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ORIGINAL ARTICLE

High frequency deep transcranial magnetic stimulation acutely increases β-endorphins in obese humans

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Abstract

Purpose In obesity, metabolic and voluntary factors regulate appetite, and a dysregulation of the reward pathway was demonstrated in all addiction disorders. Deep transcranial magnetic stimulation (dTMS) is already used to modulate cerebral dopamine activation in neuro-psychiatric diseases. We presently assess the acute effect of high frequency (HF) and low frequency (LF) dTMS on the modulation of the main neuropeptides and neurotransmitters involved in the reward pathway in obese subjects.

Methods This study was designed as a double-blind, sham-controlled, randomized clinical trial. Thirty-three obese patients (9 males, 24 females, age 48.1 ± 10.6, BMI 36.4 ± 4.7) were enrolled in the study. All patients were studied during a single dTMS session and blood aliquots were drawn before and after a single dTMS session. Metabolic and neuro-endocrine parameters were evaluated before and after: (1) 18 Hz dTMS (HF, 13 patients); (2) 1 Hz dTMS (LF, 10 patients); (3) Sham treatment (Sham, 10 patients).

Results No statistically significant variations in metabolic parameters, systolic and diastolic blood pressure, and heart rate were shown acutely. HF showed a significant increase of β-endorphin compared to other groups ($p = 0.048$); a significant increase of ghrelin in LF ($p = 0.041$) was also demonstrated.

Conclusions A single session of HF dTMS treatment determines in obese subjects an acute increase of β-endorphin level, indicating an activation of the reward pathway. The present findings constitute proof of principle for a potential application of this methodology in obesity treatment.

Keywords Obesity · Transcranial magnetic stimulation · Food craving · β-endorphin · Ghrelin

Introduction

Food craving is mainly responsible for the difficulty in maintaining weight loss in obesity. Neurophysiological mechanisms underlying food craving involve several brain regions. The prefrontal cortex (PFC), specifically the left dorsolateral prefrontal cortex (DLPFC), a brain region implicated in top-down control of behavioral responses [1], is primarily responsible for the cognitive control over eating. Compared with lean individuals, obese subjects consistently showed a lower activation in the DLPFC following a meal [2] suggesting an impaired function of PFC in the inhibitory control and decision-making ability over appetite and eating. Also, the PFC is integrated in the brain reward areas: the meso-limbic and meso-cortical dopamine systems [2].

An abnormal activation of central reward circuits, similar to drugs addiction, has also been reported in obese subjects

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[3]. The meso-cortico-limbic dopaminergic pathway is a central component of this system. Several studies suggest that exposure to palatable food induces activation in reward regions, and stimulates dopaminergic transmission mostly in the dorsal striatum [4]. On the other hand, neuroimaging studies showed a decreased striatal dopamine (DA) D2 receptor expression in obese subjects compared to lean individuals, and a correlation between the decrease in DA ligand binding in the dorsal striatum and the feeding [5, 6]. Together, these findings suggest that the reduced striatal DA D2 receptor availability [5], and the lower sensitivity of DA-based regions, may perpetuate in obese individuals pathological eating as a means to compensate for decreased activation of dopaminergic reward circuits [7, 8].

In addition to the dopaminergic system, substantial evidence also suggests an involvement of the endogenous opioid system in food and drug-induced reward and reinforcement. B-endorphin is released in the hypothalamic area following several physiological stimuli like palatable food, physical exercise, sexual intercourse etcetera, all being related to pleasant feelings and sensations. B-endorphin release is a fundamental step to sense satiety after a meal, especially if the meal is constituted by the preferred food. Dopamine release and β-endorphins levels are strictly interrelated [9, 10].

Body weight homeostasis also involves a complex and highly coordinated system of peripheral appetite hormones and centrally mediated neuronal regulation. Perturbations in circulating appetite modulators, including orexigenic peptides like ghrelin, neuropeptide Y (NPY), and a suite of anorexigenic hormones, such as leptin, insulin, cholecystokinin (CCK), peptide YY (PYY), and glucagon-like peptide (GLP)-1, have been shown to play a role in the pathophysiology of obesity [10]. Abnormalities within the food reward system may be associated with emotional and physiological stress [11].

Repetitive transcranial magnetic stimulation (TMS) is a novel technique for non-invasive, painless brain stimulation. It generates a small electromagnetic current in the brain, inducing modulation of neural excitability and promoting DA release [12, 13]. Low frequency (LF) (≤1 Hz) TMS inhibits cortical excitability, while high-frequency (HF) (≥5 Hz) TMS enhances cortical excitability by increasing neuronal depolarization [12]. For these properties, repetitive TMS has been explored as a potential tool for the treatment of several neuropsychiatric disorders. Recently, an H-coil has been developed by Zangen et al. [14] to stimulate deep brain regions; considering their deep brain localization, dopaminergic neurons could be reached by deep TMS (dTMS), with consequent impact on addiction diseases [15]. The dopaminergic pathways may also be indirectly modulated by the repetitive TMS application to the PFC, via more superficial projections of dopaminergic neurons within the DLPFC [16]. Repetitive dTMS was found to induce a significant reduction in daily alcohol consumption in alcohol-addicted patients [17, 18], and cigarette consumption in nicotine-addicted patients [19]. Cocaine craving and drug use frequency may also be reduced by repetitive TMS applied to PFC in regular cocaine users [20]. Furthermore, very recent studies suggested dTMS for the treatment of chronic pain, showing that it induced an increase of β-endorphin concentration [21–23], and suggesting this increase as a potential mechanism of action of dTMS as pain reliever.

Although dTMS has been in use for over 15 years, at present, scanty data are available on the neuroendocrinological hormones modifications taking place after a single dTMS session in humans, since most attention was posed on neurophysiological changes.

Consistent with the dysregulation of the PFC inhibitory control and brain reward system in obese subjects, and with the dTMS modulatory effect on the reward system, we hypothesized a potential role of dTMS in acutely modulating the reward pathways involved in hedonic hunger. Specifically, the present study was designed to identify acute modifications of the main neuropeptides (leptin, ghrelin) and neurotransmitters (β-endorphin, epinephrine, norepinephrine), related to the appetite/satiety balance in the reward system, in response to a single session of dTMS.

Methods

Study design

This study was designed as a double-blind, sham-controlled, randomized clinical trial, aimed to investigate the acute effects of a single dTMS session on the main neuropeptides (leptin, ghrelin) and neurotransmitters (β-endorphin, epinephrine, norepinephrine) involved in food reward system, and consequently, in food craving, comparing HF with both LF and Sham stimulation. This trial could be considered a proof-of-concept study aimed to detect a signal that the dTMS is active on pathophysiological mechanisms underlying obesity.

Eligible patients were randomly allocated either to HF or LF or Sham group. Allocation in the three groups was performed according to a randomization sequence generated by a computerized program without any restrictions (such as blocking and stratification). The randomization code was only given to the treating investigator on the day of treatment session by an independent investigator not involved with any other aspect of the trial. The independent investigator could be contacted at any time to unblind the randomization code in the case of a specific emergency.

Participants and investigators were unaware of treatment assignment.

Study approval

This study was conducted in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki declaration and its later amendments; it received approval by the local Institutional Review Board (Ethics Committee of San Raffaele Hospital, Milan, Italy). All participants provided written informed consent before participating in any study procedures.

Study participants

This study was performed at the Endocrinology and Metabolism Division, IRCCS Policlinico San Donato Scientific, Italy. Adult men and women (aged 22 to 65 years, inclusive), who referred to Endocrinology and Metabolism outpatient clinic for overweight/obesity treatment, were screened with a short telephone interview to determine eligibility. Inclusion criteria was a BMI ranging between 30 and 45 kg/m^2. If eligible, a more complete direct interview followed the telephone screening after providing signed informed consent. Patients with a personal or a family history of seizures, as well as patients with psychotic disorders, organic brain disorders, implanted metal devices, fasting blood glucose level > 150 mg/dl, abuse of substances other than nicotine, treatment with anti-obesity medications or other medications for weight reduction, or medications associated with lowered seizure threshold, were excluded from the study.

Repetitive deep transcranial magnetic stimulation (dTMS) procedure

The repetitive dTMS was performed by a trained physician using a Magstim Rapid^2 TMS (The Magstim Co. Ltd., Whitland, Carmarthenshire, United Kingdom) stimulator equipped with an H-shaped coil (H-ADD), specifically designed to bilaterally stimulate the PFC and the insula [14]. This H-coil allows direct stimulation of deeper brain regions like insula (3 cm vs 1.5 cm from the skull). Magnetic cards encoding for real or sham stimulation were used to activate the dTMS device. Both real and sham stimulation produced identical sounds and scalp sensations during the sessions.

During each dTMS session, the optimal stimulation intensity was defined by searching the individual resting motor threshold (RMT); the RMT was determined over the left primary motor cortex. Before the dTMS session, the optimal spot on the scalp was localized to stimulate the right abductor *pollicis brevis* muscle, and the RMT was defined by delivering single stimulations, applying one pulse every 5 s to the motor cortex, and gradually decreasing intensity. The RMT was defined as the stimulation with the lowest required intensity to cause the right thumb to move. Once the RMT was defined, the stimulation could begin.

High-frequency session consisted of 80 trains of 18 Hz, each lasting 2 s, with an intertrain interval of 20 s. The HF treatment duration was 29.3 min with 2880 pulses in total. Low-frequency session consisted of four trains of 1 Hz, each lasting 10 min, with an intertrain interval of 1 min. The LF treatment duration was 43 min with 2400 pulses in total. The Sham treatment was performed by a sham coil located in the same case of the real coil, producing similar acoustic artifacts and scalp sensations, inducing only negligible electric fields in the brain. In all groups receiving the real treatment, the stimulation was performed with an intensity of 100% of the RMT.

Prior to stimulation, obese subjects were either shown a series of palatable food images *(cue)* or not *(no cue)*. In the cue condition, the dTMS session was preceded by showing the patients pictures of their favorite food, identified during the interview with the subjects at the screening visit.

Measurements

Psychiatric and psychological evaluation

During the screening period, patients underwent psychiatric and psychological evaluation to rule out current major psychiatric disorders by the administration of the Structured Clinical Interview for Diagnostic and Statistical Manual (DSM) (SCID-I) [24].

Anthropometric values

Anthropometric measurements included body weight and height, in order to calculate BMI (kg/m^2). Body weight was measured without shoes, wearing light underwear, on a standing scale calibrated to the nearest 0.1 kg. Body height was measured without shoes using a stadiometer calibrated to the nearest 0.1 cm.

Blood pressure and heart rate

Systolic and diastolic blood pressure (SBP and DBP) were measured using a calibrated device. The device was a mercury sphygmomanometer with a cuff appropriate to the girth of the subject's arm. The same device was used for each subject throughout the study. Blood pressure was measured under standardized conditions (sitting position), on the same arm. Both SBP and DBP were recorded.

Table 1 Variations of parameters measured after a single dTMS session in the three groups

	HF ($n = 13$)	LF ($n = 10$)	Sham ($n = 10$)	p-value
Glucose (mg/dL)	5.54 ± 6.69	3.60 ± 7.31	4.11 ± 3.10	*0.776*
Insulin (μU/mL)	−2.91 ± 14.45	−3.25 ± 4.63	−2.12 ± 5.93	*0.995*
Cholesterol (mg/dL)	3.85 ± 6.40	0.90 ± 10.33	4.67 ± 5.43	*0.591*
Triglycerides (mg/dL)	−2.00 [−12.00; −2.00]	−8.00 [−39.00;7.00]	−12.00 [−23.00;0.00]	*0.893*
Ghrelin (ng/mL)	−1.18 ± 4.81	11.93 ± 17.92	0.02 ± 8.60	*0.041* *
Leptin (ng/mL)	−11.54 ± 11.31	−5.92 ± 10.70	−10.30 ± 15.35	*0.256*
β-Endorphin (ng/mL)	0.08 ± 0.12	−0.03 ± 0.06	0.01 ± 0.06	*0.048* *
Epinephrine (pg/mL)	14.60 ± 253.89	−5.05 ± 71.35	−72.36 ± 186.04	*0.562*
Norepinephrine (ng/mL)	0.60 ± 1.06	−0.03 ± 0.68	0.28 ± 0.72	*0.261*
SBP (mmHg)	−5.45 ± 10.11	−2.78 ± 6.67	−2.50 ± 14.77	*0.823*
DBP (mmHg)	−1.82 ± 7.83	−0.56 ± 8.08	0.50 ± 7.62	*0.900*
Heart rate (pulses/min)	−5.40 ± 4.43	−3.89 ± 5.16	−3.67 ± 3.74	*0.607*

Data are shown as mean ± standard deviation (SD)

For any outcome, variation between baseline and following a single dTMS session values is reported as Δ-variation (value ± SD) for all the three groups (HF, high frequency 18 Hz; LF, low frequency 1 Hz; Sham)

HF high frequency, *LF* low frequency, *SBP* systolic blood pressure, *DBP* diastolic blood pressure

*Results from post hoc ANOVA HF or LF vs. Sham: $*p < 0.05$

Heart rate (pulse) was measured over 1 min under the same conditions for blood pressure, and the results were recorded.

Blood pressure and heart rate were measured before the TMS treatment and immediately afterwards.

Laboratory measurements

The dTMS sessions were carried out after a 12-h overnight fast. A Venflow catheter was placed into an antecubital vein of each participant to draw blood. Blood samples were centrifuged for 15 min at 2000 × *g*. A part of the blood was immediately processed; about 10 mL of every sample were stored in aliquots at −80 °C. Neuropeptide, neurotransmitter, and metabolite determinations were performed by standardized techniques.

The laboratory assessment included: leptin (ng/mL), ghrelin (ng/mL), β-endorphins (ng/mL), epinephrine (pg/mL), norepinephrine (ng/mL). An assessment of the main metabolites was included: glucose (mg/dL), insulin (μU/mL), cholesterol (mg/dL), triglycerides (mg/dL).

Blood sample for catecholamines was performed at least 20 min after the intravenous cannula was introduced in the antecubital vein, to avoid the conditioning produced by puncture stress. The subjects were also instructed to abstain from several types of food and beverages (e.g., chocolate, licorice, bananas, citrus fruits, coffee, tea, alcohol) for 2–3 days prior to the blood sampling, and from smoking for 10 h prior to the blood sampling. Catecholamine levels in the blood can change quickly, therefore blood samples were centrifuged and processed within 5 min from sampling.

Ghrelin and β-endorphins were measured using commercially available enzyme immunoassay (EIA) kits (Phoenix Pharmaceuticals, Burlingame, CA, USA); enzyme-linked immunosorbent assay (ELISA) kits were used to assess epinephrine, norepinephrine (Elabscience Biotechnology Co. Ltd, Wuhan, China), and leptin (Diagnostic Biochem Canada Inc, London, UK).

Blood glucose was quantified with enzymatic ultraviolet method with hexokinase; serum triglycerides and total cholesterol were determined by the enzymatic colorimetric method. Insulin was determined by the electrochemiluminescence immunoassay (ECLIA).

Statistical analysis

Descriptive data are expressed as counts (percentages) for categorical data and as means ± standard deviation (SD) or median [Q1–Q3] for continuous variables, as appropriate.

The relationship between changes in parameters from baseline to a single dTMS session in glucose, insulin, cholesterol, ghrelin, leptin, β-endorphin, epinephrine, norepinephrine, SBP, DBP, and heart rate with changes into the three groups (HF, 18 Hz, and Sham) was assessed with ANOVA adjusted for baseline levels of the parameter.

Changes in median concentrations of triglycerides in the three experimental groups were compared by the

nonparametric Kruskal–Wallis test for non-normally distributed data for continuous variables.

Statistics were performed with SAS software version 9.4 (SAS Institute Inc., Cary, NC, USA). All *P*-values are two-tailed and considered significant if <0.05.

Results

Participant characteristics

Thirty-three subjects completed the study as per the protocol. The mean age of the sample group was 48.1 ± 10.6 years and the mean BMI was 36.4 ± 4.7 kg/m^2.

Patients fulfilling all inclusion/exclusion criteria were allocated to one of the three experimental groups. dTMS stimulation conditions could either be *high-frequency* (HF, 18 Hz group), *low-frequency* (LF, 1 Hz group) or *sham* (Sham group). Thirteen obese subjects were allocated in HF group, ten in LF group, and ten in Sham group. Obese subjects belonging to HF and LF groups were either shown a series of palatable food images (*cue*) or not (*no cue*); the patients in the Sham group were all exposed to the *cue*.

At baseline, no significant differences in age and BMI were found among the three groups. All patients underwent blood collection at the beginning and at the end of the single dTMS session.

Blood pressure and heart rate

After the dTMS session, no variations in SBP ($p = 0.823$), DBP ($p = 0.900$) (Table 1) and heart rate ($p = 0.607$) (Table 1) were found in HF compared to the other groups.

Metabolic and neuro-endocrine effects of acute dTMS

No significant acute variation in glucose, insulin, total cholesterol, and triglycerides was found in the three groups.

After a single HF dTMS session, β-endorphins increased by +0.08 ± 0.12 ng/mL; after a single dTMS session β-endorphin levels decreased by −0.03 ± 0.06 ng/mL; β-endorphin variation in Sham was negligible: +0.01 ± 0.06 ng/mL. Variations difference among the three groups was significant ($p = 0.048$) (Table 1, Fig. 1). The individual changes in β-endorphin levels in the three groups are reported in Fig. 2.

No significant variations were detected in all other neuro-endocrine parameters measured, with the notable exception of ghrelin hormone which increased in the LF compared to the other treatment groups (HF: −1.18 ± 4.81 ng/mL; LF: +11.93 ± 17.92 ng/mL; Sham: +0.02 ± 8.60 ng/mL; $p =$ 0.041) (Table 1, Fig. 3). The individual variations of ghrelin levels in the three groups are reported in Fig. 4.

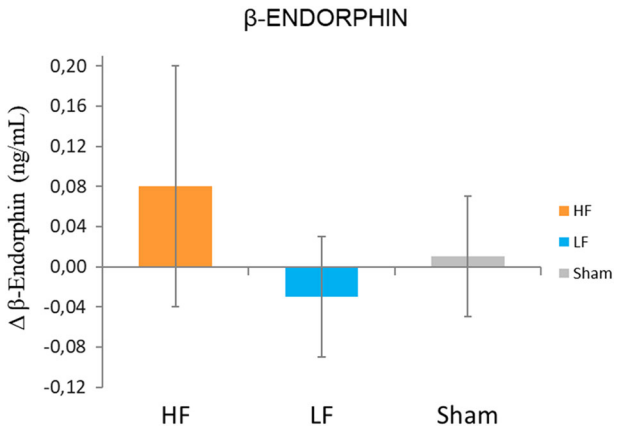

Fig. 1 Acute dTMS effects on β-endorphin levels in obese subjects. The panel presents the average variation (ng/mL) of β-endorphins after a single session of dTMS in the three differently treated groups of obese subjects: HF (high frequency 18 Hz; $n = 13$), LF (low frequency 1 Hz; $n = 10$), Sham ($n = 10$). β-endorphin increased by +0.08 ± 0.12 ng/mL and by +0.01 ± 0.06 ng/mL, respectively in HF and Sham, and decreased by −0.03 ± 0.06 ng/mL in LF, with a significant difference among the three groups ($p = 0.048$). HF high frequency, LF low frequency

No significant differences in β-endorphin release were found between *cue* and *no-cue* subgroups, when stimulating the subjects with pictures of favorite food just prior to the HF, LF, and Sham dTMS session.

Discussion

This study examined the effects of a single treatment session with dTMS over the PFC and insula, bilaterally, using either high-frequency or low-frequency stimulation in obese humans. The study comprehensively investigated acute modifications induced by repetitive dTMS on neuro-endocrine parameters related to the reward pathway. A sub-analysis was performed to investigate potential cue exposure-induced influence on acute neurophysiological effects of dTMS.

The reward pathway is a complex neuronal network connecting several areas of the brain like the hypothalamus, the substantia nigra, the Ventral Tegmental Area (VTA), the nucleus accumbens, the insula, and the PFC. The main neurotransmitter involved in the reward system is the dopamine, which plays a pivotal role in the "wanting" or desire of certain types of food, which underlie food craving [25]. The therapeutic effect of repetitive HF dTMS in addiction disorders could be explained by a facilitation effect on dopamine release via both a direct stimulation of the cortico-striatal axons, and a reduction of GABA-mediated intra-cortical inhibition, which indirectly

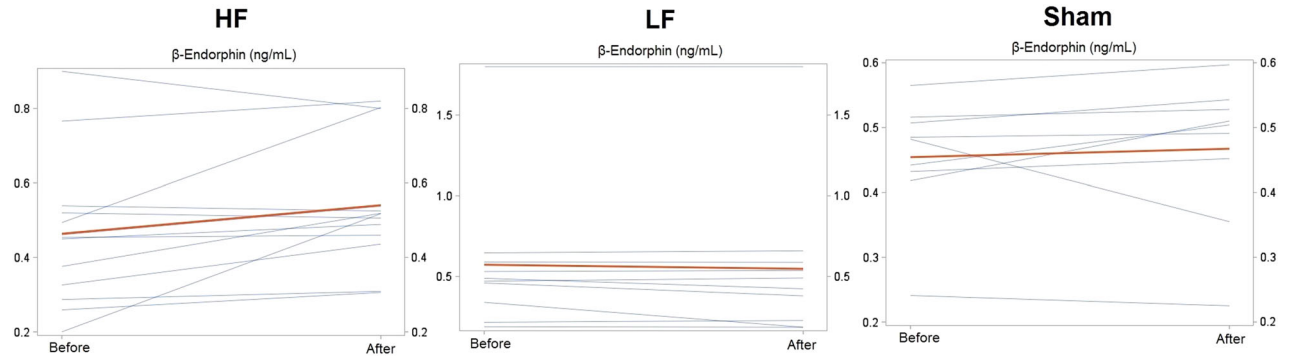

Fig. 2 Individual variations in β-endorphin levels after a single session of dTMS in the three differently treated groups of obese subjects: HF, LF, and Sham. HF high frequency, LF low frequency

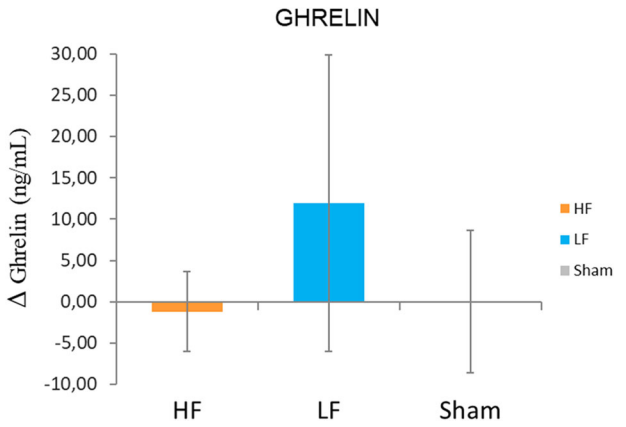

Fig. 3 Acute dTMS effects on ghrelin levels in obese subjects. The panel presents the average variation (ng/mL) of ghrelin after a single session of dTMS in the three differently treated groups of obese subjects: HF (high frequency 18 Hz; $n = 13$), LF (low frequency 1 Hz; $n = 10$), Sham ($n = 10$). Ghrelin increased by $+11.93 \pm 17.92$ ng/mL in LF, by $+0.02 \pm 8.60$ ng/mL in Sham, and decreased by -1.18 ± 4.81 ng/mL in HF, with a significant difference among the three groups ($p = 0.041$)

activates cortico-striatal neurons [18, 26]. It has been hypothesized that the dTMS-induced effect on dopaminergic system "mimics" the effect of food on these pathways, while participants are in a food restriction diet [27].

In addition to dopamine, among the neurotransmitter systems proposed to be important in controlling food-seeking behavior, there is the endogenous opioid system, which is mainly involved in the pleasurable feeling ("liking") associated with the food rewarding stimuli [28]. The anorexigenic pro-opiomelanocortin (POMC) neurons, located in the hypothalamic arcuate nucleus, secrete β-endorphin; in turn, β-endorphin neurons project to various brain regions including VTA, nucleus accumbens, amygdala, and PFC. Interaction of β-endorphin with receptors, especially μ-opioid receptors, has been demonstrate to promote DA release and to initiate processes associated with reward and reinforcement [29]. Furthermore, β-endorphin release has been shown to inhibit further POMC activation, leading to a decreased appetite and increased energy expenditure [28].

Interestingly, in our study, after a single HF dTMS session a significant increase in β-endorphins, compared to baseline and the other groups, was found. Consumption of palatable and nonpalatable meals was shown to lead to widespread endogenous opioid release in the brain [30]; we may assume that HF dTMS "mimics" the effects of food on the endogenous opioid system, as well as for the dopamine system. This finding suggests, on the one hand, an endogenous opioids-induced activation of the DA system leading to DA release ("dopamine cascade") [31], on the other hand, a role of dTMS in modulating hypothalamic hunger through a negative feedback of β-endorphins on POMC neurons. This dual effect of dTMS in regulating both "hedonic hunger" (reward system modulation) and "homeostatic hunger" (hypothalamic regulation) may suggest a potential indication of dTMS also in "food addiction".

A similar increase of β-endorphins was demonstrated by Ahmed et al. [32] when a methodology similar to ours was applied to the treatment of chronic pain with a HF protocol. This confirms the efficacy of HF stimulation in increasing endogenous opioids.

There was no evidence of the effect of dTMS on systolic/diastolic blood pressure and heart rate in any treatment group (HF, LF, and sham), suggesting a prevailing central nervous system effect without major systemic physiological changes after a single session. This is in line with the minimal side effects registered during the TMS treatments performed for addiction disorders [17–20].

In this study, LF (inhibitory) stimulation has not proven to be effective in acutely influencing β-endorphin levels, supporting the findings of a previous clinical trial testing the efficacy of dTMS in smoking [13]. In fact, LF stimulation (≤1 Hz) has been shown to inhibit cortical excitability. Our results are consistent with the model that suppression of the

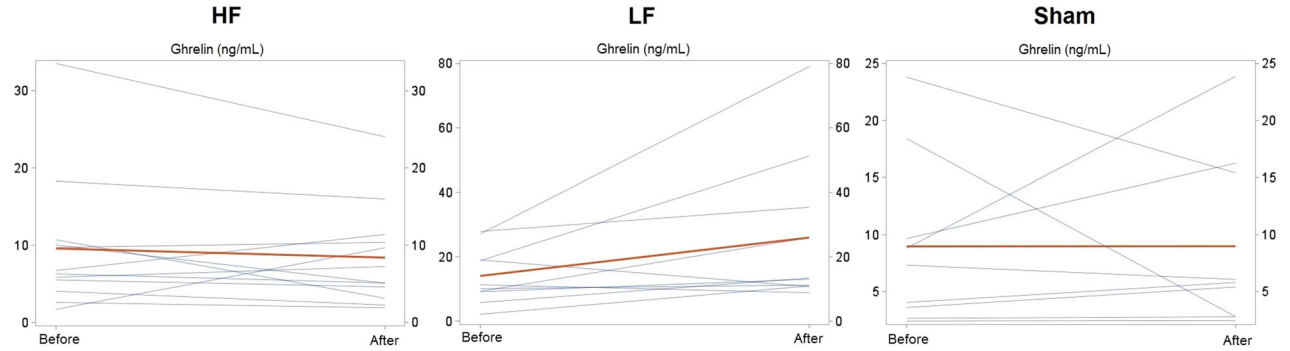

Fig. 4 Individual variations in ghrelin levels after a single session of dTMS in the three differently treated groups of obese subjects: HF, LF, and Sham. HF high frequency, LF low frequency

left DLPFC by LF repetitive TMS induces an inhibitory control reduction, leading to enhance cue-induced cravings for drugs, as observed in methamphetamine-dependent patients [33].

Interestingly, we observed a significant increase of ghrelin levels during LF dTMS stimulation in obese subjects. This trend was paralleled by an opposite trend during HF stimulation. This finding, if confirmed in a larger population, may constitute an additional potential mechanism (besides β-endorphin increase) via which dTMS modulates appetite.

In our study, no disadvantage or advantage in β-endorphin production has been found when stimulating the subjects with pictures of favorite food just prior to the high-frequency or low-frequency stimulation treatment. This is in contrast with the important role of β-endorphins in the cue-induced cocaine cravings [34]. In our study the cueing procedure was performed by showing pictures of the most favorite type of food, rather than actual food. A recent study demonstrated that food craving produced by virtual reality or picture cues was significantly lower than real food, and marginally higher than a neutral cue [35].

In conclusion, this study suggests a potential role of the HF dTMS over the PFC and insula in increasing β-endorphin production in obese subjects. It is conceivable that the main mechanism is the increased dopaminergic activity in the mesolimbic and mesostriatal pathways, which induces a positive modulation of all brain areas involved in the reward pathway. Future larger studies should determine whether this promising technique may become an obesity treatment alone or in combination with weight-lowering drugs.

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Author contributions LL and IT contributed to designing the research study. LL, AF, and CM conducted experiments; specifically, LL provided research conduct oversight; AF contributed to performing dTMS after a specific training, and to providing medical oversight; CM contributed to collecting blood samples. AF and MA contributed to acquiring data; FA and VM performed statistical analysis. AF, LL, MA, FA and VM contributed to writing the manuscript. As corresponding author, LL confirms that he had full access to all the data in the study and has final responsibility for the decision to submit for publication.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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