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 Neu... 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
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, ΔC_t represents the

threshold cycle (C_t) of the target gene minus that of the reference gene and $\Delta\Delta C_t$ represents the ΔC_t of the target group minus that of the calibrator group. Relative quantities (RQ) were determined using the equation; $RQ = 2^{-\Delta\Delta C_t}$. For the calibrator, the equation is $RQ = 2^{-0}$, which is 1; therefore, the values for the target group are expressed relative to this. Target and reference genes and assay id's are given in Table 1.

2.5. Statistics

To analyze the effect of acute ghrelin treatment or GHS-R1A knock-out on the tissue concentration of serotonin and 5-HIAA, Student's *t*-test, or Mann–Whitney *U* test when the variances were not equal, was used. In order to analyze the effect of acute central ghrelin treatment, or GHS-R1A knock-out, on gene expression, Student's *t*-test was used. *P*-values and SEM were calculated using the ΔC_t- values. Differences were considered significant at $p < 0.05$.

3. Results

3.1. Tissue concentrations of serotonin and 5-HIAA in the amygdala and the dorsal raphe

Acute central ghrelin administration significantly increased the concentration of 5-HIAA, the metabolite of serotonin, in the amygdala ($p = 0.029$, Fig. 1A). No significant difference in the 5-HIAA concentration in the amygdala was observed between the GHS-R1A knock-out and wild-type mice ($p = 0.146$, Fig. 1B). No significant changes in 5-HIAA concentrations were detected in the dorsal raphe nucleus after acute ghrelin treatment (mean ± SEM: saline: 4046 ± 850 fmol/mg, $n = 8$, ghrelin: 2887 ± 146 fmol/mg, $n = 8$, $p = 0.442$ using Mann–Whitney *U* test) or in the GHS-R1A knock-out mice (mean ± SEM: wt 2727 ± 254 fmol/mg, $n = 8$, GHS-R1A knock-out 3124 ± 215 fmol/mg, $n = 11$, $p = 0.248$ using Student's *t*-test).

The serotonin concentrations were not significantly different between the ghrelin- and saline-treated mice in either the amygdala (mean ± SEM: saline: 5484 ± 212 fmol/mg, $n = 8$; ghrelin: 5810 ± 175 fmol/mg, $n = 8$; $p = 0.255$ using Student's *t*-test) or the dorsal raphe (saline: 9956 ± 2152 fmol/mg, $n = 8$; ghrelin: 6796 ± 381 fmol/mg, $n = 8$; $p = 0.329$ using Mann–Whitney *U* test). Likewise, the serotonin concentrations did not differ between the GHS-R1A knock-out and wild-type mice in either the amygdala (mean ± SEM: wt 4414 ± 157 fmol/mg, $n = 9$; GHS-R1A knock-out 4379 ± 203 fmol/mg, $n = 11$; $p = 0.899$ using Student's *t*-test) or the dorsal raphe (wt 4423 ± 450 fmol/mg, $n = 8$; GHS-R1A knock-out 5043 ± 457 fmol/mg, $n = 11$; $p = 0.361$ using Student's *t*-test).

Table 1
Genes investigated in the amygdala and the dorsal raphe.

	Gene name	Assay ID
Receptors		
5-Hydroxytryptamine receptor 1A	Htr1a	Mm00434106_s1
5-Hydroxytryptamine receptor 1B	Htr1b	Mm00439377_s1
5-Hydroxytryptamine receptor 1D	Htr1d	Mm00434115_s1
5-Hydroxytryptamine receptor 1F	Htr1f	Mm00434122_m1
5-Hydroxytryptamine receptor 2A	Htr2a	Mm00555764_m1
5-Hydroxytryptamine receptor 2B	Htr2b	Mm00434123_m1
5-Hydroxytryptamine receptor 2C	Htr2c	Mm00434127_m1
5-Hydroxytryptamine receptor 3A	Htr3a	Mm00442874_m1
5-Hydroxytryptamine receptor 3B	Htr3b	Mm00517424_m1
5-Hydroxytryptamine receptor 4	Htr4	Mm00434129_m1
5-Hydroxytryptamine receptor 5A	Htr5a	Mm00434132_m1
5-Hydroxytryptamine receptor 5B	Htr5b	Mm00439389_m1
5-Hydroxytryptamine receptor 6	Htr6	Mm00445320_m1
5-Hydroxytryptamine receptor 7	Htr7	Mm00434133_m1
Enzymes and transporters		
Monoamine oxidase A	Maoa	Mm00558004_m1
Monoamine oxidase B	Maob	Mm00555412_m1
Serotonin transporter	Slc6a4	Mm00439391_m1
Vesicular monoamine transporter 2	Slc18a2	Mm00553058_m1
Tryptophan hydroxylase 1	Tph1	Mm00493794_m1
Tryptophan hydroxylase 2	Tph2	Mm00557715_m1
Reference genes		
Beta-actin	Actb	Mm00607939_s1
Cyclophilin A	Ppia	Mm03302254_g1

3.2. Gene expression of serotonergic receptors

In the amygdala, serotonin receptor 1a (Htr1a, RQ = 1.40, $p = 0.026$), 2c (Htr2c, RQ = 1.27, $p = 0.021$), 5a (Htr5a, RQ = 1.57, $p = 0.011$) and 7 (Htr7, RQ = 1.51, $p = 0.006$) had an increased mRNA expression after acute ghrelin treatment relative to saline-treated controls (Fig. 2A). The GHS-R1A knock-out mice had a decreased mRNA expression of serotonin receptor 2c (Htr2c, RQ = 0.80, $p = 0.017$), 5a (Htr5a, RQ = 0.85, $p = 0.024$) and 7 (Htr7, RQ = 0.84, $p = 0.046$) compared to wild-type littermates (Fig. 2B).

In the dorsal raphe, serotonin receptor 1a (Htr1a, RQ = 2.04, $p = 0.013$), 1b (Htr1b, RQ = 1.42, $p = 0.024$), 1d (Htr1d, RQ = 1.83, $p = 0.046$), 1f (Htr1f, RQ = 1.40, $p = 0.033$), 2c (Htr2c, RQ = 1.60, $p = 0.016$), 4 (Htr4, RQ = 1.63, $p = 0.012$), 6 (Htr6, RQ = 1.57, $p = 0.008$) and 7 (Htr7, RQ = 1.56, $p = 0.010$) had an increased mRNA expression after acute ghrelin treatment relative to saline-treated controls (Fig. 2C). The GHS-R1A knock-out mice had a decreased mRNA expression of serotonin receptor 2c (Htr2c, RQ = 0.86, $p = 0.025$) and 3a (Htr3a, RQ = 0.61, $p = 0.021$) compared to wild-type littermates (Fig. 2D). Serotonin receptor 2b (Htr2b) and 3b (Htr3b) mRNAs were not expressed in any of the tissues studied.

3.3. Gene expression of serotonergic enzymes and transporters

In the amygdala, the mRNA expression of monoamine oxidase A (Maoa) and monoamine oxidase B (Maob) was not changed in the ghrelin-treated mice relative to saline-treated controls (Maoa, RQ = 1.05, $p = 0.582$; Maob, RQ = 0.91, $p = 0.268$). The GHS-R1A knock-out mice had a decreased mRNA expression of monoamine oxidase A (Maoa) compared to wild-type littermates (RQ = 0.85, $p = 0.026$) (Fig. 3B), while the mRNA expression of monoamine oxidase B (Maob) was not changed (RQ = 0.90, $p = 0.111$). Tryptophan hydroxylase 1 (Tph1), tryptophan hydroxylase 2 (Tph2) the serotonin transporter (Slc6a4), and the vesicular monoamine transporter 2 (Slc18a2) mRNAs were not expressed in the amygdala.

In the dorsal raphe, monoamine oxidase A (Maoa), tryptophan hydroxylase 2 (Tph2), the serotonin transporter (Slc6a4), and the vesicular monoamine transporter 2 (Slc18a2) did not display a significantly altered mRNA expression in the ghrelin-treated mice relative to saline-treated controls (Maoa, RQ = 1.16, $p = 0.297$; Tph2, RQ = 3.68, $p = 0.181$; Slc6a4, RQ = 4.81, $p = 0.149$; Slc18a2, RQ = 1.25, $p = 0.564$) or in GHS-R1A knock-out mice relative to wild-type controls (Maoa, RQ = 0.98, $p = 0.821$; Tph2, RQ = 0.65, $p = 0.629$; Slc6a4, RQ = 0.48, $p = 0.474$; Slc18a2, RQ = 0.48, $p = 0.299$). The mRNA expression of monoamine oxidase B (Maob) was increased in the dorsal raphe after ghrelin treatment (RQ = 1.48, $p = 0.042$) (Fig. 3C), while it was not changed in the GHS-R1A knock-out mice (RQ = 0.98, $p = 0.879$). Tryptophan hydroxylase 1 (Tph1) mRNA was not expressed in the dorsal raphe.

3.4. Comparison of the mRNA expression of GHS-R1A in the amygdala, dorsal raphe and hypothalamus

The mRNA expression of GHS-R1A was 3.69 fold higher in the dorsal raphe and 13.85 fold higher in the hypothalamus than in the amygdala (Fig. 4).

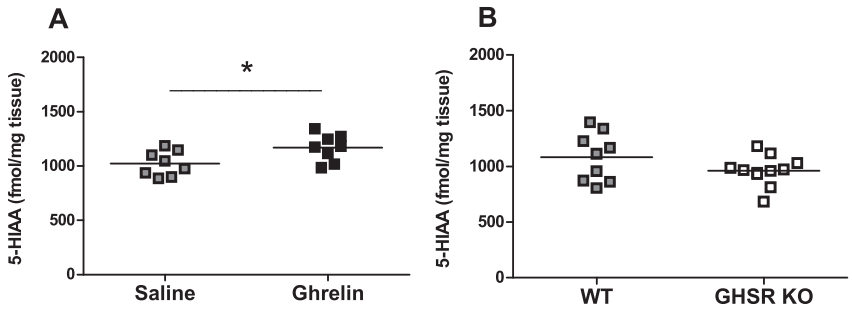

Fig. 1. Tissue concentrations of 5-hydroxyindoleacetic acid (5-HIAA) in the amygdala of (A) mice treated i.c.v. with ghrelin or saline vehicle and (B) GHS-R1A knock-out and wild-type mice. Values are expressed as fmol/mg dissected brain tissue. $^{*}p < 0.05$, Student's *t*-test.

4. Discussion

The results of the present study support the hypothesis that ghrelin targets the central serotonergic system. Our studies focused on two brain areas, the amygdala and the dorsal raphe. In the amygdala, acute central ghrelin administration increased serotonergic turnover (reflected by increased concentration of the serotonin metabolite 5-HIAA) and also increased the mRNA expression of a number of serotonergic receptors. Acute central ghrelin treatment also appeared to influence serotonergic signaling in the dorsal raphe, reflected by an increased mRNA expression of serotonergic receptors in this region. Consistent with these data, GHS-R1A knock-out mice, that lack ghrelin signaling via GHS-R1A, display reduced mRNA expression of a number of genes linked to serotonergic signaling, including several serotonergic receptors (in the amygdala and dorsal raphe) and also monoamine oxidase A (an enzyme involved in the breakdown of serotonin) in the amygdala. Given that central serotonin pathways are linked to mood and the expression of anxiety behavior, our new data suggest that serotonergic pathways provide one possible neurochemical substrate underpinning ghrelin's effects on anxiety-like behavior.

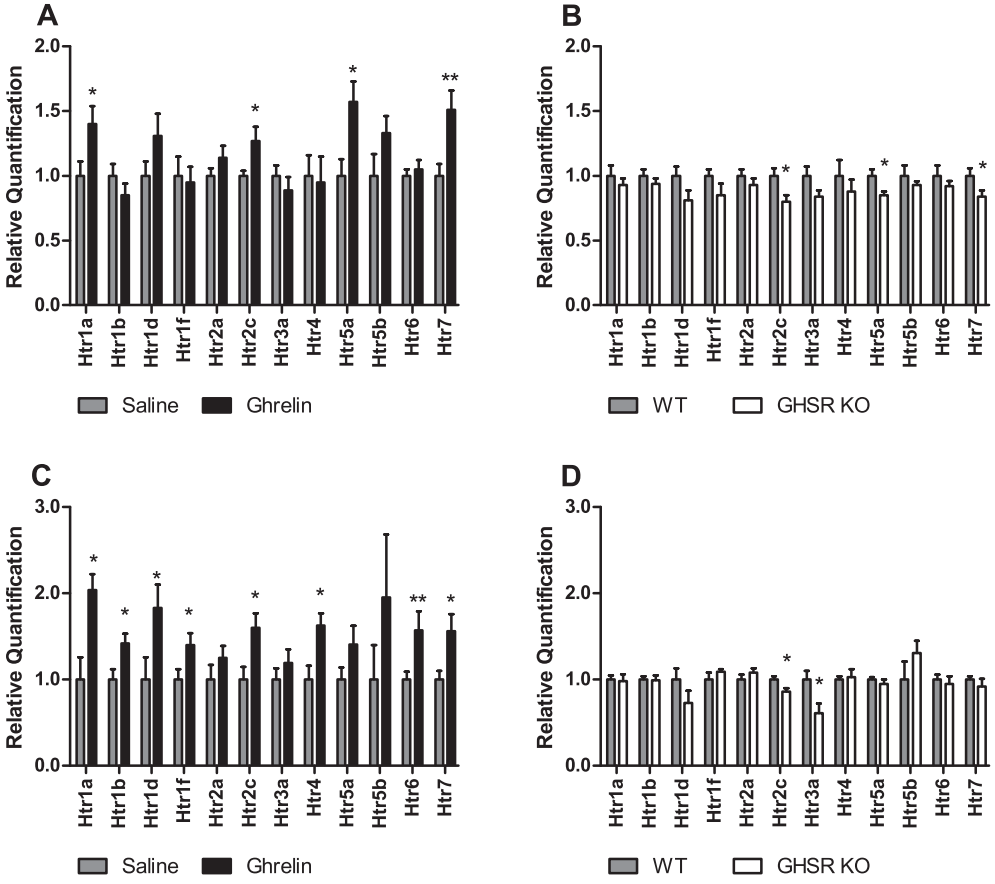

Fig. 2. mRNA expression of serotonergic receptors in the amygdala of (A) ghrelin- and saline-treated mice and (B) GHS-R1A knock-out and wild-type littermate mice and in the dorsal raphe of (C) ghrelin- and saline-treated mice and (D) GHS-R1A knock-out and wild-type littermate mice. Values are expressed as mean (+SEM) relative to saline-treated/wild-type controls. Amygdala: $n = 10$ per group. Dorsal raphe: $n = 10$ in the saline-treated group, $n = 9$ in the ghrelin-treated group. GHS-R1A knock-out: $n = 8$ per group. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared to vehicle, Student's *t*-test.

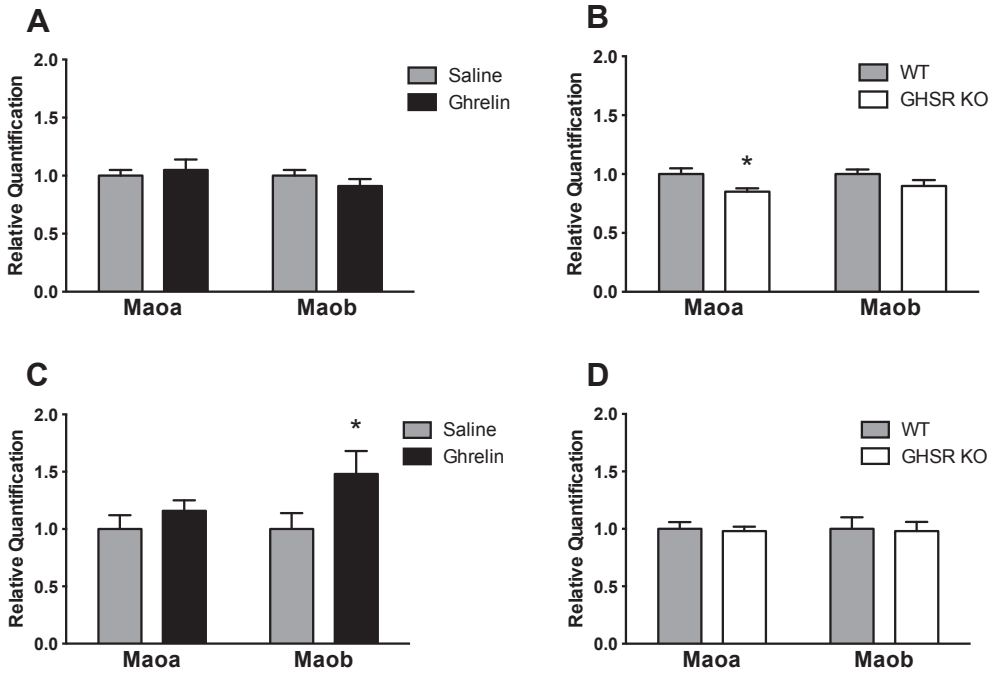

Fig. 3. mRNA expression of monoamine oxidase A (Maoa) and monoamine oxidase B (Maob) in the amygdala of (A) ghrelin- and saline-treated mice and (B) GHS-R1A knock-out and wild-type littermate mice and in the dorsal raphe of (C) ghrelin- and saline-treated mice and (D) GHS-R1A knock-out and wild-type littermate mice. Values are expressed as mean (+SEM) relative to saline-treated/wild-type controls. Amygdala: $n = 10$ per group. Dorsal raphe: $n = 10$ in the saline-treated group, $n = 9$ in the ghrelin-treated group. GHS-R1A knock-out: $n = 8$ per group. $^{*}p < 0.05$ compared to vehicle, Student's t-test.

4.1. Ghrelin increases the tissue concentration of 5-HIAA in the amygdala

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. Whereas an increased tissue concentration of the serotonin metabolite, 5-HIAA, would be indicative of an increased activity of the serotonergic neurons, an increased tissue concentration of serotonin would suggest an increased serotonergic innervation of the tissue. In our study the concentration of 5-HIAA was increased in the amygdala in the ghrelin-treated group, which could suggest an increased activity of the serotonergic neurons projecting to this area after acute ghrelin treatment. The GHS-R1A knock-out mice displayed a tendency towards decreased concentration of 5-HIAA, consistent with a previous report (Patterson et al., 2010). These authors also reported a significant increase in 5-HIAA concentration in the amygdala after 2 weeks exposure to a chronic unpredictable stress. Interestingly, these mice had significantly elevated plasma ghrelin levels, in line with our finding that ghrelin increases the tissue concentration of 5-HIAA in the amygdala.

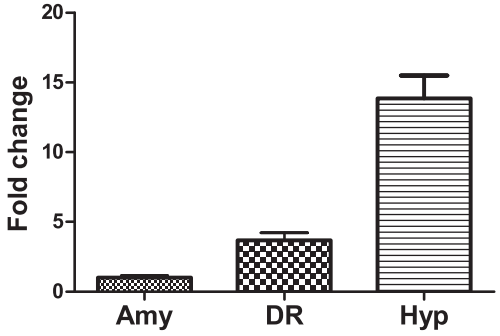

Fig. 4. mRNA expression of GHS-R1A in the amygdala (Amy) compared to the dorsal raphe (DR) and the hypothalamus (Hyp). Values are expressed as mean (+SEM) relative to amygdala. Amygdala: $n = 10$, dorsal raphe: $n = 10$, hypothalamus: $n = 10$.

The increased serotonergic activity in the amygdala may be implicated in the anxiogenic effect of ghrelin. Clinical studies have shown that individuals carrying the short variant of a polymorphism in the serotonin transporter gene have reduced transcription of the serotonin transporter, resulting in reduced serotonin reuptake, and presumably increased serotonin levels in the synaptic cleft. Interestingly, they also have higher scores on anxiety-related personality traits (Lesch et al., 1996), and increased amygdala activation in response to fearful stimuli (Hariri et al., 2002). These clinical findings are supported by animal studies showing that mice with genetic knock-out of the serotonin transporter have increased extracellular serotonin levels and increased anxiety-like behavior, while overexpression of the serotonin transporter gene results in decreased anxiety-like behavior (Holmes, 2008).

We also explored the tissue concentrations of serotonin and found that they were unaltered by acute ghrelin treatment in the amygdala as well as in the dorsal raphe. We did not expect acute ghrelin treatment to alter serotonin concentration as this would be indicative of a change in serotonin innervation in these areas. However, serotonin concentration was not altered in the GHS-R1A knockout mice either, suggesting that chronic (life-long) changes in ghrelin signaling do not alter serotonin innervation.

4.2. The effect of ghrelin signaling on the mRNA expression of serotonin-related genes

Further evidence supporting our hypothesis that the central serotonin system is a target for ghrelin is provided by our studies

exploring the mRNA expression of serotonergic receptors in the dorsal raphe and the amygdala, many of which showed increased expression following acute ghrelin treatment and conversely, showed reduced expression in GHS-R1A knock-out mice. The mRNA expression of monoamine oxidase A was also found to be decreased in the amygdala of the GHS-R1A knock-out mice compared to wild-type controls.

We found that serotonin receptor 2c, 5a and 7 showed significantly increased mRNA expression in the amygdala after acute ghrelin treatment but had significantly decreased mRNA expression in the amygdala of GHS-R1A knock-out mice. The serotonin receptor 2c is involved in the regulation of food intake as well as in mood (Serretti et al., 2004) and activation of this receptor in the amygdala has been proposed to be associated with fear responses (Drago and Serretti, 2009). Genetic knock-out studies show that this receptor is important for the regulation of anxiety-like behavior and Htr2c knock-out mice display a less anxious phenotype than wild-type controls (Heisler et al., 2007). Notably, this receptor had an increased mRNA expression after ghrelin treatment and a decreased mRNA expression in the GHS-R1A knock-out mice in both of the studied regions. The serotonin receptor 5a has not been extensively studied due to lack of specific ligands for the receptor. Interestingly, the receptors that have similar binding affinities as the 5a receptor are serotonin receptor 1a and 7 (Thomas, 2006), which also displayed an increased expression in the amygdala in our study. Regarding brain function, the serotonin receptor 5a might be involved in exploratory behavior, as serotonin receptor 5a knock-out mice have increased exploratory behavior (Grailhe et al., 1999).

The serotonin receptor 1a had an increased mRNA expression in the amygdala and the dorsal raphe after acute ghrelin administration in our study. In the amygdala, the serotonin receptor 1a is a postsynaptic receptor, while in the dorsal raphe, it functions as a somatodendritic autoreceptor, decreasing serotonin release (Barnes and Sharp, 1999). It is interesting to note that activation of presynaptic (autoreceptors) and postsynaptic 5-HT1A receptors have opposing effects in the regulation of anxiety. While administration of 5-HT1A agonists in the amygdala has anxiogenic effects, administration of a 5-HT1A agonist into the dorsal raphe is anxiolytic (Akimova et al., 2009).

In the dorsal raphe, we also observed an increased mRNA expression of serotonin receptor 1b, 1d and 1f in the ghrelin-treated animals. Serotonin receptor 1b and 1d are also autoreceptors, but in contrast to serotonin receptor 1a these are presynaptically located in the serotonergic nerve terminals (Barnes and Sharp, 1999). We also observed an increased mRNA expression of serotonin receptor 4, 6, and 7 in the dorsal raphe after ghrelin treatment, all of which are coupled to Gs proteins, stimulating adenylate cyclase (Hoyer et al., 2002). Serotonin receptor 4 agonists have previously been reported to increase the firing rate of serotonin neurons in the dorsal raphe (Lucas and Debonnel, 2002). Moreover, serotonergic neurons in the dorsal raphe of Htr4 knock-out mice display reduced spontaneous electrical activity compared to wild-type mice (Conductier et al., 2006). These data indicate that 5-HT4 receptors are important for the regulation of serotonergic activity. Regarding the functional significance of 5-HT6, there are indications that this receptor is involved in the regulation of anxiety as Htr6 knock-out mice display increased anxiety-like behavior (Barnes and Sharp, 1999). Acute stress has been shown to increase the mRNA expression of Htr7 in the hippocampus (Yau et al., 2001).

In the dorsal raphe we found a decreased mRNA expression of Htr3a in the GHS-R1A knock-out mice. Htr3a has also been implicated in anxiety. Knock-out of the Htr3a gene in mice is anxiolytic (Kelley et al., 2003). Moreover, a polymorphism in the Htr3a gene is associated with the personality trait harm avoidance in women (Melke et al., 2003). This polymorphism also modulates amygdala activation (Iidaka et al., 2005) and affects the protein levels of Htr3a (Niesler et al., 2001).

Monoamine oxidase, the enzyme responsible for metabolizing biogenic amines, exists in two forms, A and B. Monoamine oxidase A preferentially metabolizes serotonin and noradrenaline, while monoamine oxidase B preferentially metabolizes benzylamine and phenylethylamine. Dopamine is metabolized by both enzymes. Some studies suggest that serotonin can also be metabolized by monoamine oxidase B, although at a slower rate (Mitra and Guha, 1980). Monoamine oxidase A, the main enzyme responsible for metabolizing serotonin, had a decreased mRNA expression in the amygdala in the GHS-R1A knock-out mice. The decreased mRNA expression is in line with the trend towards decreased 5-HIAA concentrations in the amygdala in the GHS-R1A knock-out mice. Monoamine oxidase B is expressed in serotonergic neurons, glia cells and astrocytes. We found an increased mRNA expression of Maob in the dorsal raphe after acute ghrelin treatment. The increased mRNA expression may be indicative of an increased transcriptional activity of serotonergic neurons in the dorsal raphe after acute ghrelin treatment. As monoamine oxidase B metabolizes dopamine, it could possibly reflect increased dopamine in the dorsal raphe, although further studies would be needed to confirm this. The dorsal raphe is innervated by dopamine neurons originating in the ventral tegmental area (Kalen et al., 1988), and dopamine cells in this region are known to express GHS-R1A and be ghrelin responsive (Abizaid et al., 2006). Accordingly, stimulation of GHS-R1A in the ventral tegmental area may increase dopamine release in the dorsal raphe, an area where dopamine has been shown to activate serotonergic neurons via dopaminergic D2 receptors (Ferre and Artigas, 1993).

4.3. The amygdala as a direct or indirect target for ghrelin

To our knowledge, this is the first study quantifying the expression of GHS-R1A mRNA in the amygdala of mice and comparing it to the mRNA expression levels in other brain areas known to express GHS-R1A. Consistent with these results, we have previously identified the presence of GHS-R1A in the amygdala of rats using *in situ* hybridization (Alvarez-Crespo et al., 2012). Notably, the mRNA expression level of GHS-R1A in the amygdala was considerably lower than that observed in the dorsal raphe and the hypothalamus. Further studies, using site-specific injections, would be required to determine the extent to which ghrelin exerts direct or indirect effects on the serotonergic system in these areas. In our study, ghrelin was administered i.c.v. and the effects that we observe could be either a direct effect, via binding to GHS-R1A in the amygdala, or may involve indirect activation of afferent projections. Evidence supporting the amygdala as a direct parenchymal target for ghrelin is suggested by studies showing that site-specific administration of ghrelin into the amygdala increases anxiety-like behavior (Carlini et al., 2004). Moreover, electrophysiological studies have found ghrelin-responsive neurons in the basolateral amygdala (Toth et al., 2009). It is also possible that ghrelin increases the activity of serotonergic cells projecting to the amygdala by acting on the serotonergic cell bodies in the dorsal raphe. There are direct serotonergic pathways from the dorsal raphe to the amygdala (Wang and Aghajanian, 1977) and we have previously shown that ghrelin affects the firing rate of cells in the dorsal raphe, using extracellular recordings on brain slice preparations (Hansson et al., 2011). It should be noted however, that another study reported that ghrelin depolarizes putative serotonergic dorsal raphe cells, using whole-cell patch clamp recordings (Ogaya et al., 2011). As ghrelin seems to have an inhibitory effect on serotonin release in the hypothalamus (Brunetti et al., 2002), it

might have been expected that ghrelin would inhibit serotonin release also in the amygdala. There are indications, however, that dorsal raphe cells exert different effects in the hypothalamus and the amygdala, evidenced e.g. by the inhibitory effect of serotonergic dorsal raphe cells on the amygdala (Wang and Aghajanian, 1977), while the effect of serotonergic dorsal raphe cells is excitatory in the paraventricular nucleus (PVN) of the hypothalamus (Saphier and Feldman, 1989). The differential regulation of hypothalamus and amygdala could be due to different neurons projecting to these different target areas from the dorsal raphe. Although a minor part of the dorsal raphe cells provide branching collaterals to the central nucleus of the amygdala and to the PVN, the majority of the serotonergic cells do not branch to both of these areas, but project to either the amygdala or the hypothalamus (Petrov et al., 1994) and it is different nerve fibers projecting from the dorsal raphe that innervate the amygdala than the hypothalamus (Moore et al., 1978). The effects of ghrelin could also be mediated via other ghrelin-responsive brain areas that project to the amygdala. GHS-R1A is expressed in the external lateral parabrachial nucleus (PBN) (Zigman et al., 2006) and this subnucleus of the PBN sends neuronal projections to the amygdala (Tokita et al., 2010). GHS-R1A is also densely expressed in the hypothalamus (Zigman et al., 2006) and there are neuronal projections from the PVN to the amygdala (Sofroniew, 1980), suggesting that ghrelin responsive neurons could project to the amygdala, thereby affecting the release of serotonin by acting on presynaptic terminals, or affect gene expression by acting on cells in the amygdala.

4.4. Concluding remarks

The results of the present study suggest neurochemical convergence of ghrelin-responsive circuits with central serotonergic pathways. In particular we show that serotonin metabolism and patterns of mRNA expression of serotonin receptor subtypes in the dorsal raphe and amygdala are affected by acute central delivery of ghrelin and also by knock-out of the ghrelin receptor, GHS-R1A. In future studies, it will be interesting to determine the consequences and time course of the effects of central ghrelin action on protein levels of serotonin-linked genes in relevant brain areas. It will also be of importance to determine the extent to which the central serotonin system mediates the effects of ghrelin on behavioral endpoints including those linked to mood. Given the established role of central serotonergic systems in mood, together with the emerging data implicating central ghrelin signaling pathways at the interface between feeding control and the regulation of anxiety-like behaviors, the central serotonin system emerges as a novel candidate that could mediate ghrelin's effects on anxiety-like behavior.

Acknowledgments

This work was primarily supported by NeuroFAST (FP7-KBBE-2009-3-245009 to S.L.D.) but also by additional European Commission Seventh Framework grants (FP7-KBBE-2010-4-266408, Full4Health and FP7-HEALTH-2009-241592, EurOCHIP; both to S.L.D.), the Swedish Research Council for Medicine (2010-12208 to HN, 2011-3054 to KPS and 2012-1758 to SLD), Forskning och Utvecklingsarbete/Avtal om Läkarutbildning och Forskning Göteborg (ALFGBG-138741 to S.L.D.), the Swedish Foundation for Strategic Research to Sahlgrenska Center for Cardiovascular and Metabolic Research (A305–188), and NovoNordisk Fonden (to S.L.D.). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. We also thank the Genomics Core Facility platform at the Sahlgrenska Academy, University of Gothenburg, which was funded by a grant from the Knut and Alice Wallenberg Foundation.

Abbreviations

5-HIAA	5-hydroxyindoleacetic acid
5-HT	serotonin
GHS-R1A	growth hormone secretagogue receptor 1A
Htr1a	serotonin receptor 1A
Htr1b	serotonin receptor 1B
Htr1d	serotonin receptor 1D
Htr1f	serotonin receptor 1F
Htr2a	serotonin receptor 2A
Htr2c	serotonin receptor 2C
Htr3a	serotonin receptor 3A
Htr4	serotonin receptor 4
Htr5a	serotonin receptor 5A
Htr5b	serotonin receptor 5B
Htr6	serotonin receptor 6
Htr7	serotonin receptor 7
i.c.v.	intracerebroventricular
Maoa	monoamine oxidase A
Maob	monoamine oxidase B
PBN	parabrachial nucleus
PVN	paraventricular nucleus
RQ	relative quantification
Slc6a4	serotonin transporter
Slc18a2	vesicular monoamine transporter 2
SSRI	selective serotonin reuptake inhibitor
Tph1	tryptophan hydroxylase 1
Tph2	tryptophan hydroxylase 2

References

Abizaid, A., Liu, Z.W., Andrews, Z.B., Shanabrough, M., Borok, E., Elsworth, J.D., Roth, R.H., Sleeman, M.W., Picciotto, M.R., Tschop, M.H., Gao, X.B., Horvath, T.L., 2006. Ghrelin modulates the activity and synaptic input organization of midbrain dopamine neurons while promoting appetite. J. Clin. Investig. 116, 3229–3239.

Akimova, E., Lanzenberger, R., Kasper, S., 2009. The serotonin-1A receptor in anxiety disorders. Biol. Psychiatry 66, 627–635.

Alvarez-Crespo, M., Skibicka, K.P., Farkas, I., Molnar, C.S., Egecioglu, E., Hrabovszky, E., Liposits, Z., Dickson, S.L., 2012. The amygdala as a neurobiological target for ghrelin in rats: neuroanatomical, electrophysiological and behavioral evidence. PloS One 7, e46321.

Asakawa, A., Inui, A., Kaga, T., Yuzuriha, H., Nagata, T., Fujimiya, M., Katsuura, G., Makino, S., Fujino, M.A., Kasuga, M., 2001. A role of ghrelin in neuroendocrine and behavioral responses to stress in mice. Neuroendocrinology 74, 143–147.

Barnes, N.M., Sharp, T., 1999. A review of central 5-HT receptors and their function. Neuropharmacology 38, 1083–1152.

Brunetti, L., Recinella, L., Orlando, G., Michelotto, B., Di Nisio, C., Vacca, M., 2002. Effects of ghrelin and amylin on dopamine, norepinephrine and serotonin release in the hypothalamus. Eur. J. Pharmacol. 454, 189–192.

Carlini, V.P., Gaydou, R.C., Schioth, H.B., de Barioglio, S.R., 2007. Selective serotonin reuptake inhibitor (fluoxetine) decreases the effects of ghrelin on memory retention and food intake. Regul. Pept. 140, 65–73.

Carlini, V.P., Monzon, M.E., Varas, M.M., Cragnolini, A.B., Schioth, H.B., Scimonelli, T.N., de Barioglio, S.R., 2002. Ghrelin increases anxiety-like behavior and memory retention in rats. Biochem. Biophys. Res. Commun. 299, 739–743.

Carlini, V.P., Varas, M.M., Cragnolini, A.B., Schioth, H.B., Scimonelli, T.N., de Barioglio, S.R., 2004. Differential role of the hippocampus, amygdala, and dorsal raphe nucleus in regulating feeding, memory, and anxiety-like behavioral responses to ghrelin. Biochem. Biophys. Res. Commun. 313, 635–641.

Conductier, G., Dusticier, N., Lucas, G., Cote, F., Debonnel, G., Daszuta, A., Dumuis, A., Nieoullon, A., Hen, R., Bockaert, J., Compan, V., 2006. Adaptive changes in serotonin neurons of the raphe nuclei in 5-HT(4) receptor knock-out mouse. Eur. J. Neurosci. 24, 1053–1062.

Costall, B., Kelly, M.E., Naylor, R.J., Onaivi, E.S., Tyers, M.B., 1989. Neuroanatomical sites of action of 5-HT3 receptor agonist and antagonists for alteration of aversive behaviour in the mouse. Br. J. Pharmacol. 96, 325–332.

Currie, P.J., John, C.S., Nicholson, M.L., Chapman, C.D., Loera, K.E., 2010. Hypothalamic paraventricular 5-hydroxytryptamine inhibits the effects of ghrelin on eating and energy substrate utilization. Pharmacol. Biochem. Behav. 97, 152–155.

Dahlstroem, A., Fuxe, K., 1964. Evidence for the existence of monoamine-containing neurons in the central nervous system. I. demonstration of monoamines in the cell bodies of brain stem neurons. Acta Physiol. Scand. Suppl. 232, 231–255.
Davis, M., 1992. The role of the amygdala in fear and anxiety. Annu. Rev. Neurosci. 15, 353–375.
Diano, S., Farr, S.A., Benoit, S.C., McNay, E.C., da Silva, I., Horvath, B., Gaskin, F.S., Nonaka, N., Jaeger, L.B., Banks, W.A., Morley, J.E., Pinto, S., Sherwin, R.S., Xu, L., Yamada, K.A., Sleeman, M.W., Tschop, M.H., Horvath, T.L., 2006. Ghrelin controls hippocampal spine synapse density and memory performance. Nat. Neurosci. 9, 381–388.
Drago, A., Serretti, A., 2009. Focus on HTR2C: a possible suggestion for genetic studies of complex disorders. Am. J. Med. Genet. B Neuropsychiatr. Genet. 150B, 601–637.
Egecioglu, E., Jerlhag, E., Salome, N., Skibicka, K.P., Haage, D., Bohlooly, Y.M., Andersson, D., Bjursell, M., Perrissoud, D., Engel, J.A., Dickson, S.L., 2010. Ghrelin increases intake of rewarding food in rodents. Addict. Biol. 15, 304–311.
Ferre, S., Artigas, F., 1993. Dopamine D2 receptor-mediated regulation of serotonin extracellular concentration in the dorsal raphe nucleus of freely moving rats. J. Neurochem. 61, 772–775.
Ghersi, M.S., Casas, S.M., Escudero, C., Carlini, V.P., Buteler, F., Cabrera, R.J., Schioth, H.B., de Barioglio, S.R., 2011. Ghrelin inhibited serotonin release from hippocampal slices. Peptides 32, 2367–2371.
Graeff, F.G., Guimaraes, F.S., De Andrade, T.G., Deakin, J.F., 1996. Role of 5-HT in stress, anxiety, and depression. Pharmacol. Biochem. Behav. 54, 129–141.
Grailhe, R., Waeber, C., Dulawa, S.C., Hornung, J.P., Zhuang, X., Brunner, D., Geyer, M.A., Hen, R., 1999. Increased exploratory activity and altered response to LSD in mice lacking the 5-HT(5A) receptor. Neuron 22, 581–591.
Hansson, C., Annerbrink, K., Nilsson, S., Bah, J., Olsson, M., Allgulander, C., Andersch, S., Sjodin, I., Eriksson, E., Dickson, S.L., 2013. A possible association between panic disorder and a polymorphism in the preproghrelingene. Psychiatry Res. 206, 22–25.
Hansson, C., Haage, D., Taube, M., Egecioglu, E., Salome, N., Dickson, S.L., 2011. Central administration of ghrelin alters emotional responses in rats: behavioural, electrophysiological and molecular evidence. Neuroscience 180, 201–211.
Hariri, A.R., Mattay, V.S., Tessitore, A., Kolachana, B., Fera, F., Goldman, D., Egan, M.F., Weinberger, D.R., 2002. Serotonin transporter genetic variation and the response of the human amygdala. Science 297, 400–403.
Heisler, L.K., Zhou, L., Bajwa, P., Hsu, J., Tecott, L.H., 2007. Serotonin 5-HT(2C) receptors regulate anxiety-like behavior. Genes Brain Behav. 6, 491–496.
Holmes, A., 2008. Genetic variation in cortico-amygdala serotonin function and risk for stress-related disease. Neurosci. Biobehav. Rev. 32, 1293–1314.
Hoyer, D., Hannon, J.P., Martin, G.R., 2002. Molecular, pharmacological and functional diversity of 5-HT receptors. Pharmacol. Biochem. Behav. 71, 533–554.
Iidaka, T., Ozaki, N., Matsumoto, A., Nogawa, J., Kinoshita, Y., Suzuki, T., Iwata, N., Yamamoto, Y., Okada, T., Sadato, N., 2005. A variant C178T in the regulatory region of the serotonin receptor gene HTR3A modulates neural activation in the human amygdala. J. Neurosci. 25, 6460–6466.
Jaszberenyi, M., Bujdoso, E., Bagosi, Z., Telegdy, G., 2006. Mediation of the behavioral, endocrine and thermoregulatory actions of ghrelin. Horm. Behav. 50, 266–273.
Jerlhag, E., Egecioglu, E., Dickson, S.L., Andersson, M., Svensson, L., Engel, J.A., 2006. Ghrelin stimulates locomotor activity and accumbal dopamine-overflow via central cholinergic systems in mice: implications for its involvement in brain reward. Addict. Biol. 11, 45–54.
Jerlhag, E., Egecioglu, E., Landgren, S., Salome, N., Heilig, M., Moechars, D., Datta, R., Perrissoud, D., Dickson, S.L., Engel, J.A., 2009. Requirement of central ghrelin signaling for alcohol reward. Proc. Natl. Acad. Sci. U. S. A. 106, 11318–11323.
Kalen, P., Skagerberg, G., Lindvall, O., 1988. Projections from the ventral tegmental area and mesencephalic raphe to the dorsal raphe nucleus in the rat. Evidence for a minor dopaminergic component. Exp. Brain Res. 73, 69–77.
Kanehisa, M., Akiyoshi, J., Kitaichi, T., Matsushita, H., Tanaka, E., Kodama, K., Hanada, H., Isogawa, K., 2006. Administration of antisense DNA for ghrelin causes an antidepressant and anxiolytic response in rats. Prog. Neuropsychopharmacol. Biol. Psychiatry 30, 1403–1407.
Kelley, S.P., Bratt, A.M., Hodge, C.W., 2003. Targeted gene deletion of the 5-HT3A receptor subunit produces an anxiolytic phenotype in mice. Eur. J. Pharmacol. 461, 19–25.
Kojima, M., Hosoda, H., Date, Y., Nakazato, M., Matsuo, H., Kangawa, K., 1999. Ghrelin is a growth-hormone-releasing acylated peptide from stomach. Nature 402, 656–660.
Lesch, K.P., Bengel, D., Heils, A., Sabol, S.Z., Greenberg, B.D., Petri, S., Benjamin, J., Muller, C.R., Hamer, D.H., Murphy, D.L., 1996. Association of anxiety-related traits with a polymorphism in the serotonin transporter gene regulatory region. Science 274, 1527–1531.
Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25, 402–408.
Lucas, G., Debonnel, G., 2002. 5-HT4 receptors exert a frequency-related facilitatory control on dorsal raphe nucleus 5-HT neuronal activity. Eur. J. Neurosci. 16, 817–822.
Lutter, M., Sakata, I., Osborne-Lawrence, S., Rovinsky, S.A., Anderson, J.G., Jung, S., Birnbaum, S., Yanagisawa, M., Elmquist, J.K., Nestler, E.J., Zigman, J.M., 2008. The orexigenic hormone ghrelin defends against depressive symptoms of chronic stress. Nat. Neurosci. 11, 752–753.
Melke, J., Westberg, L., Nilsson, S., Landen, M., Soderstrom, H., Baghaei, F., Rosmond, R., Holm, G., Bjorntorp, P., Nilsson, L.G., Adolfsson, R., Eriksson, E., 2003. A polymorphism in the serotonin receptor 3A (HTR3A) gene and its association with harm avoidance in women. Arch. Gen. Psychiatry 60, 1017–1023.
Mitra, C., Guha, S.R., 1980. Serotonin oxidation by type B MAO of rat brain. Biochem. Pharmacol. 29, 1213–1216.
Moore, R.Y., Halaris, A.E., Jones, B.E., 1978. Serotonin neurons of the midbrain raphe: ascending projections. J. Comp. Neurol. 180, 417–438.
Niesler, B., Flohr, T., Nothen, M.M., Fischer, C., Rietschel, M., Franzek, E., Albus, M., Propping, P., Rappold, G.A., 2001. Association between the 5' UTR variant C178T of the serotonin receptor gene HTR3A and bipolar affective disorder. Pharmacogenetics 11, 471–475.
Ogaya, M., Kim, J., Sasaki, K., 2011. Ghrelin postsynaptically depolarizes dorsal raphe neurons in rats in vitro. Peptides 32, 1606–1616.
Patterson, Z.R., Ducharme, R., Anisman, H., Abizaid, A., 2010. Altered metabolic and neurochemical responses to chronic unpredictable stressors in ghrelin receptor-deficient mice. Eur. J. Neurosci. 32, 632–639.
Paxinos, G., Franklin, K., 2001. The Mouse Brain in Stereotaxic Coordinates. Academic Press/Elsevier, San Diego, California, USA.
Perello, M., Sakata, I., Birnbaum, S., Chuang, J.C., Osborne-Lawrence, S., Rovinsky, S.A., Woloszyn, J., Yanagisawa, M., Lutter, M., Zigman, J.M., 2010. Ghrelin increases the rewarding value of high-fat diet in an orexin-dependent manner. Biol. Psychiatry 67, 880–886.
Petrov, T., Krukoff, T.L., Jhamandas, J.H., 1994. Chemically defined collateral projections from the pons to the central nucleus of the amygdala and hypothalamic paraventricular nucleus in the rat. Cell Tissue Res. 277, 289–295.
Pinilla, L., Barreiro, M.L., Tena-Sempere, M., Aguilar, E., 2003. Role of ghrelin in the control of growth hormone secretion in prepubertal rats: interactions with excitatory amino acids. Neuroendocrinology 77, 83–90.
Salome, N., Taube, M., Egecioglu, E., Hansson, C., Stenstrom, B., Chen, D., Andersson, D.R., Georg Kuhn, H., Ohlsson, C., Dickson, S.L., 2011. Gastrectomy alters emotional reactivity in rats: neurobiological mechanisms. Eur. J. Neurosci. 33, 1685–1695.
Saphier, D., Feldman, S., 1989. Paraventricular nucleus neuronal responses following electrical stimulation of the midbrain dorsal raphe: evidence for cotransmission. Exp. Brain Res. 78, 407–414.
Serretti, A., Artioli, P., De Ronchi, D., 2004. The 5-HT2C receptor as a target for mood disorders. Expert Opin. Ther. Targets 8, 15–23.
Skibicka, K.P., Hansson, C., Egecioglu, E., Dickson, S.L., 2012. Role of ghrelin in food reward: impact of ghrelin on sucrose self-administration and mesolimbic dopamine and acetylcholine receptor gene expression. Addict. Biol. 17 (1), 95–107.
Soderpalm, B., Engel, J.A., 1990. Serotonergic involvement in conflict behaviour. Eur. Neuropsychopharmacol. 1, 7–13.
Sofroniew, M.V., 1980. Projections from vasopressin, oxytocin, and neurophysin neurons to neural targets in the rat and human. J. Histochem. Cytochem. 28, 475–478.
Thomas, D.R., 2006. 5-ht5A receptors as a therapeutic target. Pharmacol. Ther. 111, 707–714.
Tokita, K., Inoue, T., Boughter Jr., J.D., 2010. Subnuclear organization of parabrachial efferents to the thalamus, amygdala and lateral hypothalamus in C57BL/6J mice: a quantitative retrograde double labeling study. Neuroscience 171, 351–365.
Toth, K., Laszlo, K., Lukacs, E., Lenard, L., 2009. Intraamygdaloid microinjection of acylated-ghrelin influences passive avoidance learning. Behav. Brain Res. 202, 308–311.
Tschop, M., Smiley, D.L., Heiman, M.L., 2000. Ghrelin induces adiposity in rodents. Nature 407, 908–913.
Wang, R.Y., Aghajanian, G.K., 1977. Inhibition of neurons in the amygdala by dorsal raphe stimulation: mediation through a direct serotonergic pathway. Brain Res. 120, 85–102.
Yau, J.L., Noble, J., Seckl, J.R., 2001. Acute restraint stress increases 5-HT7 receptor mRNA expression in the rat hippocampus. Neurosci. Lett. 309, 141–144.
Zigman, J.M., Jones, J.E., Lee, C.E., Saper, C.B., Elmquist, J.K., 2006. Expression of ghrelin receptor mRNA in the rat and the mouse brain. J. Comp. Neurol. 494, 528–548.