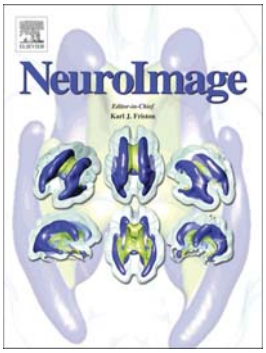

Ghrelin modulates encoding-related brain function without enhancing memory formation in humans

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Ghrelin modulates encoding-related brain function without enhancing memory formation in humans

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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.

Keywords

ghrelin, memory, cognition, enhancement, resting state, functional connectivity

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.

Introduction

The orexigenic peptide ghrelin is involved in appetite regulation (Müller et al., 2015; Tschöp et al., 2000), but also influences a number of cognitive functions in rodent models, such as fear learning, object recognition and spatial learning (Carlini et al., 2008, 2004, 2002; Kunath and Dresler, 2014). 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 (Diano et al., 2006). In animal models of neurodegenerative diseases and age-related memory decline, ghrelin appears to exert a neuroprotective effect (Bayliss and Andrews, 2013; Dhurandhar et al., 2013; Kunath et al., 2015).

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 (Moran and Gao, 2006; Olszewski et al., 2008). In humans, effects of ghrelin on appetite- and memory-related brain regions have been reported (Goldstone et al., 2014; Malik et al., 2008; Tong et al., 2014), however, the specific role of ghrelin in human cognition is yet to be defined (Kunath and Dresler, 2014; Steiger et al., 2011). Studies on the association between ghrelin serum levels and cognitive function in healthy and pathological aging have been rather contradictory so far (Gahete and Córdoba-Chacón, 2011; Spitznagel et al., 2010; Stoyanova, 2014; Theodoropoulou et al., 2012). 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 (Malik et al., 2008), whereas nocturnal ghrelin administration had no positive effect on sleep-related consolidation of a simple motor learning task (Dresler et al., 2010). 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.

Materials and Methods

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 (Lehrl, 2005)). 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.

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 (Kluge et al., 2011; Tong et al., 2013), 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 (Akamizu et al., 2004; Hosoda and Kangawa, 2012), 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 (Garin et al., 2013), 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).

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 (Hossiep et al., 1999)), a working memory task (reverse digit span (Baddeley, 1992)), a creativity task (alternative uses (Christensen and Services., 1960)), a perceptual speed test (trail making task ZVT (Tombaugh, 2004)), and tests for reaction times and psychomotor vigilance (PVT (Basner and Dinges, 2011)).

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 (Faul et al., 2007), 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).

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.

fMRI data analysis

Preprocessing: All fMRI analyses were conducted using the FMRIB Software Library (FSL) version 6.0 (Smith, 2004). 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).

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 (Pruim et al., 2014), 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 (Beckmann et al., 2009). 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 (Laird et al., 2011; Smith et al., 2009) as components to regress against. These spatial maps were then used to generate subject specific maps and time series with dual regression (Filippini et al., 2009). 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.

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 (Goldstone et al., 2014; Malik et al., 2008) 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.

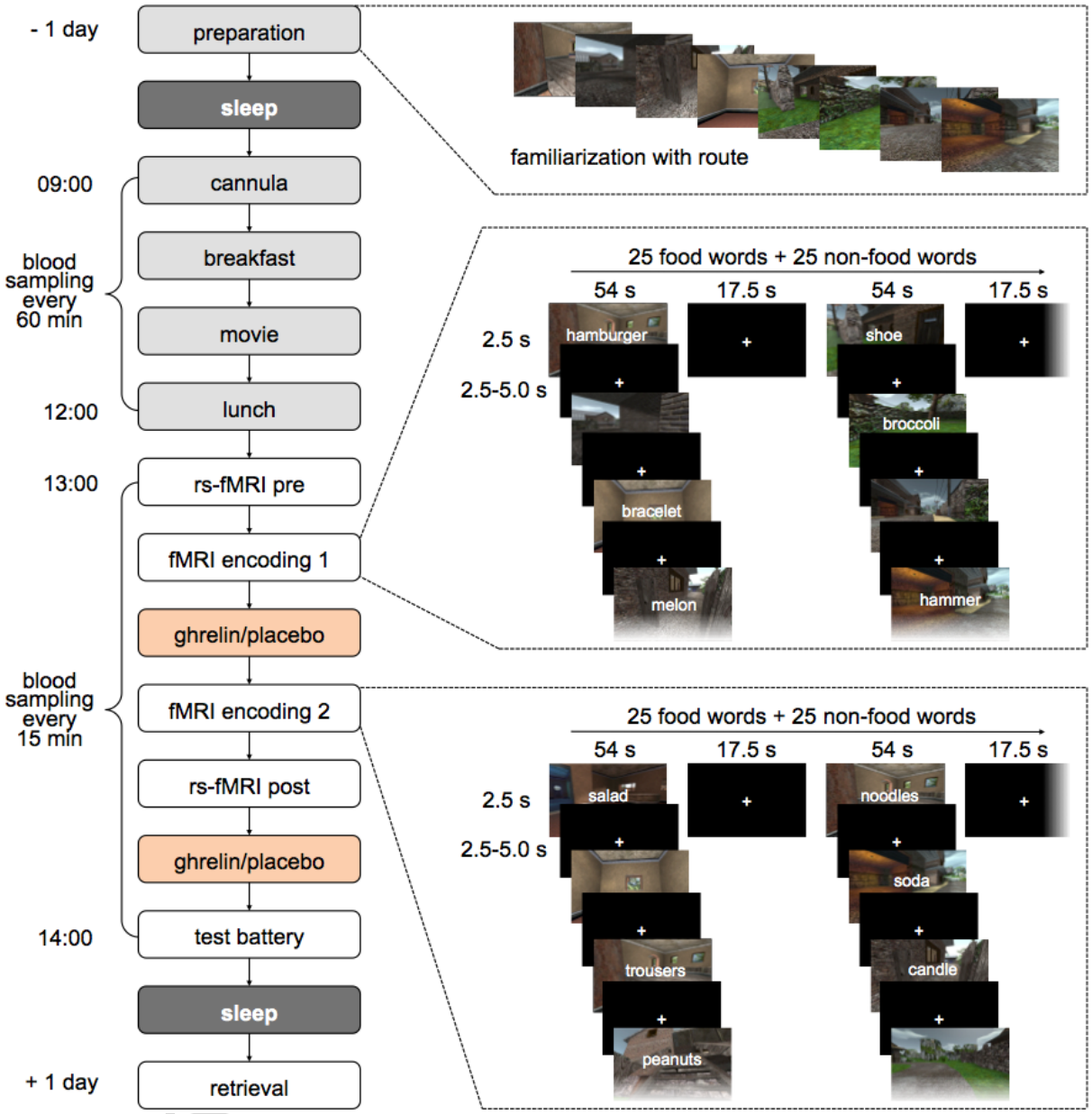

Figure 1: Overview of the test protocol. All 21 participants performed the schedule twice in a double-blind, randomized, placebo-controlled within-subject design. One hour after a standardized lunch, two encoding runs were performed under fMRI conditions, with intravenous ghrelin (or placebo) administration 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.

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 (Morris and Dolan, 2001) and after ghrelin (Malik et al., 2008), 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.

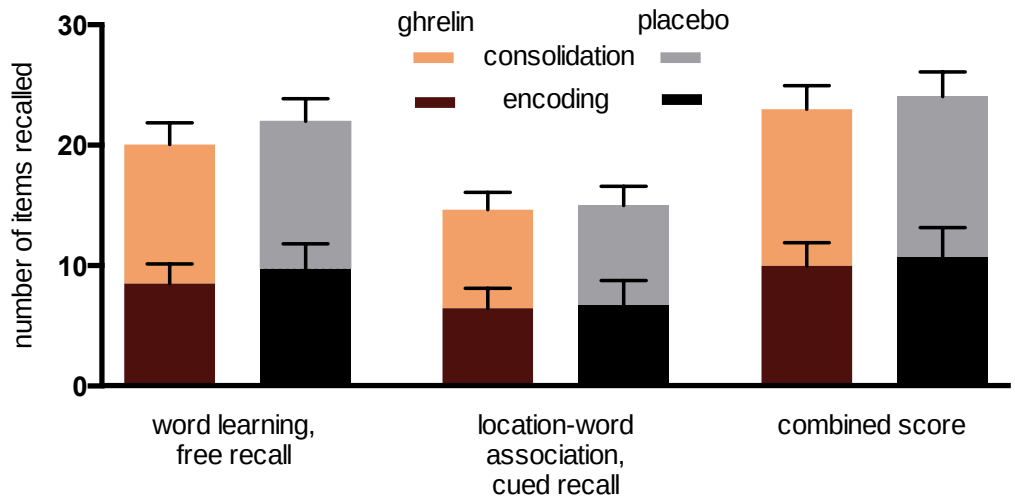

Figure 2: Ghrelin administration did not lead to improved memory encoding or consolidation for any of the outcome measures. The y-axis indicates the number of correctly recalled items out of 100

location-word associations. Combined score represents items that were correctly recalled in either free recall of words or cued recall of location-word associations. Bars indicate SEM.

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 (Kim, 2011) 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 (Goldstone et al., 2014; Malik et al., 2008): 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).

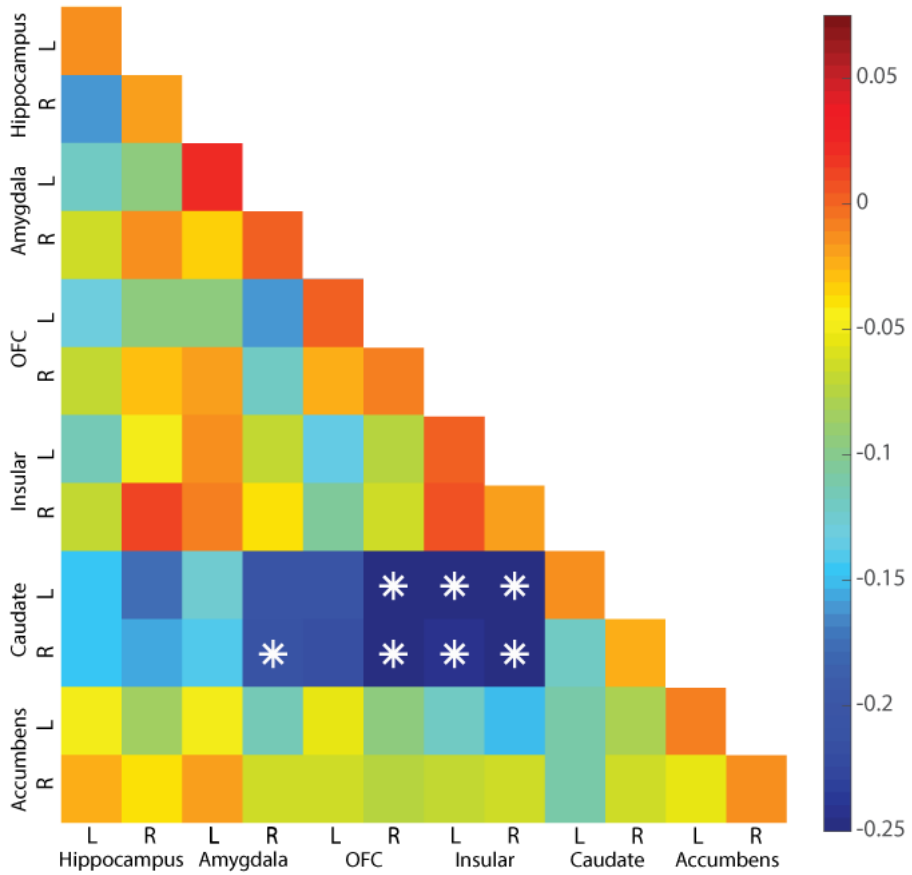

Figure 3: Comparing both resting state scans (one before, one after ghrelin application), we found decreased functional connectivity of the bilateral caudate nucleus with the bilateral insula and right orbitofrontal cortex, and of the right caudate nucleus with the right amygdala in the ghrelin vs. placebo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

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).

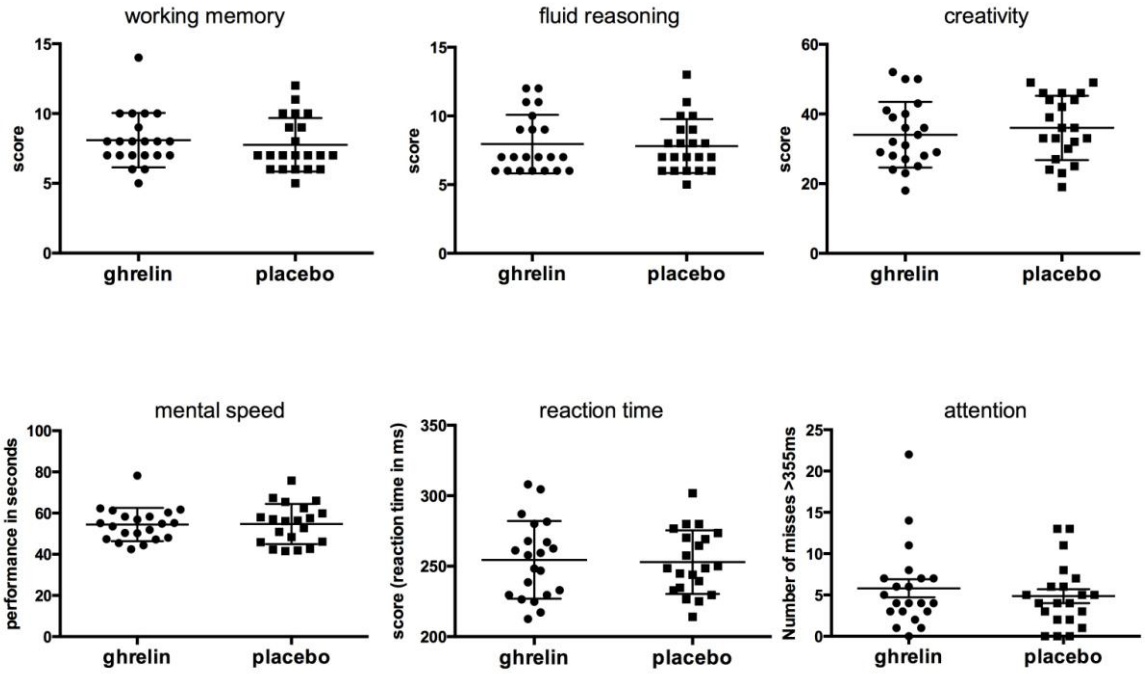

Figure 4: Performance in none of the tests used in our cognitive test battery was influenced by ghrelin administration. Results in a working memory task (reverse digit span), a fluid reasoning test (BOMAT matrices), a creativity task (alternative uses), a mental speed test (trail making), a reaction 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.

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.

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 (Kunath and Dresler, 2014). On this background, the central ghrelin receptor has been proposed as a target for cognitive enhancement interventions also in humans (Atcha et al., 2009). 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 (Malik et al., 2008), whereas nocturnal ghrelin administration had no positive effect on sleep-related consolidation of a motor sequence learning task (Dresler et al., 2010). 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 (Dhurandhar et al., 2013) or the plus maze (Diano et al., 2006). 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 (Moran and Gao, 2006; Olszewski et al., 2008). 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 (Shiiya et al., 2002), show increased connectivity of the caudate nucleus with the amygdala, insula, and prefrontal regions during presentation of appetizing pictures (Nummenmaa et al., 2012). 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 (Goldstone et al., 2014). In addition, viewing food items versus control increased ghrelin release (Schüssler et al., 2012) and activated reward and memory regions such as orbitofrontal cortex, nucleus accumbens, amygdala, insula, hippocampus and the caudate nucleus (Goldstone et al., 2014)[-] (Malik et al., 2008). Enhancing effects of ghrelin on recognition of food pictures (Malik et al., 2008) might therefore be mediated by enhanced reward processing related to food stimuli (Hamann et al., 1999; LaBar and Cabeza, 2006; McGaugh, 2004). 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 (Malik et al., 2008). 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 (Egido et al., 2002; Makovey et al., 2007). 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 (Goldstone et al., 2014; Malik et al., 2008). 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 (Hewson and Dickson, 2000; Schaeffer et al., 2013; Zigman et al., 2016). As food availability seems to play an important role when measuring cognitive effects of the peptide (Alvarez-Crespo et al., 2012; Lockie et al., 2015), 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 (Cabral et al., 2015, 2014; Diano et al., 2006). 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 (Dezaki, 2013; Reimer et al., 2003; Tong et al., 2010), 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 (Jones et al., 2012; Malik et al., 2008; Schmid et al., 2005; Schüssler et al., 2012) and its potential neuroprotective effects in long-term or pathological models (Bayliss and Andrews, 2013; Dhurandhar et al., 2013; V. dos Santos et al., 2013), at the same time thoroughly taking into account aspects of susceptibility and dosage.

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Author contributions

NK, MTo, BNK, SK, DR, KO, TDM, MTs, MC, AS, MD designed the study; NK, MTo, BNK, MP, AK, IE acquired the data; NK, NCJM, MTo, MU, MD analyzed the data; NK, NCJM, MTo, GF, MTs, MC, AS, MD interpreted the data; NK, NCJM, MD wrote the manuscript; all authors discussed and commented on the manuscript.

Competing financial interests

All authors declare no competing financial interests.