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One left dorsolateral prefrontal cortical HF-rTMS session attenuates HPA-system sensitivity to Critical Feedback in healthy females

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Abstract

Objective: Although left dorsolateral prefrontal cortical (DLPFC) repetitive Transcranial Magnetic Stimulation (rTMS) is used to treat major depression, its underlying neurophysiological working mechanism remains to be determined. Prior research suggested that the clinical effects could be mediated by affecting the hypothalamic-pituitary-adrenal (HPA) system, but experimental studies in healthy individuals did not yield clear results. However, in healthy individuals, the influence of HF-rTMS on the HPA-system may only be detected when it is challenged.

Methods: In 30 rTMS naïve healthy females we evaluated the effect of one sham-controlled high frequency (HF)-rTMS session applied to the left DLPFC on the stress hormone cortisol by collecting salivary cortisol samples. In order to increase stress levels, five minutes after stimulation, all participants performed the Critical Feedback Task (CFT), during which they were criticized on their performance. To take possible mood influences into account, all participants were also assessed with Visual Analogue Scales (VAS).

Results: The experimental procedure did not affect mood differently in the real or sham stimulation. Area under the curve (*AUC*) analysis showed that one real HF-rTMS session significantly influenced HPA-system sensitivity, as demonstrated by a decrease in cortisol concentrations. The sham procedure yielded no effects.

Conclusions: In line with former observations in major depression, one real left DLPFC HF-rTMS session significantly influenced HPA-system sensitivity in experimentally stressed females, resulting in decreases in cortisol levels.

Keywords: HF-rTMS; cortisol; healthy females; Critical Feedback Task

1. Introduction

Repetitive transcranial magnetic stimulation (rTMS) is a neuromodulation technique used in experimental neurophysiological research as well as in clinical paradigms to treat mental disorders, such as major depressive disorder (MDD) (Padberg and George, 2009). rTMS can either activate or suppress motor, sensory, or cognitive functions, depending on the brain location and parameters of its delivery (George and Belmaker, 2007): low-frequency (LF)-rTMS (≤ 1 Hz) is considered to 'inhibit' cortical regional activity, while high-frequency (HF)-rTMS (> 1 Hz) 'activates' cortical areas (Chen et al., 1997; Maeda et al., 2000). Although LF-rTMS of the right and HF-rTMS of the left dorsolateral prefrontal cortex (DLPFC) seem to both have beneficial effect in MDD patients, the strongest evidence on clinical efficacy have been reported when stimulating the left DLPFC with HF-rTMS (Schutter, 2009). However, how this application alters mood and improves symptoms remains poorly understood. Animal models suggest that an important aspect of the physiology of rTMS could be related to the endocrinological response of the hypothalamic-pituitary-adrenal (HPA) system, such as cortisol secretion (Post and Keck, 2001; Hedges et al., 2003).

The stress/mood system is mediated by neurocircuitries connecting the prefrontal cortex (PFC) and amygdala / hippocampus, and normal homeostasis is established by a negative feedback system (see figure 4 A). Here, it has been assumed that rTMS treatment results in increased neuronal activity in the (dorsolateral) prefrontal cortex, which through cortico-subcortical trans-synaptic connections suppresses (paraventricular) hypothalamic activity, resulting in corticotropin-releasing hormone (CRH) decreases and ultimately in decreased salivary cortisol concentrations (Keck, 2003). However, these prefrontal cortico-subcortical connections are likely to affect the hypothalamus through multi-synaptic indirect pathways (Rempel-Clower and Barbas, 1998; Barbas et al., 2003). Furthermore, the synaptic connections between the DLPFC and the amygdala, as depicted in Figures 4A, 4B and 5, are not assumed to represent strong direct neuronal connections able to regulate amygdala activity (for a recent overview see Ray and Zald, 2012). Given that the amygdalae have strong connections with the ventromedial prefrontal cortex, including the orbitofrontal cortex (OFC) and the subgenual parts of the anterior cingulate cortex (sgACC) (Ray and

Zald, 2012) and the amygdala's extensive connections with the hippocampus and the hypothalamus, (Jankord and Herman, 2008; Dedovic et al., 2009), the amygdalae are more functionally connected with the ventromedial parts of the forebrain than with the more dorsal parts of the prefrontal cortex. Nevertheless, HF-rTMS applied to the DLPFC may influence amygdala's functioning. For instance, in a similar but different sample of healthy females, we observed decreased neuronal amygdala activity while processing withdrawal-related visual stimuli after real HF-rTMS over the DLPFC, and not after sham (Baeken et al., 2010a).

Some studies in MDD indicated that this application is able to reduce cortisol levels, but in non-psychiatric samples these neuroendocrinological effects are far from clear (Baeken et al., 2009a, 2011a). Because the effects on the HPA-system observed in MDD could be unrelated to the underlying working mechanisms of rTMS and could be a secondary effect of treatment, it is important to gain more insight in these biological processes in non-depressed individuals. Although the relationship between negative affect and cortisol activity has been well documented (Buchanan et al., 1999), few experimental studies have examined the endocrinological effects of rTMS on the HPA-system in healthy volunteers. Using left-sided high frequency HF-rTMS, George et al (1996) found a slight increase of serum cortisol levels post left-sided prefrontal stimulation, whereas Evers et al (2001) observed a decrease of serum cortisol concentrations. However, in both studies carry-over effects could not be excluded. We reported negative results on salivary cortisol changes after one sham-controlled session of left and right-sided HF-rTMS in healthy female subjects (Baeken et al., 2009a), suggesting that simply applying one HF-rTMS session does not influence the HPA-system in non-clinical samples displaying normal functioning feedback systems. However, given the low baseline cortisol levels in healthy individuals, it might be difficult to find further decreases. Therefore, to effectively measure the impact of rTMS on the HPA-system in relation to such top-down controlling mechanisms, it might be a more optimal research design to perform neurostimulation when the participants are experimentally being stressed. To our knowledge no such studies have been performed previously.

Consequently, in the current study we wanted to evaluate whether one sham-controlled HF-rTMS session applied to the left DLPFC could influence the HPA-system when healthy female volunteers were experimentally stressed with the Critical Feedback Task (CFT). This is an oddball mental counting task where participants receive bogus negative feedback on their performance at the completion of each test-block (Rossi and Pourtois, 2012). Research has shown that this procedure is effective in inducing stress/anxiety (Nummenmaa and Niemi, 2004). It is a difficult and demanding task, with a high level of uncertainty on performance. Participants are told that, compared to peers their performance is below average, which induces stress. Furthermore, being told that task performance is related to intelligence; in particular for undergraduates this CFT can be considered as very stressful.

To evaluate the HPA-system, salivary cortisol samples were collected. To evaluate subjective changes on mood, we also assessed mood throughout the experiment with Visual Analogue Scales (VAS; McCormack et al., 1988). As gender and age could be a possible confounder in HPA-system reactivity protocols (Seeman et al., 2001), and as the intra-individual stability of baseline salivary cortisol levels is reported to be more stable in women (Kirschbaum et al., 1992), we chose to use a 'uniform' group of non-depressed young female subjects within the same age range. Furthermore, to obtain stable baseline measurements, we started stimulation after a 20 minute period of standard music relaxation.

In line with earlier research using the CFT for the induction of stress and negative affect (Rossi and Pourtois, 2012), we expected that our experimental procedure would increase negative and decrease positive mood states. However, we did not expect any differential effects on mood after real or sham stimulation. If the hypothesis is correct that left DLPFC HF-rTMS affects a stressed HPA-system, we expect that real HF-rTMS, and not sham, will attenuate cortisol concentrations.

2. Methods

2.1. Subjects

The ethics committee of the University Hospital (UZBrussel) approved the study and all subjects gave written informed consent. Subjects were financially compensated. The total sample consisted of 31 right-handed (criteria: van Strien and Van Beeck, 2000) healthy female participants (mean age= 21.53 years (SD= 2.85)) naïve to the rTMS procedure. No drugs were allowed, except birth-control pills. All participants used contraceptives at the time of the study. Psychiatric disorders were assessed by the Dutch version of the Mini-International Neuropsychiatric Interview (MINI) (Sheehan et al., 1998). A clinical psychiatric interview was performed before a subject's inclusion in the study. Subjects with a psychiatric disorder and/or a score higher than ten on the Beck Depression Inventory (BDI-II; Beck et al., 1996) were excluded. Mean BDI score= 3.45 (SD= 3.47).

Of note, in a separate pilot study we performed one sham-controlled right DLPFC stimulation session in a different but smaller group of healthy females. No effects on mood and the HPA-system were observed (unpublished results).

2.2. Assessment

On each of the salivary cortisol collections, subjects were asked to rate their mood on six horizontal 100 mm visual analogue scales (VAS; McCormack et al., 1988) in order to detect subtle changes in mood. Feelings of 'tiredness', 'vigor', 'anger', 'tension', 'depression' and 'cheerfulness' were rated "*at this moment*". The minimum score on each VAS subscale is 0 and the maximum score is 100.

As in Baeken et al. (2009a, b, 2011a), saliva samples were collected using a salivette (Sarstedt, Germany), with an insert containing a sterile polyester swab for collecting saliva, yielding a clear and particle-free sample. The salivettes were used according to the instructions provided by the manufacturer. Salivettes containing saliva were centrifuged at 2000 g for 10 min, and the filtrates were stored frozen (-20°C). Before analysis, the samples were thawed and mixed. Saliva cortisol levels were measured by RIA (Diasorin, Italy), using a modification of an unextracted RIA method for serum cortisol. Briefly, 200µL saliva was pipetted into the coated tube and incubated with ¹²⁵I cortisol for 45 minutes at 37°C. The modified cortisol assay had a measuring range from 0.5-30 µg/L and within- and between-run coefficients of variation of <5% and <10%, respectively. Salivary cortisol correlates highly with serum levels and represents the free, biologically active fraction of the hormone (Vining and McGinley, 1986). Salivary cortisol responses can be observed 5-20 min after stress induction, with peak levels after 10-30 minutes (Kudielka and Kirschbaum, 2005). It might be more indicative than serum total cortisol, because salivary cortisol, largely unbound, is independent of cortisol binding globulin variations (Lac, 2001).

2.3. Critical Feedback Task (CFT)

The CFT is an oddball mental counting task where participants receive bogus negative feedback on their performance at the completion of each test-block (Rossi and Pourtois, 2012, see Fig. 1A). The CFT is divided into one practice block (20 stimuli) and three test blocks (100 stimuli each, 80 standards and 20 targets). In each block participants are presented with a stream of identical small white tilted lines (the standards, on black background, with a duration of 250ms, ISI randomized between 900 and 1250 ms, see Fig 1B)), and are asked to detect the appearance of rare lines with a different in-plane orientation (the deviants, i.e., targets). During the practice phase participants are familiarized with the orientation of the standard line (always 35°) and they are asked to try to learn it. After the practice block, the instruction presents again the standard line, right next to an example of the two possible deviant lines. Participants are asked to covertly count the number of deviant lines, and to insert this number at the end of each test block. The angular difference between standards and targets is manipulated in order to create variation in task load: one block is difficult (standard-target difference = 3° of angle), one is intermediate (standard-target difference = 5° of angle) and one is easy (standard-target difference = 10° of angle). The participants always start with the difficult block (unknown to them) and are informed by a cover story that after each test-block they will receive a feedback on their performance. They are led to think that the difficulty of the subsequent block will depend on their performance on the current one (in a staircase design). However, the given feedback is in fact unrelated to performance (it's always negative, see Fig 1A), and the following block is always easier, to maintain motivation despite the elicitation of failure feelings and state anxiety. Every feedback consists of a neutral face with a text balloon for 20 s, stating that they performed below average as compared to the other participants. Consistently, a pseudo-randomly generated scatter plot shows their own performance against the scores of the previous (alleged) participants. To maximize the stress response to the critical feedback (CF)s, initial instructions emphasized the important role played by learning processes in this task, and alleged links between learning, performance, and intelligence. Moreover, participants were told that they would be compared with other peers at the end of each block, and of feedbacks on relative performance would be provided.

2.4. HF-rTMS experiment

A randomized sham-controlled, single blind, crossover design was used. To avoid carry-over effects from the previous stimulation, the second session was carried out after an interval of three days. See also Fig 2. The procedure of the second experiment day was exactly the same with the exception of the HF-rTMS session (real or sham) which was counterbalanced with random selection of order. We took especially care for a rigid time schedule: to avoid diurnal variations of cortisol (Hanson et al., 2000), all participants started the second experimental procedure at the same hour as the first experimental procedure. Because cortisol secretion remains reasonable steady in the late afternoon (Debono et al., 2009), all volunteers were stimulated in the afternoon within the same time frame between 16:00 and 18:00. In practice, if a given volunteer was stimulated at 17:00 at the first run, she was stimulated again at 17:00 on the second run. Subjects were kept unaware of the type of stimulation they received; they wore earplugs and were blindfolded. After finishing the final experimental day, all participants were fully debriefed.

We used a Magstim high-speed magnetic stimulator (Magstim Company Limited, Wales, UK), connected to a figure-of-eight-formed double 70mm coil. As all participants were right-handed, before each application, the resting motor threshold (MT) of the right abductor pollicis brevis muscle of each individual was determined. MT was estimated before the first stimulation session, and checked again on the second visit. In order to accurately target the left DLPFC (Brodmann area 9/46), taking into account individual anatomical brain differences, the precise stimulation site and position of the coil was determined using MRI non-stereotactic guidance (Peleman et al., 2010). Perpendicular to this point the precise stimulation site on the skull was marked and stimulated. In each high-frequency (20 Hz) stimulation session, at stimulation intensity of 110 % of the subject's MT, subjects received 20 trains of 1.9 s duration, separated by an intertrain interval of 12.1 s (1560 pulses per session). For the sham condition, the coil was held at an angle of 90°, only resting on the scalp with one edge. The study conform current safety guidelines (Rossi et al., 2009).

At the start of the experiment all subjects relaxed for 20 minutes while listening to relaxing music in a quiet and comfortable room (See Fig 2). Hereafter, subjects were asked to deliver a first

salivette and VAS, this just before the start of HF-rTMS session (T_1). All subjects then received sham or active HF-rTMS. Immediately after stimulation, subjects delivered another salivette together with the VAS questionnaire (T_2). Next, approximately five to ten minutes after HF-rTMS, all were asked to complete the CFT task, followed by the third salivette and VAS (T_3). These measurements were repeated after 15 minutes (T_4) while participants waited alone in the experiment room, without relaxing music. We analysed salivary cortisol samples at the four different time points (T_1 , T_2 , T_3 and T_4) and, as proposed by Pruessner et al (2003), we calculated the area under the curve (*AUC*) with respect to ground (*AUC_g*) and the *AUC* with respect to increase (*AUC_i*). The *AUC_g* measures, in endocrinological terms, the total 'hormonal output', whereas the *AUC_i* measures the hormonal changes over time. Therefore, the *AUC_i* index is especially suitable to evaluate HPA-system sensitivity (Fekedulegn et al., 2007).

As advised by Pruessner and colleagues (2003), in the *AUC* formulas we took into account the exact timings for each individual participant between saliva sampling throughout the experiment.

3. Statistical analysis

All collected data were analyzed with SPSS 20 (Statistical Package for the Social Sciences). Where necessary, we applied the Greenhouse-Geisser correction to ensure the assumption of sphericity. The significance level was set at $p \leq .05$ for all analyses.

First, to examine whether possible mood changes by the rTMS application could influence our results, mood changes were analyzed with a mixed 2X4 MANOVA. Within-subject factors were Stimulation (real vs. sham HF-rTMS) and Time (T₁, T₂, T₃ and T₄). The six VAS mood scales were the multiple dependent variables.

Second, the effects of HF-rTMS on the HPA-system (*AUC_g* and *AUC_i*) between real and sham stimulation were analyzed using paired *t*-tests. Analyses were performed for *AUC_g* and the *AUC_i* separately. The choice of *AUC* above repeated measures ANOVA's has several advantages. The computation of *AUC* allows simplification of the statistical analysis and increases the power of testing without sacrificing the information contained in multiple measurements (Pruessner et al., 2003). As mentioned before, the *AUC_g* measures, the total 'hormonal output', the *AUC_i* the hormonal changes over time. Based on a repeated measures ANOVA it is impossible to differentiate between these two sorts of information comprised within each measure (Fekedulegn et al., 2007). Further, if time intervals between cortisol measurements are not identical between participants, as it is seldom minute sharp in experimental research, the within-design ANOVA has no proper method to correct for these differences (Pruessner et al., 2003). Importantly, the *AUC* formulas take into account the exact timings between saliva sampling throughout the experiment.

To rule out that the timing of cortisol assessment did not differ across two experimental procedures, we performed paired *t*-tests between the time intervals on cortisol sampling in the real and the sham condition. Time intervals were calculated as the time difference in minutes between two sample points.

4. Results

For unknown reasons one female participant did not show up again for her second session after receiving sham in the first. Therefore, data from this volunteer were removed for all analyses. VAS mood ratings and salivary cortisol data of the remaining 30 participants are summarized in Table 1. Sixteen participants first received real HF-rTMS before sham and the fourteen other volunteers received sham HF-rTMS followed by the real condition.

4.1. Mood effects

For two female subjects VAS data sets were incomplete. Therefore, VAS analyses were performed on 28 volunteers.

The MANOVA showed no significant main effect for Stimulation, $F(6, 22) = 0.63, p = .70$. On the other hand, we found a significant main effect for Time, $F(18, 234) = 2.24, p < .01$. The interaction effect however between Stimulation and Time, $F(18, 234) = 1.02, p = .44$, did not reach significance.

For an overview of the main Time effects per scale see Table 2. In essence, after stimulation (T_1 vs. T_2) participants felt significantly tenser, less fatigued and less cheerful, regardless of real or sham HF-rTMS. After having performed the CFT (T_2 vs. T_3) scores on vigor diminished significantly in both stimulation conditions. These observations indicate that both experimental days resulted in similar effects on mood measurements regardless of stimulation type.

4.2. Salivary cortisol

See also Fig 3. For the AUC_g analysis, the paired t -test did not show a significant difference between the real (mean $AUC_g = 227.99$ (sd= 107.33)) and the sham (mean $AUC_g = 218.54$ (sd= 79.93)) HF-rTMS session, $t(29) = .50$, $p = .63$). This finding demonstrates that regardless of stimulation type the experimental procedure had no different effect on the global cortisol output.

Concerning the AUC_i analysis, the paired t -test showed that compared to sham (mean AUC_i : sham= -1.10 (sd= 44.51)) one real HF-rTMS session (mean AUC_i : real= -22.03 (sd= 52.33)) resulted in a significant decrease in cortisol levels, $t(29) = 2.20$, $p = .036$. A paired t -test did not show baseline (T_1) cortisol differences between one real (mean= 4.10 $\mu\text{g/L}$ (sd= 1.81) and sham (mean= 3.64 $\mu\text{g/L}$ (sd= 1.41)) HF-rTMS session ($t(29) = 1.40$, $p = .17$). This implies that AUC_i differences cannot be attributed to unequal baseline cortisol levels and indicates that during the experimental procedure one left-sided real session significantly influenced HPA-system sensitivity, resulting in decreased cortisol concentrations.

The paired t -test between real (mean= 25.10 minutes, sd= 10.91) and sham (25.13, sd= 10.66) for T_1 to T_2 showed no significant difference ($t(29) = .01$, $p = .99$). Also the paired t -test between real (16.07, sd= 2.89) and sham (17.47, sd= 5.82) for T_2 after stimulation to T_3 just after performing the CFT showed no significant difference ($t(29) = 1.14$, $p = .26$). Finally, the paired t -test between real (17.30, sd= 3.56) and sham (16.93, sd= 3.47) for T_3 to T_4 ca 15 minutes after performing the CFT also did not reach significance ($t(29) = .41$, $p = .69$). These results show that the timings of cortisol assessment were not significantly different between the real and the sham procedure, and that the differences observed in our AUC_i analyses cannot be attributed to confound in timing of salivary cortisol sampling.¹

¹ Finally, to exclude that the AUC_i results cannot simply be explained by changes on the HPA-system prior to the CFT (due to the HF-rTMS procedure), we performed several ANCOVA analyses (controlling for inter-individual differences in time intervals) taking into account the separate salivary cortisol samplings before and after the different procedures (HF-rTMS/sham; CFT; 15 minutes waiting) within the experiment. These analyses were performed for the sham and real HF-rTMS separately, with Time (T_{pre}

vs T_{post} for a given procedure within the experiment) as within-subjects variable, Order (first sham versus first rTMS) as the between-subjects factor, and the time Interval (the time difference in minutes between two sample points) as covariate. We used the same time Intervals as described in the paragraph above. See also Fig 2. In short, when taking into account the individual salivary cortisol samplings in the real HF-rTMS condition, only after performing the CFT (between T₃ and T₄) the ANCOVA showed a significant main effect of Time ($F(1,27) = 4.05, p = .05$). No main effects of Interval ($F(1,27) = 1.81, p = .19$) was observed. The main effect of Order ($F(1,27) = 3.59, p = .07$) showed a significant trend. The mean cortisol levels appeared to be higher when participants received real HF-rTMS in the first session (T₃: 3.87 (sd= 1.50) and T₄: 4.19 (sd= 3.16) compared to those who received first the sham stimulation (T₃: 3.39 (sd= .76) and T₄: 2.98 (sd= .70)). The interaction effects between Time and Order ($F(1,27) = .78, p = .39$) was not significant, the interaction effect between Time and Interval ($F(1,27) = 3.33, p = .08$) showed a trend towards significance. However, the Pearson correlation analysis between Interval and the change in cortisol concentrations between T₃ and T₄ was not significant ($r = <-.01, n=30, p = .96$). Although only trend-like, these extra ANCOVA analyses may point to some effect of order: cortisol levels appeared to be higher when participants received real HF-rTMS in the first session, which might be caused by some habituation to the procedure in the second session. Importantly, the ANCOVA examining the effect of real HF-rTMS on cortisol concentrations (salivary cortisol sampling between T₁ and T₂) did not show a main effect of Time ($F(1,27) = .04, p = .84$), Order ($F(1,27) = 2.85, p = .10$), or Interval ($F(1,27) = .60, p = .45$). Also the interaction effects between Time and Order ($F(1,27) = .08, p = .78$), and between Time and interval ($F(1,27) = .21, p = .65$) were not significant. No other significant main or interaction effects were observed for the other time points, not for the real HF-session nor for sham (p 's > .05). These extra analyses show that the cortisol attenuation occurred just after the CFT in the real HF-rTMS condition. Together with the non-significant effects of HF-rTMS on cortisol levels just after stimulation, regardless of real or sham, the latter analyses in particular underscores that the positive effect on the HPA-system only occurs after healthy participants are being stressed, in our case with the CFT, and only after real stimulation.

5. Discussion

In this study, part of a larger project investigating the influence of HF-rTMS on different neurocognitive markers, we evaluated the effect of one sham-controlled left DLPFC HF-rTMS session on subjective mood changes and salivary cortisol during an experimental stressful oddball mental counting task. Subjective mood was not found to be different for the sham and real HF-rTMS experimental procedure. This corroborates with previous sham-controlled findings in healthy subjects of no different mood effects after one session of HF-rTMS applied to the left DLPFC (Mosimann et al., 2000; Baeken et al., 2006, 2008). Nevertheless, after both stimulation conditions participants felt tenser and less cheerful, indicating stress-related responses and increases in negative affect. In addition, also regardless of stimulation type, reduced scores on vigor were observed only after the CFT. This further increase in negative effect can be attributed to the critique of a bad cognitive performance (Rossi and Pourtois, 2012). However, subjectively experienced mood gives only limited insight into the neurophysiology of emotion and physiological responses might operate independently of verbal reports (Buck, 1999; Campbell & Ehlert, 2012).

In terms of global cortisol secretion, one HF-rTMS session did not affect the HPA-system differently between stimulation types (*AUCg*). This finding is not unexpected in an unimpaired cortico- limbic system with normal feedback (FB) mechanisms (see also Fig 4 A and B). During stress and/or increased negative affect, enhanced amygdala activity results in hypothalamic (paraventricular nucleus) release of corticotrope releasing hormone (CRH), which activates the release of adenocorticotrope hormone (ACTH) in the anterior pituitary gland. This activates the adrenal cortex to release the stress hormone cortisol into the blood stream (Gold and Chrousos, 2002; Erickson et al., 2003). Indeed, it has already been demonstrated that stress and negative affect was associated with higher salivary cortisol levels (Smyth et al. 1998; Herman et al., 2005). Furthermore, the paraventricular nucleus of the hypothalamus receives neural inputs from many regions of the brain, including the hippocampus (Aihara et al., 2007). Under normal conditions, the hippocampus inhibits amygdala activity, and regulates the HPA-axis (Herman et al., 2003). In a normal functioning negative

feedback system, when emotional homeostasis is reached again, this negative FB loop down-regulates activity via glucocorticoid (GR) receptors. Notwithstanding that global cortisol output was not different between a real and a sham HF-rTMS session, one real stimulation session only significantly influenced HPA-system sensitivity, resulting in decreases in cortisol levels (*AUCi*).

Our current results lend support to our assumption that one left-sided HF-rTMS session is able to attenuate the HPA-system while experimentally being stressed. Because cortisol effects are frequently observed in stress induction paradigms (i.e. Dickerson and Kemeny, 2004), it could be argued that some kind 'discomfort' due to different sensations during the real session versus sham would have interfered with our endocrinological measurements. However, if this would be the case we would have detected an increase in tension or other mood changes only in the real condition and not after sham. Because we used a sham-controlled counterbalanced design and all of our volunteers were stimulated twice within the same time frame on separate days, the differences in HPA-system sensitivity cannot be attributed to the circadian variation of cortisol (Hanson et al., 2000). Furthermore, in order to obtain more valid baseline cortisol and mood measurements, all volunteers participated in a standard relaxation session before starting the experimental procedure. Importantly, salivary cortisol levels did not differ between the two stimulation days at T_1 . We used salivettes as this has certain advantages over blood samples: sampling is non-invasive, it can frequently be repeated, and it avoids stress induction (painless) (Castro et al., 2000).

So how can we interpret these results? First of all, the *AUCg* analyses showed that the salivary cortisol measurements at T_1 , T_2 , T_3 and T_4 , were not related to real or sham stimulation. However, as the *AUCi* contains the important information whether any changes of the HPA-system as indexed by saliva cortisol occurred over time (examining changes in the events during the observation period over the entire experimental procedure over T_1 , T_2 , T_3 and T_4), our findings revealed that only real HF-rTMS significantly decreased cortisol concentrations. Our extra ANCOVA analyses showed that this attenuation of cortisol concentrations occurred directly after having performed the CFT in the real HF-rTMS experimental procedure. Although only trend-like, these extra ANCOVA analyses may point to some effect of order. Cortisol levels appeared to be higher when participants received real HF-rTMS in

the first session, which might be caused by some habituation to the procedure in the second session. Because there were significant differences between T_3 and T_4 more ca 15 minutes after having performed the CFT, these findings indicate that the effects of one real HF-rTMS session are transient and have short term effects in healthy individuals. Although one would expect cortisol increases after having performed the CFT in the sham condition, we can only speculate that for all participants the entire experimental procedure was a stressful event and performing the CFT did not add significantly more stress to the procedure. Nevertheless, the crucial finding here is that real HF-rTMS attenuated cortisol concentrations after participants performed this critical feedback task. Despite that no subjective mood changes were detected, this attenuation after real stimulation suggests that on the endocrinological level healthy females may become less sensitive to stressful events or negative experiences.

Further, our current *AUCi* results do agree with Keck's hypothesis that the (left) prefrontal cortex participates in the rTMS-induced blunted response of HPA-activity as found in individuals documented to have high cortisol concentrations over longer periods of time, such as depressed patients (Keck, 2003) (see also Fig 5). In addition, it also indicates that in healthy individuals the HPA-system may need to be challenged in order to detect such influences (in the current experiment the negative feedback to the CFT after HF-rTMS). Indeed, our previous negative results investigating the effect of HF-rTMS on the HPA-system might be attributed to an already relatively low baseline level of cortisol in an 'unchallenged' healthy group of women (Baeken et al., 2009a, 2011a). Of course, the interpretation of our results is limited to relatively young healthy females and cannot be generalized to a broader or psychiatric population.

Nevertheless, our endocrinological observations in experimentally stressed participants agree with the neurophysiological effects of this neurostimulation tool in stress-related disorders such as MDD: HF-rTMS initially disrupts neural processes in the stimulated area (Paus et al., 2001), thereafter resulting in higher neural activity in the DLPFC (Nahas et al., 2001). Through synaptic connections, metabolic changes in the connected subcortical structures influence the HPA-system (Paus & Barrett, 2004). Keck (2003) proposed that the influence of rTMS over the DLPFC during depressed emotional

states indirectly affects the hypothalamus (paraventricular nucleus), resulting in blunted responses of HPA-axis activity (see Fig 5). Some studies have examined this hypothesis in depressed patients (Schutter and van Honk, 2010). Compared to negative mood states in non-psychiatric samples, in (melancholic) depression there is an even more pronounced shift in the homeostasis with diminished activity in the DLPFC, enhanced activity in the amygdala and activation of the core stress system. In depressed states this excitatory system results in dramatic CRH and ACTH increases and in cortisol elevations (Gold and Chrousos, 2002). In these kinds of patients, the failing negative feedback system may result in chronic hypercortisolemia. However, it has to be noted that in depressed patients HPA-system abnormalities are not consistently observed (Schutter and van Honk, 2010). Nevertheless, in a sample of severely depressed patients, salivary cortisol concentrations decreased immediately after one active left DLPFC HF-rTMS session and not after sham (Baeken et al., 2009b). Pridmore (1999) observed normalization of the dexamethasone suppression test in a small sample of medicated depressed subjects after multiple sessions of left prefrontal HF-rTMS. Of interest, depressed patients who fail to respond to several pharmacological interventions show unchanged enhanced HPA-system activity (Wolkowitz & Reus, 2001) and HF-rTMS non-responders continue to display a more sensitive HPA-system (Baeken et al., 2010b).

However, through which exact pathway left DLPFC HF-rTMS affects the HPA-axis remains to be clarified and without concomitant neuroimaging techniques the interpretation of our endocrinological results remains to some extent speculative. As shown in Fig 5, a possible working mechanism points to a DLPFC / anterior cingulate cortical (ACC) pathway. Indeed, besides the dorsolateral prefrontal regions, in brain imaging studies examining negative affect, the dorsal (d)ACC areas are often involved as well (Pizzagalli, 2011). Diminished connectivity between the DLPFC and dACC might also result in the failing of the ACC's inhibitory role in amygdala regulation of emotional processing in major depression. Different brain imaging studies in MDD lend support to the assumption that left HF-rTMS affects and 'normalizes' DLPFC and ACC metabolic and functional neuronal activities (Baeken et al., 2009; Kito et al., 2008, 2012; Fox et al., 2012 a). However, the subgenual (sg)ACC, part of the ventromedial prefrontal cortex, and strongly connected to the amygdalae (Barbas et al., 2003; Ray and

Zald, 2012) may be a critical region to be involved in the response to the rTMS application. The sgACC, implicated in “visceromotor” functions and in modulating affect, has consistently been shown to be metabolically hyperactive during depressive episodes and successful antidepressant treatment results in neuronal attenuation of this ventromedial prefrontal cortical area (Drevets et al., 2008). Further, also HF-rTMS treatment has been shown to affect deregulated sgACC neurocircuits in depressed patients (Fox et al, 2012b; Baeken et al., *in press*).

Because the DLPFC and the hippocampus are synaptically connected via (glutamatergic) pyramidal neurons (Puig et al., 2003), from an electrophysiological point of view, HF-rTMS may also directly influence neuronal activity in hippocampal regions. Albeit several different signalling synapses may be involved (for an overview see the recent review of Marsden (2013)), animal models found that rTMS and electrical stimulation of (medio)frontal cortical areas influenced serotonergic neurotransmission in the hippocampus (Juckel et al., 1999; Ogiue-Ikeda et al., 2003). Serotonin (5-HT) is an important excitatory transmitter involved in HPA-system regulation (Neumeister and Charney, 2002). Of interest, HF-rTMS applied to the left DLPFC leads to decreased serotonin synthesis in the parahippocampal areas in healthy subjects (Sibon et al., 2007). Serotonin receptors such as the post-synaptic 5-HT_{2A} receptor located in the amygdala and hippocampus regulate the HPA-system during stress (Leonard, 2005). In a recent study, examining the effect of left DLPFC HF-rTMS treatment on 5-HT_{2A} receptor binding indices in medication-resistant depressed patients we found a significant hippocampal 5-HT_{2A} receptor down-regulation only when treatment was clinically successful (Baeken et al., 2011b).

In conclusion, our results show that in experimentally stressed female participants one real left-sided HF-rTMS session attenuates HPA-system sensitivity, resulting in decreases in cortisol levels. The additional use of stressful experimental procedures might be especially critical to influence stress responses and negative affect in laboratory settings evaluating the effects of HF-rTMS on the HPA-system (Nummenmaa and Niemi, 2004). Our observations explain to some extent as to why successive left prefrontal HF-rTMS sessions could be beneficial in patients with deregulated HPA-

systems, such as major depression. From a neurophysiological point of view, it might be interesting to examine whether LF-rTMS applied to the left DLPFC increases HPA-system sensitivity while healthy females are experimentally being stressed, as opposite neuro-endocrine effects can be anticipated. Albeit based on ethical grounds we do not advocate to perform multiple HF-rTMS sessions in healthy populations, one can speculate that successive sessions may influence the HPA-system to a larger extent than only one stimulation session. Future studies evaluating the HPA-system, in healthy participants as well in stress-related psychiatric disorders, might do well to combine experimental stress tasks with brain imaging techniques.

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Table and figure legends

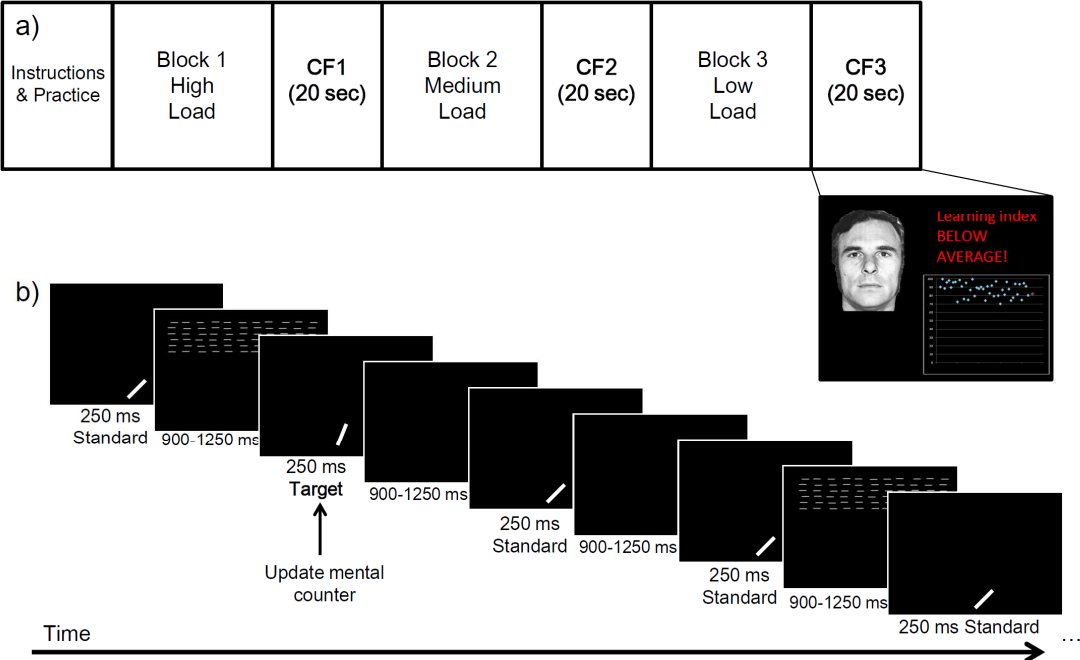
| | <u>Real HF-rTMS</u> | | | | |
|---|---------------------|-------------|-------------|-------------|-------------|
| | <i>T1</i> | <i>T2</i> | <i>T3</i> | <i>T4</i> | <i>T1</i> |
| Salivary cortisol ($\mu\text{g/L}$) | 4.10 (1.81) | 3.66 (1.23) | 3.58 (1.24) | 3.63 (2.40) | 3.64 (1.41) |
| VAS Fatigue | 4.00 (2.66) | 3.40 (2.62) | 3.24 (2.36) | 3.60 (2.54) | 3.79 (2.66) |
| VAS Vigor | 5.42 (2.30) | 5.45 (2.18) | 5.12 (2.41) | 5.15 (2.16) | 5.11 (2.28) |
| VAS Anger | .86 (1.10) | 1.08 (1.15) | 1.31 (1.57) | 1.04 (1.48) | .73 (.69) |
| VAS Tension | 1.46 (1.41) | 1.95 (1.61) | 1.75 (1.76) | 1.56 (1.60) | 1.44 (1.35) |
| VAS Depression | .66 (.69) | .70 (.74) | .78 (1.17) | .61 (.77) | .59 (.65) |
| VAS Cheerfulness | 6.33 (2.25) | 5.69 (2.27) | 5.64 (2.27) | 5.80 (2.11) | 6.08 (1.97) |

Table 1. Mean ratings and standard deviations for the VAS subscales before (T_1) and immediately after left-sided HF-rTMS (T_2). Immediately after the Critical Feedback Task (CFT) (T_3) and 15 min later (T_4) on the left DLPFC.

| | <u>Experimental procedure</u> | | | |
|--------------------------------------|-------------------------------|-------------|-------------|-------------|
| | <i>T1</i> | <i>T2</i> | <i>T3</i> | <i>T4</i> |
| VAS Fatigue ^a | 3.90 (0.46) | 3.33 (0.43) | 3.25 (0.40) | 3.55 (0.40) |
| VAS Vigor ^b | 5.26 (0.40) | 5.43 (0.38) | 5.13 (0.42) | 5.11 (0.40) |
| VAS Anger | 0.79 (0.15) | 0.90 (0.17) | 1.03 (0.19) | 0.90 (0.19) |
| VAS Tension ^a | 1.45 (0.15) | 1.87 (0.20) | 1.74 (0.21) | 1.41 (0.16) |
| VAS Depression | 0.62 (0.11) | 0.72 (0.14) | 0.66 (0.15) | 0.56 (0.12) |
| VAS Cheerfulness ^a | 6.21 (0.38) | 5.87 (0.38) | 5.85 (0.37) | 5.79 (0.37) |

Table 2. Mean ratings and standard errors for the VAS subscales of the entire experimental procedure before (*T*₁) and immediately after HF-rTMS (*T*₂). Immediately after the Critical Feedback Task (CFT) (*T*₃) and 15 min later (*T*₄). Significant pairwise comparisons ($p < .05$): ^a between *T*₁ and *T*₂, ^b between *T*₂ and *T*₃, ^c between *T*₃ and *T*₄.

Figure 1:



(A) Task design. Three blocks of descending load level (preceded by a practice block) were intermixed with bogus critical feedbacks (CF, always negative) on task performance. Inset: The critical feedbacks contained a neutral face, a message clearly stating that performance (learning index) was below average as compared to a group of previous participants, and a pseudo randomly generated scatterplot with the performance of all the (alleged) participants, in which the current one was clearly in the lower part of the distribution.

(B) Trial sequence. Standard (80%) and target (20%) lines were intermixed in a RSVP at fixation. Participants had to silently count the targets, and report their number at the end of each block. During the ISI, randomly jittered between 900 and 1250 ms, a peripheral texture of horizontal lines was presented in 50% of the trials (no predictive value for the central stimuli).

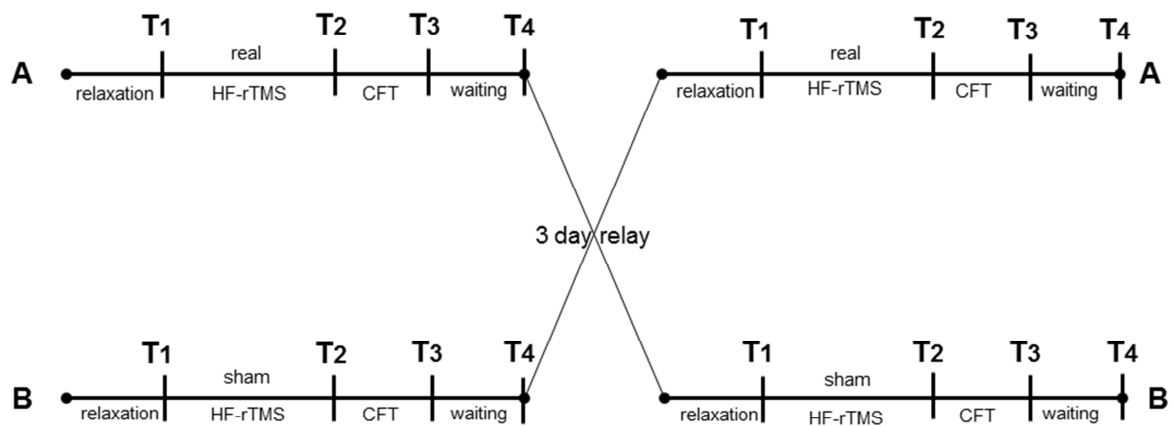


Figure 2: Visualization of the experimental sham-controlled HF-rTMS cross-over design.

AB= a female subject who first receives real HF-rTMS then receives sham. BA= a subject that first receives sham then receives real HF-rTMS. The experiment starts with 20 minutes relaxation. Five to 10 min after stimulation participants performed the Critical Feedback Task (CFT). At T₁ = just before HF-rTMS, T₂ = just after HF-rTMS, at T₃= just after the CFT and 15 min hereafter (T₄) all volunteers were assessed with 6 visual analogue scales and delivered a salivette at each of these time points. To avoid carry-over effects the second HF-rTMS sessions was performed three days later.

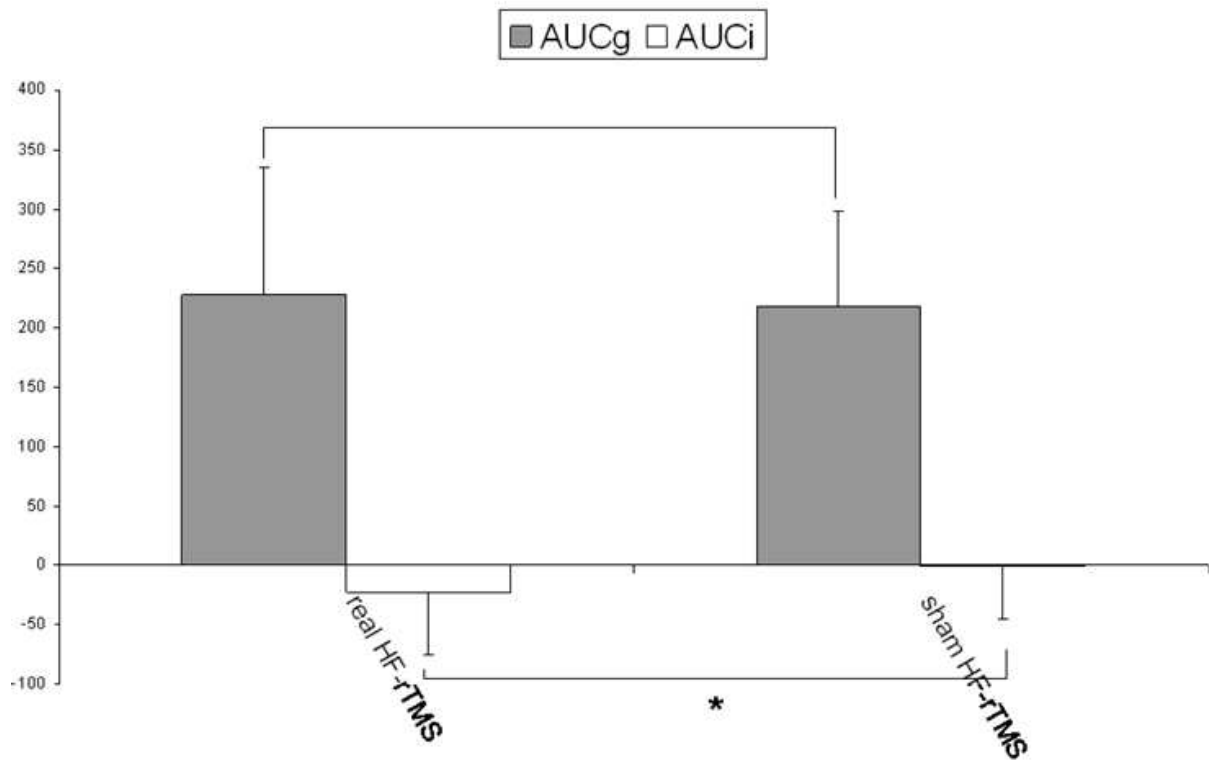


Figure 3: Results of the area under the curve (*AUC*) with respect to ground (*AUCg*) and the *AUC* with respect to increase (*AUCi*) for the real and the sham left DLPFC HF-rTMS session (means with standard error) separately represented in bar graphs. *Significant differences between one left-sided real and sham HF-rTMS session at $p < .05$.

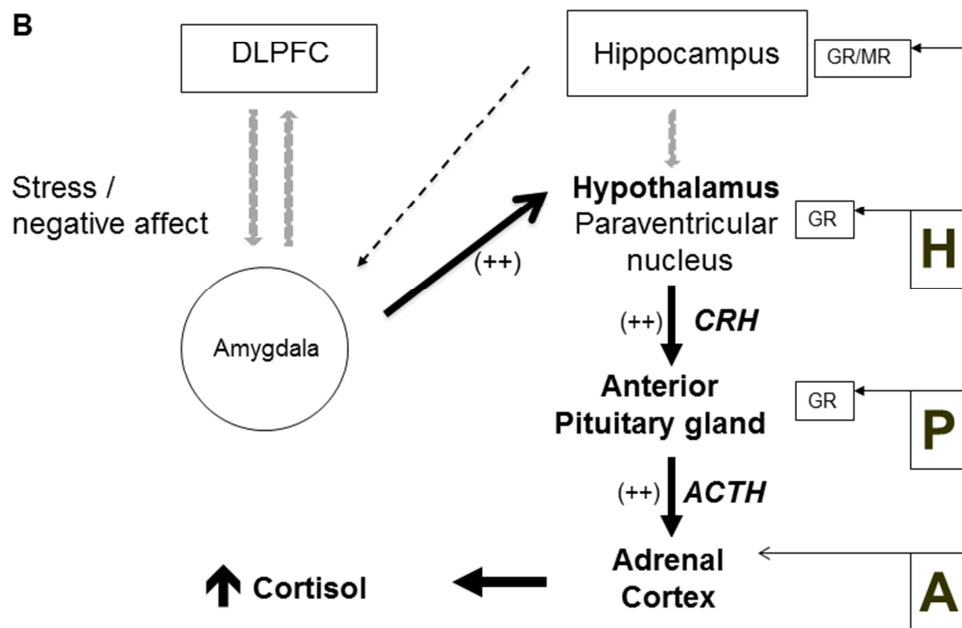
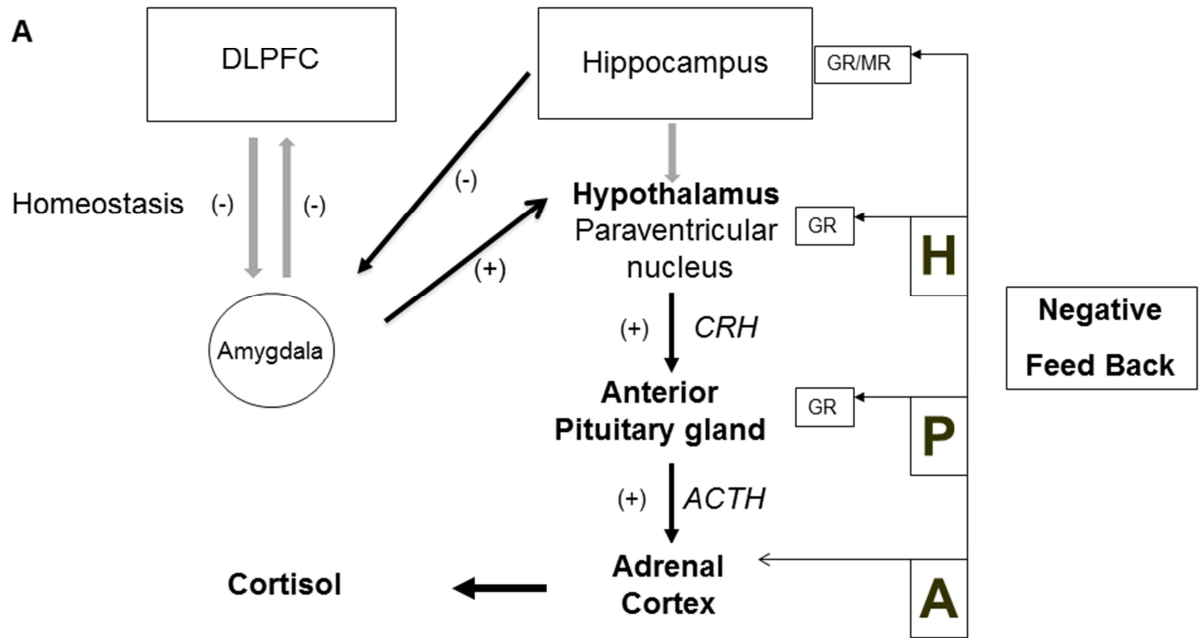


Figure 4: The Hypothalamus- Pituitary-Adrenal cortex (HPA) system and negative feedback system in normal mood state.

- A) Interrelation of the stress/mood system mediators and circuitries between the prefrontal cortex (PFC) and amygdala / hippocampus in normal homeostasis and negative feedback system.
- B) During stress or increased negative affect enhanced amygdala activity results in hypothalamic (paraventricular nucleus) release of corticotrope releasing hormone (CRH), which activates the release of adenocorticotrope hormone (ACTH) in the anterior pituitary gland. This activates the adrenal cortex to release the stress hormone cortisol into the blood stream.

Smaller or larger circles and rectangles indicate shifts in neuronal activities. Full lines represent strong functional connections between structures, dotted lines represent decreased functional connections.

CAVE: The grey lines between the presented anatomical regions do not by definition imply direct synaptic connections. For instance, the neuronal connections between the DLPFC and amygdala are presumably indirect. The neuronal routes to the hypothalamus are multi-synaptic.

H: Hypothalamus. P: Pituitary gland. A: Adrenal cortex. MR: mineralocorticoid receptor. GR: glucocorticoid receptor. (-) inhibitory. (+) excitatory.

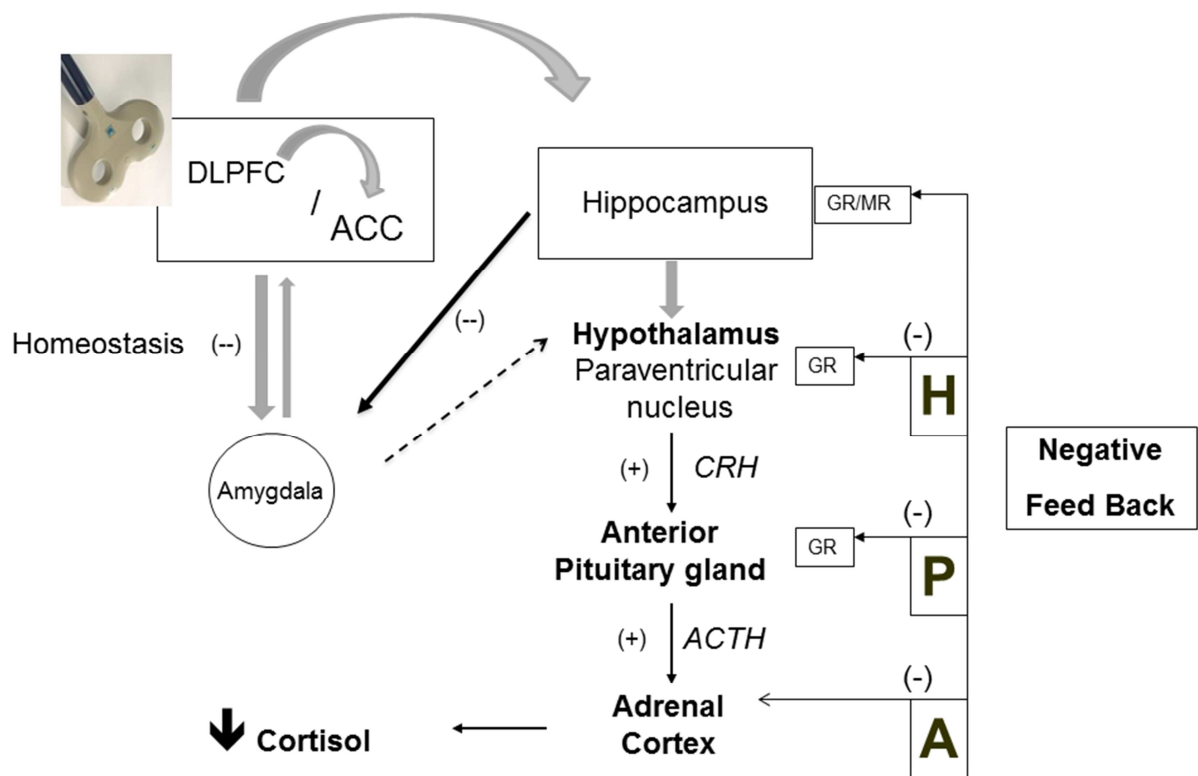


Figure 5: Theoretical framework of HF-rTMS applied to the left DLPFC on the Hypothalamus-Pituitary-Adrenal cortex (HPA) system in negative mood states.

In the left hand corner a figure-of-eight shaped rTMS coil is depicted. The HF-rTMS application is thought to result in increased neuronal activity in the stimulated area (DLPFC), which through cortico-subcortical transsynaptic connections suppresses hypothalamic and/or indirectly amygdala hyperactivity, resulting in CRH decreases and ultimately in decreased salivary cortisol concentrations, returning to the initial homeostasis. Although HF-rTMS effects on the HPA-system act presumably through fronto-cingulate networks, the direct connection between the DLPFC and hippocampus may add to this effect (full black arrows). A decreased functionality in the latter might contribute to a diminished inhibition of the amygdalae, resulting in continuous amygdala hyperactivity.

Full lines represent strong functional connections between structures, dotted lines represent decreased functional connections. Grey lines represent multi-synaptic neuronal connections, which are not by definition direct routes (for an overview see Ray and Zald, 2012).

DLPFC: dorsolateral prefrontal cortex. ACC: anterior cingulate cortex. GR: glucocorticoid receptors
MR: mineral-corticoid receptor. CRH: corticotrope releasing hormone. ACTH: adenocorticotrope
hormone. H: Hypothalamus. P: Pituitary gland. A: Adrenal cortex. (-) inhibitory. (+) excitatory.