Aus dem Max-Planck-Institut für Psychiatrie

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Ghrelin and Cognition

Dissertation
zum Erwerb des Doktorgrades der Medizin
an der Medizinischen Fakultät der
Ludwig-Maximilians-Universität zu München

vorgelegt von

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aus

Würzburg

Jahr

2017

Mit Genehmigung der Medizinischen Fakultät
der Universität München

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04.05.2017

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1. Formal statements

1.1 Affidavit

Eidesstattliche Versicherung

Kunath, Nicolas

Name, Vorname

Ich erkläre hiermit an Eides statt, dass ich die vorliegende Dissertation mit dem Thema

„Ghrelin and Cognition“

selbständig verfasst, mich außer der angegebenen keiner weiteren Hilfsmittel bedient und alle Erkenntnisse, die aus dem Schrifttum ganz oder annähernd übernommen sind, als solche kenntlich gemacht und nach ihrer Herkunft unter Bezeichnung der Fundstelle einzeln nachgewiesen habe.

Ich erkläre des Weiteren, dass die hier vorgelegte Dissertation nicht in gleicher oder in ähnlicher Form bei einer anderen Stelle zur Erlangung eines akademischen Grades eingereicht wurde.

Ort, Datum

Unterschrift des Doktoranden

1.2 Abbreviations

AEBSF	4-(2-Aminoethyl)benzensulfonylfluorid
ANOVA	Analysis of Variance
AUC	Area under the curve
Aβ	Amyloid Beta
BET	Brain extraction tool
BOLD	Blood oxygen level dependent
BOMAT	Bochumer Matritzentest
CNS	Central Nervous System
COG	Center of gravity
CREB	cAMP responsive element-binding (protein)
DAB	3,3'-Diaminobenzidine
DCX	Doublecortin
DPX	Distrene, Plasticiser, Xylene
EGTA	Ethylene glycol-bis-N,N,N',N'-tetraacetic acid
fMRI	Functional magnetic resonance imaging
G-Protein	GTP binding protein
GH	Growth hormone
GHS-R	Growth hormone secretagogue receptor
GI	Glycemic index
GLM	General linear model
IACUC	Institutional Animal Care and Use Committee
IBA	Ibandronate
ICV	Intracerebroventricular
JNK	Janus kinase
MCFLIRT	Motion correction FMRIB's Linear Image Registration Tool
MWT-B	Mehrfachwahl-Wortschatz-Intelligenztest Version B
NIH	National institute of health
NMDA	N-Methyl-D-aspartate
(p-)IRS	(phosphorylated) insulin receptor substrate
PET	Positron emission tomography
SAP	Stress-activated phospho(-kinase)
PSD	Post-synaptic density
PVT	Psychomotor vigilance task
QMR	Quantitative magnetig resonance (imaging)
RDS	Reverse digit span
RIPA	Radioimmunoprecipitation assay
ROI	Region of interest
SD	Standard deviation
SEM	Standard error of the mean
TE	Echo time
TgAPPSwDI	Transgenic amyloid precursor protein Swedish-Dutch-Iowa
TNF	Tumor necrosis factor
TR	Relaxation time
Tris	Tris(hydroxymethyl)aminomethane
ZVT	Zahlenverbindungstest

1.3 List of publications

1.3.1 Ghrelin agonist does not foster insulin resistance but improves cognition in an Alzheimer's disease mouse model

Published in:	Scientific Reports
Date of publication:	19th of June 2015
Journal Impact Factor:	5.578 (Thomson Reuters 2014)

1.3.2 Ghrelin alters encoding-related brain activity without enhancing memory formation in humans

Published in:	NeuroImage
Date of acceptance:	7th of July 2016
Journal Impact Factor:	5,463 (Thomson Reuters 2016)

1.4 Confirmations of Co-Authors

All co-authors signed the confirmation pursuant to §4a Paras. 3 and 5 Doctoral Degree Regulations for Dr. med., Dr. med. dent. and Dr. rer. biol. hum. and pursuant to §7 Para. 4 Doctoral Degree Regulations for Dr. rer nat. at the Medical Faculty. The signatures are listed in the section “Appendix”.

1.4.1 Co-authors to publication 1

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2. Summary

2.1 Zusammenfassung (deutsch)

2.1.1 Hintergrund

Ghrelin ist ein Peptid mit einer Länge von 28 Aminosäuren und besitzt in seiner aktiven Form eine charakteristische Acyl-Seitenkette, die durch das Enzym „Ghrelin-O-Acyltransferase“ am dritten Serin-Rest angefügt wird. Es wurde 1999 in einer japanischen Arbeitsgruppe als Ligand des “Growth Hormone Secretagogue”-Rezeptors entdeckt. Der Name „Ghrelin“ ist sowohl Akronym (**gr**owth **h**ormone **rel**ease **in**ducing) als auch Anspielung auf die proto-indo-europäische Wortwurzel “ghre” für “wachsen”.

Außer seiner Rolle in der Freisetzung von Wachstumshormon ist Ghrelin das einzige bislang bekannte periphere orexigene Peptidhormon und scheint fester Bestandteil zirkadianer Rhythmen der Nahrungsaufnahme zu sein. Der Rezeptor findet sich an vielen Orten in Säugetierorganismen und besitzt zudem die Eigenschaft, Dimere mit Rezeptoren anderer Transmittersysteme wie z.B. jenes des Serotonins oder des Dopamins zu bilden. Dies zeigt die breite Relevanz Ghrelins oder vielmehr des „Ghrelin-Systems“, das mannigfach auf unseren Energiehaushalt aus kognitiver genauso wie metabolischer Stoßrichtung einzuwirken scheint – sofern diese Trennung überhaupt aus dem Blickwinkel der Ghrelin-Forschung vertretbar ist: Sehr bald legten Forschungergebnisse nahe, dass die Bedeutung dieses Peptids weit über die reine Regulation des Energiehaushalts hinausgeht.

Es scheint vielmehr auch komplexe kognitive Prozesse zu beeinflussen. An Nagetiermodellen konnte konsistent gezeigt werden, dass Ghrelin die Gedächtnisbildung unterstützt, allen voran in den Bereichen Objekterkennung, räumliches Lernen und aversives Gedächtnis. Es wird daher als eine mögliche Brücke

zwischen Energiehaushalt und Kognition diskutiert und erfüllt daher, bildlich ausgedrückt, in mancher Hinsicht die Rolle eines „Eichhörnchenhormons“: Diese Tiere erinnern sich in Zeiten des Fastens mehr oder minder präzise an die Orte, an denen Nahrung zuvor versteckt wurde. In der Tat zeigten jüngste Projekte an Wildtieren dieser Gattung eine mögliche Relevanz Ghrelins im Stoffwechsel dieser Tiere auf. Im angelsächsischen Sprachgebrauch ist es vor allem das Bild des „belly-brain-links“, das den gleichen Sachverhalt verdeutlichen soll. Nun verbietet sich allerdings die direkte Extrapolation dieser Daten aus Nagetiermodellen und Hörnchen auf den Menschen allein schon wegen der ungleich komplexeren Zusammenhänge menschlicher Kognition. Dennoch stellt sich die Frage, ob Ghrelin möglicherweise einen ebenso positiven Einfluss auf kognitive Prozesse des Menschen hat – ein Sachverhalt mit möglicher Relevanz im Verständnis sowohl der Entwicklung von Übergewicht als auch Anorexie. Zwar bestand in der Arbeitsgruppe Prof. Axel Steiger (Principal Investigator der Studie 2) bereits vor Studie 2 eine mehrjährige Erfahrung mit der Gabe von Ghrelin bei menschlichen Probanden unter Fragestellungen der Schlafforschung im Allgemeinen und Schlafendokrinologie im Speziellen. Eine Untersuchung der Auswirkungen Ghrelins auf die menschliche Gedächtnisleistung war jedoch unseres Wissens nach zuvor noch nie erfolgt. Damit stellt Studie 2 die erste Studie ihrer Art dar, die die menschliche kognitive Leistungsfähigkeit unter und nach Gabe von Acyl-Ghrelin untersucht.

Aufgrund seiner neuroprotektiven Wirkung bei neurodegenerativen Erkrankungen könnte Ghrelin in Zukunft eine Rolle als Therapeutikum bei Alzheimer-Demenz und Parkinson spielen. Arbeiten der Forschungsgruppe von Dr. Inga Kadish (Principal Investigator der Studie 1) zeigten gar eine niedrigere Belastung mit A-Beta-Plaques im Alzheimer-Mausmodell unter chronischer Behandlung mit einem Ghrelinagonisten bei gleichzeitig besserer kognitiver Leistung der Nagetiere im

Vergleich zur Placebo-Kontrollgruppe. Nicht abschließend geklärt ist nach wie vor, über welchen Mechanismus Ghrelin diese protektive Wirkung entfaltet, einige mögliche Hypothesen z.B. über eine Beeinflussung der Signalwege des Insulin-Systems, werden in Studie 1 verfolgt und diskutiert.

Gleichzeitig hat Ghrelin insulinostatische Eigenschaften und ist daher möglicherweise ein relevanter Faktor in der Pathogenese von Diabetes mellitus. Sehr oft postuliert wurde ein diabetogener Effekt Ghrelins durch die Begünstigung hoher Serum-Glukosewerte mittels Unterdrückung der Sekretion von Insulin. An dieser Stelle ergibt sich ein Widerspruch zu der mittlerweile nachgewiesenen und weithin akzeptierten pathoätiologischen Verbindung von Diabetes und Neurodegeneration: Ist Ghrelin nun wie bereits erwähnt aufgrund seiner neuroprotektiven Wirkung ein potentielles Therapeutikum für neurodegenerative Erkrankungen? Oder begünstigt die chronische Ghrelingabe schlussendlich die Entwicklung einer diabetogenen Stoffwechsellage und führt im Gegenteil nicht nur zu einem erheblich erhöhten Diabetesrisiko sondern auch langfristig zu einer Schädigung des Nervensystems? An dieser Stelle setzt Studie 1 an, indem sie eine chronische Gabe eines Ghrelinagonisten nicht nur im Hinblick auf kognitive sondern auch metabolische Effekte untersucht.

2.1.2 Studie 1

Aus entwicklungsgeschichtlicher Perspektive dauerten bzw. dauern Fastenperioden selten nur einige Stunden. Krankheiten wie Alzheimer-Demenz und Diabetes sind chronische Erkrankungen, die sich über Jahre und Jahrzehnte entwickeln. Nichtsdestotrotz schlossen frühere Ghrelin-Studien selten Zeiträume von mehr als zwei Wochen in ihre Beobachtungen ein. Daher war es Anliegen von Studie 1, die langfristigen Effekte eines Ghrelinagonisten auf gleichermaßen Kognition und

Stoffwechsel zu beleuchten (s.o.). Dazu wurde ein APPSwDI-Mausmodell mit einem Ghrelinagonisten (LY444771) mehr als vier Monate lang behandelt. Gleichzeitig wurde ein Teil der Versuchstiere mit eigens entwickeltem Futter mit einem hohen glykämischen Index gefüttert. Während die Studie die positive Wirkung des Agonisten auf kognitive Endpunkte bestätigen konnte, zeigte sich überraschender Weise keinerlei Einfluss des Agonisten auf die Glukosetoleranz. Kaum ein Unterschied bestand zwischen den unterschiedlichen Futtersorten, auch die Kombination aus Ghrelin und hohem glykämischem Index führte zu keinerlei Verschlechterung der Glukosetoleranz.

Zudem kam die Studie überraschender Weise zu dem Schluss, dass eine chronische Gabe des Ghrelinagonisten nicht zu einer chronisch erhöhten Nahrungsaufnahme und damit zu chronischer Gewichtszunahme bei der Versuchstieren führte, ohne jedoch eine überzeugende Erklärung für diese Beobachtung vorweisen zu können.

2.1.3 Studie 2

Die zweite Studie war die erste, die systematisch den Einfluss von Ghrelin auf menschliche Kognition untersuchte. Aufgrund der vielversprechenden Ergebnisse aus Nagetiermodellen lautete die Arbeitshypothese dieser Studie, dass die Einmalgabe von Acyl-Ghrelin die Gedächtnisleistung gesunder Menschen möglicherweise verbessert. Die Ergebnisse konnten dies jedoch nicht bestätigen. 21 gesunde männliche Probanden mussten in einem Paradigma zum räumlichen Lernen geschriebene Begriffe in einer dreidimensionalen virtuellen Umgebung mit ihrer Lokalisation erlernen.

Ghrelin veränderte zwar die Hirnaktivität (BOLD fMRI) in Hirnregionen, die bekannter Weise eine Rolle in der Verarbeitung von wortbezogenen Assoziationen spielen und beeinflusste auch die Konnektivität in neuronalen Netzwerken z.B. zwischen dem beidseitigen Nucleus caudatus, dem rechten orbitofrontalen Kortex und der

beidseitigen Inselrinde. Überraschenderweise fand sich jedoch kein Effekt des Hormons in den getesteten kognitiven Disziplinen: Arbeitsgedächtnis, Bochumer Matrizentest, Kreativität, Zahlenverbindungstest, Reaktionsgeschwindigkeit und Aufmerksamkeit. Eine weitere Teilhypothese lautete, dass Ghrelin möglicherweise dazu führt, dass sich Probanden nahrungsbezogene Begriffe besser einprägen und dies umso mehr, je attraktiver oder kalorienreicher die Nahrung ist. Ein solcher Zusammenhang konnte ebenfalls nicht nachgewiesen werden.

Letztlich scheint der Einfluss von Ghrelin auf menschliche Kognition komplexer zu sein als erwartet, doch sind auch die Einschränkungen der Studie zu erwägen: Statt geschriebener Begriffe sollten künftige Studien Bilder von Gegenständen und Nahrungsmitteln verwenden, die erfahrungsgemäß in neurokognitiven Experimenten eine höhere Salienz besitzen. Zudem verbessert Ghrelin sehr wahrscheinlich nach Einmalgabe auch nicht die Denkleistung gesunder Versuchspersonen. Doch stellt die Untersuchung von längeren Fastenperioden oder chronischen Ghrelingaben beim Menschen einen interessanten Ansatz dar, die Auswirkungen des Hormons auf kognitive Prozesse am Menschen weiter zu beleuchten.

2.2 Summary (English)

2.2.1 Background

Ghrelin is a 28-aminoacid peptide with a distinct acyl-chain at its third serine residue, added by the enzyme ghrelin-O-acyl-transferase. It was discovered in 1999 as a ligand of the growth hormone secretagogue receptor by a Japanese research group. Its name is both an acronym (**gr**owth **h**ormone **rel**ease **in**ducing) and an allusion to the Proto-Indo-European word fragment "ghre", meaning "to grow".

Besides its role in growth hormone release, it has been identified as the only peripheral orexigenic hormone and appears to be an integral part of circadian rhythms of food intake. The receptor is widely spread in mammal organisms and further has the capacity of forming heterodimers with other transmitter systems such as the serotonin or dopamine system. This shows the broad relevance of ghrelin or rather the "ghrelin system" interacting in a myriad of ways with aspects of energy homeostasis both from a metabolic and a cognitive perspective – if this separation can be validly upheld in ghrelin research.

Soon it became clear that its importance and impact goes far beyond the regulation of energy homeostasis. It rather seems to influence and shape cognitive processes, consistently improving memory formation in different rodent models, mainly in the fields of object recognition, spatial learning and aversive memory. It is being discussed as a link between metabolism and cognition and therefore has in many ways, metaphorically speaking, the role of a "belly-brain link" or "squirrel hormone": In times of fasting, these animals remember more or less accurately where their food is hidden. Indeed recent projects show a relevance of ghrelin in metabolic aspects in sciurid hibernators. However, a direct extrapolation of data from rodent models or sciurid

hibernators to humans cannot be validly done due to the much more complex context of human cognition. Nonetheless, it is an interesting and very relevant question whether ghrelin has a positive impact on human cognitive performance as well since it may help explain the cognitive aspects of feeding in humans with implications in understanding both obesity and anorexia.

There has been a longstanding experience in experiments involving the administration of ghrelin to human volunteers even before study 2 in Prof. Axel Steiger's (principal investigator study 2) research group in the context of sleep research in general and more specifically sleep endocrinology. However, to our knowledge, no study had systematically looked at the impacts of ghrelin on human cognitive performance. Thus, study 2 is the first of its kind looking into the cognitive aspects of ghrelin action in humans during and after the administration of acyl ghrelin.

Due to its beneficial impact in neurodegenerative diseases ghrelin might play a role as a therapeutic agent in conditions such as Alzheimer's and Parkinson's disease. Projects of Dr. Inga Kadish's (principal investigator study 1) research group even showed a lower A-beta-plaque load and better cognitive performance after chronic treatment with a ghrelin agonist in an Alzheimer's disease mouse model compared to controls treated with placebo. A conclusive mechanism for this protective effect has not been identified yet, some possible hypotheses e.g. via influencing signaling pathways of the insulin system are presented and discussed in study 1.

At the same time, ghrelin has insulinostatic properties, making it a relevant hormonal player in diabetes, possibly helping to explain the link between both conditions. It has often been postulated that ghrelin might be a diabetogenic factor as it raises serum glucose levels via a reduction of insulin release. This idea stands in harsh contrast to the well-proven and widely accepted connection of diabetes and

neurodegeneration: Is ghrelin as mentioned before a possible therapeutic agent in neurodegenerative diseases due to its neuroprotective effect shown in numerous studies? Or does ghrelin after all favor a diabetogenic metabolic situation leading to a higher risk of developing diabetes and thus to a long-term threat to the integrity of the nervous system? This is the idea of study 1 looking into the chronic administration of a ghrelin agonist not only with respect to cognitive but also to metabolic effects.

2.2.2 Study 1

Seen from an evolutionary perspective, periods of fasting were and are rarely short-term events of a few hours and diseases such as Alzheimer's and diabetes are intrinsically chronic diseases with a pathoetiological onset of potentially years and decades. Nonetheless, early studies looking into ghrelin's effects in these conditions hardly ever covered periods of more than a couple of weeks. Thus, the first study aimed at creating a paradigm looking into the long-term effects of a ghrelin agonist on both cognitive and metabolic endpoints by treating an APPSwDI mouse model with a ghrelin agonist (LY444771) for more than four months.

At the same time, some animals were fed with a specifically developed high glycemic index diet. While ghrelin's positive influence on cognition in this mouse model could be confirmed in this study, it surprisingly showed no negative impact of the agonist on glucose tolerance when given in a long-term regimen. There was hardly any difference between the different diets, even the combination of ghrelin and a high glycemic index diet did not lead to a significant deterioration of glucose tolerance. Surprisingly, one of the study's conclusions was that chronic treatment with a ghrelin agonist did not lead to a chronically elevated food intake and consequently to a chronic weight gain, however, without finding a convincing reason for this observation.

2.2.3 Study 2

The second study was the first one to systematically look at the hormone's effects on human cognition. With the promising results from rodent models in mind, the study's hypothesis was that a single administration of acyl ghrelin could improve memory formation in healthy volunteers. Results however did not show any improvement of memory in a spatial learning paradigm in which 21 healthy male volunteers had to memorize written words with their location in a three-dimensional virtual environment. Ghrelin altered brain activity as measured by BOLD fMRI in brain areas known to be involved in verbal association processing and also influenced connectivity between several brain regions such as the bilateral caudate nucleus and the right orbitofrontal cortex and the bilateral insula. Surprisingly, it did not affect any of the cognitive disciplines tested in this study: working memory, fluid reasoning, creativity, mental speed, reaction time and attention.

Another hypothesis postulated a differential effect of ghrelin on the memorization of food and nonfood items with a better effect for food items, also dependent on their attractiveness and caloric value. However, we did not see any significant difference between food and nonfood items dependent on ghrelin administration, nor was there a difference between food items regarding their caloric value. After all, ghrelin's impacts on human cognition appear to be more complex than anticipated. However, we do see the limitations of our study: Future projects should use pictures instead of written words as they usually have a higher salience in neurocognitive experiments.

Looking at the results of this study, a single administration of ghrelin most likely does not act as a cognitive enhancer in healthy subjects. However, it certainly is a promising approach for future studies to look at prolonged periods of fasting, or even a

chronic administration of ghrelin in human volunteers in order to further characterize the hormone's properties as a neuropeptide in humans.

3. Introduction

3.1 Food for thought: Cognitive aspects of feeding

Regarding the pivotal role of food intake in the quest to survive and to progenerate, it appears evident why virtually all aspects of feeding behaviour are intrinsically finely regulated cognitive processes. The act of looking for food, the choice of what to eat, when and where as well as the questions of how to prepare for periods of absence of food, of how to cooperate for the common goal of feeding or of how to defend one's food once it is obtained are probably not only aspects of cognition but possibly, at least in part, at the origin of what we define today as the abstract concept of cognition. Or plainly, as we put it in a previously published book chapter[1] on ghrelin's role in memory related processes: As the act of eating in evolution has only recently become as easy as to open a fridge filled with delicious treats, there is a fundamental need for all living organisms to establish a close link between energy needs and thinking, between craving for food and behaviour, between belly and brain.

3.2 Thought for food: the complexity of energy homeostasis

However, when it comes to defining the different elements of this link that is rather a delicate network of neuroendocrine processes, the role of each piece in the mosaique is usually complex and rarely unequivocally clear, thinking of the many impacts on cognition of factors such as leptin, insulin, glucagone and cortisol just to name a few. In the quest to define the role of each element, researchers are facing the difficulty to standardize their studies for all other factors, although they may still be unknown to a certain extent. At the same time, to make sense of the results, the puzzle as a whole has

to be taken into consideration. Even more so in the case of ghrelin whose interaction with and embedding in the signalling systems of other peptides involved in the regulation of energy homeostasis (see below) is only starting to emerge.

Discovery	1999 (Kojima et al.[2])
Characteristics	28 aminoacids, acylation (n-octanoylation) at Serin3-residue characteristic for active form
Characterization as an orexigen	2000 (Tschöp et al.[3])
Characterization as a neuropeptide relevant in behaviour/cognition	From 2002 onwards (most notably Carlini et al., Diano et al.[4–7])
Relevance in sleep	From 2003 onwards (Weikel, Rosenhagen, Steiger et al.[8])
Main place of production in mammals	Oxyntic cells of the stomach; receptor apparently ubiquitous in mammal organism
Receptor	Growth-hormone secretagogue receptor (GHS-R)
Agonists for use in humans (clinically approved/in phase III)	Pralmorelin (approved, Kaken Pharma, Sella Pharma; GH deficiency diagnostic), Macimorelin (phase III, Aeterna Zentaris; GH deficiency diagnostic), Anamorelin (phase III, Helsinn, cancer cachexia/anorexia, for a comprehensive list see review “Ghrelin” by Müller et al.[9])

Table A: Ghrelin at a glance

3.3 The story of ghrelin: a hunger hormone with a taste for memory enhancement?

Shortly after its discovery as a growth hormone secretagogue produced predominantly in the stomach[2], first studies in rodents attributed an orexigenic role to the peptide, increasing food intake and weight gain when given regularly over a period of a few days[3]. Soon after, rodent experiments could show an effect on behaviour, mainly on aversive memory as demonstrated by Carlini et al.[4] This group administered ghrelin ICV in a rat model, the main behavioural read-out was a step-down inhibitory avoidance task. Carlini and colleagues could also demonstrate a positive influence of ghrelin on object recognition in a mouse model[10]. Ghrelin's enhancing effects on aversive memory and object recognition were confirmed by other groups: Goshadrou et al. observed that ghrelin can prevent the negative impacts of an NMDA-receptor antagonist on cognition in a passive avoidance task[11]. Atcha et al. showed better performance in object recognition for a ghrelin agonist[12].

However, looking at ghrelin's behavioural effects, some questions and inconsistencies remain: Carlini's study on object recognition used a food-restricted mouse model in which negative impacts of food restriction on memory performance were counteracted by ghrelin. Further, one landmark study by Diano et al. observed impairments in spatial memory but not in aversive memory in ghrelin receptor knock-out mice[7]. While different ways of ghrelin administration – oral[12], subcutaneous[7], ICV[4] – all seem to be effective, only memory acquisition and not retrieval appears to be positively influenced by ghrelin at least in a rodent model[6]. On top of that, one study observed better memory performance in GHS-R1a knock-out mice[13], others reported impaired memory performance in neonatal chicks[5] after central ghrelin and in correlation with endogenous ghrelin in humans[14]. Many of these points may be

explained with regard to the caveats of each individual study design and model organism used. However, they clearly show that ghrelin's unquestionable influence on cognition in general might reach beyond the complexity of a mere cognitive enhancer and that all extrapolation between species has to be done with utmost care. However, with further studies also reporting a positive effect on spatial memory and even spine synapse density and long-term potentiation[7,15], a possible role of ghrelin as a neurocognitive enhancer was increasingly discussed, further supported by the henceforth emerging role of ghrelin in neurodegenerative diseases[16–18] such as Alzheimer's and Parkinson's disease.

Looking beneath these behavioural results, the transmitter systems and biochemical signaling pathways involved in the mediation of ghrelin's actions form a rather complex picture. First, ghrelin has an impact on different transmitter systems such as serotonin[19], nitric oxide[15], glutamate[20] and dopamine[21]. Second, ghrelin appears to act via a G-protein coupled intracellular signaling pathway[22], leading to changes in intracellular calcium availability via the second messenger insositol trisphosphate[23], which is a rather common biochemical cascade. Further, CREB seems to be influenced by ghrelin signaling[24]. Currently, these pieces of information (discussed in detail in a previously published book chapter[1]) form a piecemeal mosaic that still needs more studies in order for us to deduce a real mechanistic relevance and understanding from it. Key to understanding the extremely widespread actions of the ghrelin system may be the capacity of the GHS-R to form heterodimers with other receptor systems[25,26].

Two of the main neurophysiological correlates of ghrelin's impact on memory formation are increased hippocampal long term potentiation and spine synapse density, both identified by Diano and colleagues[7]. More recent studies further consolidate the

notion of ghrelin as a neuropeptide relevant in hippocampal memory formation by showing that ghrelin's orexigenic actions are to a certain degree mediated via neural pathways involving hippocampal subfields[27,28].

As mentioned before, ghrelin influences a number of different neuroendocrine systems. It also appears to play a role in the regulation of the hypothalamic-pituitary-adrenal axis[29], thus becoming relevant in sleep and mental health[8,30–36]. The two studies of this dissertation were not designed to address these issues and rather focused on endpoints relating to memory, cognition as well as energy homeostasis.

3.4 Does ghrelin act as a cognitive enhancer in young, healthy, male humans?

A peptide enhancing memory in feeding-related processes in a state of hunger could be seen as an evolutionary advantage. In our book chapter, we used a squirrel that has to remember in winter where its acorns are hidden as an illustrating example[1]. Although current studies indeed confirm ghrelin's relevance in the metabolism of sciurid hibernators caught in the wilderness of Colorado[37,38], its relevance for memory processes in this species has yet to be assessed. Nontheless, we pursued the to a certain extent counterintuitive idea of an orexigen improving cognitive performance and included the hormone in the multicentre research project "Comparing Apples with Oranges: A Differential View on Neuroenhancement" supported by Volkswagen Stiftung[39] looking into the cognitive effects of a number of substances. Furthermore, few studies had and have addressed the effects of ghrelin on human memory performance so far. Although a number of clinically tested ghrelin agonists is available[9] (see table A), we decided to design a study employing the natural, active (acylated) form of the hormone to stay as close to natural conditions as possible. The problems arising from this

decision such as the time-sensitive administration[40] and the handling of the fragile peptide are pointed out in the methods part of the second publication.

Topic	Publication/Review article
Overview: Ghrelin	Müller et al. 2015
Ghrelin & Memory	Kunath/Dresler 2014
Ghrelin & Insulin	Chabot et al. 2014, Tong et al. 2010, Dezaki 2013
Ghrelin & Sleep	Steiger et al. 2011
Ghrelin & Mental Health	Wittekind/Kluge 2015
Ghrelin & Neurodegeneration	Gahete et al. 2011, Bayliss et al. 2013, Shi et al. 2016
Ghrelin as a belly-brain link	Hsu et al. 2016

Figure A: (left) Golden mantled ground squirrel (Spermophilus lateralis, photo: Eborutta 2003/Creative Commons license) – metabolism of this sciurid hibernator indeed appears to be affected by ghrelin; (right) key topics in ghrelin research and recommended reading

It is important to note that this study dubbed "GHREEN" (**ghre**lin and cognitive **en**hancement) did not seek to define the effects of hunger on cognition as this would be far beyond the scope of a single study. It rather aimed at characterizing the acute effects of acyl ghrelin on certain aspects of human cognition (see "methods" study 2) directly in the aftermath of administration.

3.5 The methodological challenge of designing a suitable paradigm for the question

As measuring the amount of acyl ghrelin actually reaching the CNS would hardly be ethically justified in a sample of healthy volunteers, we decided to monitor peripheral bioavailability instead. We were aware of consequently leaving the question of

interindividual differences in ghrelin's central bioavailability to future studies and to accept the uncertainty of differences in this respect within our sample of volunteers – despite the great efforts to standardize as much as possible for environmental and biometric variability. A number of studies have addressed the questions of how ghrelin crosses the blood-brain barrier[41], what factors influence the existing transport mechanisms[42] and what central networks mediate the subsequent response to ghrelin signalling[27]. In future studies, it will be crucial to define the exact relevance of each of these pathways – active bidirectional transport, vagal afferences, passive diffusion e.g. via circumventricular organs – not only with respect to ghrelin's orexigenic actions but also to other aspects of cognition as far as they can be separated from each other. Further adding to the complexity of characterizing ghrelin signalling, recent studies show that ghrelin release and action appear to be regulated in a circadian manner and influenced by the current metabolic state of the individual as well as food anticipation[28,43–45].

In designing the in-fMRI memory task, we tried to create a paradigm for visuo-spatial memory in humans, capable of making a difference between food- and non-food items realistically embedded in a virtual surrounding imitating a walk in an everyday environment. While at the beginning, a considerable effort was made to use a real walk in a local park as a setting for the task, we later decided to abandon this idea for the sake of better standardization possibilities in a virtual surrounding and created a custom-built virtual, three-dimensional memory task based on the freeware virtual gaming software "Sauerbraten" (see "Methods" publication 2).

However, with the recall task after one day showing merely screenshots of the virtual environment and with our volunteers often reporting a memorizing strategy employing certain landmarks such as houses, rooms or streets associated with the items

to be recalled, our task rather turned out to be a cued word-location association task. Furthermore, it needs to be pointed out that, for a better graphical embedding of items into the virtual task, we used written words instead of pictures, which rises the very valid question of how salient the items we used actually were.

Nonetheless, within the limits described above, we are certain that our conclusion that ghrelin does not globally enhance cognitive performance in young, male humans is valid, especially with Bayesian analyses being in favour of the null model (see publication 2). First of all, our paradigm tested a large number of cognitive disciplines. Secondly, differences between treatment groups were, in most cases, virtually non-existent and did not even come close to a statistical trend or significance. Thirdly, volunteers showed an extraordinarily high adherence to the study design, with an overall high motivation to perform well in the cognitive tasks and a low drop-out rate once the study was entered.

However, with another group's work pointing in this direction[46] and with our study's limitations in mind, we do think that there is a possibility of finding a difference for memory performance in food- and non-food items once a study design involving e.g. more salient stimuli is employed.

	Study 1	Study 2
Drug used	Ghrelin agonist LY444711	Natural active peptide
Timeframe	Long-term (months)	Acute (intra-day)
Organism	Mouse model (C57/BL6 APPSwDI)	Young, healthy, male humans (20-30 years of age)
Main question	Long-term effect of a ghrelin agonist on Alzheimer's disease pathology and glucose homeostasis under the influence of a high-glycemic-index diet	Effects of acute administration of acyl ghrelin on human different disciplines of human cognition
Main paradigms	Water maze test, oral glucose tolerance test, immunohistochemical stainings of brain slices	Custom-built fMRI-monitored virtual cued location-word association task; cognitive test battery (see methods study 2)
Ghrelin monitoring	Once at the end of the study	Constantly (hourly to every 10-15min)
Ghrelin assay used	2-site sandwich assay[47]	Radioimmunoassay
N=	36	21
Institute	Department of Cell, Developmental and Integrative Biology, University of Alabama at Birmingham, USA	Max-Planck-Institute of Psychiatry, Munich, Germany
Principal Investigator	Dr. Inga Kadish	Prof. Dr. Axel Steiger/Dr. Martin Dresler
Financial support	NIH grants R01AG043972, P30DK056336 and P30NS47466; LY444711 provided at no cost by Eli Lilly, Indianapolis, USA; AMIOCA starch provided at no cost by Ingredion Inc., Bridgewater, NJ, USA;	Volkswagen-Stiftung, "Comparing Apples with Oranges: A Differential View on Neuroen-hancement", 2011

Table B: The two publications at a glance

3.6 Neurodegeneration and the role of insulin, glucose – and ghrelin?

Ever since the Rotterdam study provided convincing evidence of a direct association between Alzheimer's disease and diabetes[48] (a link between cognitive decline and diabetes had been suggested long before), science tries to find a mechanistic explanation for this correlation. Early studies had long contradicted the assumption of the brain being an organ insensitive to insulin signalling[49,50]. While the hormone's actions in the brain go well beyond mere regulatory effects on metabolism[51], it is now becoming clear that central insulin signalling also has an impact on peripheral glucose homeostasis[52]. Further, a deficiency in insulin signalling appears to be an important factor in the neurodegenerative cascade leading to the clinical appearance of Alzheimer's disease[53]. There are two main reasons to believe that ghrelin also plays a role in the interplay of glucose homeostasis and neurodegeneration. First, ghrelin has insulinostatic properties when administered acutely[54], probably in order to keep glucose levels high in a situation of energy deficiency. Second, there is a strong body of evidence of ghrelin being neuroprotective in several entities of neurodegenerative diseases[16,55]. The latter led to a study performed in Inga Kadish's lab showing that a ghrelin agonist given chronically is capable of reducing the Aβ-plaque load in an Alzheimer's disease mouse model[17].

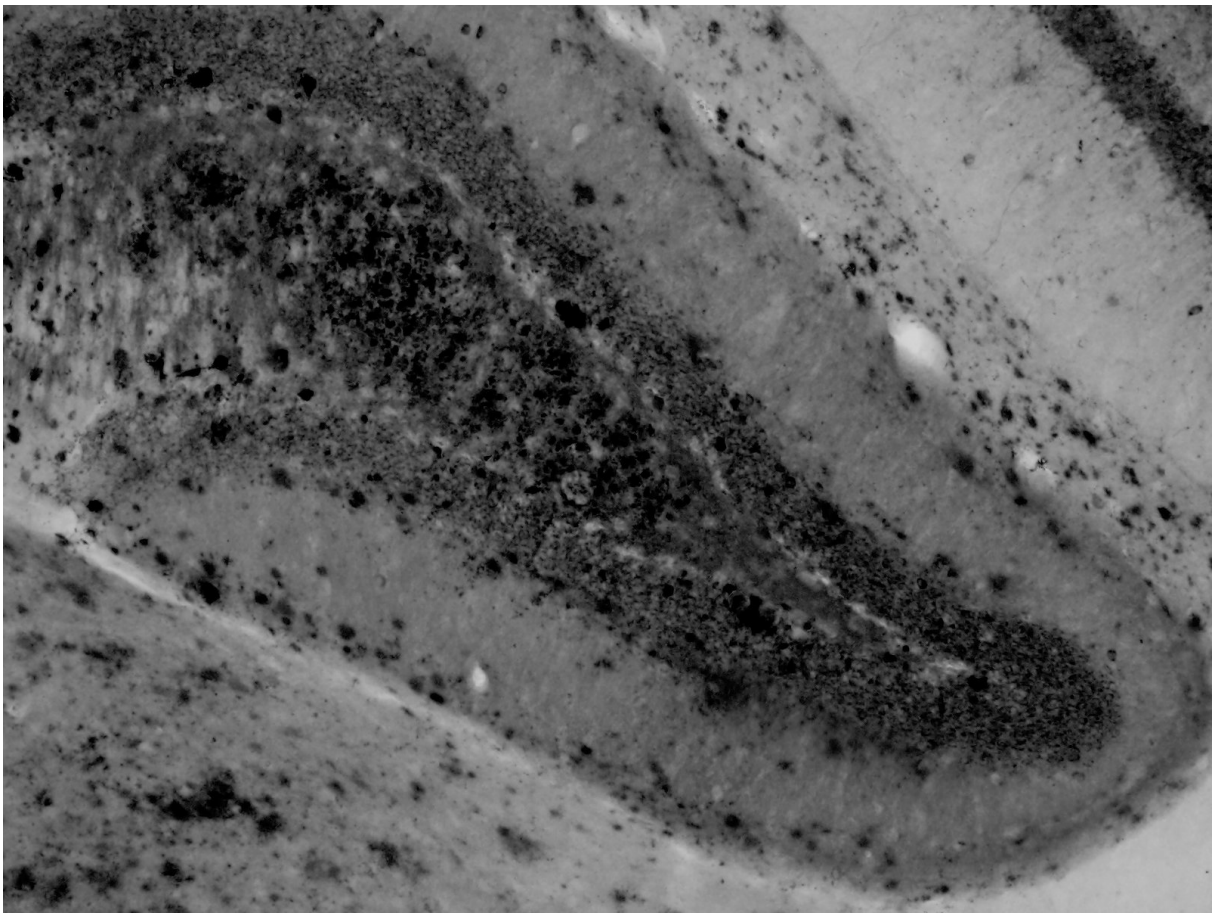

Figure A: Example of a W02-staining for Aβ-plaques in a heavily affected animal (C57/BL6 APPSwDI mouse) in study 1 (see methods study 1 and immunohistochemistry results).

3.7 Chronic disease, chronic administration: The idea behind publication 1

With this study in mind, we aimed at designing a study looking into the effects of a high glycemic index diet on Alzheimer's disease pathology in the same mouse model – and the impact ghrelin has in this setting. If ghrelin is indeed insulinostatic, there is reason to believe that it actually even deteriorates the possibly negative effects of a high glycemic index diet on Alzheimer's disease pathology[56]. This, however, would contradict the findings of the many studies showing the positive properties of ghrelin in

neurodegenerative diseases (see hypotheses described in publication 1). At the same time, neither the effects of ghrelin agonist administration on insulin secretion and glucose tolerance nor the resulting impact on Alzheimer's disease pathology had previously been addressed in a long-term treatment model. This is surprising as Alzheimer's as well as other neurodegenerative diseases are intrinsically chronic diseases and thus, any therapeutic approach is necessarily a long-term approach.

	High glycemic index diet	**Control diet**
Protein (% kcal from)	20.8	18.8
Carbohydrate (% kcal from)	60.2	63.9
Fat (% kcal from)	19.0	17.2
Caloric density (kcal/g)	3.4	3.8
Main carbohydrate ingredients	AMIOCA waxy maize starch, maltodextrin	Corn starch, maltodextrin

Table C: Together with specialists from Ingredion Inc. and Harlan/Teklad, a custom research high glycemic index diet was developed – with the amount of calories stemming from each macronutrient as well as caloric density being close to the standard control diet (see methods study 1).

4. Conclusions

4.1 Publication 1: Surprising results in long-term ghrelin agonist treatment

In our setting, a high glycemic index diet did not deteriorate performance in a water maze task after four months of treatment nor did it worsen immunohistochemical parameters in an Aβ-plaque mouse model compared to other groups on a different diet. This may be in part because our mice were still not old enough when they were sacrificed to show a detrimental effect in these measures caused by their sugary diet (see methods study 1). Neither did we see an improvement in immunohistochemical parameters after ghrelin agonist treatment as in a previous study with a different design[17].

There are, however, two important messages contained in the results of this study: First, the cognitive enhancement seen after long-term ghrelin agonist treatment appears to be robust, that is consistent over both studies in the same mouse model[17,18] and independent from the feeding regimen used.

Second, the long-term effects of ghrelin agonist treatment differ greatly from the short-term effects of ghrelin (agonist) treatment in measures such as food intake, body weight development, body composition and, above all, glucose tolerance (see results and discussion study 1). The effects in weight development and body composition – a glucose tolerance test was not performed at that time – seen in Dr. Kadish's previous study with the same mouse model but different diets were not as radical as in the study presented in this dissertation. Nonetheless, the weight gain effects that were thus far regarded as typical of ghrelin (agonists) could not be observed either.

Although the possibilities of extrapolating the results of our study to other mouse models or even other mammal organisms are very limited – a very specific mouse model with a very specific diet and a very specific ghrelin agonist were used – it should make us sensitive to possibly big differences in the impacts of ghrelin on mammal organisms depending on whether it is give acutely or on a long-term feeding regimen (see discussion study 1).

How do we explain the improvements in cognitive performance if no differences in immunohistochemical endpoints could be detected? Although finding a precise answer to this question resembles the search for the notorious needle in a haystack, the positive effects of ghrelin agonist treatment on glucose tolerance hint in the direction of insulin signalling as a possible endpoint to look at. The data we found in this respect – a lower expression of p-IRS-1 Ser636, which has been shown to be associated with both Alzheimer's disease and diabetes[57], after ghrelin agonist treatment – is far from robust and fails to fully explain the behavioural changes we saw in our sample of mice. Nonetheless, it is little surprising and opens up a new pathway of thinking when it comes to explaining ghrelin's beneficial effects on both glucose tolerance and cognitive performance.

One important finding that may be seen as a confounder in this study is the higher level of activity during the active period in mice treated with the ghrelin agonist (see figure 3 of publication 1). Therefore, one might regard the positive effects of the ghrelin agonist on cognitive parameters as a mere consequence of the already known positive effects of exercise on cognitive performance both in rodents and humans[58,59]. Although we regard this as a valid limitation to the interpretation of our results, we do not think that exercise alone can explain our findings regarding food intake and weight

gain after ghrelin agonist treatment. Nonetheless, especially in future long-term studies, this possible confounder has to be taken seriously.

4.2 Publication 2: What ghrelin does not do

The result of the second study are, at least as far as behavioural paradigms are concerned, negative. The cognitive enhancing effects we hypothesized but could not detect appear to be either utterly absent or, more likely, limited to tasks involving more salient stimuli relevant to a person in a state of hunger[46]. The fMRI results appear to be more robust but a lack of behavioural results makes any interpretation relatively difficult.

Despite the overall negative results of this study, a number of conclusions and ideas for future studies can be drawn from it. First of all, a study with a similar design but more salient stimuli could look into the difference of memory performance for food and non-food items. Although our study failed to show significant differences, this still remains a promising approach which could help to further define the role ghrelin has to play in the "belly-brain-link" described above.

Second, with the fMRI BOLD signal essentially relying on a vascular response and with the vascular contributions to the development of Alzheimer's and other forms of dementia[60], it should be asked to what extent ghrelin could be able to selectively improve blood flow[61], oxygenation and consequently metabolic integrity in brain areas relevant to memory formation. Depending on the model organism and the technique used, a study addressing this question may find interesting results in a short term as well as a long-term paradigm.

Further, also thinking of the results from study 1, the long-term effects of ghrelin and its agonists on glucose homeostasis, weight development and cognition need to be thoroughly defined. As some agonists will probably soon be used in larger samples of patients for chronic conditions such as cancer cachexia[62], it would be both relatively simple and highly important to include these endpoints in large-scale clinical trials.

As the current evidence suggests that ghrelin (agonists) have both short-term and long-term cognitive enhancing effects at least in rodents, it should be asked what effect is more robust and reliable. To what extent are the cognitive enhancing effects of the early studies with ghrelin, often given ICV[4] due to a state of arousal more or less independent of the substance administered? In part, this question is answered by the efficacy of other routes of administration (see above). Are the long-term results replicable in other (non-pathological) mouse models, rats and possibly even primates or are they rather restricted to a very specific setting?

And, as a last point, it needs to be asked what relevance studies using supraphysiologically high levels of ghrelin can have in everyday life – both for humans as well as other mice and other mammals. What natural secretion pattern does ghrelin show in long-term settings involving situations of exercise, different food compositions and different rhythms of food intake and sleep? Current studies looking at the circadian character of ghrelin secretion are already following this train of thought[28]. Are there differences between small and large animals or between small and large specimens of the same species, also thinking of ghrelin's character as a growth hormone secretagogue? What exactly happens to ghrelin and insulin signalling (and essentially the nutrients and substances for whose regulation our bodies synthesize these hormones such as glucose, proteins and fatty acids) at the very moment the body adapts

to longer periods of fasting or, almost more relevantly, to prolonged periods of overeating?

How can we define the course of anorexia nervosa[63], bulimia nervosa[64] and obesity[65] in terms of ghrelin (and insulin) signalling[9] and possibly develop pharmaceutical ways to prevent the patient from crossing a "point of no return"? And, with all the questions asked in the last paragraph: What exactly is cognition in the different settings and how is it affected? With regard to the complexity of the ghrelin system, verbal accuracy amongst researchers, a clear differentiation of what aspect of cognition is examined in relation to what exact part of ghrelin signalling will be crucial in future ghrelin research. With ghrelin and its agonist still being at a relatively early stage of widespread clinical use, we still have a chance to better understand ghrelin's role and relevance in human and other organisms as well as its delicate interactions with other hormonal systems before we try to interfere with it therapeutically.

5. Publication 1

Ghrelin agonist does not foster insulin resistance but improves cognition in an Alzheimer's disease mouse model

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5.1 Abstract

The orexigenic hormone ghrelin, a potential antagonist of the insulin system, ensures sufficient serum glucose in times of fasting. In the race for new therapeutics for diabetes, one focus of study has been antagonizing the ghrelin system in order to improve glucose tolerance. We provide evidence for a differential role of a ghrelin agonist on glucose homeostasis in an Alzheimer's disease mouse model fed a high–glycemic index diet as a constant challenge for glucose homeostasis. The ghrelin agonist impaired glucose tolerance immediately after administration but not in the long term. At the same time, the ghrelin agonist improved spatial learning in the mice, raised their activity levels, and reduced their body weight and fat mass. Immunoassay results showed a beneficial impact of long-term treatment on insulin signaling pathways in hippocampal tissue. The present results suggest that ghrelin might improve cognition in Alzheimer's disease via a central nervous system mechanism involving insulin signaling.

Supported in part by NIH grants R01AG043972, P30DK056336 and P30NS47466. The opinions expressed are those of the authors and do not necessarily represent those of the NIH or any other organization.

5.2 Introduction

Ever since the discovery of ghrelin as a ligand of the growth hormone secretagogue receptor in 1999[2], our understanding of the versatile role of ghrelin in mammals has constantly expanded. The characterization of ghrelin has spanned its actions as an orexigenic hormone leading to weight gain and adiposity in rodents[3,66], to the stimulation of appetite in humans[67], its impacts on cognitive processes in rodents[4,7] and humans[46,68,69], and its role as a neuroprotective agent in neurodegenerative diseases[8,16,17,70,71]. Ghrelin's involvement in glucose metabolism became apparent very early[72,73], with evidence for a differential role of des-acyl ghrelin[74,75]. Recently, many groups have focused on the interactions of ghrelin with the insulin system in humans[54,69]. Antagonizing the insulinostatic ghrelin system has repeatedly been suggested as a novel mechanism by which to improve glucose homeostasis in humans. However, to our knowledge, none of the studies of the interactions of ghrelin with glucose homeostasis have addressed the long-term impact of ghrelin administration on a mammal.

Our group showed previously that administration of a ghrelin agonist leads to improved cognition and improved markers of pathology in an Alzheimer's disease mouse model, even in the absence of caloric restriction[17]. The pathophysiological correlations between Alzheimer's disease, impaired glucose metabolism, and diabetes are well established[76–78], and elevated serum glucose levels have been shown to be an independent risk factor for dementia in humans[79]. In the present study, therefore, we aimed to investigate the long-term effects of a ghrelin agonist given for 4 months on Alzheimer's disease pathology, cognition, and metabolism in the same mouse model fed a high–glycemic index (GI) diet as a constant challenge for glucose homeostasis. We

hypothesized to see either (i) a detrimental effect of ghrelin agonist treatment in combination with this diet on cognitive and metabolic endpoints owing to interference with insulin signaling and consequently higher overall blood glucose levels or (ii) a protective effect as seen in our previous study via a thus far unknown mechanism.

5.3 Results

5.3.1 Ghrelin agonist acts as a long-term cognitive enhancer in spatial learning

Other groups have previously reported increased levels of anxiety in neonatal chicks and rats in the open field test after ghrelin administration[4,5]. In several preliminary tests we performed to exclude any a priori differences between groups, we did not observe any statistically significant differences between groups in categories such as anxiety or exploration activity (open field, zero maze, dark-light-box; see methods; data not shown). We also did not detect any significant group differences in performance in an object recognition task, which had been observed to be improved by short-term ghrelin treatment by another research group[10].

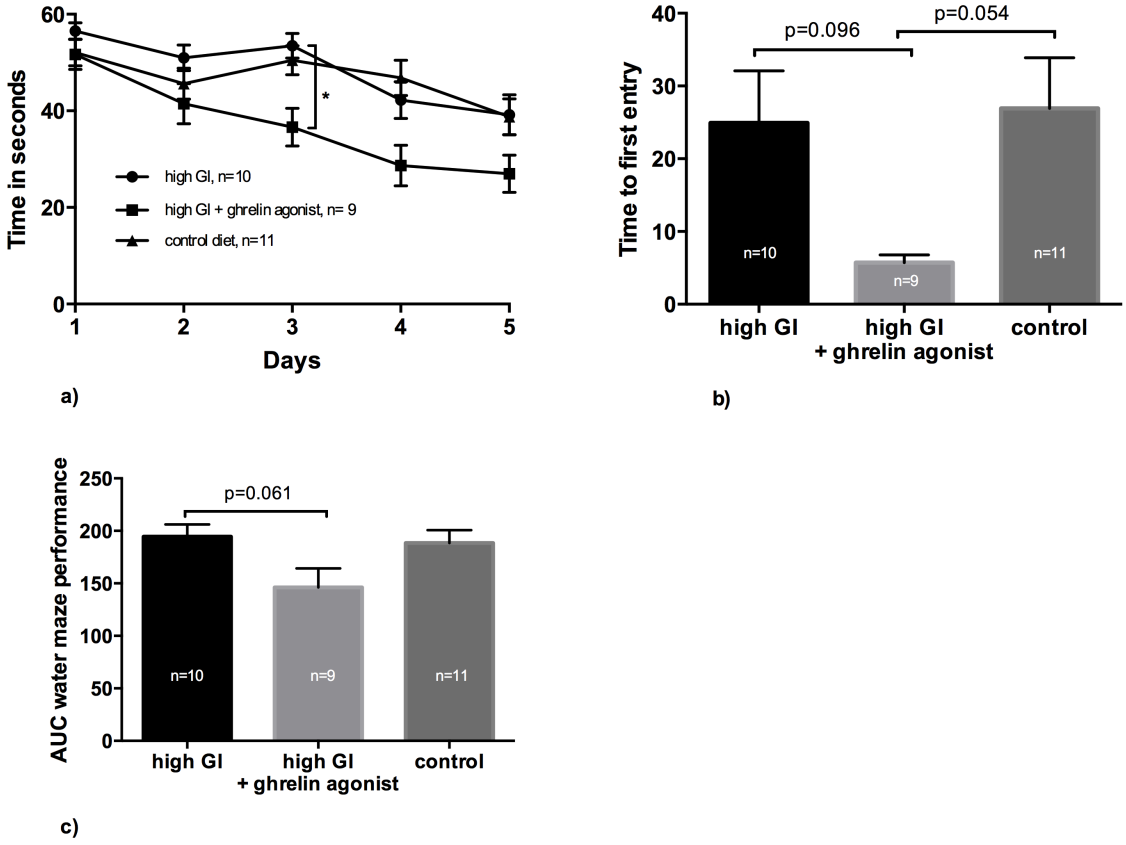


Figure 1: Ghrelin-agonist-treated animals performed better in a water maze test. They showed a faster learning curve than did the group fed a high-GI diet alone. Intra-day differences between high-GI and high-GI + ghrelin agonist groups were significant for day 3 ((a), one-way ANOVA followed by post-hoc Tukey's multiple comparisons test, p=0.026), an Area-Under-The-Curve (AUC)-comparison for the graphs in (a) revealed that ghrelin agonist treated animals showed a strong tendency to perform better over the entire experiment ((c), p=0.061, one-way ANOVA/Tukey's). During probe trials (time to first entry in the correct quadrant), the difference between high-GI and high-GI + ghrelin agonist were significant at tendency level only ((b), p=0.096 for high GI vs. high GI + ghrelin agonist, p=0.054 for high GI + ghrelin agonist vs. controls, one-way ANOVA/Tukey's). Bars indicate SEM.

Among the three study groups (the group fed a high-GI diet, the group fed a high-GI plus ghrelin agonist, and the control group, which was fed an AIN-93G purified diet), the group fed a high-GI diet plus ghrelin agonist showed the best memory performance in the water maze (figure 1). Both in its learning dynamics in the course of the test days and in its performance in the probe trial, this group outperformed the other groups. However, the group fed a high-GI diet was not impaired in its cognitive performance compared with the control group as we originally hypothesized.

5.3.2 Ghrelin agonist does not significantly affect Aβ plaque load or microglia activation

In a previous study we reported a positive influence of ghrelin on Alzheimer's disease pathology markers such as Aβ plaque load (human Aβ4-10; see methods) and activated microglia[17]. In the current study, however, we did not observe any significant differences between the treatment groups in either of these immunohistochemical endpoints in the stratum oriens and dentate gyrus of the dorsal hippocampal area (figures 2, (a) and (b)). Because the olfactory epithelium has been shown to be involved at an early stage in Alzheimer's disease[80], we included the olfactory bulb in our immunohistochemical measurements. Microglia activation in the olfactory bulb was less in the group fed a high-GI diet plus ghrelin agonist than in the group fed a high-GI diet alone ($p=0.057$, figure 2, (c)). The Aβ plaque load in the olfactory bulb, however, did not differ significantly between these groups as measured in a grayscale density assessment (figure 2, (c); see methods). Other research groups have reported an increased number of doublecortin (DCX)-positive cells after ghrelin treatment in the hippocampus of 2-month-old 5XFAD mice[81]. We did not observe any significant differences between groups in DCX-positive cell count in the dentate gyrus (data not shown).

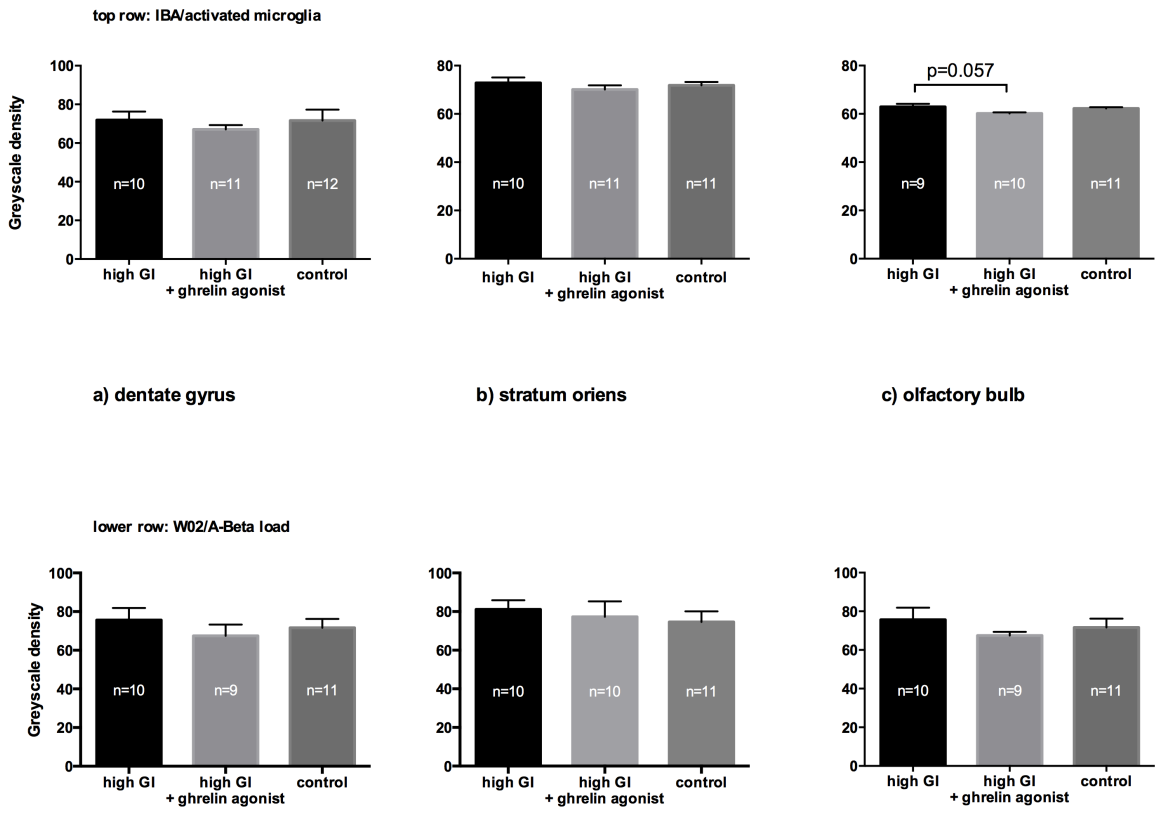


Figure 2:

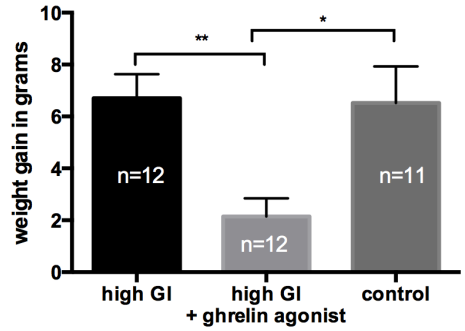
Neither markers for activated microglia (IBA, top row) nor for Aβ-load (W02, lower row) were significantly different after long-term ghrelin agonist treatment in the dentate gyrus (a) and stratum oriens (b). Only the level of activated microglia in the olfactory bulb of ghrelin-agonist-treated animals showed a tendency to be lower than in animals fed the high-GI diet alone ((c), Kruskal-Wallis test, followed by post-hoc Dunn's multiple comparisons test, p=0.057). Bars indicate SEM.

5.3.3 Long-term ghrelin agonist treatment leads to less weight gain, less overall food consumption, and more activity

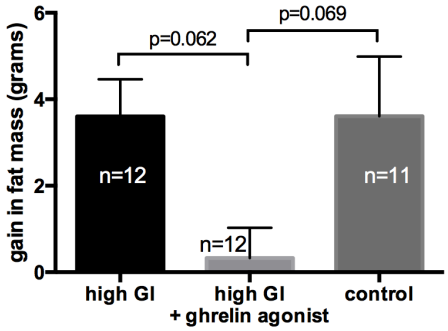
Ghrelin and its agonists lead to overeating and obesity when food intake is unlimited[3,82]. Interestingly, the group fed a high-GI diet plus ghrelin agonist did not gain as much weight as did the other treatment groups (figure 3, (a)). Only weight gain in the two groups not treated with the agonist was highly significant (figure 3, (a), $p=0.009$ for high-GI vs. high GI + ghrelin agonist group, $p=0.015$ for controls vs. high-GI + ghrelin agonist group, ANOVA/Tukey's). Of note, the increase in fat mass was particularly low in the group fed a high-GI diet plus ghrelin agonist (figure 3, (b)).

Because the food consumption of agonist-treated animals was limited to the average amount consumed by the group fed the high-GI diet alone (see methods), overeating triggered by the ghrelin agonist was not possible in this group. We observed a strong feeding response in our animals after the administration of the ghrelin agonist; however, the attempt to quantify this response in CLAMS metabolic cage measurements failed. The mice did not tolerate the procedure, mainly because of an accidentally shifted dark-night cycle. As a proof of concept, we have included CLAMS data from previous studies with C57/BL6 mice that clearly show the immediate feeding response after the administration of the same agonist LY444711 (figure 3, (e) and (f)).

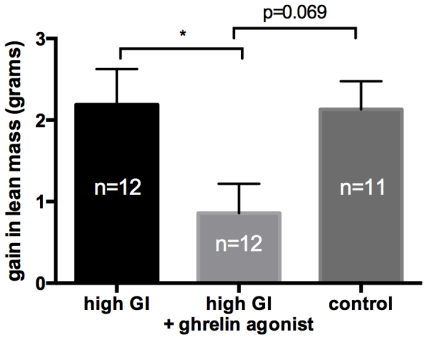
Interestingly, daily recording of food intake in the group fed a high-GI diet plus ghrelin agonist over 8 weeks showed that the animals did not consume the full amount of food given to them daily (figure 3, (g)). This finding and the overall elevated activity levels in agonist-treated animals compared with those fed the high-GI diet alone ($p<0.001$ for high-GI/controls vs. ghrelin agonist treated group, ANOVA/Tukey's, figure 3, (d)) can explain the lesser weight gain in this treatment group.


weight gain in grams
10
8
6
4
2
0
**
*
n=12
n=12
n=11
high GI
high GI
+ ghrelin agonist
control

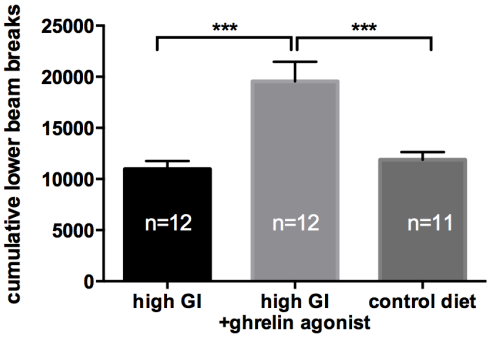
a)


gain in fat mass (grams)
6
4
2
0
p=0.062
p=0.069
n=12
n=12
n=11
high GI
high GI
+ ghrelin agonist
control

b)


gain in lean mass (grams)
3
2
1
0
*
p=0.069
n=12
n=12
n=11
high GI
high GI
+ ghrelin agonist
control

c)


cumulative lower beam breaks
25000
20000
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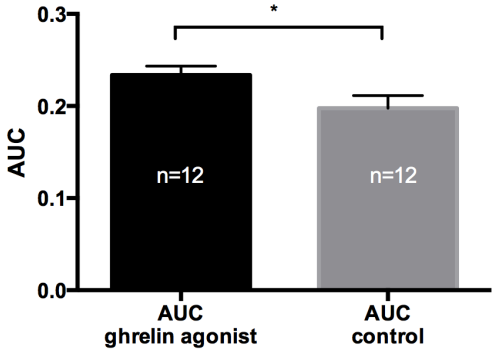
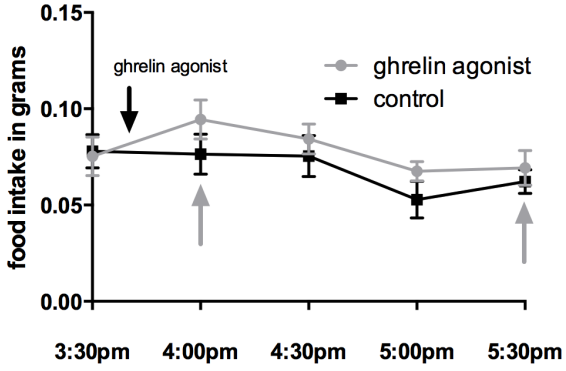
5000
0
***
***
n=12
n=12
n=11
high GI
high GI
+ghrelin agonist
control diet

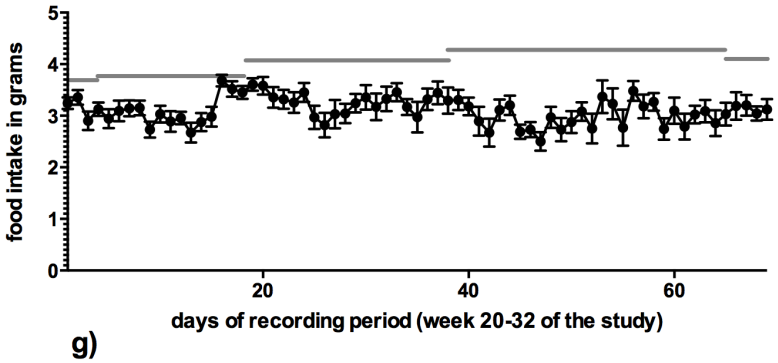
d)


AUC
0.3
0.2
0.1
0.0
*
n=12
n=12
AUC
ghrelin agonist
AUC
control

e)


food intake in grams
0.15
0.10
0.05
0.00
ghrelin agonist
ghrelin agonist
control
3:30pm
4:00pm
4:30pm
5:00pm
5:30pm

f)


food intake in grams
5
4
3
2
1
0
20
40
60
days of recording period (week 20-32 of the study)

g)

Figure 3:

Over a period of 3 months ((a)-(c), compare timepoints "week 8" and "week 21" of the study), animals not treated with the ghrelin agonist gained significantly more weight than ghrelin agonist treated animals ((a), p=0.009 for high-GI group vs. ghrelin agonist group, p=0.015 for controls vs. ghrelin agonist group, one-way ANOVA/ Tukey's). The same groups showed a tendency to gain more fat mass ((b), p=0.062 for high GI vs. ghrelin agonist group, p=0.069 for controls vs. ghrelin agonist group, one-way ANOVA/Tukey's) than ghrelin agonist treated animals. The high-GI group gained significantly more lean mass than the ghrelin-agonist treated group ((c), p=0.048), the controls showed a tendency ((c), p=0.069, one-way ANOVA/Tukey's). Activity levels during the mice's active period (measurements taken in week 21) were higher in ghrelin-agonist-treated animals than in the high-GI and control diet groups ((d), p<0.001 for both comparisons, one-way ANOVA/Tukey's). Immediately after administration, the ghrelin agonist led to significantly higher food intake during the 2 subsequent hours ((e), p=0.045 for AUC between gray arrows in (f), data for a sample of 12-month-old C57/BL6 mice from a different study, t-test for unpaired samples). However, cumulative food intake as measured for an entire day hardly ever reached the maximum of food assigned to ghrelin-agonist-treated animals as indicated by the gray lines ((g), days refer to the period while food intake was recorded). Bars indicate SEM.

5.3.4 Long-term ghrelin agonist treatment does not impair glucose tolerance

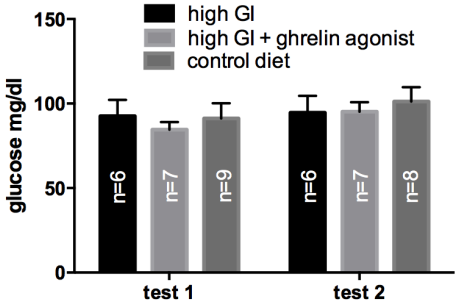
In order to characterize the impacts of a high-GI diet and long-term ghrelin agonist treatment on glucose metabolism, we performed an oral glucose tolerance test after 3 and 4 months of treatment. Baseline glucose levels after 6 hours of fasting (see methods) did not differ significantly between the groups in either test (figure 4, (a)).

A comparison of the area under the time curve (AUC) for both high-GI groups as well as the controls during the first test, which was performed shortly before the daily administration of the ghrelin agonist, did not reveal any differences. This suggests that neither the high-GI diet on its own nor in combination with long-term ghrelin agonist treatment impaired glucose tolerance (figure 4, (b)). In order to clarify the ghrelin agonist's short-term effects on glucose homeostasis, in the second glucose tolerance test, we treated animals with the ghrelin agonist immediately before administering the glucose load. In this experiment, the agonist-treated animals showed a significantly higher AUC than during the first test ($p=0.010$, t-test for paired samples, figure 4, (b)), whereas the mean AUC for the other groups did not change significantly. There were also no significant differences in the second test between groups. This result illustrates the differential effect of the ghrelin agonist on short-term and long-term glucose homeostasis.

We expected to see overall lower endogenous acyl ghrelin levels after long-term treatment with a ghrelin agonist, hypothesizing that artificially high ghrelin levels over a long period of time would lead to a down-regulation of endogenous production of the active peptide. However, both serum acyl and desacyl ghrelin levels as measured after a 6-hour fast were significantly higher in the group fed a high-GI diet plus ghrelin agonist than in the group fed the high-GI diet alone (figure 4, (f) and (h)). A cross-reactivity in the assay between ghrelin agonist and endogenous ghrelin cannot be excluded with

absolute certainty but appears both highly unlikely and probably insignificant because the last administration of the agonist took place 24 hours before the blood samples were taken. Insulin levels measured at the same time did not differ significantly between groups (figure 4, (g)).

It could be speculated that the long-term ghrelin agonist treatment led to a lower amount of ghrelin receptors in peripheral tissues, requiring higher circulating active ghrelin levels for the same metabolic effects. However, the differential analysis of peripheral tissues for endpoints relevant to insulin and ghrelin signaling was beyond the scope of this project. Possible future results of currently ongoing measurements will be discussed in a separate publication.



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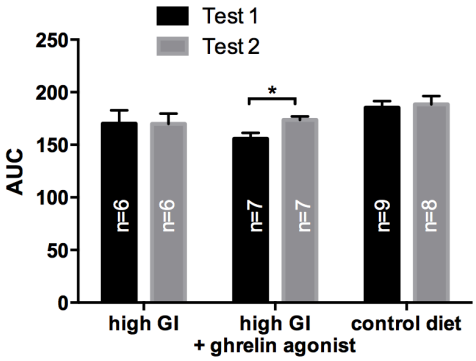

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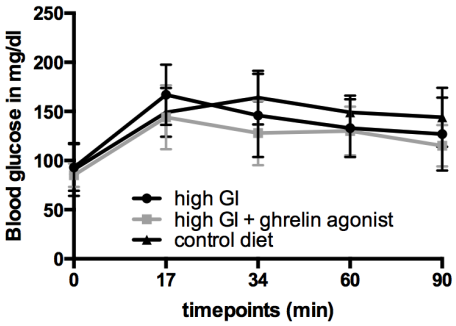
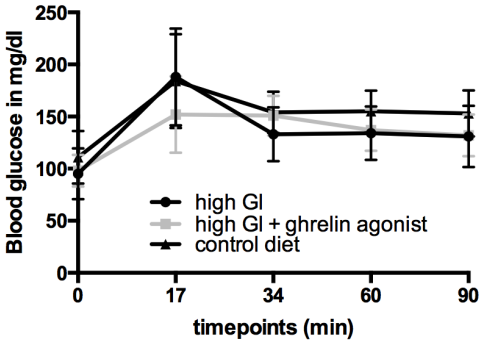
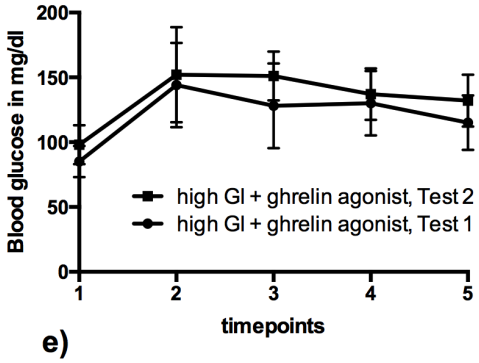


c) first glucose tolerance test



d) second glucose tolerance test



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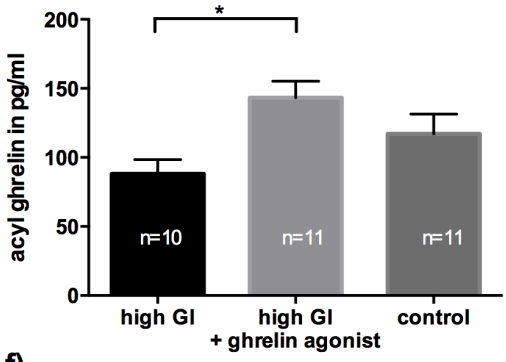

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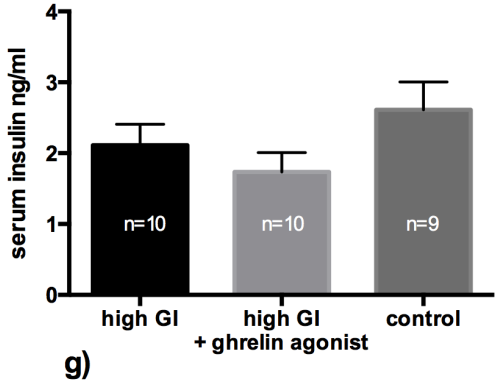

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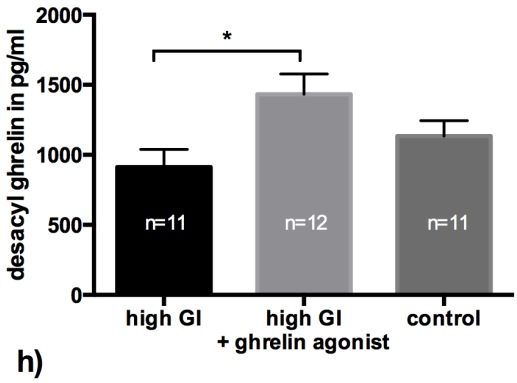


h)

Figure 4:

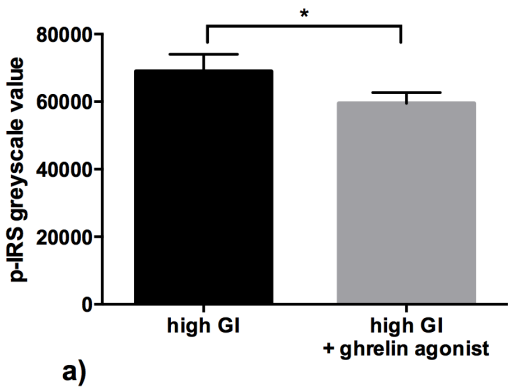
Baseline blood glucose levels after a six hours fasting period did not differ significantly between treatment groups ((a), one-way ANOVA/Tukey's). Overall, the results of an oral glucose tolerance test were not influenced by long-term ghrelin agonist treatment (AUC = area under the curve, (b)-(e)). In the second test, animals from the high GI + ghrelin group were treated with the ghrelin agonist immediately before the glucose tolerance test and showed significantly higher blood glucose levels than in the first test (p=0.010, t-test for paired samples, (b) and (d)). Both serum acyl ((f), p=0.020, Kruskal-Wallis/Dunn's) and desacyl ghrelin ((h), p=0.020, ANOVA/Tukey's) levels measured after a 6-hour fasting period were significantly higher in animals treated with the ghrelin agonist. There were no significant differences in serum insulin levels in the same samples ((g), one-way ANOVA/Tukey's 4.7). Bars indicate SEM.

5.3.5 Ghrelin agonist treatment beneficially influences central insulin signaling pathway

Because other authors suggested an involvement of the tumor necrosis factor α (TNF-α)/c-Jun n-terminal kinase (JNK) pathway in Alzheimer's disease triggered by Aβ-oligomers[57], we measured TNF-α, pSAP-JNK, and phosphorylated insulin receptor substrate 1 (p-IRS Ser636) as well as synaptophysin and PSD-95 as synaptic markers in hippocampal brain tissue from the groups fed the high-GI diet (figure 5). We found a significant difference in p-IRS levels between the groups (fig. 5, (a), p=0.039, nonparametric Kolmogorov-Smirnov test), indicating a possible interaction of long-term ghrelin agonist treatment with central insulin signaling.

There was a moderate negative correlation in a linear regression analysis between behavioral results and p-IRS levels for both groups (r=-0.41); however, this correlation was not significant (p=0.175, data not shown). We did not observe any

differences in structural synaptic markers, neither presynaptically (synaptophysin) nor postsynaptically (PSD-95). Because there were no group differences in TNF-α or JNK-levels, we could not reproduce the TNF-α/JNK interrelations in Alzheimer's disease in our mice.



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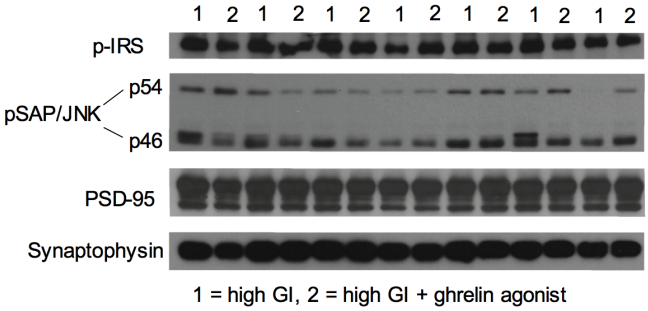

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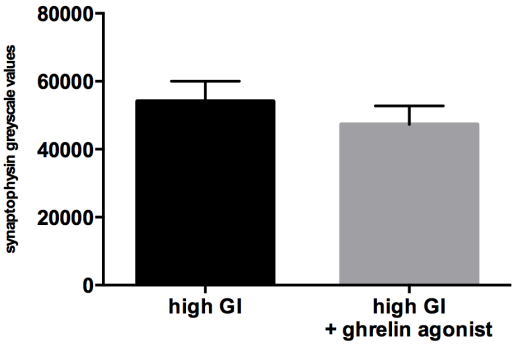

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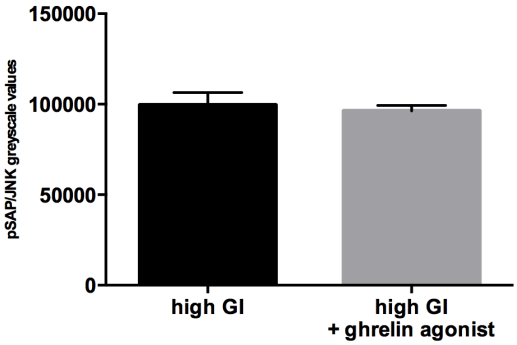

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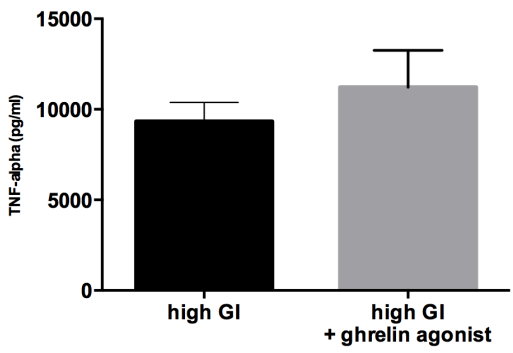

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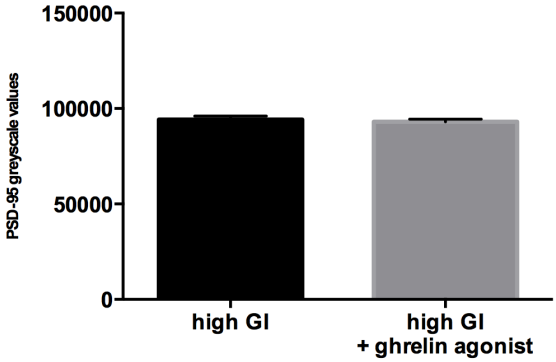

f)

Figure 5:

Animals treated with a ghrelin agonist showed a significantly lower amount of phosphorylated IRS (pIRS Ser636), which has been shown to be associated with impaired glucose tolerance ((a), p=0.039, nonparametric Kolmogorov-Smirnov test). However, we did not detect any significant differences in hippocampal tissue between the high GI and high GI + ghrelin agonist groups for synaptophysin (c), pSAP/JNK (d), TNF-α (e) or PSD-95 (f). Bars indicate SEM.

5.4 Discussion

Type 2 diabetes and Alzheimer's type dementia are chronic diseases; consequently, all symptomatic treatments are intrinsically long-term. However, most studies of the interactions of ghrelin and insulin, which partly aimed to derive novel therapeutic pathways in diabetes, have looked at fairly short time frames of hours, days, or weeks[83–85]. In our study, we chose long-term ghrelin agonist administration in order to model the impacts of therapeutically influencing this system in a mammal over a period of several months. First, we could reproduce the previously known cognitive-enhancing effects of ghrelin and ghrelin agonists[8], and at the same time we showed that this effect is seen even under the influence of a high-GI diet despite the ghrelin agonist's short-term insulinostatic effect. The cognitive-enhancing effects were seen in the water maze test (figure 1), which is mainly a hippocampus-dependent spatial learning task[86]. This finding underlines the relevance of this ghrelin agonist's cognitive effects in the Alzheimer's type of dementia, which most prominently affects hippocampal brain areas and functions.

Most interestingly, we could show a long-term effect of ghrelin agonist treatment on metabolism that differed from its short-term actions on food consumption, weight development, and glucose tolerance. At the same time, we observed the well-known short-term orexigenic and insulinostatic effects of this endogenous peptide. These findings indicate a differential metabolic role of the ghrelin system in short-term and long-term treatment and call for a further differentiation of ghrelin's long term role on glucose homeostasis, e.g. by including glucose clamp techniques in a long-term study design. Further, the observations in metabolic endpoints were made using the ghrelin agonist in combination with a high glycemic index diet. To what extent the results presented in this manuscript depend on this specific combination and to what extent they are also valid for a combination of a normal diet with a ghrelin agonist will be addressed in future and ongoing studies.

Given ghrelin's differential interactions with insulin signaling, possibly also via mTORC1-dependent pathways[84,87,88], we hypothesized a potentially protective effect of ghrelin agonist treatment on insulin signaling in the central nervous system. In agonist-treated animals, we found a lower expression of p-IRS-1 Ser636, which has been shown to be associated with both peripheral insulin resistance[89], obesity[90] and Alzheimer's disease[57]. We therefore speculate that ghrelin and insulin signaling in the central nervous system are, to an extent, synergistic. On the one hand, the hormone reduces peripheral glucose uptake in periods of fasting, whereas on the other hand it improves or at least does not reduce glucose uptake in the central nervous system in situations of energy deficiency[91].

A limitation of the interpretation of the present results is that the data are based on a mouse model for Alzheimer's disease under the influence of a very specific high-GI diet.

The latter might explain why we could not replicate the immunohistochemistry results of our previous study[17]. All extrapolation of these findings to other animal models must be done with care. Furthermore, we did not observe any structural differences in immunohistochemical markers for Aβ plaque load or central nervous system inflammation or in synaptic markers, which essentially leaves the task of identifying an immediate correlate of cognitive enhancement by ghrelin to future studies.

The present findings do suggest that any new therapeutic approaches in both diabetes and neurodegenerative diseases that are based on a manipulation of the ghrelin system must be addressed with utmost care. Counteracting ghrelin signaling for better glucose control or enhancing ghrelin signaling in the central nervous system for neuroprotection and cognitive enhancement are two tempting therapeutic pathways in neuroscience and endocrinology. However, both have to withstand long-term testing and the potentially contrasting effects of ghrelin and ghrelin agonists in peripheral tissues and in the brain.

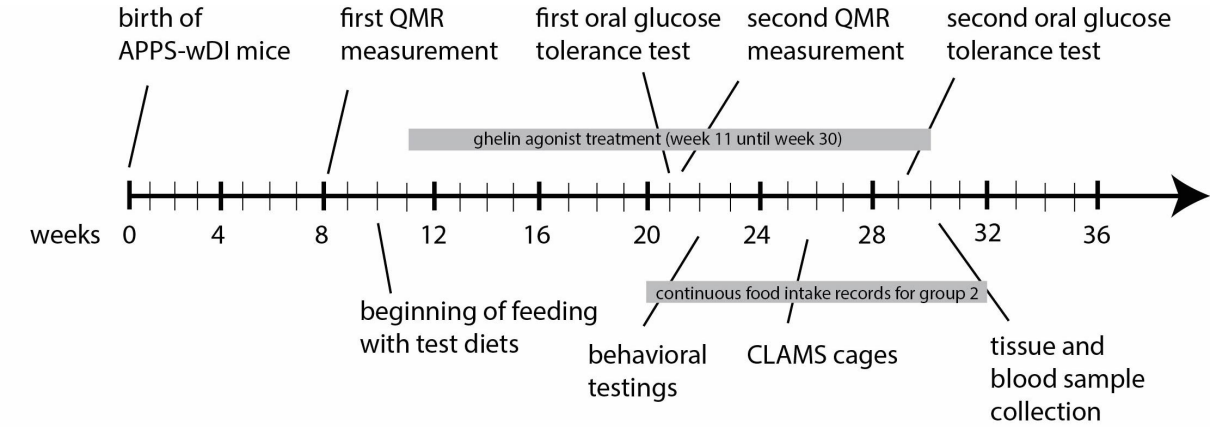

Figure 6:

Timeline of the study

5.5 Methods

5.5.1 Ethics statement

All animal protocols were approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC). All methods were carried out in accordance with the approved guidelines and protocols.

5.5.2 Animals, diets, and treatment

The study timeline is shown in figure 6. A total of 36 male Tg APPSwDI (human APP with Swedish, Dutch, and Iowa mutations on a C57BL/6 background) were raised under equal dietary conditions for 2 months. At 10 weeks of age, the animals were divided into three groups of 12 animals each and received a diet consisting of 60% of kcal in carbohydrates with equal amounts of maltodextrin and sucrose plus either waxy maize starch (high-GI diet groups) or AIN-93G purified diet (controls). For detailed diet composition, see the supplementary material. During the first week of dietary acclimatization, all animals received a 45-mg sucrose pellet daily. After that, the group fed the high-GI diet plus ghrelin agonist received a 45-mg sucrose pellet containing 1.66% ghrelin agonist[92] (LY444711; Eli Lilly, Indianapolis, IN) every day (30mg/kg/day, parallel to our previous study[17], dose determined according to previous work by Giddings et al. 2008, abstract added to supplement); the other groups continued to be treated with sucrose pellets as placebos. Treatment took place daily at the same time between 2:00 and 4:00 pm during the animals' light cycle and continued until the animals were sacrificed (treatment period: week 11 until week 30). Staff watched all animals take and eat the pellets and noted the days when the pellet was not consumed.

This was only the case for few animals during dietary acclimatization. During the treatment period all animals ate the pellets. The amount of food consumed by all groups was measured every 2 weeks and the threshold of food restriction for the ghrelin-agonist-treated group was set at the average level of food consumption of the group fed the high-GI diet alone.

5.5.3 Behavioral and cognitive assessments

All behavioral and cognitive assessments took place between weeks 22 and 24 (see fig. 6). All tests took place during the light cycle. Feeding times were not changed throughout the assessments.

5.5.4 Open field test

The maze consisted of a 42 by 42 cm^2 arena with clear sides (20 cm high). The animal was placed in the arena and observed for 4 minutes with a camera-driven tracker system (Ethovision 9.5, Noldus, The Netherlands). The arena was subdivided into the open center area and the sides. The system recorded the position of the animal at 5 frames/s.

5.5.5 Water maze

The water maze apparatus and procedure were described in detail before[93]. Briefly, we used a blue plastic pool, 120 cm in diameter, and a see-through round platform, 10 cm in diameter, located 0.5 cm below the water surface. During days 1 through 5 of the testing period, the mice were trained to find the hidden platform, which was kept in a constant

position throughout these 5 days. Three trials were run per day; all starting positions were used equally in a pseudo-random order. The mice were given 60s to find the platform and 10s to stay on the platform. If the mouse did not find the platform in the assigned time, it was manually put onto the platform. The inter-trial interval during which the mouse was placed in a towel-bedded drying cage lasted 1 minute. Learning of the task was evaluated by recording the latency time to find the platform. At the end of the four trials on day 5 of the testing period, the mice were tested in a 60-s probe trial with no escape platform present. Mice that had learned the platform position predominantly searched in the "correct" quadrant of the pool during the probe trial or entered the correct quadrant faster. Trials were recorded by using a camera-driven tracker system (Ethovision 9.5, Noldus, The Netherlands).

5.5.6 Zero maze

For the zero maze test, we used a round maze with a diameter of 61 cm designed for mice (SD Instruments, San Diego, CA). At the beginning of the trial, all mice were placed on the same open part facing in the same direction. Velocity, distances moved, and time spent in the open and closed parts were recorded for 4 minutes by using a camera-driven tracker system (Ethovision 9.5, Noldus, The Netherlands).

5.5.7 Light-dark-box

We used a custom-built plastic light-dark box (46.5 cm length, 22 cm width, 28x22 cm light part, 18.5x22 cm dark part). Time spent in the light and dark parts as well as the number of entries into the dark part were recorded for 5 minutes by using a camera-

driven tracker system (Ethovision 9.5, Noldus, The Netherlands). Mice were placed in the light part of the box facing away from the entrance to the dark part.

5.5.8 Immunohistochemistry

Animals were sacrificed at week 30 for immunohistochemical, Western blot, and ELISA analyses. Mice were anesthetized with ketamine/xylazine (100/10 mg/kg) and perfused with cold saline. The brains were removed and cut in half sagittally, and the right hemisphere of the brain was placed in 4% paraformaldehyde overnight. The left hemisphere was dissected into four pieces (rostral cortex, caudal cortex, hippocampus, and midbrain/brainstem) and stored frozen at -80°C for protein analysis (ELISA, Western blot). The right half and the intact whole brains from 12 animals, 4 per group, were put in 30% sucrose for cryoprotection, and 30-µm thick coronal sections were cut on a freezing-sliding microtome.

Sections from 29 brains were stained for Aβ with the W0-2 antibody (human $A\beta_{4\text{-}10}$; 1:2000; The Genetics Company, Schlieren, Switzerland). Another series of sections from the same 29 brains was stained for Iba-1 (1:1000; Wako, Richmond, VA) as a marker for activated microglia. For Aβ staining, sections were pretreated for 30 minutes in 85°C sodium citrate solution (pH=6.5). Following incubation with the primary antibody in TBS-T overnight at room temperature, tissues were rinsed three times and incubated with the appropriate biotinylated secondary antibody for 2 hours at room temperature. Sections were again rinsed three times and put for 2 hours with the tertiary antibody, extra Avidin-peroxidase. After another three rinses, metal-enhanced DAB staining was used for visualization. For each antibody, all sections were processed in one staining tray. All slides were air-dried, cleared in xylene, and coverslipped with DPX.

ImageJ software (NIH open source; http://imagej.nih.gov/ij/) was used to analyze the area occupied by Aβ and glial reactivity in stratum oriens of the dorsal hippocampus and in the dorsal dentate gyrus. Images of the appropriate brain areas were acquired with an Olympus DP70 digital camera. All images were acquired in one session to avoid changes in light levels. ImageJ measurements were performed by a scientist who was blind to the study design. Few images had to be excluded due to staining/tissue preparation problems (see fig. 2).

5.5.9 Oral glucose tolerance test

In order to avoid a priori differences in baseline blood glucose levels, mice had no access to food for a period of six hours before the glucose tolerance test. For the oral glucose tolerance test, 300 µl of a solution of 16.7 g glucose in 100 ml of purified water was administered directly into the mice's stomach via gavage needles. Blood samples were taken from tail veins and immediately measured with the TRUE2Go blood measurement system[94] for one baseline time point and then after 17, 34, 60, and 90 min. The mice were placed in a plastic retainer system during the procedure. One mouse was excluded from the analysis because it did not tolerate the gavage process.

5.5.10 Protein extraction and Western blotting

For ELISA and Western blots, brain tissue was homogenized in RIPA (150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP-40, 50 mM Tris, pH 8, 20 mM NaF, 2 mM EGTA, 0.5% levamisole, 1 mM NaVO4) plus protease inhibitor cocktail (p2714 Sigma-Aldrich, St Louis, MO) by use of the fast homogenization process Minilys® (Precellys,

Bertin, France). After protein estimation with the Bradford method[95], samples were diluted to an appropriate concentration.

For Western blotting, p-IRS Ser636 antibody (Santa Cruz Biotechnology, Dallas, TX), synaptophysin antibody clone SVP-38 (Sigma-Aldrich, St Louis, MO), pSAPK/JNK Thr183/Tyr185 (Cell Signaling Technologies, Danvers, MA) and PSD-95 antibody (Upstate/Millipore, Billerica, MA) were used. After electrophoresis and transfer to nitrocellulose, samples were incubated with the primary antibody overnight and were then incubated with the suitable secondary antibody for 90 minutes. For measuring TNF-alpha, a commercial ELISA kit was used (EMTNFA, ThermoScientific, Rockford, IL).

5.5.11 Blood samples

Blood samples were taken after a 6-hour fasting period via intracardial puncture from the left ventricle shortly before the animals were perfused. Samples of 250 µl of blood were collected in chilled EDTA tubes (Becton, Dickinson and Company, Franklin Lakes, NJ) that were prefilled with 5 µl of 200 mM AEBSF stock yielding a final concentration of 4 mM AEBSF. Samples were centrifuged for 20 minutes at 17000 rpm and 4°C and the plasma collected was immediately acidified with 200 µl of 1 M HCl per 1 ml of plasma. pH was adjusted accordingly before ELISA measurements for insulin and ghrelin. Acyl ghrelin and des-acyl ghrelin was measured with a custom-built 2-site sandwich ELISA[47]. For the measurement of insulin a commercially available ELISA kit was used (EZRMI-13K, EMD-Millipore, Billerica, MA).

5.5.12 Quantitative magnetic resonance imaging

In vivo body composition (total body fat and lean tissue) of mice was determined by using an EchoMRI™ 3-in-1 quantitative magnetic resonance (QMR) machine (Echo Medical Systems, Houston, TX). A system test was performed by using a known fat standard before the measurements were taken. Mice were weighed and then placed into a clear holding tube capped with a stopper that restricted vertical movement but allowed constant airflow. The tube was inserted into the machine and the mouse was scanned by using Normal Precision mode.

5.5.13 Metabolic cages

Twenty-four-hour patterns of food intake, energy expenditure (indirect calorimetry), and physical activity were measured by using CLAMS (Columbus Instruments Inc., Columbus, OH). This instrument also enforced the feeding regimens in an automated, computer-controlled manner. Body weight was monitored weekly.

5.5.14 Activity measurements

Additional activity measurements over a period of five consecutive light and dark cycles were performed at week 21 by using a custom-built infrared-based beam-breaking system that recorded horizontal and vertical movements. Mice were placed in the system in their home cages with reduced bedding in order to not disrupt the continuous infrared measurements. Only data recorded on days 2 to 4 were included in the analysis.

5.5.15 Statistical methods

All datasets were tested for Gaussian distribution using a D'Agostino & Pearson omnibus normality test. Whenever a normal (Gaussian) distribution could be validly assumed, a one-way ANOVA, then a post-hoc Tukey's test for multiple comparisons was used to test for significant differences between groups (referred to as "ANOVA/Tukey's"). Nonparametric samples were tested using the Kruskal-Wallis test and Dunn's test for multiple comparisons as a post-hoc test (referred to as "Kruskal-Wallis/Dunn's"). Whenever only two groups were involved in the measurements, differences were tested using a t-test for paired/unpaired samples in parametric distributions or a Kolmogorov-Smirnov test for nonparametric distributions. Being aware of the nested data problem[96], we only compared values on the same level of analysis to decrease the likelihood of type-1 errors. All analyses were performed with GraphPad Prism software version 6.05 (GraphPad Software, Inc., La Jolla, CA).

5.6 Acknowledgements

We particularly thank our students Rebecca White and William McGilberry for their dedicated help with everyday lab work. We also thank Dr. Daniel L. Smith, Rachel Brewer, and Nathan Miyasaki for their valuable critical input and constructive ideas. The ghrelin agonist LY444711 was kindly provided at no cost by Eli Lilly, Indianapolis, IN. AMIOCA waxy maize starch was provided at no cost by Ingredion Inc., Bridgewater, NJ.

5.7 Author contributions

N.K. and I.K. are responsible for the idea, concept and design of the study, supported by the critical input of T.v.G. I. K. performed all immunohistochemical measurements in collaboration with T.v.G. N.K. performed all behavioral assessments, under assistance and supervision of T.v.G. and A.K. M.D.S. was responsible for the long-term feeding regimen, animal health and both oral glucose tolerance tests, assisted by N.K. A.K. was responsible for Western Blots, in particular quality management and data aggregation. D.B.A. substantially guided and supported the conception and writing of the final publication manuscript. All authors reviewed the manuscript.

5.8 Additional Information

All authors declare no competing financial interests.

6. Publication 2

Ghrelin alters encoding-related brain activity without enhancing memory formation in humans

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6.1 Abstract

Ghrelin regulates energy homeostasis in various species and enhances memory in rodent models. In humans, the role of ghrelin in cognitive processes has yet to be characterized. Here we show in a double-blind randomized crossover design that acute administration of ghrelin alters encoding-related brain activity, however does not enhance memory formation in humans. Twenty-one healthy young male participants had to memorize food- and non-food-related words presented on a background of a virtual navigational route while undergoing fMRI recordings. After acute ghrelin administration, we observed decreased post-encoding resting state fMRI connectivity between the caudate nucleus and the insula, amygdala, and orbitofrontal cortex. In addition, brain activity related to subsequent memory performance was modulated by ghrelin. On the next day, however, no differences were found in free word recall or cued location-word association recall between conditions; and ghrelin's effects on brain activity or functional connectivity were unrelated to memory performance. Further, ghrelin had no effect on a cognitive test battery comprising tests for working memory, fluid reasoning, creativity, mental speed, and attention. In conclusion, in contrast to studies with animal models, we did not find any evidence for the potential of ghrelin acting as a short-term cognitive enhancer in humans.

6.2 Highlights

- Effects of ghrelin on memory for food-related words-location associations were tested.
- Functional connectivity during post-encoding rest was altered after ghrelin injection.
- Acute ghrelin administration had no behavioral effects on long-term memory retention.
- Acute ghrelin administration had no behavioral effects on several other cognitive tasks.
- Ghrelin's effects on memory markedly differ between animal models and human subjects.

6.3 Introduction

The orexigenic peptide ghrelin is involved in appetite regulation[3,9], but also influences a number of cognitive functions in rodent models, such as fear learning, object recognition and spatial learning[1,4,10,97]. The hippocampus appears to be a central structure in ghrelin's effects on memory, with the peptide leading to a lower threshold for long term potentiation in the dentate gyrus and to an increase in hippocampal spine synapse density[7]. In animal models of neurodegenerative diseases and age-related memory decline, ghrelin appears to exert a neuroprotective effect[16–18].

Due to its dual role in appetite regulation and hippocampus-related memory formation, an evolutionary role of ghrelin in foraging processes was suggested: ghrelin might support learning of food-associated locations[98,99]. In humans, effects of ghrelin on

appetite- and memory-related brain regions have been reported[46,68,69], however, the specific role of ghrelin in human cognition is yet to be defined[1,8]. Studies on the association between ghrelin serum levels and cognitive function in healthy and pathological aging have been rather contradictory so far[14,71,100,101]. Also for younger human subjects, inconclusive results have been reported for the role of ghrelin in memory processing: memory for food- compared to non-food-related pictures was enhanced after administration of ghrelin in a simple recognition paradigm[46], whereas nocturnal ghrelin administration had no positive effect on sleep-related consolidation of a simple motor learning task[102]. Effects of ghrelin on more complex cognitive processes including encoding or consolidation of hippocampus-dependent memories of spatial or verbal information have not been studied yet.

In this study, 21 healthy young male participants performed two subsequent runs of a spatial-verbal learning task while undergoing functional magnetic resonance imaging (fMRI). They had to learn food and non-food words presented on the background of a spatial navigation environment (figure 1). After each run, acyl ghrelin or placebo was administered in a double-blind, randomized, placebo-controlled within-subject design, thereby testing ghrelin effects on both pure consolidation (pre-injection encoding run) and encoding (post-injection encoding run) processes. Memory performance was tested one day later in both a location-independent free recall task and a cued location-word association recall task using screenshots of the potential word presentation locations as spatial cues. Immediately before and after the encoding runs, participants underwent a resting state fMRI scan.

Our hypothesis was that ghrelin would enhance both memory encoding and consolidation, particularly for food-related information associated with spatial locations.

We further hypothesized that these memory-enhancing effects would be reflected by specific activation changes in appetite- and memory-related brain regions such as the orbitofrontal cortex, insula, nucleus caudatus, nucleus accumbens, amygdala, and hippocampus, both in task-related and resting state fMRI. In addition, we exploratively tested the effects of ghrelin on a cognitive test battery including working memory, fluid reasoning, creativity, mental speed and attention tasks.

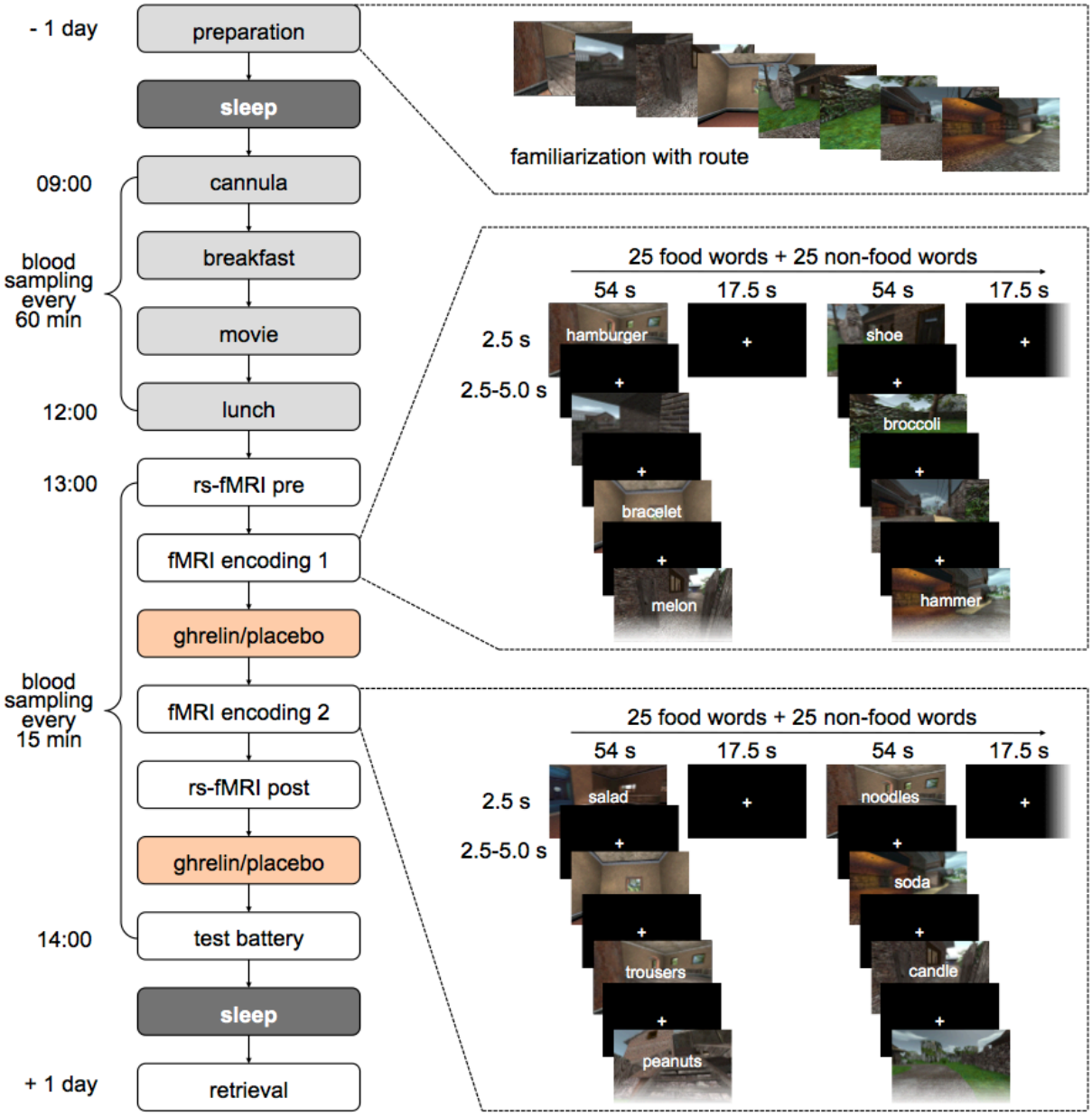


Figure 1: Overview of the test protocol. All 21 participants performed the schedule twice in a double-blind, randomized, placebo-controlled within-subject design. One hour after a standardized lunch, two encoding runs were performed under fMRI conditions, with intravenous ghrelin (or placebo) administration shortly before the second run. Before the first and after the second run, an eyes-closed resting state scan (rs-fMRI) was recorded. Immediately after the inside-fMRI sessions, a second dose of ghrelin (or placebo) was given and participants underwent a cognitive test battery. Memory performance was tested one day after encoding with free word recall and cued location-word association recall.

6.4 Materials and Methods

6.4.1 Participants

Twenty-one male, healthy, right-handed volunteers at the age of 23±3 years (mean±SD, range: 20-30) years and with a bodyweight of 72±7 kg (range: 60-80 kg) participated in our study. Their health status was confirmed with a medical screening including psychiatric interview; blood screening (full blood count, urea and electrolytes, liver function parameters, thyroid function parameters, inflammatory markers); urine screening for infections and drugs; comprehensive questionnaire covering eating and sleeping habits and intake of alcohol and caffeine, and assessment of verbal competence via a standardized German vocabulary test (MWT-B[103]).

Exclusion criteria were as follows: 1) irregular eating patterns or dietary restraints including vegetarian/vegan/lactose-free or non-Western diet; 2) history of or ongoing inflammatory, degenerative, neoplastic, endocrine, metabolic, cardiovascular, neurological or psychiatric disease or serious injuries; 3) history of or ongoing drug abuse; 4) irregular chronobiological rhythm including shift work or late-night work; 5) ferromagnetic objects inside the body, claustrophobia or other conditions that are not compatible with fMRI procedures; 6) non-right handedness according to the Edinburgh Handedness Inventory; 7) non-native German language use. For a period of one week before the first test block and until the last test block, participants were asked to stick to a three-meals-a-day rhythm. During test blocks, participants were asked to completely refrain from caffeine and alcohol consumption. Ethical approval was granted by the ethics committee of the University of Munich. Accordingly, all participants gave written informed consent.

6.4.2 Experimental design and procedures

Participants were tested in a randomized, placebo-controlled, within-subject crossover design. All participants completed two two three-day test blocks (each consisting of a pre-test, a learning trial and a re-test; see figure 1), which were about two weeks (14±4 days) apart. The nights in between the test days were spent at home. On pre-test days, we explained the general procedure of the main learning trials to our participants in order to avoid unnecessary delays particularly after the time-sensitive administration of ghrelin.

During the main test day, participants arrived at our institute at 09.00 a.m. with no previous breakfast. Right after arrival, a standard venous cannula (18G or 21G, B.Braun, Germany) was inserted into an antecubital vein. Via this cannula, 5ml of blood were taken every 60 min, during the in-scan learning session and during the cognitive test battery, a blood sample was taken every 15 min (figure 1). The blood was first filled into tubes containing 150µg of Aprotinin/150µg EDTA and put on ice for a maximum of 60 min before centrifugation and freezing of the serum samples. In order to prevent the blood in the cannula from clotting, participants received a constant infusion of NaCl 0,9% (B.Braun, Germany) with 400 I.U./500ml NaCl of high molecular weight heparin (Ratiopharm, Germany) at a controlled speed of 50-70ml per hour, reaching a total of 500 – 700 ml per test day. Serum ghrelin levels were measured via radioimmunoassay by the Max Planck Institute of Psychiatry clinical chemistry core unit (Ghrelin active RIA kit, DRG Instruments GmbH, Marburg, Germany).

Volunteers received a standard breakfast of two wheat rolls, butter and jam, a small sausage and 200ml of orange juice (in total approx. 520kcal/2200kJ, proteins 11g, fat 21g, carbohydrates 70g) right after intravenous catheterization, and a standard lunch of turkey steak with mushroom sauce, boiled rice and vegetables plus a chocolate

pudding as a desert (in total approx. 550kcal/2300kJ, proteins 27g, fat 13g, carbohydrates 80g) between 12.00 and 12.30 p.m. Water was offered ad libitum to participants throughout the entire test day. All participants reported sufficient satiety levels after lunch. Before the beginning of the in-MRI learning sessions at around 1.00 p.m., a 45mins break was taken beginning at the start of lunch. The time between breakfast and lunch was filled with a movie. All trainings and tests were performed in the same rooms supervised by the same lab personell.

Before the second encoding session, participants received a semi-bolus of 100µg acyl ghrelin (Bachem, Switzerland) diluted in 5ml aqua ad injectabilia (B.Braun, Germany) or a placebo of 5ml NaCl 0,9% (B.Braun, Germany). The ghrelin dose, representing a quantity in the middle of the spectrum given in previous studies[32,40], was given over a period of 2-3 min, injecting 1ml of the solution every 30-45 sec. To avoid losing any ghrelin in the blood withdrawal system, the volume of the tubes was measured in advance and pre-filled with ghrelin solution before the 30-45 sec injection intervals were started and flushed with several milliliters of saline right after the ghrelin injection. There was a delay of about 10 min from the end of the injection period until the beginning of the second encoding session in order to ensure a sufficient central bioavailability during the learning process. Due to acyl ghrelin's short half-life time of about 8-12 min[104,105], after the second resting state scan we injected another 100µg of ghrelin intravenously to ensure approximately the same amount of ghrelin being measurably available in the participant's organism during the subsequent cognitive test battery (see also supplemental figure S1). Ghrelin or placebo was administered consistently within test days, i.e. participants received either two ghrelin or two placebo injections on a given test day.

None of the participants reported any adverse effects of ghrelin administration such as nausea, vomiting, headache, dizziness or worse. As some of these side effects have been reported in a previous systematic study on ghrelin's pharmacological properties in humans[67], we suspect that possibly administration as a semi-bolus may be beneficial. Although our cognitive test battery did not include e.g. explicit hunger ratings as a subjective indicator of ghrelin efficacy, participants were we able to indicate their assumption about receiving ghrelin with relatively high acuity on a visual analogue scale (74+/-22 vs. 26+/-24 in the ghrelin vs. placebo condition, respectively).

6.4.3 Cognitive testing

For preparation of the learning task, participants trained on two three-dimensional virtual tracks before every test day. Similar simulations of spatial navigation have been successfully used in fMRI studies of spatial and grid cell-like processes in the human medial temporal lobe before (Doeller et al., Nature 2010; Kunz et al., Science 2015). Every participant had to walk these virtual tracks (Sauerbraten/Cube 2, sauerbraten.org) marked by black boxes four times, once with the help of a test assistant, once on his own and twice counting black boxes. These boxes were placed exactly where screenshots were taken of the track and where the words to be learned the next day would appear during the learning sessions. Screenshots were taken in approximately the same virtual distance and presented in the order of the track. The number of boxes counted by the subject were noted and compared to the actual number placed on the track in order to control training compliance.

On the test day, the spatio-verbal learning task consisted of two encoding runs with 50 words each (25 food-related, 25 non-food-related), in order to test ghrelin effects on both consolidation (first run) and encoding (second run). All words were

common German nouns (note that in figure 1, nouns are shown in English for better understanding only); encoding difficulty was matched between lists and tested in pilot trials in different subjects. The words were presented on screenshots of the two tracks the volunteers had walked the day before in the order of the black boxes, imitating the very same virtual walks. Screenshots were presented in blocks of eight images for 2500ms each, separated by a jittered (2500–5000ms) fixation cross. Each encoding block was started with a brief instruction and contained 4-7 screenshots with words and 1-4 empty screenshots in pseudo-random order. For each encoding run, in sum, 50 words were placed on 80 screenshots (i.e. including 30 word-free screenshots). In between the encoding blocks, there was a rest block (fixation cross) of 17.5 seconds, during which participants had been instructed not to rehearse.

Participants´ memory was tested on the following day in a two-steps retrieval test. First, a free recall session of 7 min was held in which participants were asked to write down on a blank sheet any of the words they still remembered from any of the two tracks from the previous day without any cueing. In a second step, empty screenshots of the tracks used the day before were presented via the program E-Prime. Each screenshot was presented for a duration of 3 sec followed by a 30 sec response time (black screen) in which participants were supposed to write down using a computer keyboard what item they think was placed on the screenshot of this particular location. All of the 2x80 screenshots were presented during the E-Prime session regardless of whether a word had been shown on them or not.

A cognitive test battery of about 60 min immediately followed the in-fMRI learning session and subsequent second ghrelin administration. It comprised a nonverbal fluid reasoning test (BOMAT, 10 min version[106]), a working memory task (reverse digit span[107]), a creativity task (alternative uses[108]), a perceptual speed test

(trail making task ZVT[109]), and tests for reaction times and psychomotor vigilance (PVT[110]).

6.4.4 Statistical analysis

For free word recall, cued location-word association recall, and a combined score including all words correctly recalled during free or cued recall independent of position, repeated measures ANOVAs were performed, each comprising the factors *condition* (ghrelin or placebo), *time* (consolidation vs. encoding), and *stimulus* (food vs. non-food items) for the spatial-verbal learning task. For the cognitive test battery, a repeated measures ANOVA with the factor *condition* (ghrelin or placebo) was performed. All behavioral data was analyzed using IBM SPSS Statistics Version 22 (IBM, Armonk, NY), an α of $p<.05$ was considered significant. Separate power calculations for *condition* main effects, *condition* × *time* interactions, and *condition* × *stimulus* interactions were performed for each free recall, cued location-word association recall, and a combined score of these with G*Power 3[111], assuming medium effect sizes of $f=.25$. We further performed Bayesian repeated measures ANOVAs with default prior scales for the free/cued recall combined score and the cognitive test battery using JASP Version 0.7.5.6 (jasp-stats.org).

6.4.5 fMRI data acquisition

Whole-brain functional images were acquired on a 3T (GE Discovery MR750) scanner using a 2D gradient echo planar image sequence. For both the task and the resting state scans we used a repetition time (TR) of 2.5 s, an echo time (TE) of 30 ms and a flip angle of 90°. For the resting state scans we acquired 34 interleaved slices with a field of view

(FOV) of 24 cm x 24 cm, a matrix size of 64 x 64, resulting in an in-plane spatial resolution of 3.75 mm, and a slice thickness of 3 mm and a slice gap of 1 mm. In total 192 volumes were acquired. For the learning session scans we acquired 42 interleaved slices with a FOV of 24 cm x 24 cm, a matrix size of 96 x 96, resulting in an in-plane spatial resolution of 1.875 mm, and a slice thickness of 2mm and a slice gap of 0.5mm. In total we acquired 312 volumes.

6.4.6 fMRI data analysis

Preprocessing: All fMRI analyses were conducted using the FMRIB Software Library (FSL) version 6.0[112]. For preprocessing, the functional images were corrected for effects of head motion using MCFLIRT and the brain was extracted using BET. Slice time correction was done using Fourier-space time-series phase-shifting. For spatial smoothing we used a Gaussian kernel with full width half maximum of 6mm. The whole 4D Volume was normalized by multiplication by a single factor. To remove temporal drifts in the data we applied high pass filter with a sigma of 50s. 4 Dummy volumes were acquired and discarded.

Task-based analyses: All the different task-based analysis used a hierarchical general linear model (GLM) approach with three levels: a run level, a subject level and finally a group level. On the first level we modeled the events during each individual run: stimulus onsets as well as fixation effects were modeled. The stimulus events were split into later remembered and later forgotten items to be contrasted in a subsequent memory analysis. On the second level the data of the four runs (encoding 1 and 2 in the ghrelin or placebo conditions) were combined using a fixed effect model. This combination was either done by averaging all runs (task main effect), only contrasting the second run placebo versus ghrelin (drug main effect) or contrasting the second

versus the first run across the days (interaction run x drug). Then we used a mixed effect model to combine the results on the subject level to create the group statistics. Next to the regressors of interest all first level GLMs contained nuisance regressors for the white matter and cerebrospinal fluid signal (1 each, compartments were estimated using the segmentation tool of FSL fast), and 24 motion parameters (3 parameters for rotation, 3 for translation, 6 derivatives of these, 12 squares of all of these). All GLM contrasts were corrected for multiple comparisons using clusters determined by Z>2.3 and a (corrected) cluster significance threshold of $p < 0.05$. Of note, while it has recently been stressed that some cluster correction methods lead to inflated false positive rates, FSL FLAME as used here was reported to be largely exempt from these problems (Eklund et al., 2015).

For analyzing the design in a block fashion, we modeled the onset and duration of the blocks and contrasted encoding blocks with baseline fixation blocks. To investigate whether ghrelin modulates the BOLD response associated with the viewing of food vs. non-food words we used regressors for the onsets of food and no-food items and contrasted them within run and across runs. To assess the task-related brain activity associated to successful memory formation we performed a subsequent memory analysis using the later remembered items (either in the free or the cued recall) and contrasted them with the later forgotten items, independent of the type of item (food or non-food).

6.4.7 Resting state preprocessing

For the resting state data we applied the same preprocessing as for the task scans except that we removed two additional volumes at the start. For the ROI-based connectivity analysis we used ICA-AROMA[113], an ICA based denoising method that filters

out noise components from the data, also we regressed the global signal out as it would confound ROI to ROI correlation estimates. For the dual regression approach ICA-AROMA is not necessary as the noise components end up in separate ICA components.

Dual Regression analysis: To investigate ghrelin-induced changes in resting state networks we used dual regression[114]. Since we were most interested in changes of the default mode network and the salience network, we used the 20 dimensional ICA results of BrainMap[115,116] as components to regress against. These spatial maps were then used to generate subject specific maps and time series with dual regression[117]. The spatial maps were then compared between the conditions using the randomize permutation test implemented in FSL.

As a control analysis we repeated the dual regression, but this time instead of using the established networks of BrainMap we used Melodic to estimate independent components on the resting state data itself. To have an unbiased estimate we used FSL Melodic to estimate the ICs during post-encoding rest in the placebo condition and then regressed those components against post-encoding rest in the drug and the placebo condition. The number of dimensions of the ICA was estimated using the Laplace approximation to the Bayesian evidence of the model order.

6.4.8 ROI based analysis

For analyzing whether ghrelin induced changes in functional connectivity not on a network level but on a smaller scale, we conducted an ROI based resting state analysis. The ROIs were based on previous studies[46,68] and included the amygdala, hippocampus, caudate nucleus, nucleus accumbens, insula and the orbitofrontal cortex. We created the ROIs from the Harvard Oxford Cortical and subcortical atlas included in FSL. For each region we extracted the time series for each voxel. Between regions correlations were

calculated by correlating the mean time series per region. The correlation of each region with the rest of the brain was calculated by correlating the mean time series of the ROI with the mean time series of the rest of the brain. To test differences for significance we used a permutation test.

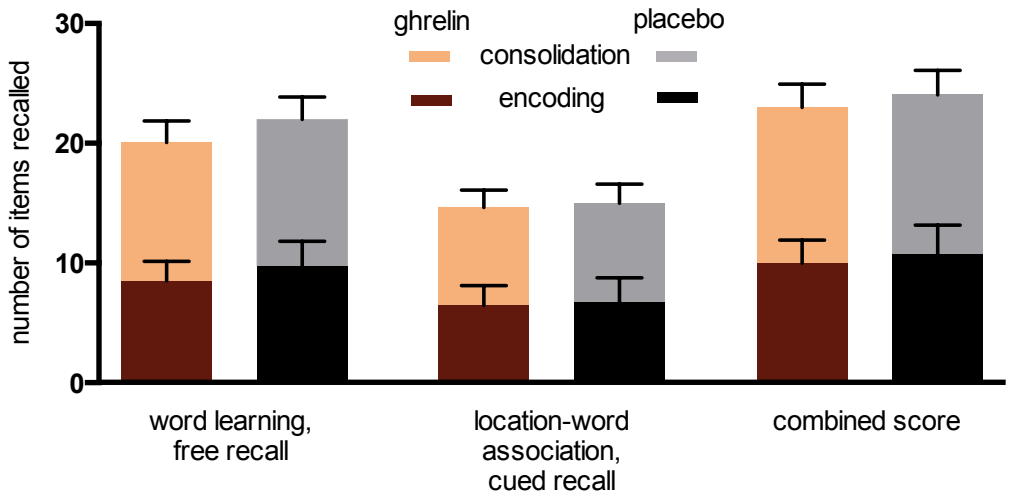


Figure 2: Ghrelin administration did not lead to improved memory encoding or consolidation for any of the outcome measures. Combined score represents items that were correctly recalled in free recall of words or cued recall of location-word associations. Bars indicate SEM.

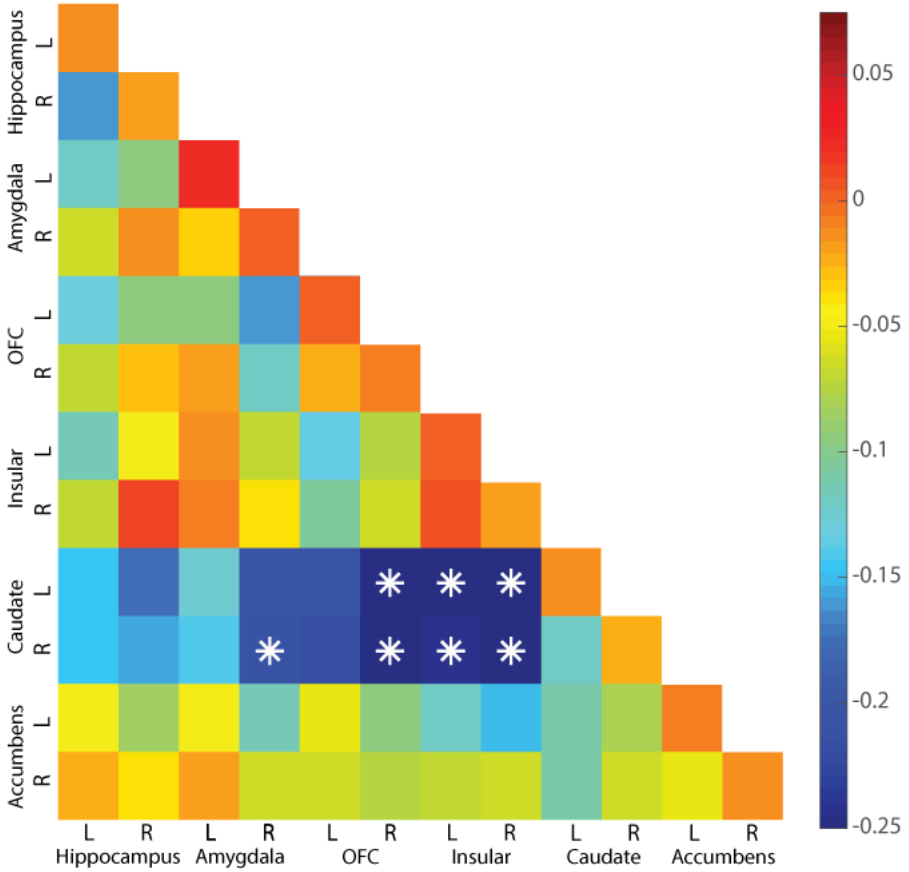


Figure 3: Comparing both resting state scans (one before, one after ghrelin application), we found decreased functional connectivity of the bilateral caudate nucleus with the bilateral insula and right orbitofrontal cortex, and of the right caudate nucleus with the right amygdala in the ghrelin condition. Significant effects on an FDR-corrected p<.05 level are indicated by an asterix.

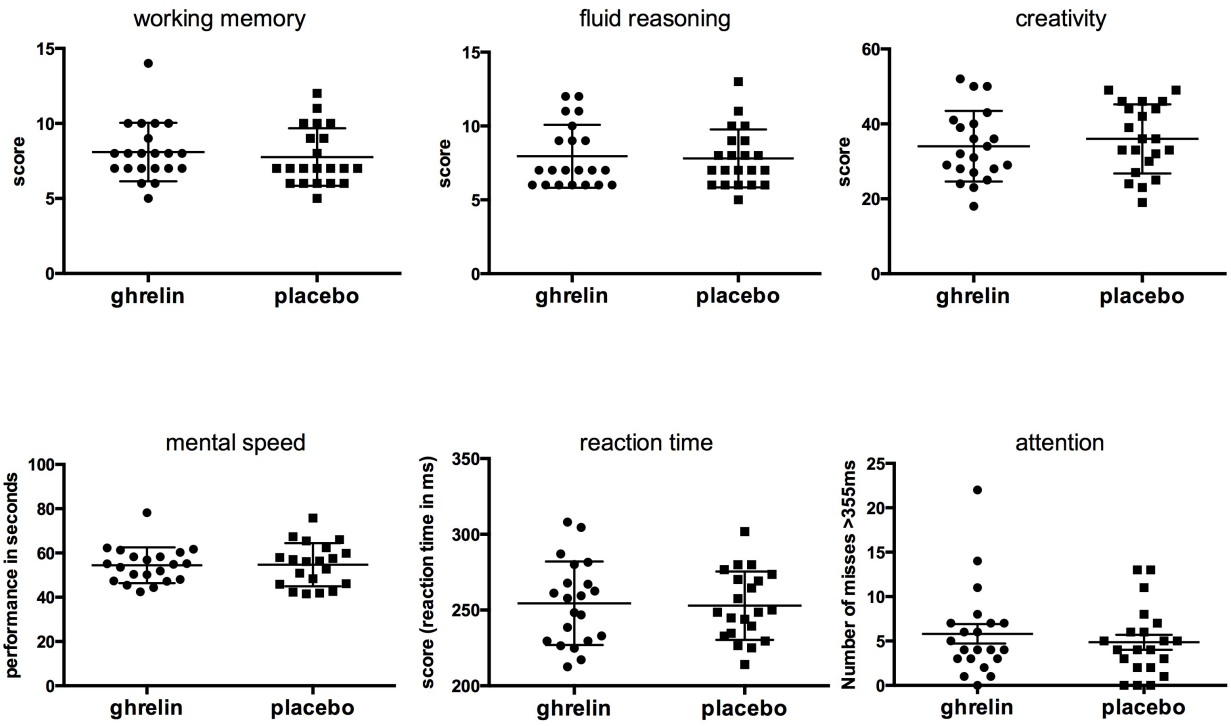


Figure 4: Performance in none of the tests used in our cognitive test battery was influenced by ghrelin administration. Results in a working memory task (reverse digit span), a fluid reasoning test (BOMAT matrices), a creativity task (alternative uses), a mental speed test (trail making), a reaction time task (psychomotor vigilance task, PVT: mean reaction times of the fastest 10 reactions in ms) and an attention task (PVT: number of misses defined as reaction time over 355ms) were not different between conditions (all $F<2.13$, $p>0.16$). Bars indicate SEM.

6.5 Results

In contrast to our hypotheses and a body of animal research, we did not find any positive effects of ghrelin administration on a spatial-verbal learning task (figure 2). As we injected ghrelin/placebo between two subsequent learning runs, we aimed to differentiate between potential ghrelin effects on pure consolidation processes (first run, before ghrelin application) and encoding processes (second run, after ghrelin application). Given that previous findings show better memory performance for food versus nonfood items in physiological states of hunger[118] and after ghrelin[46], we used food and non-food items as stimuli. In a repeated measures ANOVA comprising the factors *condition* (ghrelin vs. placebo), *time* (consolidation vs. encoding) and *stimulus* (food vs. non-food), we observed no significant main effect of *condition* on free word recall ($F_{1,20}$=.356, p=.558, η^2=.017), cued location-word association recall ($F_{1,20}$=.014, p=0.906, η^2=.001) or a combined score comprising all words remembered in both free and cued recall ($F_{1,20}$=.271, p=.608, η^2=.013). We further observed no significant *condition* × *time* interaction, *condition* × *stimulus* interaction, or *condition* × *time* × *stimulus* interaction for any of the outcome measures (all F<1.08, p>.311, η^2<.051; see figure 2 and supplemental table T1). Given our sample size and within-subject correlations of test scores, medium-sized main effects of ghrelin and medium-sized *condition* × *stimulus* interactions would have been detected with >95% probability for each free recall, cued recall or a combined score of these. Medium-sized *condition* × *time* interactions would have been detected with >90% probability for free recall, and with >95% probability for cued recall or the combined score. Bayesian analyses of the combined score were in favor of the Null model (*condition* BF_{10}=0.25; *condition* × *time* interaction BF_{10}=0.33). Since memory was nominally even worse under ghrelin as

compared to placebo, positive effects of ghrelin on the performed memory tasks can be excluded with considerable confidence.

To test the effects of ghrelin on a neurobiological level, we first analyzed the interaction of *condition* (ghrelin vs. placebo) and *time* (consolidation vs. encoding run) on task-related fMRI BOLD response for the contrast between encoding vs. rest blocks. We found the right occipital cortex, right lingual gyrus and right fusiform gyrus to be more activated in the ghrelin as compared to the placebo condition (see supplemental figure S2/supplemental table T2), however, effects in neither of these regions were related to memory performance (all p>.2). To further test whether ghrelin affected the task-related fMRI BOLD response associated with successful memory formation, we conducted a subsequent memory analysis and then tested whether the activation was modulated by ghrelin. Contrasting all correctly remembered items with the forgotten ones per subject across all sessions revealed activation in regions known to be related with subsequent memory for words and verbal associations[119] such as the left intraparietal sulcus, bilateral fusiform gyrus, left parahippocampal gyrus, and left superior frontal gyrus, and deactivations in the right frontal pole and right lateral occipital cortex (see supplemental figure S3/supplemental table T3), which is congruent with our design employing words presented in front of scenes of a virtual route. In a next step, we tested if ghrelin modulates this subsequent memory effect by contrasting ghrelin and placebo conditions. We found increased activation of the left intraparietal sulcus, bilateral occipital cortex and precuneus and decreased activation in the left frontal pole under ghrelin (figure S3/table T3). Again, however, these differences between ghrelin and placebo conditions in the subsequent memory effect did not correlate with memory performance (all p>.4). In an additional analysis of the fMRI BOLD response associated with the viewing of food stimuli, we found altered encoding-

related brain processing for food words as compared to non-food words in the precuneus, occipital cortex and left superior frontal gyrus (see supplemental figure S4). However, we did not find any enhancing or modulating effect of ghrelin on the behavioral or neurobiological effects of stimulus type, i.e. food vs. non-food items.

To test whether ghrelin modulated brain activation during rest, we first performed an independent component analysis (ICA) with subsequent dual regression on the fMRI resting state data in order to search for ghrelin-induced differences in large-scale functional brain networks. Setting the focus on memory- and appetite-related changes, we restricted our analysis to the default mode network and the salience network. A comparison of functional connectivity within these networks did not yield any significant differences between conditions.

In addition to the ICA dual regression approach, we also performed a connectivity analysis of the fMRI resting state data between the following regions of interest (ROI) of each hemisphere based on previous literature[46,68]: hippocampus, amygdala, orbitofrontal cortex (OFC), insula, caudate nucleus, and nucleus accumbens. In the post- as compared to pre-encoding resting state, we found a reduction of functional connectivity of the bilateral caudate nucleus with the right orbitofrontal cortex and bilateral insula, and between the right caudate nucleus and the right amygdala under ghrelin compared to placebo (all $p_{FDR}<.05$; see figure 3).

We did not detect any influence of ghrelin on other cognitive domains. Performances in a working memory task (reverse digit span), a fluid reasoning test (BOMAT matrices), a creativity task (alternative uses), a mental speed test (trail making), and a reaction time and attention task (psychomotor vigilance) did not differ significantly under the influence of ghrelin vs. placebo (all $p>0.160$; figure 4). All

Bayesian analyses of the cognitive test battery were in favor of the Null model (BF_{10} between 0.3 and 0.8).

Throughout both test days, blood samples were first taken hourly, then every 15 minutes (figure 1). In the ghrelin condition, serum ghrelin levels both during the encoding block and during the cognitive test battery (see supplemental figure S1) were markedly higher than baseline, demonstrating that participants performed all cognitive tasks under strong ghrelin influence in the ghrelin condition.

6.6 Discussion

Besides its role in metabolic processes, accumulating evidence from animal models points to an enhancing role of ghrelin on fear learning, object recognition and spatial memory, in particular when given before the encoding phase of memory formation[1]. On this background, the central ghrelin receptor has been proposed as a target for cognitive enhancement interventions also in humans [12]. In contrast to animal research, however, evidence for a role of ghrelin in human memory is sparse. Memory for food- compared to non-food-related pictures was enhanced after administration of ghrelin in an item recognition memory paradigm [46], whereas nocturnal ghrelin administration had no positive effect on sleep-related consolidation of a motor sequence learning task[102]. Effects of ghrelin on more complex cognitive processes including encoding or consolidation of hippocampus-dependent memories of spatial or verbal information have not been studied yet.

Many of the cognitive enhancing effects of ghrelin in rodents were observed in hippocampus-dependent spatial learning tasks such as the water maze[17] or the plus maze[7]. Due to its dual role in appetite and memory regulation, ghrelin has been

suggested to enhance spatial memory for food-associated locations, possibly supporting evolutionary functions related to foraging[98,99]. Our spatial learning task was designed to associate appetitive and non-appetitive verbal material with a background of a naturalistic environment based on a three-dimensional navigational computer game, thereby testing this foraging function hypothesis. In contrast to both animal research and our hypothesis, we did not observe any enhancing effects of ghrelin administration on either the encoding or consolidation phase of a spatial-verbal association task. This was true for both food and non-food related items, and both for free and spatially cued recall. As all learned stimuli had to be recalled one day after encoding, these effects are independent from potentially modulating effects of ghrelin on retrieval.

On the neurobiological level, ghrelin increased activity in the right occipital cortex, right lingual gyrus and right fusiform gyrus during encoding (see supplemental figure S2/supplemental table T2), however this effect was unrelated to memory performance. Ghrelin also modulated the subsequent memory effect in the left intraparietal sulcus, bilateral occipital cortex, precuneus, and left frontal pole. This suggests that successful memory formation was achieved differently under ghrelin as compared to placebo, however without any effect on overt behavioral memory performance.

During post-encoding rest, ghrelin administration led to decreased functional connectivity of the caudate nucleus with the amygdala, insula and orbitofrontal cortex (see figure 3). Generally, ghrelin's interaction with dopaminergic brain circuits is well established, and a negative association of the connectivity of these brain regions with ghrelin levels has been demonstrated for task-related fMRI data before: Obese individuals, who are known to exhibit decreased ghrelin levels[120], show increased connectivity of the caudate nucleus with the amygdala, insula, and prefrontal regions

during presentation of appetizing pictures[121]. However, due to the lack of behavioral ghrelin effects on encoding or consolidation in our study, these functional connectivity changes are unlikely to be related to memory processes.

Previous studies found ghrelin effects on pleasantness ratings of food items that mimicked fasting[68]. In addition, viewing food items versus control increased ghrelin release[122] and activated reward and memory regions such as orbitofrontal cortex, nucleus accumbens, amygdala, insula, hippocampus and the caudate nucleus[68-46]. Enhancing effects of ghrelin on recognition of food pictures[46] might therefore be mediated by enhanced reward processing related to food stimuli[123–125]. In our study, we found better free recall performance on the behavioral level and altered encoding-related brain processing for food words as compared to non-food words on the neurobiological level (see supplemental table T1 and figure S4). However, we did not find any enhancing or modulating effect of ghrelin on the behavioral or neurobiological effects of stimulus type, possibly due its abstraction level or salience: food names in contrast to pictures of food. Instead of profiting from the intrinsically rewarding effects of appetizing stimuli, participants might have utilized the food category as a cue that helped to prime food words, thus leading to better free recall in contrast to non-food words that did not form a single congruent category. This interpretation is supported by the fact that no significant difference between food and non-food stimuli was found for cued recall.

Ghrelin's role in memory processes might thus be restricted to simple tasks with a clear appetitive component that activates the reward system. In contrast, it does not increase memory performance for more abstract or non-appetitive information. A general memory enhancing effect of ghrelin on human memory would also be inconsistent with earlier findings that only recognition of food pictures but not scenes

profited from ghrelin administration[46]. In animal studies, memory tasks generally involve appetitive stimuli or other highly salient components such as fear in order to motivate the animals to perform the task, which might lead to performance enhancing effects in a broader range of memory tasks in animal models.

Baseline ghrelin levels after an overnight fast as well as ghrelin levels immediately before the administration of the first dose of ghrelin varied considerably, despite matching of our study participants regarding age/weight and thorough standardization of all test meals, possibly due to factors we did not standardize for in our study such as our participants' exact body composition[126,127]. However, hyperghrelinemia achieved after intravenous administration of ghrelin in our study reached considerably beyond the range of endogenous ghrelin levels (supplemental figure S1), thereby clearly overcompensating inter-individual differences in anthropometric and metabolic parameters. Cognitively modulating effects of ghrelin reported in other studies were achieved in different metabolic states, across sexes and different age groups on the basis on similarly supraphysiological levels of ghrelin[46,68]. Nonetheless, future studies need to address the question of susceptibility to exogenous ghrelin administration, e.g. by defining relevant metabolic predictors, in order to discern the subtle effects of ghrelin on central nervous processes which have been shown to depend on metabolic state in rat models[128–130]. As food availability seems to play an important role when measuring cognitive effects of the peptide[131,132], we strictly standardized food intake during test days. Further, order effects can be a concern in within-subject crossover designs, since improvements in cognitive tasks from first to second session might occur and interact with the drug. Including the order of placebo vs. ghrelin injections as a between subject factor into the repeated measures ANOVA,

however, we did not find any order × drug condition interaction effects on encoding or consolidation as assessed by either free or cued recall (all $F<.2$, $p>.6$).

It is important to note that recall was tested one day after memory acquisition. While early studies on ghrelin's role in memory formation and cognition almost exclusively looked at short-term processes (Carlini et al., 2002; Diano et al., 2006), recent evidence suggests that robust findings that are also independent from arousal effects by acute administration are found in long-term treatment studies (Dhurandhar et al., 2013; Kunath et al., 2015) and likely depend on neurogenic effects (Cahill et al., 2014; Kent et al., 2015; Hornsby et al., 2016).

A further crucial aspect in the interpretation of the lack of behavioral effects is the possibility that i.v. ghrelin did not reach those brain regions relevant for learning and memory. In animal models, divergent findings suggest that there may be differences between species concerning the amount of ghrelin crossing the blood-brain barrier and the relevant binding sites[7,133,134]. We can present only indirect indicators as to what extent active ghrelin actually crossed the blood-brain barrier and became available to learning-related brain regions. Whereas we observed amygdala connectivity to be modulated by ghrelin during post-encoding resting state, we did not find hippocampal activity to be affected by ghrelin during either task or rest. Future studies in humans involving technologies such as MR-spectroscopy, PET-MRI or the measurement of cerebrospinal fluid levels may draw a clearer picture of how and where exactly centrally available ghrelin modulates brain metabolism. Given ghrelin's considerable interactions with glucose homeostasis[54,135,136], such studies should also consider the possibility that indirect effects mediated by systemically higher or lower glucose levels made available for brain metabolism may be more important than the actual direct binding of ghrelin to the GHS-R1a itself.

The aim of this study was to draw a more comprehensive picture of ghrelin's short-term effects on human memory and general cognitive performance. As we observed no improvement in any cognitive domain tested in our trial, we conclude that ghrelin does not generally act as a short-term cognitive enhancer in humans. Differences in the fMRI subsequent memory effect suggest that successful memory formation might have been achieved differently under ghrelin, however without any effect on overt behavioral memory performance. It will have to be tested if this lack of behavioral effect in humans will also hold for information with stronger appetitive valence or fear/stress components and under a long-term perspective. We suggest that future studies aiming at transferring the promising data on ghrelin's memory effects in rodents on human samples should make a clear-cut differentiation of ghrelin's short-term actions as an orexigenic neuropeptide possibly modulating certain cognitive functions such as food preference and appetitive behavior[46,122,137,138] and its potential neuroprotective effects in long-term or pathological models[16,17,70], at the same time thoroughly taking into account aspects of susceptibility and dosage.

6.7 Supplemental data

	free word recall	cued association recall	combined score
drug	$F_{1,20}$=.356, p=.558, η^2=.017	$F_{1,20}$=.014, p=.906, η^2=.001	$F_{1,20}$=.271, p=.608, η^2=.013
time	$F_{1,20}$=6.415, p=.020, η^2=.243	$F_{1,20}$=3.237, p=.087, η^2=.139	$F_{1,20}$=8.700, p=.008, η^2=.303
stimulus	$F_{1,20}$=8.273, p=.009, η^2=.293	$F_{1,20}$=.816, p=.377, η^2=.039	$F_{1,20}$=3.720, p=.068, η^2=.157
drug × time	$F_{1,20}$=.059, p=.811, η^2=.003	$F_{1,20}$=.018, p.896=, η^2=.001	$F_{1,20}$=.023, p=.882, η^2=.001
drug × stimulus	$F_{1,20}$=.828, p=.374, η^2=.040	$F_{1,20}$=.028, p=.868, η^2=.001	$F_{1,20}$=.545, p=.469, η^2=.027
time × stimulus	$F_{1,20}$=.247, p=.625, η^2=.012	$F_{1,20}$=1.850, p=.189, η^2=.085	$F_{1,20}$=.070, p=.794, η^2=.003
drug × time × stimulus	$F_{1,20}$=.589, p=.452, η^2=.029	$F_{1,20}$=1.079, p=.311, η^2=.051	$F_{1,20}$=.328, p=.573, η^2=.016

Supplemental Table T1: For the three different outcome measures free word recall, cued location-word association recall, and a combined score of these two, repeated measures ANOVAs comprising the factors drug (ghrelin vs. placebo), time (consolidation vs. encoding) and stimulus (food vs. non-food) did not reveal any significant main effect of drug and no significant interaction of drug with any of the other factors. The analyses did reveal significant main effects of time, probably due to more interference in the second as compared to the first encoding run. Further, the analyses did reveal a significant stimulus effect for free recall, suggesting that participants could utilize the food category as a cue that helped to recall food words. Non-food word did not stem from a single congruent category, hence no categorical cue could be utilized for these. This interpretation is supported by the fact that no significant stimulus effect was found for cued location-word association recall.

Voxels	P	Z max	Z-max X	Z-max Y	Z-max Z	Z-COG X	Z-COG Y	Z-COG Z
645	0.000938	3.49	32	-82	24	25.8	-78	34.1
408	0.0195	3.66	24	-60	-10	24.1	-70.6	-6.67

Supplemental Table T2: Cluster showing a significant interaction of condition (ghrelin vs. placebo) and time (consolidation vs. encoding) in the positive contrast between encoding vs. baseline blocks. Effects are cluster-corrected at p<0.05 with Z>2.3. For each significant cluster, the number of voxels, the p-value, the maximum z-value, MNI space coordinates of the maximum z-value voxel, and coordinates of the center of gravity (COG) are given.

Subsequent memory effect, positive contrast:

Voxels	P	Z max	Z-max X	Z-max Y	Z-max Z	Z-COG X	Z-COG Y	Z-COG Z
1815	3.57E-10	3.71	-40	-50	62	-28.1	-60.9	52.7
817	2.06E-05	3.48	-34	-30	-20	-46.9	-58.6	-2.7
812	2.20E-05	3.95	10	10	66	-1.61	11.6	61.7
671	0.000141	3.4	-28	-2	64	-41.8	4.76	43.2
347	0.0181	3.48	50	-42	-20	47.5	-50.1	-11.8

Subsequent memory effect, negative contrast:

Voxels	P	Z max	Z-max X	Z-max Y	Z-max Z	Z-COG X	Z-COG Y	Z-COG Z
803	2.47E-05	3.47	54	-60	42	50.6	-59.6	42.8
422	0.00534	3.82	46	46	-8	44.3	49.5	-4.82
346	0.0184	3.35	14	70	18	16.6	62.5	25.4
337	0.0215	3.62	4	-48	22	5.51	-47	26.9

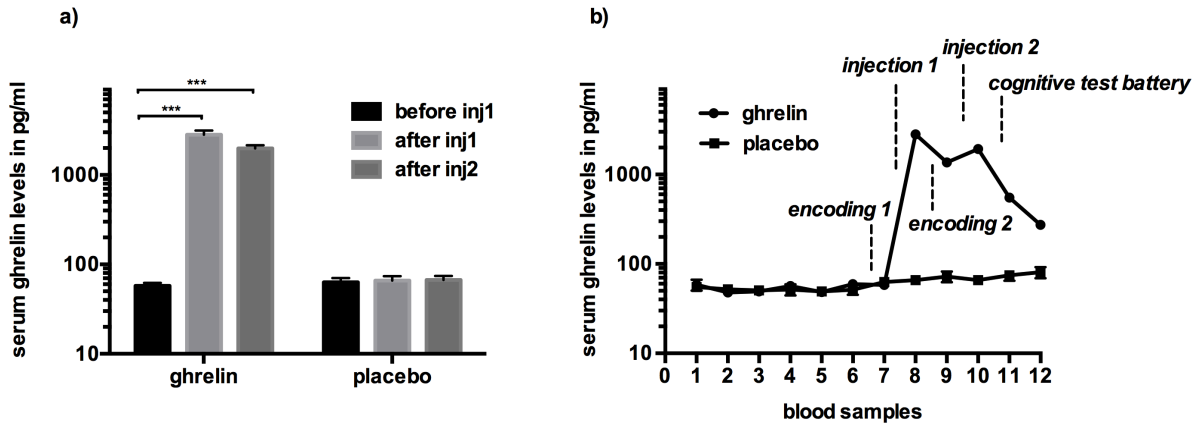
Ghrelin modulation of the subsequent memory effect, positive contrast:

Voxels	P	Z max	Z-max X	Z-max Y	Z-max Z	Z-COG X	Z-COG Y	Z-COG Z
1824	4.65E-10	3.75	20	-88	16	15.9	-72.1	22.7
611	0.000381	3.75	-36	-48	70	-37.7	-49.6	63.2
442	0.00446	3.37	-34	-84	16	-31.1	-80.3	19.2

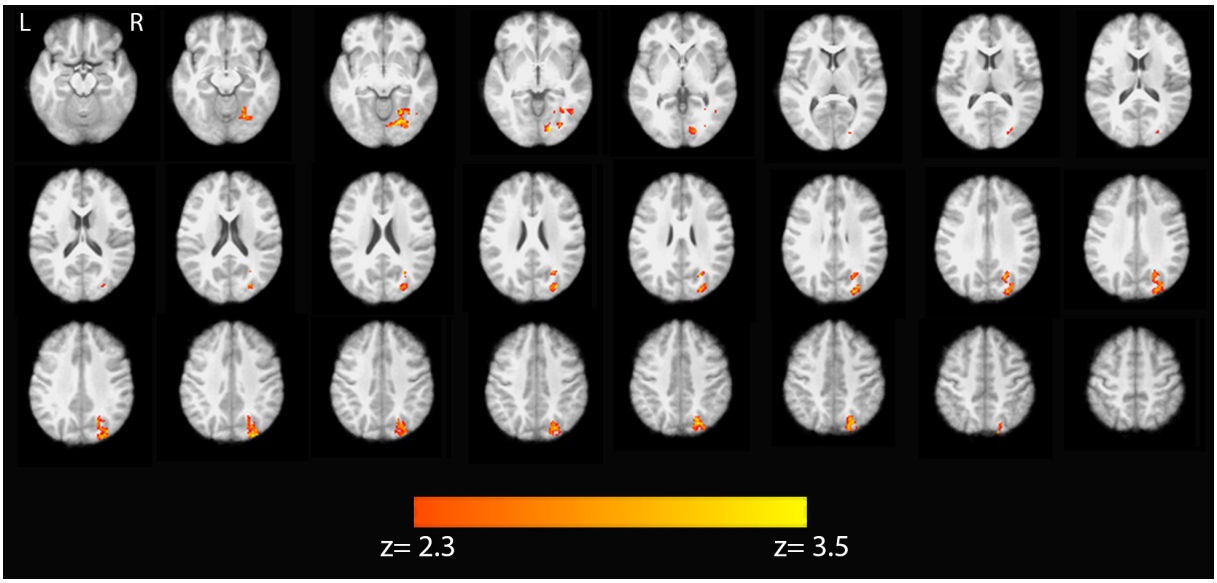
Ghrelin modulation of the subsequent memory effect, negative contrast:

Voxels	P	Z max	Z-max X	Z-max Y	Z-max Z	Z-COG X	Z-COG Y	Z-COG Z
406	0.00781	3.47	-20	58	8	-15	64	5.27

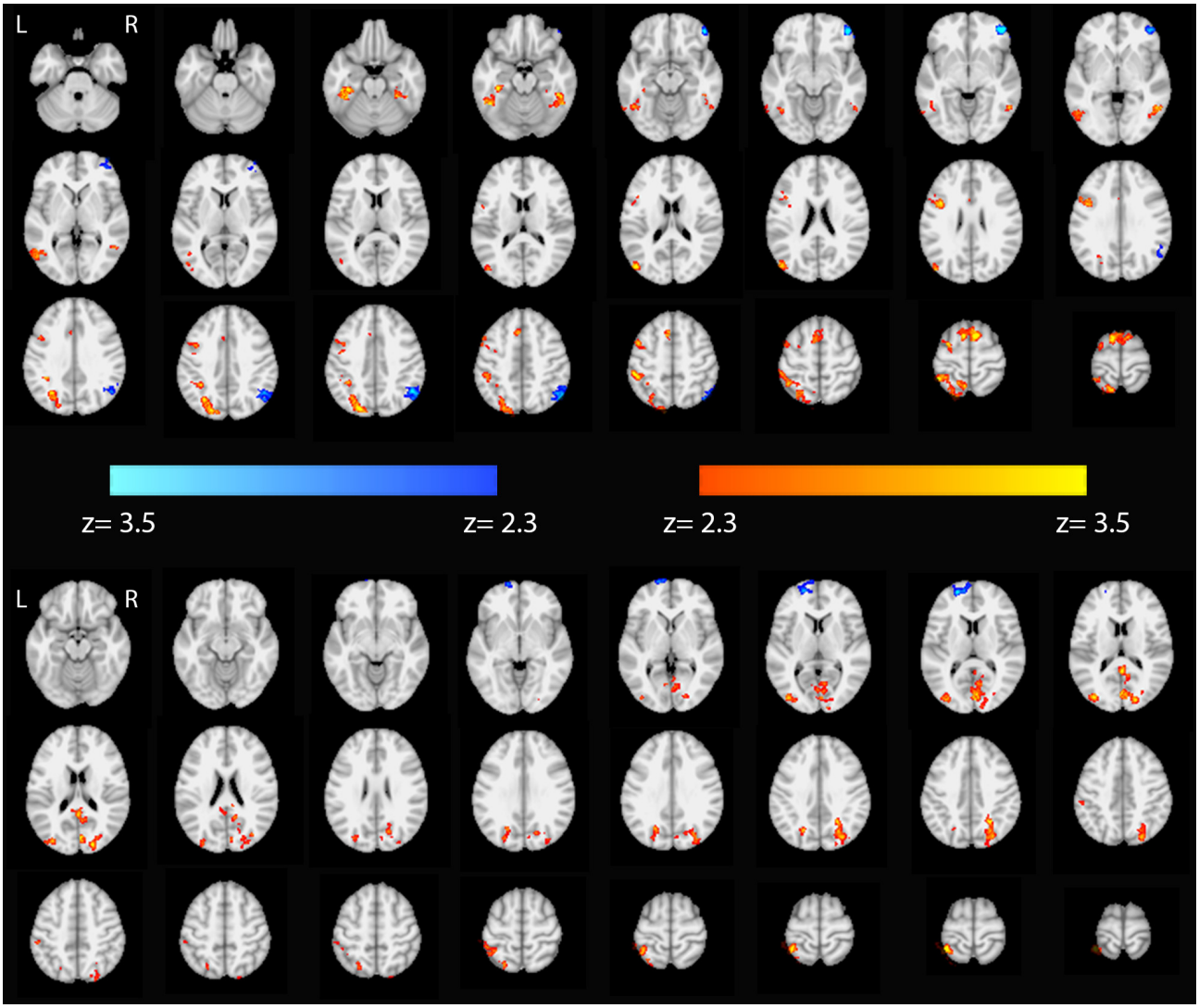
Supplemental Table T3: Cluster showing a significant subsequent memory effect (words remembered vs. words forgotten after 24h, combined score comprising all words recalled in free or cued recall), and a significant modulation by ghrelin of the significant subsequent memory effect. Effects are cluster-corrected at p<0.05 with Z>2.3. For each significant cluster, the number of voxels, the p-value, the maximum z-value, MNI space coordinates of the maximum z-value voxel, and coordinates of the center of gravity (COG) are given.



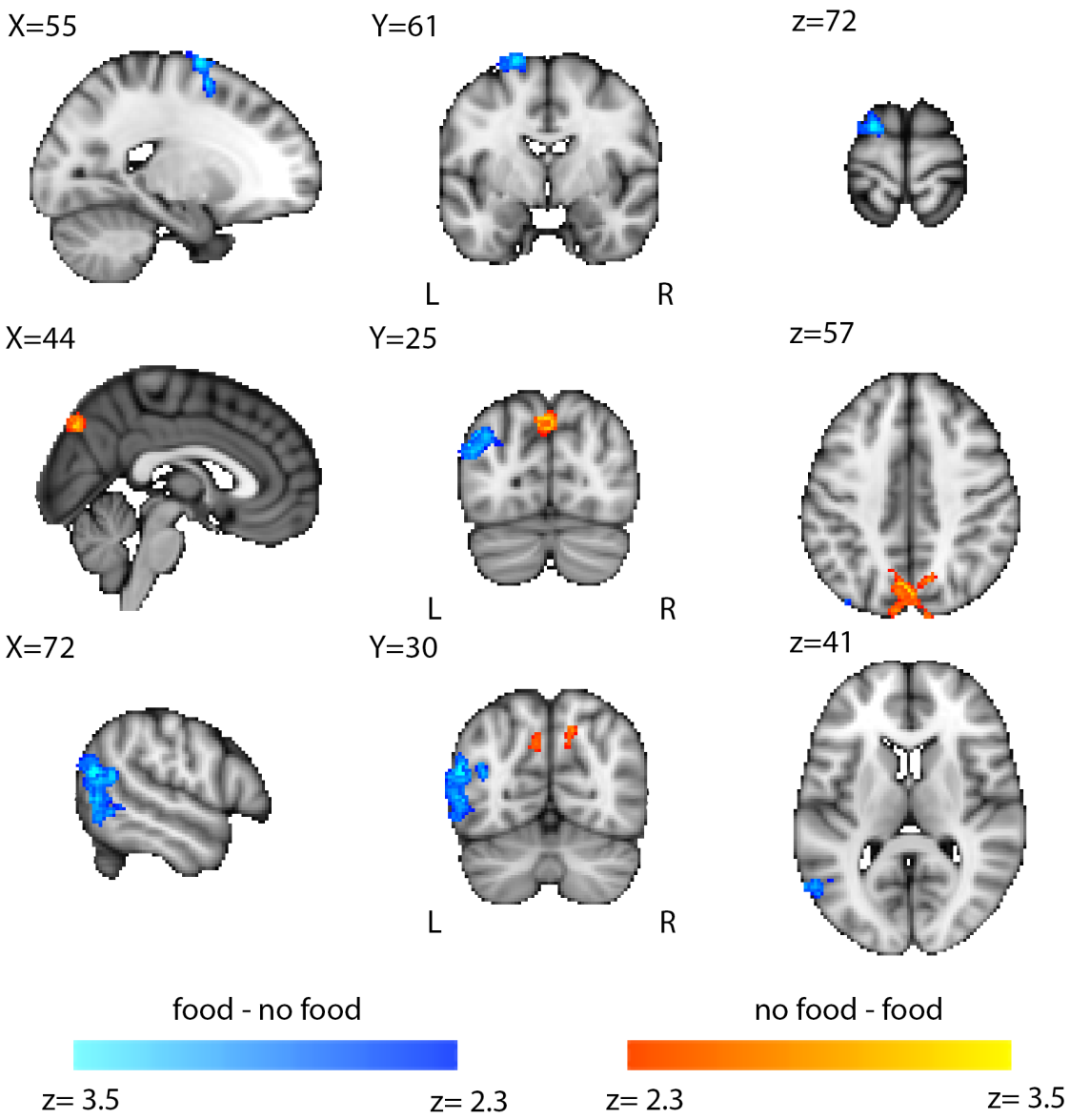
Supplemental Figure S1: a) Group average. Serum ghrelin levels were significantly higher (p<0.0001 each) both after the first and after the second injection than at baseline („before inj1", averaged values of samples taken before the first injection). Bars indicate SEM. b) Serum acyl ghrelin levels rose sharply after the first injection, then took a short dip due to ghrelin's short half-life time and rose again after the second injection before vanishing towards the end of each test day. Both during the second learning phase inside the MRI scanner and during the cognitive test battery, supraphysiological serum acyl ghrelin levels could be measured in all participants.



Supplemental Figure S2: Interaction of condition (ghrelin vs. placebo) and time (consolidation vs. encoding) in the contrast between encoding vs. baseline blocks. Effects are cluster-corrected at p<0.05 with Z>2.3. See supplemental table T2 and text for details.



Supplemental Figure S3, top: Subsequent memory analysis of words remembered vs. words forgotten after 24h (combined score comprising all words recalled in free or cued recall). Bottom: Subsequent memory effect as modulated by ghrelin. Effects are cluster-corrected at p<0.05 with Z>2.3. See supplemental table T3 and text for details.



Supplemental Figure S4: Brain activation related to the presentation of food vs. non-food words (main effect). Effects are cluster-corrected at p<0.05 with Z>2.3. See text for details.

7. References

1. Kunath, N. & Dresler, M. in *Central functions of the ghrelin receptor* (eds. Portelli, J. & Smolders, I.) 167–175 (Springer New York, 2014).
2. Kojima, M. *et al.* Ghrelin is a growth-hormone-releasing acylated peptide from stomach. *Nature* **402,** 656–60 (1999).
3. Tschöp, M., Smiley, D. L. & Heiman, M. L. Ghrelin induces adiposity in rodents. *Nature* **407,** 908–13 (2000).
4. Carlini, V. P. *et al.* Ghrelin increases anxiety-like behavior and memory retention in rats. *Biochem. Biophys. Res. Commun.* **299,** 739–43 (2002).
5. Carvajal, P., Carlini, V. P., Schiöth, H. B., de Barioglio, S. R. & Salvatierra, N. A. Central ghrelin increases anxiety in the Open Field test and impairs retention memory in a passive avoidance task in neonatal chicks. *Neurobiol. Learn. Mem.* **91,** 402–7 (2009).
6. Carlini, V. P., Ghersi, M., Schiöth, H. B. & de Barioglio, S. R. Ghrelin and memory: differential effects on acquisition and retrieval. *Peptides* **31,** 1190–3 (2010).
7. Diano, S. *et al.* Ghrelin controls hippocampal spine synapse density and memory performance. *Nat. Neurosci.* **9,** 381–8 (2006).
8. Steiger, A., Dresler, M., Schüssler, P. & Kluge, M. Ghrelin in mental health, sleep, memory. *Mol. Cell. Endocrinol.* **340,** 88–96 (2011).
9. Müller, T. D. *et al.* Ghrelin. *Mol. Metab.* **4,** 437–460 (2015).
10. Carlini, V. P. *et al.* Decreased memory for novel object recognition in chronically food-restricted mice is reversed by acute ghrelin administration. *Neuroscience* **153,** 929–934 (2008).
11. Goshadrou, F., Kermani, M., Ronaghi, A. & Sajjadi, S. The effect of ghrelin on MK-801 induced memory impairment in rats. *Peptides* **44,** 60–5 (2013).
12. Atcha, Z. *et al.* Cognitive enhancing effects of ghrelin receptor agonists. *Psychopharmacology (Berl).* **206,** 415–427 (2009).
13. Albarran-Zeckler, R. G., Brantley, A. F. & Smith, R. G. Growth hormone secretagogue receptor (GHS-R1a) knockout mice exhibit improved spatial memory and deficits in contextual memory. *Behav. Brain Res.* **232,** 13–9 (2012).
14. Spitznagel, M. B. *et al.* Serum ghrelin is inversely associated with cognitive function in a sample of non-demented elderly. *Psychiatry Clin. Neurosci.* **64,** 608–11 (2010).
15. Carlini, V. P. *et al.* Ghrelin induced memory facilitation implicates nitric oxide synthase activation and decrease in the threshold to promote LTP in hippocampal dentate gyrus. *Physiol. Behav.* **101,** 117–23 (2010).
16. Bayliss, J. a & Andrews, Z. B. Ghrelin is neuroprotective in Parkinson's disease: molecular mechanisms of metabolic neuroprotection. *Ther. Adv. Endocrinol. Metab.* **4,** 25–36 (2013).
17. Dhurandhar, E., Allison, D., Groen, T. Van & Kadish, I. Hunger in the absence of caloric restriction improves cognition and attenuates Alzheimer's disease

pathology in a mouse model. *PLoS One* **8,** 2–9 (2013).

18. Kunath, N. *et al.* Ghrelin agonist does not foster insulin resistance but improves cognition in an Alzheimer's disease mouse model. *Sci. Rep.* **5,** 11452 (2015).

19. Ghersi, M. S. *et al.* Ghrelin inhibited serotonin release from hippocampal slices. *Peptides* **32,** 2367–71 (2011).

20. Fuente-Martín, E. *et al.* Ghrelin Regulates Glucose and Glutamate Transporters in Hypothalamic Astrocytes. *Sci. Rep.* **6,** 23673 (2016).

21. Jiang, H., Betancourt, L. & Smith, R. G. Ghrelin amplifies dopamine signaling by cross talk involving formation of growth hormone secretagogue receptor/dopamine receptor subtype 1 heterodimers. *Mol. Endocrinol.* **20,** 1772–85 (2006).

22. Howard, A. D. *et al.* A receptor in pituitary and hypothalamus that functions in growth hormone release. *Science* **273,** 974–7 (1996).

23. Holst, B., Cygankiewicz, A., Jensen, T. H., Ankersen, M. & Schwartz, T. W. High constitutive signaling of the ghrelin receptor - identification of a potent inverse agonist. *Mol. Endocrinol.* **17,** 2201–10 (2003).

24. Cuellar, J. N. & Isokawa, M. Ghrelin-induced activation of cAMP signal transduction and its negative regulation by endocannabinoids in the hippocampus. *Neuropharmacology* **60,** 842–51 (2011).

25. Jiang, H., Betancourt, L. & Smith, R. G. Ghrelin amplifies dopamine signaling by cross talk involving formation of growth hormone secretagogue receptor/dopamine receptor subtype 1 heterodimers. *Mol. Endocrinol.* **20,** 1772–1785 (2006).

26. Schellekens, H., Dinan, T. G. & Cryan, J. F. Taking two to tango: a role for ghrelin receptor heterodimerization in stress and reward. *Front. Neurosci.* **7,** 148 (2013).

27. Hsu, T. M. *et al.* Hippocampus ghrelin signaling mediates appetite through lateral hypothalamic orexin pathways. *Elife* **4,** 1–20 (2015).

28. Hsu, T. M., Suarez, A. N. & Kanoski, S. E. Ghrelin: A link between memory and ingestive behavior. *Physiology and Behavior* **162,** 10–17 (2016).

29. Spencer, S. J., Emmerzaal, T. L., Kozicz, T. & Andrews, Z. B. Ghrelin's role in the hypothalamic-pituitary-adrenal axis stress response: Implications for mood disorders. *Biological Psychiatry* **78,** 19–27 (2015).

30. Nakashima, K. *et al.* Ghrelin gene polymorphism is associated with depression, but not panic disorder. *Psychiatr. Genet.* **18,** 257 (2008).

31. Lutter, M. & Elmquist, J. Depression and metabolism: linking changes in leptin and ghrelin to mood. *F1000 Biol. Rep.* **1,** 63 (2009).

32. Kluge, M. *et al.* Effects of ghrelin on psychopathology, sleep and secretion of cortisol and growth hormone in patients with major depression. *J. Psychiatr. Res.* **45,** 421–426 (2011).

33. Kluge, M. *et al.* Ghrelin enhances the nocturnal secretion of cortisol and growth hormone in young females without influencing sleep. *Psychoneuroendocrinology* **32,** 1079–1085 (2007).

34. Weikel, J. C. *et al.* Ghrelin promotes slow-wave sleep in humans. *Am. J. Physiol. Endocrinol. Metab.* **284,** E407–15 (2003).

35. Kluge, M. *et al.* Ghrelin plasma levels are not altered in major depression. *Neuropsychobiology* **59,** 199–204 (2009).

36. Yi, C.-X., Heppner, K. & Tschöp, M. H. Ghrelin in eating disorders. *Mol. Cell. Endocrinol.* **340,** 29–34 (2011).

37. Healy, J. E., Ostrom, C. E., Wilkerson, G. K. & Florant, G. L. Plasma ghrelin concentrations change with physiological state in a sciurid hibernator (Spermophilus lateralis). *Gen. Comp. Endocrinol.* **166,** 372–378 (2010).

38. Healy, J. E., Bateman, J. L., Ostrom, C. E. & Florant, G. L. Peripheral ghrelin stimulates feeding behavior and positive energy balance in a sciurid hibernator. *Horm. Behav.* **59,** 512–519 (2011).

39. Volkswagen-Stiftung. Volkswagen-Stiftung. *European Platform* 1 (2011). at <https://www.volkswagenstiftung.de/foerderung/schluesselergebnisse/herausforderung/european-platform-for-life-sciences-mind-sciences-and-the-humanities/bewilligungen-2011.html>

40. Tong, J. *et al.* The pharmacokinetics of acyl, des-acyl, and total ghrelin in healthy human subjects. *Eur. J. Endocrinol.* **168,** 821–828 (2013).

41. Banks, W. A., Tschöp, M., Robinson, S. M. & Heiman, M. L. Extent and direction of ghrelin transport across the blood-brain barrier is determined by its unique primary structure. *J. Pharmacol. Exp. Ther.* **302,** 822–7 (2002).

42. Banks, W. A., Burney, B. O. & Robinson, S. M. Effects of triglycerides, obesity, and starvation on ghrelin transport across the blood–brain barrier. *Peptides* **29,** 2061–2065 (2008).

43. Cummings, D. E., Frayo, R. S., Marmonier, C., Aubert, R. & Chapelot, D. Plasma ghrelin levels and hunger scores in humans initiating meals voluntarily without time- and food-related cues. *Am. J. Physiol. Endocrinol. Metab.* **287,** E297–304 (2004).

44. LeSauter, J., Hoque, N., Weintraub, M., Pfaff, D. W. & Silver, R. Stomach ghrelin-secreting cells as food-entrainable circadian clocks. *Proc. Natl. Acad. Sci. U. S. A.* **106,** 13582–7 (2009).

45. Ott, V. *et al.* Meal anticipation potentiates postprandial ghrelin suppression in humans. *Psychoneuroendocrinology* **37,** 1096–1100 (2012).

46. Malik, S., McGlone, F., Bedrossian, D. & Dagher, A. Ghrelin modulates brain activity in areas that control appetitive behavior. *Cell Metab.* **7,** 400–9 (2008).

47. Liu, J. *et al.* Novel ghrelin assays provide evidence for independent regulation of ghrelin acylation and secretion in healthy young men. *J. Clin. Endocrinol. Metab.* **93,** 1980–1987 (2008).

48. Ott, A. *et al.* Diabetes mellitus and the risk of dementia: The Rotterdam Study. *Neurology* **53,** 1937–1937 (1999).

49. Baskin, D. G. *et al.* Insulin and leptin: dual adiposity signals to the brain for the regulation of food intake and body weight. *Brain Res.* **848,** 114–23 (1999).

50. Schwartz, M. W. *et al.* Inhibition of hypothalamic neuropeptide Y gene expression by insulin. *Endocrinology* **130,** 3608–16 (1992).

51. Heni, M., Kullmann, S., Preissl, H., Fritsche, A. & Haring, H.-U. Impaired insulin action in the human brain: causes and metabolic consequences. *Nat Rev Endocrinol* **11,** 701–711 (2015).

52. Plum, L. Central insulin action in energy and glucose homeostasis. *J. Clin. Invest.* **116,** 1761–1766 (2006).

53. Luchsinger, J. A. Type 2 diabetes, related conditions, in relation and dementia: An opportunity for prevention? *Journal of Alzheimer's Disease* **20,** 723–736 (2010).

54. Tong, J. *et al.* Ghrelin suppresses glucose-stimulated insulin secretion and deteriorates glucose tolerance in healthy humans. *Diabetes* **59,** 2145–51 (2010).

55. Shi, L., Du, X., Jiang, H. & Xie, J. Ghrelin and Neurodegenerative Disorders-a Review. *Mol. Neurobiol.* (2016).

56. Solfrizzi, V. *et al.* Diet and Alzheimer's disease risk factors or prevention: the current evidence. *Expert Rev. Neurother.* **11,** 677–708 (2011).

57. Bomfim, T. R. *et al.* An anti-diabetes agent protects the mouse brain from defective insulin signaling caused by Alzheimer's disease- associated Aβ oligomers. *J. Clin. Invest.* **122,** 1339–53 (2012).

58. van Praag, H., Shubert, T., Zhao, C. & Gage, F. H. Exercise enhances learning and hippocampal neurogenesis in aged mice. *J. Neurosci.* **25,** 8680–5 (2005).

59. Kramer, A. F. & Erickson, K. I. Capitalizing on cortical plasticity: influence of physical activity on cognition and brain function. *Trends Cogn. Sci.* **11,** 342–348 (2007).

60. Snyder, H. M. *et al.* Vascular contributions to cognitive impairment and dementia including Alzheimer's disease. *Alzheimer's Dement.* **11,** 710–717 (2015).

61. Nagaya, N. *et al.* Hemodynamic and hormonal effects of human ghrelin in healthy volunteers. *Am J Physiol Regul Integr Comp Physiol* **280,** R1483–7 (2001).

62. Takayama, K. *et al.* Anamorelin (ONO-7643) in Japanese patients with non-small cell lung cancer and cachexia: results of a randomized phase 2 trial. *Support. Care Cancer* **24,** 3495–3505 (2016).

63. Keski-Rahkonen, A. *et al.* Epidemiology and course of anorexia nervosa in the community. *Am. J. Psychiatry* **164,** 1259–1265 (2007).

64. Fairburn, C. G., Cooper, Z., Doll, H. A., Norman, P. & O'Connor, M. The natural course of bulimia nervosa and binge eating disorder in young women. *Arch Gen Psychiatry* **57,** 659–665 (2000).

65. El-Sayed Moustafa, J. S. & Froguel, P. From obesity genetics to the future of personalized obesity therapy. *Nat Rev Endocrinol* **9,** 402–413 (2013).

66. Wren, A. M. *et al.* Ghrelin Causes Hyperphagia and Obesity in Rats. *Diabetes* **50,** 2540–2547 (2001).

67. Garin, M. C., Burns, C. M., Kaul, S. & Cappola, A. R. Clinical review: The human experience with ghrelin administration. *J. Clin. Endocrinol. Metab.* **98,** 1826–37 (2013).

68. Goldstone, A. P. *et al.* Ghrelin mimics fasting to enhance human hedonic, orbitofrontal cortex, and hippocampal responses to food. *Am. J. Clin. Nutr.* **99,** 1319–30 (2014).

69. Tong, J. *et al.* Acute administration of unacylated ghrelin has no effect on Basal or stimulated insulin secretion in healthy humans. *Diabetes* **63,** 2309–19 (2014).

70. V. dos Santos, V. *et al.* Ghrelin as a Neuroprotective and Palliative Agent in Alzheimer's and Parkinson's Disease. *Curr. Pharm. Des.* **19,** 6773–6790 (2013).

71. Gahete, M. & Córdoba-Chacón, J. Role of ghrelin system in neuroprotection and cognitive functions: implications in Alzheimer's disease. *Peptides* **32,** 2225–2228 (2011).

72. Date, Y. *et al.* Ghrelin Is Present in Pancreatic -Cells of Humans and Rats and Stimulates Insulin Secretion. *Diabetes* **51,** 124–129 (2002).

73. Broglio, F. *et al.* Ghrelin, a natural GH secretagogue produced by the stomach, induces hyperglycemia and reduces insulin secretion in humans. *J. Clin. Endocrinol. Metab.* **86,** 5083–6 (2001).

74. Gauna, C. *et al.* Ghrelin stimulates, whereas des-octanoyl ghrelin inhibits, glucose output by primary hepatocytes. *J. Clin. Endocrinol. Metab.* **90,** 1055–60 (2005).

75. Sun, Y., Asnicar, M. & Smith, R. G. Central and peripheral roles of ghrelin on glucose homeostasis. *Neuroendocrinology* **86,** 215–28 (2007).

76. Kloppenborg, R. P., van den Berg, E., Kappelle, L. J. & Biessels, G. J. Diabetes and other vascular risk factors for dementia: which factor matters most? A systematic review. *Eur. J. Pharmacol.* **585,** 97–108 (2008).

77. Ferreira, S. T., Clarke, J. R., Bomfim, T. R. & De Felice, F. G. Inflammation, defective insulin signaling, and neuronal dysfunction in Alzheimer's disease. *Alzheimers. Dement.* **10,** S76–83 (2014).

78. Hofman, a *et al.* Atherosclerosis, apolipoprotein E, and prevalence of dementia and Alzheimer's disease in the Rotterdam Study. *Lancet* **349,** 151–4 (1997).

79. Crane, P. K. *et al.* Glucose levels and risk of dementia. *N. Engl. J. Med.* **369,** 540–8 (2013).

80. Attems, J., Walker, L. & Jellinger, K. a. Olfactory bulb involvement in neurodegenerative diseases. *Acta Neuropathol.* **127,** 459–75 (2014).

81. Moon, M., Cha, M.-Y. & Mook-Jung, I. Impaired hippocampal neurogenesis and its enhancement with ghrelin in 5XFAD mice. *J. Alzheimers. Dis.* **41,** 233–41 (2014).

82. Tsubone, T. *et al.* Ghrelin regulates adiposity in white adipose tissue and UCP1 mRNA expression in brown adipose tissue in mice. *Regul. Pept.* **130,** 97–103 (2005).

83. Dezaki, K. in *Endocrine Development* **25,** 135–143 (2013).

84. Chabot, F., Caron, A., Laplante, M. & St-Pierre, D. H. Interrelationships between ghrelin, insulin and glucose homeostasis: Physiological relevance. *World J. Diabetes* **5,** 328–41 (2014).

85. Sangiao-Alvarellos, S. & Cordido, F. Effect of ghrelin on glucose-insulin homeostasis: Therapeutic implications. *International Journal of Peptides* **2010,**

(2010).

86. Morris, R. Developments of a water-maze procedure for studying spatial learning in the rat. *J. Neurosci. Methods* **11,** 47–60 (1984).

87. Laplante, M. & Sabatini, D. M. mTOR signaling in growth control and disease. *Cell* **149,** 274–93 (2012).

88. Laplante, M. & Sabatini, D. M. mTOR signaling at a glance. *J. Cell Sci.* **122,** 3589–94 (2009).

89. Hotamisligil, G. S. *et al.* IRS-1-mediated inhibition of insulin receptor tyrosine kinase activity in TNF-alpha- and obesity-induced insulin resistance. *Science* **271,** 665–8 (1996).

90. Hirosumi, J., Tuncman, G. & Chang, L. A central role for JNK in obesity and insulin resistance. *Nature* **2,** 10–13 (2002).

91. Pinkney, J. The role of ghrelin in metabolic regulation. *Curr. Opin. Clin. Nutr. Metab. Care* **17,** 497–502 (2014).

92. Lugar, C. W. *et al.* Synthesis and biological evaluation of an orally active ghrelin agonist that stimulates food consumption and adiposity in rats. *Bioorg. Med. Chem. Lett.* **14,** 5873–6 (2004).

93. Liu, L. *et al.* The effects of long-term treatment with metrifonate, a cholinesterase inhibitor, on cholinergic activity, amyloid pathology, and cognitive function in APP and PS1 doubly transgenic mice. *Exp. Neurol.* **173,** 196–204 (2002).

94. Kipnes, M. S., Joseph, H., Morris, H., Manko, J. & Bell, D. E. Clinical performance of the TRUE2go blood glucose system--a novel integrated system for meter and strips. *Diabetes Technol. Ther.* **11,** 649–55 (2009).

95. Kruger, N. J. The Bradford method for protein quantitation. *Methods Mol. Biol.* **32,** 9–15 (1994).

96. Aarts, E., Verhage, M., Veenvliet, J. V, Dolan, C. V & van der Sluis, S. A solution to dependency: using multilevel analysis to accommodate nested data. *Nat. Neurosci.* **17,** 491–6 (2014).

97. Carlini, V. P. *et al.* 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 (2004).

98. Moran, T. H. & Gao, S. Looking for food in all the right places? *Cell Metab.* **3,** 233–234 (2006).

99. Olszewski, P. K., Schiöth, H. B. & Levine, A. S. Ghrelin in the CNS: from hunger to a rewarding and memorable meal? *Brain Res. Rev.* **58,** 160–70 (2008).

100. Theodoropoulou, A., Metallinos, I. C., Psyrogiannis, A., Vagenakis, G. A. & Kyriazopoulou, V. Ghrelin and leptin secretion in patients with moderate Alzheimer's disease. *J. Nutr. Health Aging* **16,** 472–477 (2012).

101. Stoyanova, I. I. Ghrelin: A link between aging, metabolism and neurodegenerative disorders. *Neurobiol. Dis.* **72,** 72–83 (2014).

102. Dresler, M., Kluge, M., Genzel, L., Schüssler, P. & Steiger, A. Nocturnal administration of ghrelin does not promote memory consolidation.

Pharmacopsychiatry **43,** 277–8 (2010).

103. Lehrl, S. *Manual zum MWT-B*. (Spitta-Verlag, 2005).
104. Akamizu, T. *et al.* Pharmacokinetics, safety, and endocrine and appetite effects of ghrelin administration in young healthy subjects. *Eur. J. Endocrinol.* **150,** 447–455 (2004).
105. Hosoda, H. & Kangawa, K. in *Methods in enzymology* **514,** 113–126 (2012).
106. Hossiep, R., Turck, D. & Hasella, M. *Bochumer Matrizentest (BOMAT) advanced*. (Hogrefe, 1999).
107. Baddeley, A. Working memory. *Science* **255,** 556–559 (1992).
108. Christensen, P. R. & Services., S. P. *Alternate uses*. (Sheridan Psychological Services, 1960).
109. Tombaugh, T. N. Trail Making Test A and B: Normative data stratified by age and education. *Arch. Clin. Neuropsychol.* **19,** 203–214 (2004).
110. Basner, M. & Dinges, D. F. Maximizing sensitivity of the psychomotor vigilance test (PVT) to sleep loss. *Sleep* **34,** 581–591 (2011).
111. Faul, F., Erdfelder, E., Lang, A.-G. & Buchner, A. G*Power 3: a flexible statistical power analysis program for the social, behavioral, and biomedical sciences. *Behav. Res. Methods* **39,** 175–191 (2007).
112. Smith, S. M. Overview of fMRI analysis. *Br. J. Radiol.* **77 Spec No,** S167–75 (2004).
113. Pruim, R. H. R. *et al.* ICA-AROMA: A robust ICA-based strategy for removing motion artifact from fMRI data. *Submitt. Publ.* **112,** 267–277 (2014).
114. Beckmann, C. F., Mackay, C. E., Filippini, N. & Smith, S. M. Group comparison of resting-state FMRI data using multi-subject ICA and dual regression. *Neuroimage* **47,** S148 (2009).
115. Smith, S. M. *et al.* Correspondence of the brain's functional architecture during activation and rest. *Proc. Natl. Acad. Sci. U. S. A.* **106,** 13040–5 (2009).
116. Laird, A. R. *et al.* Behavioral Interpretations of Intrinsic Connectivity Networks. *J. Cogn. Neurosci.* **23,** 4022–4037 (2011).
117. Filippini, N. *et al.* Distinct patterns of brain activity in young carriers of the APOE-epsilon4 allele. *Proc. Natl. Acad. Sci. U. S. A.* **106,** 7209–7214 (2009).
118. Morris, J. S. & Dolan, R. J. Involvement of human amygdala and orbitofrontal cortex in hunger-enhanced memory for food stimuli. *J. Neurosci.* **21,** 5304–10 (2001).
119. Kim, H. Neural activity that predicts subsequent memory and forgetting: a meta-analysis of 74 fMRI studies. *Neuroimage* **54,** 2446–61 (2011).
120. Shiiya, T. *et al.* Plasma ghrelin levels in lean and obese humans and the effect of glucose on ghrelin secretion. *J. Clin. Endocrinol. Metab.* **87,** 240–244 (2002).
121. Nummenmaa, L. *et al.* Dorsal striatum and its limbic connectivity mediate abnormal anticipatory reward processing in obesity. *PLoS One* **7,** e31089 (2012).
122. Schüssler, P. *et al.* Ghrelin levels increase after pictures showing food. *Obesity (Silver Spring).* **20,** 1212–7 (2012).

123. Hamann, S. B., Ely, T. D., Grafton, S. T. & Kilts, C. D. Amygdala activity related to enhanced memory for pleasant and aversive stimuli. *Nat. Neurosci.* **2,** 289–293 (1999).

124. McGaugh, J. L. The amygdala modulates the consolidation of memories of emotionally arousing experiences. *Annu. Rev. Neurosci.* **27,** 1–28 (2004).

125. LaBar, K. S. & Cabeza, R. Cognitive neuroscience of emotional memory. *Nat. Rev. Neurosci.* **7,** 54–64 (2006).

126. Egido, E. M., Rodriguez-Gallardo, J., Silvestre, R. a & Marco, J. Inhibitory effect of ghrelin on insulin and pancreatic somatostatin secretion. *Eur. J. Endocrinol.* **146,** 241–4 (2002).

127. Makovey, J., Naganathan, V., Seibel, M. & Sambrook, P. Gender differences in plasma ghrelin and its relations to body composition and bone - an opposite-sex twin study. *Clin. Endocrinol. (Oxf).* **66,** 530–7 (2007).

128. Hewson, A. K. & Dickson, S. L. Systemic administration of ghrelin induces Fos and Egr-1 proteins in the hypothalamic arcuate nucleus of fasted and fed rats. *J. Neuroendocrinol.* **12,** 1047–1049 (2000).

129. Schaeffer, M. *et al.* Rapid sensing of circulating ghrelin by hypothalamic appetite-modifying neurons. *Proc. Natl. Acad. Sci.* **110,** 1512–1517 (2013).

130. Zigman, J. M., Bouret, S. G. & Andrews, Z. B. Obesity Impairs the Action of the Neuroendocrine Ghrelin System. *Trends Endocrinol. Metab.* **27,** 54–63 (2016).

131. Lockie, S. H., Dinan, T., Lawrence, A. J., Spencer, S. J. & Andrews, Z. B. Diet-induced obesity causes ghrelin resistance in reward processing tasks. *Psychoneuroendocrinology* **62,** 114–120 (2015).

132. Alvarez-Crespo, M. *et al.* The Amygdala as a Neurobiological Target for Ghrelin in Rats: Neuroanatomical, Electrophysiological and Behavioral Evidence. *PLoS One* **7,** e46321 (2012).

133. Cabral, A., Valdivia, S., Fernandez, G., Reynaldo, M. & Perello, M. Divergent neuronal circuitries underlying acute orexigenic effects of peripheral or central ghrelin: critical role of brain accessibility. *J. Neuroendocrinol.* **26,** 542–54 (2014).

134. Cabral, A., De Francesco, P. N. & Perello, M. Brain circuits mediating the orexigenic action of peripheral ghrelin: narrow gates for a vast kingdom. *Front. Endocrinol. (Lausanne).* **6,** 44 (2015).

135. Reimer, M. K., Pacini, G. & Ahrén, B. Dose-dependent inhibition by ghrelin of insulin secretion in the mouse. *Endocrinology* **144,** 916–21 (2003).

136. Dezaki, K. Ghrelin function in insulin release and glucose metabolism. *Endocr. Dev.* **25,** 135–143 (2013).

137. Jones, R. B. *et al.* Functional neuroimaging demonstrates that ghrelin inhibits the central nervous system response to ingested lipid. *Gut* **61,** 1543–51 (2012).

138. Schmid, D. A. *et al.* Ghrelin stimulates appetite, imagination of food, GH, ACTH, and cortisol, but does not affect leptin in normal controls. *Neuropsychopharmacology* **30,** 1187–92 (2005).

8. Acknowledgements

Particular thanks to Dr. Inga Kadish and Dr. Martin Dresler who were both outstanding supervisors

Thanks to Dr. Thomas van Groen for his marvellous introduction to behavioural experiments in mice

Thanks to Prof. Axel Steiger for his more than valuable input as an experienced researcher

Thanks to Prof. Florian Holsboer for giving me the opportunity to work and publish at his institute

Thanks to Ashish Kumar and Matthias Tonon who supported the projects not only as great colleagues but also as friends

Thanks to all doctors in our team, especially Anna Kopczak and her baby son who supported the project even in difficult times

Special thanks to Sinja Heger for her incredible patience in sustaining frequent periods of stress-induced grumpiness