Neuropharmacology 79 (2014) 498–505

Contents lists available at ScienceDirect

Neuropharmacology

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

# Influence of ghrelin on the central serotonergic signaling system in mice☆

Caroline Hansson [a,*], Mayte Alvarez-Crespo [a], Magdalena Taube [b], Karolina P. Skibicka [a], Linnéa Schmidt [b], Linda Karlsson-Lindahl [b], Emil Egecioglu [a], Hans Nissbrandt [c], Suzanne L. Dickson [a]

[a] Department of Physiology/Endocrinology, Institute of Neuroscience and Physiology, The Sahlgrenska Academy at the University of Gothenburg, Gothenburg, Sweden
[b] Department of Molecular and Clinical Medicine, Institute of Medicine, The Sahlgrenska Academy at the University of Gothenburg, Gothenburg, Sweden
[c] Department of Pharmacology, Institute of Neuroscience and Physiology, The Sahlgrenska Academy at the University of Gothenburg, Gothenburg, Sweden

ARTICLE INFO

*Article history:*
Received 19 April 2013
Received in revised form
22 November 2013
Accepted 14 December 2013

*Keywords:*
Serotonin
Serotonin receptors
Amygdala
Dorsal raphe
GHS-R1A
Anxiety

ABSTRACT

The central ghrelin signaling system engages key pathways of importance for feeding control, recently shown to include those engaged in anxiety-like behavior in rodents. Here we sought to determine whether ghrelin impacts on the central serotonin system, which has an important role in anxiety. We focused on two brain areas, the amygdala (of importance for the mediation of fear and anxiety) and the dorsal raphe (i.e. the site of origin of major afferent serotonin pathways, including those that project to the amygdala). In these brain areas, we measured serotonergic turnover (using HPLC) and the mRNA expression of a number of serotonin-related genes (using real-time PCR). We found that acute central administration of ghrelin to mice increased the serotonergic turnover in the amygdala. It also increased the mRNA expression of a number of serotonin receptors, both in the amygdala and in the dorsal raphe. Studies in ghrelin receptor (GHS-R1A) knock-out mice showed a decreased mRNA expression of serotonergic receptors in both the amygdala and the dorsal raphe, relative to their wild-type littermates. We conclude that the central serotonin system is a target for ghrelin, providing a candidate neurochemical substrate of importance for ghrelin's effects on mood.



## 1. Introduction

The circulating hormone ghrelin (Kojima et al., 1999) has an established role in food intake and body fat accumulation (Tschop et al., 2000). Increasing evidence suggests that the neurobiological effects of ghrelin extend beyond energy balance, to include reward-motivated behavior (Abizaid et al., 2006; Jerlhag et al., 2009; Egecioglu et al., 2010; Perello et al., 2010; Skibicka et al., 2012), memory (Diano et al., 2006) and mood (Asakawa et al., 2001). There are indications that the central ghrelin signaling system could also be involved in the regulation of anxiety. Ghrelin has been shown to increase anxiety-like behavior in rodents when administered acutely, both peripherally or centrally (Asakawa et al., 2001; Carlini et al., 2002, 2004), although it has also been reported to reduce the depressive and anxiogenic effects of acute stress in mice (Lutter et al., 2008). We have also shown that chronic central ghrelin administration increases both anxiety- and depression-like behavior in rats (Hansson et al., 2011). Consistent with this, administration of antisense for ghrelin decreases anxiety-like behavior in rats (Kanehisa et al., 2006) and gastrectomy surgery, that substantially reduces circulating ghrelin levels, was associated with a decrease in anxiety- and depression-like behavior in rats (Salome et al., 2011). We have also shown that panic disorder is associated with a polymorphism in the preproghrelin gene in humans (Hansson et al., 2013), further enhancing the connection between ghrelin and anxiety-related disorders.

One potential candidate target system for these neurobiological effects of ghrelin on anxiety-like behavior is the central serotonergic system. Destruction of serotonergic neurons as well as depleting the brain of serotonin (5-HT) decreases anxiety-like behavior (Soderpalm and Engel, 1990), and activation of the 5-HT1A



* Corresponding author. Current address: Department of Molecular and Clinical Medicine, Institute of Medicine, The Sahlgrenska Academy at the University of Gothenburg, Box 425, SE-415 30 Gothenburg, Sweden. Tel.: +46 31 786 6747.
*E-mail address:* caroline.hansson@gu.se (C. Hansson).

0028-3908/$ – see front matter © 2013 The Authors. Published by Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.neuropharm.2013.12.012

autoreceptor, that decreases serotonergic release, induces an anxiolytic effect (Akimova et al., 2009), while increased serotonergic signaling increases anxiety (Graeff et al., 1996). There are several studies proposing an interaction between ghrelin and the serotonergic system. Ghrelin has been shown to decrease serotonergic release from hypothalamic synaptosomes (Brunetti et al., 2002) as well as from hippocampal slices (Ghersi et al., 2011). Administration of serotonin or a serotonin receptor agonist into the hypothalamus decreases ghrelin-induced food intake as well as ghrelin-induced increases in respiratory quotient (Currie et al., 2010). In addition, ghrelin-induced increases in food intake and memory retention are attenuated by administration of the selective serotonin reuptake inhibitor (SSRI) fluoxetine (Carlini et al., 2007). Other effects of ghrelin that have also been suggested to be mediated via a serotonergic pathway include activation of the HPA-axis, regulation of body temperature and the secretion of growth hormone (Pinilla et al., 2003; Jaszberenyi et al., 2006). Serotonergic cell bodies located in the dorsal and medial raphe nuclei send serotonergic projections to the forebrain, including the limbic midbrain area (Dahlstroem and Fuxe, 1964). We have recently shown that ghrelin alters the firing rate of cells in the dorsal raphe *in vitro* (Hansson et al., 2011).

In a recent study, we identified the amygdala as a target for ghrelin, involving neuroanatomical, electrophysiological and behavioral studies. We also showed that intra-amygdala ghrelin administration affects feeding as well as anxiety-like behavior (Alvarez-Crespo et al., 2012). The amygdala is an important brain region for the regulation of fear and anxiety (Davis, 1992). There are indications that the serotonergic system is involved in mediating fear and anxiety in this region; blockade of serotonergic receptors in the amygdala is anxiolytic, while intra-amygdaloid injection of a serotonin receptor agonist has the opposite effect (Costall et al., 1989). It is not yet known whether ghrelin's effects on anxiety-like behavior in rodents involves increased serotonin signaling in the amygdala, forming a key aim of our study.

In the present study, we sought to explore the impact of: I) acute central ghrelin administration and II) knock-out of the ghrelin receptor, GHS-R1A, on serotonergic turnover and the mRNA expression of serotonergic receptors, enzymes and transporters in relevant brain areas.

## 2. Materials and methods

### 2.1. Animals

For the acute ghrelin treatment, male C57/Bl6J mice (8–10 weeks, Taconic, Denmark) were used. Upon arrival in the animal facility they were group housed and allowed to acclimatise for one week before the experiment. After surgery, they were housed individually. Male GHS-R1A knock-out mice (9–11 weeks) and their wild-type littermates were also used in the study. The derivation of these mice has been described previously (Egecioglu et al., 2010). All animals were kept under a 12:12 LD cycle (lights on at 0700) with free access to standard food pellets and tap water. The studies were approved by the local Ethics Committee at the University of Gothenburg, Sweden.

### 2.2. Surgical procedure for central ghrelin administration

For intracerebroventricular (i.c.v.) catheter placement, mice were anaesthetized using isoflurane (induction 4%, maintenance 3–4%; air flow, 260 ml/min) and placed in a stereotactic frame (Kopf Instruments, Tujunga, CA, USA). A steel guide cannula (AG-8, code 806302 from Agntho's, Lidingö, Sweden) was implanted into the third dorsal ventricle using the following coordinates from bregma: AP −0.9 mm, ML 0.0 mm, DV −1.0 mm (Paxinos and Franklin, 2001). The cannula was anchored to the skull with one jeweller's screw (Agntho's) and dental cement (Dentalon, Agntho's, fluid 7509, powder 7508) attached to the cannula and to the stabilizing screw. Romefen (5 mg/kg) was given as a postoperative analgesic and saline (0.5 ml per mouse) was given subcutaneously to prevent dehydration. After surgery, the mice were allowed to recover for one week, before any experimental procedures were undertaken.

### 2.3. Determination of the tissue concentration of serotonin and its metabolite 5-hydroxyindolacetic acid (5-HIAA)

To explore the impact of acute central ghrelin administration on the tissue concentration of serotonin and the serotonin metabolite 5-HIAA in the amygdala and dorsal raphe, dissected tissue was collected from ghrelin- and saline-treated mice. Thus, mice bearing chronically implanted i.c.v. catheters were injected with acylated ghrelin (Tocris, Bristol, UK; 1 μg/μl, flow rate 1 μL/min, this dose was based on (Jerlhag et al., 2006)) or an equal volume (1 μL) vehicle saline solution, using CMA infusion pumps (CMA Microdialysis AB, Solna, Sweden) and Hamilton syringes (Genetec, Västra Frölunda, Sweden). Thirty min after the i.c.v. infusion, mice were anaesthetized using isoflurane and decapitated. Brains were rapidly removed and the amygdala and dorsal raphe nucleus were dissected manually using a brain matrix, using coordinates and visual landmarks. The coordinates used were (i) for the amygdala: −0.82 to −2.80 mm anterior–posterior relative to Bregma, −4.5 to −5.8 mm depth and 1.5–3.0 mm lateral to the midline and (ii) for the dorsal raphe: −4.1 to −5.1 mm anterior-posterior relative to Bregma, −2.6 to −3.5 mm depth and 0.0 ± 0.8 mm lateral to the midline (Paxinos and Franklin, 2001). All dissections were performed during daytime and used a balanced design with respect to the time of injection. Dissected brain areas were frozen on dry ice and stored in −80 °C for later determination of serotonin and 5-HIAA concentration.

We also investigated whether GHS-R1A knock-out mice have altered tissue concentration of serotonin and 5-HIAA in the amygdala and dorsal raphe. GHS-R1A knock-out mice and wild-type littermates were anaesthetized using isoflurane and decapitated. Brains were rapidly removed and the amygdala and dorsal raphe nucleus were dissected and stored as described above.

#### 2.3.1. Serotonin and metabolite measurements

To explore the effects of ghrelin on the activity of the serotonergic neurons, we measured the tissue concentrations of serotonin and its metabolite 5-HIAA after acute ghrelin treatment and in GHS-R1A knock-out mice in the amygdala and the dorsal raphe.

Individual brain tissue samples were homogenized (using a Sonifier Cell Disruptor B30; Branson Sonic Power Co. Danbury, CT, USA) in a solution of 0.1 M perchloric acid, 5.37 mM EDTA and 0.65 mM glutathione. After centrifugation (14000 rpm, 4 °C, 10 min) the supernatant was collected and immediately analyzed for serotonin and 5-HIAA using a split fraction HPLC-ED system. Serotonin was analyzed on an ion-exchange column (Nucleosil, 5 μ SA 100 A, 150 × 2 mm, Phenomenex, Torrance, CA, USA) with a mobile phase consisting of 13.3 g citric acid, 5.84 g NaOH, 40 mg EDTA, and 200 ml methanol in distilled water to a total of 1000 ml. 5-HIAA was analyzed on a reverse phase column (Nucleosil, 3 μ, C18, 100 A, 50 × 2 mm, Phenomenex) with a mobile phase consisting of 11.22 g citric acid, 3.02 g dipotassium phosphate, 40 mg EDTA, and 60 ml methanol in distilled water to a total volume of 1000 ml.

### 2.4. Gene expression

We explored the impact of acute ghrelin treatment on gene expression of serotonin-related genes in dissected amygdala and dorsal raphe tissue. All procedures for infusion, brain removal and dissection were identical to those described above. For the gene expression however, the mice were anaesthetized and brains dissected 100 min after the ghrelin infusion. In addition, hypothalami of the saline-treated mice were dissected for determination of the GHS-R1A expression in order to assess relative levels of this receptor in different tissues. Dissected brain areas were frozen on dry ice and kept in RNA later in +4 °C over night. The next day, the RNA later was removed and the brain tissue samples stored in −20 °C for later determination of mRNA expression.

We also assessed the expression of serotonin-related genes in GHS-R1A knock-out mice and wild-type littermate mice. The brain areas of these mice were dissected and stored as described above.

#### 2.4.1. RNA isolation and mRNA expression

Individual brain samples were homogenized in Qiazol (Qiagen, Hilden, Germany) using a TissueLyzer (Qiagen). Total RNA was extracted using RNeasy Lipid Tissue Mini Kit (Qiagen), with additional DNAse treatment (Qiagen). RNA quality and quantity were assessed by spectrophotometric measurements (Nanodrop 1000, NanoDrop Technologies, USA). For cDNA synthesis, total RNA was reversed transcribed using random hexamers (Applied Biosystems, Sundbyberg, Sweden), and Superscript III reverse transcriptase (Invitrogen Life Technologies, Paisley, UK), according to the manufacturer's description. Recombinant RNaseout® Ribonuclease Inhibitor (Invitrogen) was added to prevent RNase-mediated degradation. All the cDNA-reactions were run in triplicate and the triplicates were pooled for the RT-PCR.

Real-time RT PCR was performed using TaqMan® Custom Arrays. They were designed with TaqMan probe and primer sets for target genes and reference genes chosen from an on-line catalog (Applied Biosystems). The sets were factory-loaded into the 384 wells of TaqMan® Arrays. Each port on the TaqMan® Arrays was loaded with cDNA corresponding to 100 ng total RNA, combined with nuclease free water and 50 μl TaqMan® Gene Expression Master Mix (Applied Biosystems) to a final volume of 100 μl. The TaqMan® Arrays were analyzed using the 7900HT system with a TaqMan Array Upgrade (Applied Biosystems). Thermal cycling conditions were: 50 °C for 2 min, 94.5 °C for 10 min, followed by 40 cycles of 97 °C for 30 s and 59.7 °C for 1 min. A combination of β-actin and cyclophilin A were used as reference genes. Gene expression values were calculated based on the $\Delta\Delta C_t$ method (Livak and Schmittgen, 2001). Briefly, $\Delta C_t$ represents the