting in malnutrition and worsening clinical outcome and long-term prognosis (Arezzo di Trifiletti et al., 2013). Both psychiatric and non-psychiatric diseases may be accompanied by severe anorexia and cachexia such as in anorexia nervosa (AN) and some types of cancer. Low appetite can also accompany major depression, senile dementias as well as chronic cardiac and renal failure (Ebner et al., 2013). Therefore, improving appetite and food intake is one of the desired therapeutic approaches in these patients, however, no established orexigenic pharmacological treatment is currently available (Thomas, 2006).

For obtaining orexigenic effects, stimulation of the ghrelin system become recently one of the promising candidate molecular pathways (DeBoer, 2012; Garcia et al., 2015; Kamiji and Inui, 2008). Ghrelin is a 28 amino acid acylated peptide hormone produced by the enteroendocrine cells in gastric mucosa which via the circulation reaches the brain and stimulates ghrelin's specific receptors (GHSR1) leading to increased appetite and food intake (Kojima et al., 1999). Thus, ghrelin is a key peripheral signal to the brain to increase hunger sensation which is integrated by the hypothalamic and brainstem circuitries receiving also an input from satiety hormones (Berthoud, 2008). Based on this physiological role, administrations of ghrelin may represent the first choice for pharmacotherapy of anorexia. In fact, several studies tested effects of synthetic ghrelin in anorexia nervosa and cancer anorexia but the therapeutic effect was modest and inconsistent among the patients (Hotta et al., 2012; Miljic et al., 2006; Ogiso et al., 2011). Possible underlying causes can be related to central ghrelin resistance and/or its fast degradation (De Vriese et al., 2004). Indeed, the acylated active form of ghrelin is unstable and the majority of circulating ghrelin is degraded by plasma enzymes to des-acyl-ghrelin lacking orexigenic effects (Asakawa et al., 2005; Tong et al., 2013).

We have recently found that plasmatic IgG protect ghrelin from degradation and that such IgG from obese subjects and mice enhanced ghrelin's orexigenic effects (Takagi et al., 2013). It suggests that plasmatic IgG may play a physiological role in ghrelin's orexigenic signaling which can be altered in anorectic patients. Indeed, decreased plasma levels of ghrelin-reactive IgG were found in AN patients (Terashi et al., 2011) and, furthermore, these IgG were characterized by increased dissociation rates (Takagi et al., 2013). Moreover, typically elevated plasma levels of ghrelin and des-acyl-ghrelin found in AN patients (Méquinion et al., 2013; Otto et al., 2001) and in some other chronic conditions of malnutrition, suggest that increased expression of ghrelin precursor induced by negative energy balance (François et al., 2015) is not sufficient to provide physiological signal of hunger leading to nutrient intake. Taken together, these data suggest that trying to treat anorexia using ghrelin together with IgG that would protect it from degradation may provide a better orexigenic effect than ghrelin alone.

In the present study we tested such possibility using a rodent model of anorexia associated with increased physical activity and restricted access to food known as activity-based anorexia (ABA) (Carrera et al., 2001; Routtenberg and Kuznesof, 1967). We also studied effects of ghrelin and IgG in mice with the same feeding time restriction (FTR) but without physical activity. We analyzed effects of ABA and FTR on plasma levels of ghrelin and des-acyl-ghrelin and their corresponding IgG levels and affinities. IgG used in this study were purified from plasma of lean and obese mice, the latter characterized by their increased affinity for ghrelin (Takagi et al., 2013).

## 2. Experimental procedures

### 2.1. Animals

Animal care and experimentation complied with both French and European Community regulations (Official Journal of the European Community L 358, December 18, 1986), and M.C. was authorized by the French government to use animal models (authorization no. 76e107). Male C57Bl/6 mice (Janvier Labs, Le Genest St Isle, France) were acclimatized in individual cages at 23 °C during 7 days. During this period and all the experiment, the 12-h light-dark cycle was inverted (dark phase: 9:30 AM to 9:30 PM). At day (D) 1 of the experience, mice were randomised into 3 groups: ABA ($n$=22), FTR ($n$=29) and ad libitum fed controls $n$=8). ABA mice were placed in individual cages with an activity wheel with RunningWheel software (Intellibio, France). Wheel activity was continuously recorded. FTR and control mice were placed in individual cages without activity wheel.

### 2.2. Experimental procedure

From D1 to D5, mice had free access to water and standard diet. Food access was progressively limited in ABA and FTR groups from 6 h at D6 to 3 h at D9 until the end of experiment (Figure 1A). Food was given at the beginning of the dark phase (9:30 AM). Food consumption was measured when food was removed. Water remained in free access. At D10 until the end of the experiment, ABA and RFA group were divided into 4 groups to receive different treatment 15 min before the food provision. They were injected with 0.9% NaCl ($n$=6 ABA, $n$=7 FTR), with ghrelin at 1 nM ($n$=5 ABA, $n$=7 FTR), with ghrelin at 1 nM coupled to lean mice IgG at 1 nM ($n$=5 ABA, $n$=7 FTR) and with ghrelin at 1 nM coupled to *ob/ob* mice IgG at 1 nM ($n$=6 ABA, $n$=8 FTR). Co-injected IgG from lean and *ob/ob* mice were characterised for their affinity for ghrelin in our previous study (Takagi et al., 2013). At D17, mice were euthanized.

### 2.3. Ghrelin and des-acyl ghrelin assays

During the sacrifice, blood from the cave vein was collected into Aprotinin tubes (BD Vacutainer) containing EDTA (1 mg/ml), aprotinin (500 U/ml). Plasma was separated by centrifugation 3000 rpm at 4 °C (Eppendorf). Before freezing, plasma was acidified with HCl 1 N (10% of the plasma volume) to preserve ghrelin from deacylation. Ghrelin and des-acyl ghrelin concentrations in all experiments including human and mouse plasma were measured using EIA kits (Mitsubishi Chemical Med Corp, Tokyo, Japan) according to the manufacturer instructions.

### 2.4. Ghrelin and des-acyl ghrelin reactive IgG assay

Plasma levels of IgG reactive with ghrelin and des-acyl-ghrelin were measured using ELISA. They were done in normal and dissociating conditions to measure free and total IgG, respectively (Fetissov, 2011). 100 μL of ghrelin and des-acyl ghrelin peptide were coated at 2 μg/mL in 0.5 M carbonate-bicarbonate buffer pH 9.6 containing 0.02% sodium

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