Ghrelin & Circuit Formation

By Got My Ion You
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Outline

What we’ll cover today

- **Background: what is ghrelin?**
  - Overview
  - Biochemical pathway of ghrelin in hypothalamic circuit
- **Related studies**
  - Vulnerability of ghrelin circuitry to perturbations
  - Neonatal overnutrition & ghrelin
- **Collden et al. study**
  - Experimental set-up & procedures
  - Results
- **Tying it all together - conclusions**
  - Implications and discussion of results from Collden et al. study
Background: What is Ghrelin?
**Background: What is ghrelin?**

**Ghrelin**: a metabolic hormone secreted from mainly the stomach that acts centrally to promote **feeding behavior** by binding to **growth hormone secretagogue receptors** in the **arcuate nucleus of the hypothalamus**.

‘Hunger hormone’ - stimulates appetite, increases food intake and promotes fat storage.
Background: What is ghrelin?

- 28 amino acid Orexigenic peptide and hormone
- Made predominantly by your stomach’s X/A shaped cells
- Active form = acylated-ghrelin (AG) - Synthesized from preproghrelin, (acylated by enzyme ghrelin O-acyltransferase (GOAT))
- Ghrelin levels increase before meals and decrease after meals.
Eating causes blood levels of ghrelin to decrease.

Hunger stimulates the release of ghrelin from the stomach.

Ghrelin travels in the blood to the hypothalamus where it acts.

Stimulates appetite.
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Ghrelin travels in the blood to the hypothalamus where it acts.

Stimulates Appetite.
Background: What is ghrelin?

- Ghrelin is the yin to leptin’s yang
- Leptin inhibits ghrelin (it suppresses the expression of ghrelin receptors in the NPY system, therefore preventing the stimulation of feeding behaviors)
Key Players

- Ghrelin
- GHS-R1a – Growth Hormone Secretagogue Receptor 1a
  - Expressed in NPY/AgRP neurons in the ARC
- Hypothalamus
- GOAT (Ghrelin O-acyl transferase)
- POMC (peptide precursor) ---> GABA (inhibitory neurotransmitter)---->Alpha MSH (inhibitor of appetite)
Biochemical Pathway of Ghrelin in the Hypothalamic Circuit
Biochemical pathway of ghrelin in hypothalamus

- No food, low glucose, low leptin → release of ghrelin

- Ghrelin: bloodstream → hypothalamus.

- In the Hypothalamus:
  - Ghrelin binds to **GHS-1a** in the **arcuate nucleus**
  - Expressed in NPY/AgRP neurons (in ARC)
    - Inhibits POMC and Alpha MSH (inhibitor appetite)

Ghrelin increases AgRP and NPY expression → Orexigenic effect (Hungry)

Martinez de Morentin et al., Metabolic Syndrome and Neurological Disorders, 2013
-we need to understand Ghrelin’s role in this circuit in order to understand where it influences within the hypothalamus
-remember that Ghrelin has orexigenic effects, and is linked to other pathways in the brain that produce orexigenic effects
-also remember that there is peripheral ghrelin circulating outside of the CNS and it may travel to the brain
Related studies
Vulnerability of Ghrelin Circuitry to Perturbations
Ghrelin is not a hormone “in isolation” → it involves a dynamic biochemical interplay in both the peripheral and central nervous system.
So when we say "ghrelin," we are really referring more broadly to its many biochemical derivatives.
AG is responsible “typical” orexigenic effects we commonly associate w/ ghrelin

- Once synthesized in gastric mucosa, AG needs to be transported to ARH via tanycytes, where it binds to and activates GHSR1a receptors
- Desacyl ghrelin (DAG) can counterbalance this effect
  - Tends to be lower in obese individuals (look at AG/DAG ratio for more nuanced picture)
WTF
-so much activation/deactivation/pathways
1) many types of ghrelin
2) acyl-ghrelin activates GHSR1a receptors
3) DAG counterbalances this
From this we gather that although the obese population has lower levels of ghrelin they *should* feel less hungry, but perhaps they have lower levels of DAG. The literature does not specify.
Ghrelin-reactive immunoglobins (Igs) → prevent degradation, ↑ action

an overview of the ghrelin system

Basic nutritional investigation

High-fat diet increases ghrelin-expressing cells in stomach, contributing to obesity

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cells, we do not imply an effect of HFD on cell proliferation, but on the number of detectable cells expressing preproghrelin mRNA and revealed by the in situ hybridization technique. Accordingly, a 15% increase of ghrelin precursor-expressing cells in HFD-fed mice may signify that in control mice such extra cells may express ghrelin mRNA below the detection level. These data reflect the plasticity of the ghrelin synthesis following nutritional modulations. The number of cells in the stomach expressing ghrelin precursor are also greatly affected by the negative energy balance: our recent study showed that chronic food restriction in
KEY TAKEAWAY: ghrelin system is vulnerable to perturbations at multiple points along its biochemical pathway (e.g., synthesis, transport from periphery, degradation, etc.) especially during development when the neurons along this pathway are just forming.

Sources of perturbation to the ghrelin circuitry:

- Obesity / T2D
- Stress
- Anorexia nervosa

Obesity is just one of many conditions that causes alterations to ghrelin circuitry!
Stress

Experimental paradigm:

- Operationalize “stress-eating” in mice w/ Conditioned Place Preference (CPP) task
  - Will gravitate towards area it has associated w/ receiving a HFD reward
- In rats - peripheral injection of ghrelin “preferentially enhances fat intake over carbohydrate intake” (p. 2685)
- GHSR-deficient mice / GHSR antagonist-treated rats → consume less PB/Ensure but NOT regular chow

Research article

Ghrelin mediates stress-induced food-reward behavior in mice

Jen-Chieh Chuang,1 Mario Perello,1 Ichiro Sakata,1 Sherri Osborne-Lawrence,1 Joseph M. Savitt,2 Michael Lutter,1,3 and Jeffrey M. Zigman1,3,4
Stress

Chronic social defeat stress (CSDS) - model psychosocial stress in humans

Depression-like behavioral deficits and ↑↑ plasma ghrelin levels

Bind to GHSRs + induce hyperphagia (increased appetite), particularly HF foods

Research article

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Prolonged psychosocial stress

Depression

Hedonic eating behaviors

Hyperphagia

Intake of comfort foods & Increased body weight

Ghrelin

catecholaminergic neuron

GHSR

stomach

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Anorexia nervosa

Experimental paradigm:

- Use ABA mice (activity-based anorexia) - subject to caloric restriction + increased energy expenditure (running wheel activity) to model anorexia nervosa (AN)
- Immunohistochemical analysis of mRNA preproghrelin levels in:
  - Stomach
  - Hypothalamus
- Immunohistochemical analysis of levels of:
  - Total ghrelin
  - Acyl ghrelin (AG)
  - Desacyl ghrelin (DAG)
  - Ghrelin-reactive immunoglobins (IgG, IgM & IgA)
Anorexia nervosa

- Higher overall plasma levels of peripheral & central ghrelin for AN
- Delayed or absent postprandial decrease of ghrelin, higher # of Igs → (impaired modulation)
- Central resistance to ghrelin

**Figure 1.** Hypothesized alterations of ghrelin’s signaling in anorexia nervosa.

- ↑ growth hormone secretagogue receptor 1a; ↓ mechanisms contributing to insufficient stimulation of food intake irrespective of high ghrelin levels; = no alteration; ↑ increase/stimulation; ↓ decrease/inhibition, - - - > indirect effect; ACTH, adrenocorticotropic hormone; BBB, brain-blood barrier; BMI, body mass index; GH, growth hormone; IGF-1, insulin-like growth factor-1; T₃, triiodothyronine.
The overall takeaway...

- Ghrelin circuitry / pathway is similar to other hormonal circuits we have looked at so far re: complexity
- Susceptible to perturbations at multiple points along the pathway ESPECIALLY early in neural development
Neonatal Overnutrition & Ghrelin
How Does Ghrelin Affect the Body?

GHS-R1A Locations:
- Stomach and intestines (GI tract)
- CNS (primarily in the hypothalamus)

Suggests that the hormone integrates the central and peripheral systems
Neonatal Nutrition

Postnatal nutrition is incredibly important in proper organ development!

- In rodents, the postnatal period has been shown to be crucial in hypothalamus development
  - Influenced by intrinsic AND environmental factors
- Hormone levels can be influenced by nutrition
  - Nutrition manipulation can affect hypothalamic development
  - Early nutritional challenges can change the body’s sensitivity and tolerance to certain hormones
HOW DOES THIS CIRCUITRY DEVELOP?
The Effects of Overnutrition

- Studies show that overfed mice have significantly **decreased circulating ghrelin levels** compared to a control group.
- GHSR expression in the **arcuate nucleus** of the hypothalamus (ARH) is also **reduced**.
- Less ghrelin = less signaling?
  - Yes but there’s more to the story.
The Effects of Overnutrition

- Direct exposure of ghrelin to the arcuate nucleus in the hypothalamus showed similar results in control and obese populations
  - Neurons are still able to respond to ghrelin even when exposed to overnutrition
    - Where’s the problem???
    - Suggests neonatal overnutrition alters the ability of peripheral ghrelin to reach the neurons and activate them
  - Western blot analysis ⇒ injected with peripheral ghrelin ⇒ yields increased ghrelin levels in the ARH of normal mice
    - HOWEVER, obese/overfed mice do not show this effect

- Ghrelin transport likely involves alteration in ghrelin uptake by tanycytes
Tanycytes

- Transport ependymal (glial-like) cells in the brain
- Interacts with cerebrospinal fluid (CSF)
  - Transports signals from CSF to the central nervous system
  - Helps cross BBB
- Overnutrition may alter tanycytes’ function
Studies show that overfed mice display increased GHS-R1A levels in white adipose tissue (WAT) compared to a control group.

- Downstream pathways of ghrelin signaling are affected.
- Increased GHSR-1α ⇒ suggested that it can be part of adaptation of decreased hormone levels.
- Changes in ghrelin signaling pattern may be central for an adaptive response of adipocytes.
- Reducing GHSR expression reduces adiposity (being severely overweight) and improves insulin sensitivity by regulating fat metabolism in adipose tissues.
The Effects of Overnutrition

- Higher GHS-R1A
  $\Rightarrow$ phosphorylation of proteins
  $\Rightarrow$ higher PI3K, AKT
  $\Rightarrow$ higher GLUT4
  $\Rightarrow$ increase adipocyte glucose uptake
  - Can lead to fatty acid accumulation
GHS-R1A (growth hormone secretagogue receptor)

GHS-R1A can be activated even in the absence of ghrelin through its dimer partners (sometimes):

- D1R/D2R (dopamine receptors)
- MC3R (melanocortin-3 receptor)
- 5-HT2CR (serotonin 2C receptor)
IT IS IMPORTANT TO NOTE THAT THE FULL MECHANISM OF GHRELIN SECRETION AND REDUCTION HAS NOT BEEN FULLY REALIZED
- neonatal nutrition is important because it alters levels of ghrelin through adulthood

-we need to understand these complicated pathways ie. GHSR-1A in order to target specific pathways through experiments ie. where can we attach fluorescent markers to make sure our experiment is working
From the paper:

- Postnatal overnutrition $\Rightarrow$ central resistance to peripheral ghrelin during hypothalamic development
- Ghrelin signaling is involved in neonatal programming of metabolism
  - Changes can contribute to metabolic defects observed in postnatally overnourished mice
- But how did the authors come to these conclusions?
  - Through measuring hormone levels and gene expression levels
Original article

**Neonatal overnutrition causes early alterations in the central response to peripheral ghrelin**

Gustav Collden², Eglantine Balland²,³, Jyoti Parkash²,³, Emilie Caron²,³, Fanny Langlet²,³, Vincent Prevot², Sebastien G. Bouret¹,²,³.

Collden et al. study
Experimental Set-Up & Procedure
Experimental Set-Up & Procedure

Raise 2 litters of mice (“pups”):

- “Normal litter” (NL) vs. “small litter” (SL)
  - NL pups were fed a “standard” diet
  - SL pups were fed a diet higher in volume
- Only male pups in each litter examined in experiment.
- Litters included offspring from at least three different litters.
- Later (Day 120): High-fat/high-sucrose challenge administered to subgroups of each litter
  - Control mice fed standard diet for 8 weeks
  - Experimental group fed the HFHS diet for 8 weeks
    - HFHS: 60% fat + water containing 20% sucrose

Image Credit: University of Louisville
Physiological Measurements

- Weighing
- Measuring fat/lean mass
- Food intake
  - Sensors on containers
- Respiratory exchange ratio
  - O2 used vs. production of CO2
- Blood glucose levels
Measuring Ghrelin Levels

- Assayed total and acylated ghrelin levels.
  - Acylated ghrelin can activate GHSR (because acylation allows it to cross blood-brain barrier).
  - Gives view of deacylated ghrelin, which plays a role in cardiovascular system, cell proliferation.
Measuring Gene Expression

- mRNA extracted from arcuate nucleus of hypothalamus (ARH), the dorsal medial hypothalamic nucleus (DMH), and stomach.
- Used mRNA to generate complementary DNA (cDNA).
  - Using reverse transcriptase enzyme.
- Performed real-time quantitative polymerase chain reaction (qPCR) on cDNA samples.
Measuring Gene Expression (cont)

- Real-time qPCR assayed for multiple substances of interest:
  - GHSR (Growth hormone secretagogue receptor)
  - Ghrelin
  - GOAT (Ghrelin O-acyltransferase)
  - POMC (pro-opiomelanocortin)
  - AgRP (Agouti-related peptide)
  - NPY (Neuropeptide Y)
Measuring Effects of Ghrelin Injection

- Separated mice into two groups: Those injected with ghrelin in stomach, and those receiving control injections
  - Other group received intracerebroventricular ghrelin injections.
- Froze and sliced brains into thin sections, then treated with two rounds of antibodies.
  - First antibodies target Fos domain.
  - Second antibodies fluoresce first antibodies.
- Counted cFOS-immunopositive cells.
Assessing Ghrelin Uptake

- Fluorescence microscopy in recently sacrificed mice to measure ghrelin uptake by tanycytes.
  - Mice injected with fluorescent bioactive ghrelin
  - Two sets of antibodies used
- Used western blotting to determine ability of ghrelin to be transported to mediobasal hypothalamus (MBH) 45 min after ghrelin injection
  - Applied antibodies to quantify ghrelin levels in MBH
Results & Figures
Early Over Nutrition & Obesity

Small litter (SL) mice = over nutrition

- Have **higher body weights** pre & post weaning (adulthood) (A/B)
- **Eat more** (C)
- Slightly higher respiratory exchange rate (D)
- **More fat** mass as adults (E)
- Increased susceptibility to **weight gain** on HFHS diet (F)
- **Increase in fat mass** on HFHS diet (G)
- Higher fasting glycemia in HFHS diet (H)
Ghrelin Production

In small litter mice:

- Less ghrelin (A)
- Less Acyl ghrelin (B)
- Ghrelin and ghrelin activating enzyme mRNA changes in controls (C)
- Ghrelin levels don’t change in SL (D)
- Ghrelin & Ghrelin mRNA expression restricted to early postnatal life

Adults aren’t expressing ghrelin!

Less ghrelin, less E expenditure, higher weight, more eating!
Early Life Nutrition Importance

- NL pups on day 16 injected with ghrelin = significantly higher serum acyl ghrelin levels (A)

- SL pups, whether injected with saline or ghrelin, STILL show increased body weight compared to NL pups injected with ghrelin or serum = no change in body weight or composition from fig. 1 (B-E)

- SL mice neonatally injected with ghrelin = significantly lower fasting glycemia than saline treatment

Central response to peripheral ghrelin

Are brain regions showing hormonal resistance? YES

- SL mice ARH/ME Ghσr mRNA similar to NL mice (A)
- Neonatal overnutrition caused a reduction in Ghσr mRNA levels at P16 and P22 (A)
Central response to peripheral ghrelin

EARLY ghrelin matters for activating ARH neurons!

- Peripheral ghrelin = increase in cFos immunoreactive cells in the ARH of control (NL) mice at P14, P16, and P22
- cFos immunoreactivity significantly reduced in the ARH of SL pups
- no differences in cFos expression between SL and NL mice at P60 (Altered response to ghrelin ONLY postnatal)
Ghrelin and POMC

- Neonatal ghrelin = significant increase in Ghsr mRNA levels in the ARH/ME of both NL and SL mice (C)

- AT DAY 14:
  - Peripheral ghrelin to NL mice increased expression of Agrp & Npy mRNA (D/E)
  - No change in Agrp and Npy mRNA levels in ARH of SL mice (D/E)
  - Ghrelin injection = no change in Pomc mRNA expression (both NL & SL mice) (F)
Ghrelin and DMH

- Ghsr mRNA unchanged in DMH of SL neonates (A)
- Peripheral ghrelin administration = cFos induction in the DMH of NL or SL neonates? NO (B)

Neonatal: peripheral ghrelin does not activate neurons in the dorsomedial hypothalamic nucleus (DMH: has lots of ghrelin receptors).
Intracerebroventricular injection of ghrelin:

- NL & SL mice have similar cFos immunoreactivity in the ARH

Therefore:

- ARH neurons of SL mice still respond to ghrelin
- Ghrelin transport across BBB may be defective in SL (overnourished) mice

Can ARH neurons of SL mice respond to ghrelin? Inject ghrelin centrally to activate cFos immunoreactivity in the ARH.

Normal central response to ghrelin.
Ghrelin Transport in MBH

- Ghrelin exclusively present in ME tanycytes 5 min after injection (A)

- After 15 min tanycytes internalized ghrelin (B/C)

Are tanycytes important for ghrelin uptake?
Altered ghrelin transport in mediobasal hypothalamus of SL mice.
With same ghrelin treatment:
- Significant external ghrelin in MBH of NL mice (D)
- No ghrelin in MBH of SL mice (D)

Therefore:
- Neonatal overfeeding reduces ghrelin access to the MBH (ghrelin receptors)
Key Findings Summary

SL (overnourished) mice show:
● High body weight, fat, feeding, susceptibility to weight gain, & fasting glycemia
● Reduced ghrelin & ghrelin mRNA activity
● Consistent body tendencies, whether injected with saline or ghrelin
● Resistance to hormones in arcuate nucleus of the hypothalamus (ARH)/median eminence (ME).
● Exclusive postnatal changes in response to ghrelin (suggesting postnatal feeding is key influence)
● No activation of neurons in the dorsomedial hypothalamic nucleus (DMH) to neonatal peripheral ghrelin
● Normal central response to ghrelin (ARH neurons respond to ghrelin, defective transport across BBB)
● Altered ghrelin transport in mediobasal hypothalamus, reduced ghrelin access
● Altered ghrelin transport to the ARH
For those of us who are not microbiology majors.......
<table>
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<tr>
<th>Inactive Ghrelin</th>
<th>Active acylated Ghrelin</th>
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<tr>
<td>-majority of Ghrelin (90%)</td>
<td>-present after exercise</td>
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<td>-creates the “hunger” feeling</td>
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Not many studies on the differences between the two but a great direction for the field to take: comparing levels of the two types across obese and controls, how much active ghrelin can individuals with weight problems produce...etc.
Across the BBB...

-noted that neonatal overfeeding leads to difficulty transporting Ghrelin across BBB

-this is shown through those with lower levels of Ghrelin who continue exhibit higher levels of feeding (mice studies)

-NPY and AgRP circuit MUST be present for Ghrelin to have effect

Implications?

-we can target circuitry in the brain to treat obesity rather than peripheral ghrelin

-orexigenic effects happen in the brain rather than peripheral areas
It all starts at birth...

- Mice study shows that overfeeding leads to decreased Ghrelin levels.
- Lower ghrelin = higher body weight (weight does not mean hunger).
- There are less ghrelin receptors meaning less ability for Ghrelin to activate neurons.
- Overfeeding interferes with ghrelin transport through BBB.

What if I’m not a mouse?
- We can use this research to look towards neonatal nutrition in humans.
- Lower chances for obesity at a young age.
Ghrelin has other friends...

- leptin & ghrelin: high amounts of ghrelin and low amounts of leptin signal hunger, the tables are turned after eating

- studies when losing weight show that ghrelin is increased and leptin is decreased (of course! Leptin is produced in fat cells)

But I thought there was less Ghrelin in obese individuals?

--this suggests that ghrelin could be inversely related to calorie intake

- again neonatal formation of receptors plays a role in this because if there is
The Real World...

- Diet induced weight loss increases ghrelin levels which is why the weight may be hard to keep off.

- Prader-Willi syndrome is a genetic disease in which patients have obesity, extreme hunger, learning disability but high levels of ghrelin.

- Anorexia also has high levels of ghrelin.

- Are obese individuals truly “hungry” all the time?
References
References


