

BIOLOGICAL SCIENCES

Physiology

Ultradian rhythmicity of plasma cortisol is necessary for normal emotional and cognitive responses in man

SHORT TITLE: Glucocorticoid rhythmicity and the human brain

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Abstract

Glucocorticoids are secreted in an ultradian, pulsatile pattern which emerges from delays in the feedforward-feedback interaction between the anterior pituitary and adrenal glands. Dynamic oscillations of glucocorticoids are critical for normal cognitive and metabolic function in the rat and have been shown to modulate the pattern of glucocorticoid-sensitive gene expression, modify synaptic activity and maintain stress responsiveness. In man, current cortisol replacement therapy does not reproduce physiological hormone pulses and is associated with psychopathological manifestations -especially apathy and attenuated motivation in engaging with daily activities. In this paper, we test the hypothesis that the pattern of glucocorticoid dynamics in the brain is of crucial importance for regulating cognitive and behavioural processes. We provide evidence that exactly the same dose of cortisol administered in different patterns alters the neural processing underlying the response to emotional stimulation, the accuracy in recognition and the attentional bias towards/away from emotional faces, the quality of sleep and the working memory performance of healthy male volunteers. These data indicate that the pattern of the glucocorticoid rhythm differentially impacts human cognition and behaviour under physiological, non-stressful conditions, and has major implications for the improvement of cortisol replacement therapy.

Significance Statement

The hypothalamic-pituitary-adrenal axis is a critical neurohormonal network, regulating homeostasis and coordinating stress responses. In the current studies, we demonstrate that an oscillating pattern of plasma cortisol is important for maintenance of healthy brain responses as measured by functional neuroimaging and behavioural testing. Our data highlight the crucial role of glucocorticoid rhythmicity in (i) modulating sleep behaviour and working memory performance, and (ii) regulating the human brain's responses under emotional stimulation. Current optimal cortisol replacement therapies for patients with primary or secondary adrenal insufficiency are associated with poor psychological status and these results suggest that more attention to aspects of chronotherapy will benefit these patients and may also have major implications for improved glucocorticoid dynamics in stress and psychiatric disease.

Author contributions

G.M.R. and S.L.L. conceived and designed the study. C.J.H., M.R.M., J.C.B., N.J.T. and K.K. optimized the study protocol. N.J.T. and K.K. optimized the technical equipment's capacity to perform the neurobehavioural investigation of the study. K.K., G.M.R., J.C.B. and N.J.T. optimized the functional and perfusion neuroimaging protocol. K.K. and N.M. enrolled the participants. N.M. was interacting with participants during the periods of treatment intervention. K.K., N.M., A.W. and P.M. performed the neurobehavioural investigation. K.K., C.J.H., M.R.M., J.C.B., C.D., P.M., J.T. and S.L.L. analysed the data. All authors contributed to the writing of the manuscript and approved the final version.

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Introduction

Glucocorticoids (GCs), predominantly cortisol in man, are critical for life and are key regulators of cognitive, metabolic and immunological homeostasis (1). The lipophilic nature of these hormones allows them easy access to all tissues of the body including the central nervous system where they can cross the blood-brain barrier to act upon glucocorticoid receptors (GRs) and mineralocorticoid receptors (MRs) found in both neuronal and glial populations (2). In the rat, there is an extensive literature on the importance of GCs for short- and long-term neurocognitive adaptation to stressful conditions (3–6), and clinical studies in healthy human subjects, using functional neuroimaging techniques, clearly demonstrate the importance of GCs in the neural response to stress (7–16).

During the basal “unstressed” physiological state, plasma cortisol varies across the 24-hour cycle with a circadian rhythm that reaches a peak soon after waking and falls to low levels late at night. This circadian rhythm is actually made up from an underlying ultradian rhythm, which can be modified by internal or external stressors (17). This ultradian rhythm of glucocorticoid secretion has been observed in all mammalian species studied, including rodents (18), sheep (19), horses (20), deer (21), cows (22, 23), monkeys (24, 25) and man (26–35). Mathematical biomodelling also provides a very strong rationale for the importance of this oscillating rhythm (36–39).

Current regimens of cortisol replacement therapy for patients lacking adrenal function do not replace ultradian rhythmicity, and result in considerable morbidity. Several centres have confirmed that these treatment regimens result in impaired health related quality of life (40–45), adverse metabolic and cardiovascular risk profiles (46), increased levels of pro-inflammatory cytokines (47), and reduced activity, low motivation and mental fatigue with associated high levels of unemployment and disability benefit (41, 48–50). This was initially assumed to be a direct consequence of the inability of oral medication to provide the normal circadian variation of cortisol, and that this could be improved by continuous subcutaneous infusions of the hormone. This has proved not to be the case and Gagliardi et al. have convincingly shown that “non-pulsatile” continuous subcutaneous infusion does not improve subjective health status (51) whilst Oksnes et al. in an open label study, showed only small changes in a limited number of domains in a quality of life questionnaire and suggested that this might be due to the lack of ultradian pulses in their infusion protocol (52).

There are also clear theoretical reasons why pulsatile replacement might be necessary for normal physiological responses. We know GCs not only pulse in the blood, but also in peripheral tissues such as the brain (53). Furthermore, the brain responds dynamically to these oscillations (54) with differential genomic (55) and rapid non-genomic responses including the accumulation of glutamatergic receptors into synapses and induction of long-term potentiation (56). Even the behavioural responses of rodents to a mild stressor are dependent on endogenous pulses (57).

Motivated by the clinical need for improved glucocorticoid-based therapeutics, and the preclinical evidence, we hypothesized that different ultradian glucocorticoid rhythms, under normal, non-stressful, non-pathological conditions, might be translated differently in relevant glucocorticoid responsive human brain regions, and this differential processing should be detectable using well-designed experimental protocols. We developed a “block and replace” protocol (combined administration of metyrapone with hydrocortisone infusion) in which we could reliably impose definitive patterns of plasma hydrocortisone (58). This has allowed us to provide three predetermined patterns of cortisol replacement therapy: (i) normal circadian rhythmicity lacking any physiological ultradian rhythm (subcutaneous-continuous hydrocortisone infusion, SCC), (ii) normal circadian and ultradian rhythmicity (subcutaneous-pulsatile hydrocortisone infusion, SCP) and (iii) current optimal oral replacement therapy (PO), characterized by suboptimal circadian and ultradian rhythms. We have used these three treatment regimens in a double-blind, placebo-controlled, crossover study on healthy male volunteers to assess the importance of cortisol rhythmicity for normal brain activity in man,

utilizing a combination of fMRI and psychological tasks (Fig. 1), based on the stimulation with emotionally valenced cues (implicit facial expression processing task, IFEPT, and parts of the p1vital® emotional test battery, respectively), that recruit glucocorticoid responsive brain regions we have previously shown to be sensitive to changes in glucocorticoid infusion patterns in our preclinical studies (56, 57). We also gathered dynamic measurements of affective state throughout these interventions.

Results

Ecological momentary assessment (EMA) data taken throughout the study showed no difference in either positive or negative affect between the SCC and SCP groups [$F(1.587, 22.221) = 0.196$, $p = 0.773$, and $F(1.321, 18.493) = 2.303$, $p = 0.141$, respectively]. However, individuals on oral hydrocortisone replacement showed higher negative mode ratings compared to the SCP group (mean difference of 3.154, 95% CI 0.754-5.554, $p=0.009$) (Fig. 2A).

The non-pulsatile GC rhythm is associated with poorer quality of sleep. An interaction of cortisol dynamics was found in one of the four domains of sleeping behaviour assessed by the Leeds sleep evaluation questionnaire (LSEQ) [$F(1.914, 26.801) = 4.137$, $p = 0.029$, $\omega^2 = 0.12$]. Based on the volunteers' responses in the visual analogue scale (VAS), quality of sleep is poorer (more and/ or longer periods of restlessness and wakefulness) when undergoing the subcutaneous-continuous hydrocortisone infusion in comparison to the other two modes of hydrocortisone replacement (Fig. 2B and Fig. S1). Post-hoc analysis with a Bonferroni adjustment however didn't reveal any pairwise differences between any of the combinations of groups.

The n-back task reveals an effect of optimal pulsatile GC replacement in retention of working memory capacity under increased cognitive demands. The n-back task is considered to reflect working memory processes, when n equals 2 or more; in the latter cases, the working memory buffer must be updated continuously to keep track of what the current stimulus must be compared to, necessitating maintenance and manipulation of information in the working memory. Zero- and 1-back tasks are used as control sessions. Two subjects performed poorly (significantly low %accuracy scores, with the values of studentized residuals being lower than -3) in these control sessions in at least one of the study arms. This poor performance indicates a systematic bias (see Materials and Methods section), unrelated to cortisol dynamics or other experimentally controlled factors, requiring removal from the data analysis. Due to the crossover nature of the study, these subjects were excluded completely from further analysis for this task ($N=13$ per study group).

Analysis of variance (ANOVA) elicited a two-way interaction of [cognitive load] x [cortisol dynamics] on participants' performance in the n-back task [$F(1.525, 18,304) = 4.437$, $p = 0.035$]. Volunteers in the SCP group retain the same performance across the 2- and 3-back sessions, in contrast to their performance in the other two treatment modes, where performance in 3-back sessions is poorer compared to the 2-back sessions, especially in the non-pulsatile group (continuous infusion group mean drop in %accuracy score from 2- to 3-back sessions 7.9% with 95% CI 2.7-13%, $p = 0.006$, $\omega^2 = 0.28$, mean difference in the %accuracy in 3-back sessions between the subcutaneous pulsatile and the continuous infusion groups 6.6% with 95% CI -0.3% to 13.5%, $p = 0.063$, $\omega^2 = 0.10$) (Fig. 3).

ANOVA did not demonstrate any two-way interaction of [cognitive load] x [cortisol dynamics] on participants' reaction times across correct responses [$F(1.403, 16.835) = 0.663$,

$p = 0.477$], nor any significant interactions for the main effects of each factor. Therefore, the differences in %accuracy reported above have not been confounded by differences in reaction times.

The SCP group shows a reduced accuracy in recognizing negatively valenced emotional input, as assessed by p1vital® face emotion recognition task (FERT). This task measures the individuals' accuracy and speed for interpreting facial expressions. After each brief exposure to a human face (for details see Materials & Methods), subjects need to make one of seven choices, indicating that they encountered either a neutral expression, or a positive expression (happy, surprise), or a negative one (angry, fear, disgust, sad). %Accuracy scores for recognizing neutral faces did not differ between treatment groups [$F(1.780, 24.914) = 0.463$, $p = 0.612$], and were higher compared to those for recognizing faces with an emotional valence. ANOVA elicited a two-way interaction of [valence] x [cortisol dynamics] on the %accuracy of the volunteers' judgment about the emotional aspect of the presented faces [$F(1.629, 22.809) = 3.747$, $p = 0.047$]; across treatment groups, participants show a greater accuracy in recognizing positive emotions compared to negative emotions, in agreement with earlier research (59, 60). While the accuracy in recognizing positive emotions doesn't change between treatments, this is not the case with negative emotions [$F(1.480, 20.714) = 6.492$, $p = 0.011$, $\omega^2 = 0.20$]. The perception of negative facial expressions was reduced in the SCP group compared to the SCC (mean difference of 4.9%, 95% CI 0.2-9.6%, $p=0.039$) and the PO group (mean difference of 4%, 95% CI -0.2 to 8.2%, $p=0.067$) (Fig. 4A).

These treatment-related perceptual variations were confirmed by the ANOVA on the %origin of misclassifications [$F(1.637, 22.916) = 4.120$, $p = 0.036$, $\omega^2 = 0.12$], since subjects undergoing the optimal pulsatile hydrocortisone replacement are more likely to misclassify negative emotional faces compared to when undergoing the other two modes of hydrocortisone replacement, as well as by the main effect of treatment on the %destination of misclassifications [$F(1.665, 23.312) = 6.522$, $p = 0.008$, $\omega^2 = 0.20$], since the SCP group misclassified towards emotional faces with a significantly higher frequency than the other groups (Fig. S2). Overall, the optimal pulsatile treatment results in lower accuracy for recognizing negatively valenced faces, and consequently more misclassifications of negatively-valenced faces towards other negative or positive faces.

There was not a two-way interaction of [valence] x [cortisol dynamics] by ANOVA on the reaction time of subjects' responses [$F(1.927, 26.971) = 1.959$, $p = 0.162$]. The main effect of treatment also did not show an interaction with the reaction time of responses between study groups [$F(1.668, 23.351) = 1.026$, $p = 0.361$]. The main effect of emotional valence though showed an interaction [$F(1, 14) = 48.857$, $p < 0.001$], similar to the one found in earlier studies (59, 60); independent of treatment group and on average, participants tend to respond faster to faces with a positive emotional valence compared to faces with a negative emotional valence (the mean difference is 211 ms with 95% CI 164-257 ms, $p<0.001$).

The p1vital® emotional face-related attentional bias task (FDOT) indicated that the SCP group shows facilitation of attention deployment towards positive stimuli. This task assesses attention to positive and negative stimuli using a reaction time measure, the vigilance score (for details see Materials & Methods). While subjects have their attention concentrated in the middle of a screen, concurrent flashing of emotionally valenced and neutral stimuli takes place at the top and bottom of it, leaving behind, either top or bottom, two dots, vertically or horizontally oriented. The subjects need to guide their attention and correctly identify the orientation of the two dots, as quick as possible. The facial stimuli are unattended during all trials, and this lack of top-down attentional amplification, prohibits conscious processing (i.e. recruits preconscious processing mechanisms). Moreover, the facial stimuli are presented for either 100 ms or 16 ms and replaced for the remaining 84 ms with a jumbled

face (mask). In the latter case, the bottom-up stimulus strength is very weak, recruiting even weaker neural mechanisms than preconscious processing (i.e. subliminal mechanisms) (61). In all three treatment groups, the mean %accuracy scores for identifying the orientation of the two dots were very high and very similar (97.5%, 97.1% and 96.7%). ANOVA elicited a three-way interaction of [masking] x [valence] x [cortisol dynamics] on the vigilance score of the subjects' responses [$F(1.771, 24.778) = 4.039, p = 0.035$], driven by the differential, treatment-dependent deployment of attention towards or away from emotional faces when moving from the subliminal (masked faces, Fig. 4B) to the preconscious (unmasked faces, Fig. 4C) perceptual level.

Across treatments and emotional valences (happy and fearful faces), we can observe a pattern, in which subjects show a negative vigilance score (i.e. attentional bias away from emotional faces) at a subliminal level, transformed to a positive one (i.e. attentional bias towards emotional faces) at a preconscious level. This pattern is not only reversed for fearful faces in subjects receiving oral hydrocortisone replacement, but the difference in the vigilance scores between the two perceptual levels is notable [$F(1.879, 26.301) = 3.456, p = 0.049, \omega^2 = 0.08$, mean difference 25.1 with 95% CI -2 to 52.3, $p = 0.067$], especially due to the deployment of these subjects' attention away from unmasked fearful faces [absolute attentional bias: $t(14) = -2.363, p = 0.033, d = 0.31$]. At a preconscious level, subjects on the optimal pulsatile treatment tend to be strongly attracted by happy faces, not only *per se* [absolute attentional bias: $t(14) = 3.872, p = 0.002, d = 1.00$], but also in comparison to fearful faces [$F(1, 14) = 5.874, p = 0.030, \omega^2 = 0.14$, mean difference in vigilance score 23.1 with 95% CI 5.5-40.7, $p = 0.014$]. Overall, GC rhythmicity seems to interact with the subliminal and preconscious neural pathways which coordinate the deployment of attention in the presence of emotional stimuli.

Different GC rhythms change the neural processing of emotional input. Our fMRI protocol assessing the processing of emotional faces (IFEPT) provoked significant activations from most of the predefined ROIs (Table S3) and the combined evaluation of the functional neuroimaging (fMRI) experiments (on emotional face processing and on non-specific visual stimulation) with the ROI analysis on the regional perfusion data revealed the presence of GC-susceptible brain areas, whose underlying neural processing of emotional input is rhythm-sensitive. These rhythm-dependent neural responses specifically relate to facial expression processing, rather than non-specific differences due to neural reactivity or neural coupling or due to differences in the resting perfusion of these brain areas between the groups.

Whole-brain analysis of the fMRI data acquired during the IFEPT, identified brain region clusters (family-wise error corrected, Z-threshold = 2.3, $p < 0.05$) whose blood oxygen level dependent (BOLD) signal activity patterns corresponding to emotional face discrimination showed notable variations. These included some of our predefined ROIs, and were observed in the differential processing of happy and sad faces, and fearful and sad faces, between the SCC and SCP groups (Fig. S4), and in the differential processing of happy and sad faces between the SCC and PO groups (Fig. S5). The five ROIs specified were parts of the right amygdala, right striatum, right orbitofrontal cortex, right and left insula, and the corresponding data are presented in Figs. 5, 6 and S6.

During our control task, visual stimulation (flashing checkerboard), whole-brain analysis did not reveal any notable BOLD signal variations between the treatment groups in areas responsible for visual processing (occipital and temporal lobes) or any of the ROIs, predefined for this study (Fig. S7). The variations detected in those five ROIs in the IFEPT fMRI experiment should therefore reflect differences in the processing of facial traits and emotion, rather than non-specific differences due to neural reactivity or neural coupling.

At another level, resting perfusion has been shown to have an inverse relationship with the BOLD responses and is thus an important confounding factor in fMRI studies (62). Our

arterial spin labelling (ASL) data showed comparable regional resting perfusion (for pairwise comparisons, see Fig. S6 and Table S8) across the five GC-sensitive ROIs responding differently to emotional faces during the IFEPT fMRI experiment, further strengthening the concept that these variations reflect existing differences in the underlying neural processing of facial traits and emotion.

Different GC rhythms change the functional connectivity of glucocorticoid-sensitive brain regions and their functional role during the processing of emotional input. Further to our FERT data on the neural processing of happy and sad faces we investigated whether there is a functional link between these glucocorticoid-rhythm sensitive neural and psychological processes, and how different GC rhythms might alter that link. We conducted a multiple post-hoc correlation analysis between the absolute values of the effect estimates (absolute %BOLD signal changes) of the brain regions, which were differentially responsive to viewing happy and sad faces between the treatment groups, an index of ambiguity in recognizing emotion corresponding to happy and sad faces, derived from the FERT data, and the EMA data illustrating the positive and negative affective state of individuals, presented before (Fig. 7).

This post-hoc analysis was decided in the light of the existing strong evidence about the role of (i) amygdala in the detection of ambiguous, emotion-relevant cues (63, 64), and (ii) the insular cortex in the integration of internal cues, like the affective state (65), and empathic processing (66, 67) to the cognitive evaluation of emotional input, as well as the involvement of (iii) orbitofrontal cortex and (iv) striatum in the neural systems mediating emotional processing (68, 69).

In order to define the ambiguity in recognizing emotional faces we created an index derived from the data of the FERT. We divided the number of misclassifications from or towards each valence (sad and happy faces) by the number of emotional faces of that valence correctly recognized for each mode of hydrocortisone replacement such that the higher the index, the higher the degree of ambiguity in recognizing each emotion. This index was chosen, because of its concurrent very high correlation coefficient with both, the percentage accuracy scores (for correctly identifying) and the number of misclassifications involving the corresponding emotional valence (Fig. S9).

Among the SCC and SCP groups, only in the latter were the %BOLD signal changes of the right amygdala and right insula, responding to happy and sad faces, highly correlated with the monitoring of ambiguity in recognizing the corresponding emotion [$r(30) = 0.430$, $p = 0.018$] or the affective state [$r(30) = 0.521$, $p = 0.003$], respectively. Moreover, in the SCP group, these two brain structures showed an increased in-between functional connectivity compared to the SCC group [$r_{SCP}(30) = 0.312$ over $r_{SCC}(30) = 0.160$] (Fig. 7A). Furthermore, subjects on the oral replacement showed an increased functional connectivity between the right orbitofrontal cortex with bilateral insulae [$r_{PO}(30) = 0.501$, for the right insula, and $r_{PO}(30) = 0.428$, for the left insula] during the neural processing of the happy and sad faces, in comparison to the SCC group [$r_{SCC}(30) = 0.110$, and $r_{SCC}(30) = 0.046$, respectively] (Fig. 7B).

Relationship of sleep quality with outcomes of the study. Since cortisol impacts on sleep physiology, and the latter influences emotional and memory processing in man (70–73), we plotted our LSEQ derived sleep evaluations against the rest of our data (Fig. S6 and Table S10). Quality of sleep had (i) a moderate positive correlation with the %BOLD signal changes of the pulsatile and continuous infusion groups reported in the right insula in the context of discriminating between fearful and sad faces [$r(30) = 0.344$, $p = 0.063$, accounting for the 11.8% of the statistical variance of these BOLD signal responses], and (ii) a moderate negative correlation with the number of misclassifications originating from negatively valenced faces

[$r(45) = -0.366$, $p = 0.014$, accounting for the 13.4% of the statistical variance on the number of misclassifications]. In all other cases, no significant correlations were specified.

Discussion

We have investigated the neural processing and behavioral responses related to emotional perception and cognitive performance in healthy male volunteers on three patterns of the same total dose hydrocortisone replacement therapy. The SCP group was characterized by physiological circadian and ultradian GC rhythms (Fig. S11), the SCC group was characterized by a normal circadian but no ultradian GC variation, and the PO group (where we used current optimal GC replacement recommendations) was characterized by a delayed circadian peak of cortisol, two further pulses during the day and very low levels at awakening. It is the low quality of life, reduced activity, low motivation and mental fatigue reported in patients on oral replacement therapy or continuous infusion therapy, that motivated the current study.

The pattern of glucocorticoid replacement differentially modulates processes related to working memory and sleep physiology. In particular, lack of physiological cortisol pulsatility is associated with poorer working memory performance at times of increased cognitive demands. Moreover, the absence of ultradian rhythmicity correlates with a poorer self-perceived quality of sleep. Our data demonstrate that different patterns of plasma cortisol oscillations have a differential impact on the GC-sensitive brain regions underlying emotional processing, with distinct consequences for (i) the accuracy of recognizing emotional faces (in particular the negatively valenced ones), (ii) the direction of this perceptual bias, and (iii) the attentional bias towards or away from emotional faces. These GC rhythm-dependent changes could reflect functional modifications among corticolimbic brain regions underlying the differential recognition of negatively valenced faces observed in FERT, or the differential deployment of attention across perceptual levels, in the presence of emotional stimuli, as observed in FDOT.

While the %BOLD signal change of the right amygdala of the SCP group in response to emotional faces relates to the difficulty of the individuals to recognize emotions correctly, the same brain region becomes dissociated from monitoring emotional ambiguity in the SCC group. In this context, it is worth noting that amygdala %BOLD signal changes in the SCP group were significantly higher when subjects were viewing sad (negatively valenced) faces compared to happy (positively valenced). This is in line with the FERT data, supporting the notion that the decreased accuracy (i.e. increased uncertainty) of subjects on the optimal pulsatile infusion in recognizing emotional faces is negative valence-specific. Similarly, while the right insula of the SCP group seems to integrate internal cues, like the affective state, into the process of encoding facial expressions, it becomes dissociated from that process in the SCC group. Lastly, the functional connectivity between the right orbitofrontal cortex and insular cortices during emotional face presentation appears to be very strong in the PO group, which is not the case for the SCC group.

Previous studies using FERT and FDOT for the assessment of neuropsychological processes involved in depression and anxiety (75) have shown similar responses of healthy subjects receiving antidepressant and anxiolytic regimes, to the responses we find in our patients on the pulsatile SCP regime (59, 60, 75–78). This implies an involvement of GC rhythmicity in the psychophysiological mechanisms regulating mood and anxiety.

Earlier neuroimaging experiments have provided evidence that high levels of exogenous GCs, mimicking stress-associated states, alter both the neural processing in response to emotional or cognitive stimulation and the resting state functional connectivity (7–14). These studies suggest a specific role for MRs in some of these stress-related changes in both the neural processing and functional connectivity (15, 16) while MR or GR antagonism impact the amygdala-dependent processing of emotional faces (79). Our study findings now

provide evidence that even in the absence of a stressful stimulus, the ultradian GC rhythm is critical in regulating neural dynamics and, consequently, behavioural and cognitive phenotypes. Future studies in patients with adrenocortical insufficiency are now needed not only to help reduce the morbidity of current replacement regimens but also to provide evidence from longer term modification of replacement cortisol rhythmicity for improved brain function and a more personalized approach to glucocorticoid therapeutics.

Materials & Methods

Study design. A randomized, double-blind, placebo-controlled, crossover study of three different modes of hydrocortisone replacement in healthy subjects. The trial was registered under the UK Clinical Research Network (IRAS Ref: 106181, UKCRN-ID-15236, 23 October 2013). The study followed the CONSORT guidelines for randomized controlled trials (Fig. S12).

Participants. Fifteen right-handed, healthy, male volunteers aged between 20-33 years were included in the study (Table S13). Subjects had no history of neuropsychiatric disease as confirmed by clinical assessment and were excluded if they'd received a diagnosis or had a family history of a psychiatric disorder. The ethics committee of the University of Bristol approved the study. Participants gave informed consent in written form. Volunteers passed a detailed screening session, part of which was the acquisition of a high resolution, anatomical MRI scan.

Pharmacological interventions. The cortisol biosynthesis blocking agent (metyrapone), as taken orally in all three arms of the study, together with 3 different methods for replacing GCs with a fixed daily dose of 20 mg hydrocortisone: (i) subcutaneously via a pump, delivering normal circadian but no ultradian rhythmicity (ii) Normal circadian and ultradian rhythmicity or (iii) per os, 3 times daily (after waking up, during lunch and dinner) (58).

Randomisation & blinding procedures. Block randomisation schedules were generated by staff members not directly involved in data collection for this study; each subject was randomly assigned to one of the six possible orders of treatments, with the limitation that that the difference in the final number of participants between any order of treatments should not exceed one, when the total number of participants reaches fifteen. This provision was designed to avoid significant imbalances between the number of individuals eventually assigned to each of those 6 options.

Dispensing and processing of all medication/placebo was managed by Bristol Royal Infirmary University Hospital Pharmacy, ensuring that study researchers remained blinded to the conditions. To ensure that the study remained double-blind, all participants were required to take the same daily regime of tablets and remain connected to a subcutaneous pump. Each week one of these treatments was placebo (either placebo tablets or 0.9 % saline infusion via the pump). In addition, the study investigators responsible for care of the pumps had no input into the psychological testing.

Anonymized data from all outcome measures were stored to the University of Bristol central servers. The data have been cleaned at individual level without knowledge of which session corresponds to which subject, and consequently, further postprocessed and compared statistically at a group-level, without knowledge of which group corresponds to which mode of hydrocortisone substitution.

EMA. Throughout each 5-day treatment period, participants had to reply to a fixed set of nine questions (via an android phone given to them), like those contained in the positive and negative affect schedule (80), in the form of a VAS (with 0 being the absolute negative response and 100 the absolute positive), at multiple time-points during each day (Fig. S14).

Overall, 1018 assessments were collected. Principal component analysis was used to reduce the nine items of the questionnaire by identifying related groups of variables. Two factors, (i) positive affect, and (ii) negative affect, were extracted based on the examination of the eigenvalues, the scree plot and the interpretability of the factors. A varimax rotation to the factor loading matrix was applied to achieve a simpler loading pattern. Only rotated factor loadings with a magnitude of 0.4 or greater were retained for the computation of the factor scores. The factor scores are a weighted sum of the loaded factors for each participant. The mean scores from all factor values, corresponding to the positive and negative affect, collected per subject per treatment mode were calculated.

Neuroimaging study. Prior the fMRI experiments, shimming along the longitudinal axis of the main magnetic field has been performed to reduce the impact of geometric distortions and improve signal acquisition from the areas of the orbitofrontal cortices. Moreover, field maps have been acquired and used during the pre-processing stage of the functional image analysis, improving the co-registration of the functional images to the corresponding high-resolution anatomical ones (Table S15). This study follows the guidelines for reporting fMRI data suggested by Poldrack et al. (81).

Overview of the neuroimaging analysis. Neuroimaging data analysis was carried out using FMRIB software library 5.0 (82) and Statistical Parametric Mapping v.8 (Figs. S16 and S17). The high-resolution, anatomical, T1-weighted images acquired during the screening phase for each healthy male individual were used for spatially normalizing the low-resolution functional and perfusion images, and for anatomical localization. They were pre-processed to fit into the co-registration process with the functional and perfusion images, and standard space. Bias field correction has been applied, before removing the non-brain tissue.

Functional neuroimaging data analysis. The regions of interest (ROIs) for the fMRI studies have been defined *a priori* (58). The functional image pre-processing steps consisted of (i) brain intensity normalization, (ii) 3D motion correction, (iii) B_0 unwarping with assistance from the B_0 fieldmap images, (iv) brain extraction, (v) spatial smoothing, (vi) temporal high pass filtering, and (vii) co-registration of functional images with corresponding high-resolution anatomical images and with MNI152 standard space.

For each individual/session fMRI dataset, a regression analysis was performed using a general linear model fitting the temporal evolution corresponding to the paradigm (IFEPT or visual stimulation). A fraction of the temporal derivative of the blurred original waveform was added to the model. Temporal filtering was also applied. The form of the hemodynamic response function convolution method to be applied to the basic waveform was the Gamma variate. In the case of the IFEPT three different effects were modelled (original exploratory variables); (i) visual exposure to fearful human faces, (ii) visual exposure to happy human faces and (iii) visual exposure to sad human faces. This analysis produced individual session/subject level maps of activity indicating which brain regions were responding to the emotional face recognition (contrasting the baseline, resting state condition). Various contrasts were used representing either each separate emotional valence processing (fear, happy, sad), or emotion discrimination processing (fear > happy, fear > sad, happy > sad, and

vice versa). In the case of the visual stimulation task, one effect was modelled; the visual exposure to the flashing checkerboard. This analysis produced individual session/subject level maps of activity indicating which brain regions were responding to the visual stimulation (contrasting the baseline, resting state condition). In the context of these individual/session-level timeseries analyses, pre-whitening was applied.

To test whether the IFEPT was inducing the (expected) activation of the ROIs, we've performed a whole-brain single group average, carried out by using a mixed effects model, by inputting all 45 individually-analyzed fMRI datasets. This type of analysis produced thresholded z-score maps showing whose brain regions' mean activation in response to each valence of emotional faces was significantly different from resting condition.

For the between-group comparisons, whole-brain group level analyses (separately for the emotional and the visual stimulation experiments) were carried out using a mixed effects model. Each group-level analysis produced thresholded z-score brain region clusters highlighting between-treatment groups' statistically significant variations in the activation pattern per contrast used (1x3 repeated measures ANOVA, FEAT is fitting such a mixed effects model with ordinary least squares, requiring the assumption of compound symmetry). In all cases, corrections for multiple comparisons were performed at the cluster level using Gaussian random field theory (minimum $z > 2.3$, cluster p threshold < 0.05).

Only data from the predefined ROIs, if contained in contrasts that exhibit a cluster-corrected, between-treatment groups' statistically significant variance, are presented in full detail. Both, the contrast of parameter estimate (arbitrary values) and two measures of the effect size (%BOLD signal change values and extent of the ROIs' activation) with standard deviations (S.D.) are shown in the corresponding Graphs, according to latest guidelines for reporting fMRI data (83).

Perfusion neuroimaging data analysis. For the resting state ASL data, pre-quantification processing involved (i) construction of a pseudo-4D dataset by stacking up the different ASL flow images along the existing time axis in order of their T1 values, (ii) motion correction, (iii) pairwise subtraction of TAG and CONTROL volumes to generate time-averaged perfusion-weighted images, (iv) brain extraction, (v) generation of transformation matrices between the brain-extracted high-resolution brain image and the M0 calibration image, and (vi) spatial normalisation to reduce arterial contamination.

For the session-level quantification of brain perfusion, the head coil image was divided by a pre-scan normalized version to give an estimate of the receive coil sensitivity map. This map was used to correct the perfusion and head coil calibration images to prevent bias in the final quantitative parameter maps. The explicit expected bolus arrival time was set to 1.3 seconds. Signal calibration was performed using cerebrospinal fluid (CSF) as a reference. The mean CSF signal within the ventricles was calculated and corrected for the T1 and T2* of CSF (4.3 seconds and 400 ms respectively), to determine its equilibrium magnetization, $M_{0,CSF}$. This value was corrected for the T2* (50 ms) and relative proton density of blood, the density of brain tissue, and the inversion efficiency of the ASL pulse train ($\alpha = 85\%$), to obtain an estimate of the effective equilibrium magnetization of blood, $M_{0,b}$. Absolute values of brain perfusion in mL/100g tissue/min were eventually created (84). In that process, it was taken into consideration that the T2* of blood depends on whether the labeled water still resides in the vascular compartment, or whether it has exchanged into tissue, a feature proportional to the permeability-surface area for water, which is smaller in white matter (80 mL/100g tissue/min) and bigger in grey matter (ranging from 100 mL/100g tissue/min in subcortical regions to 150 mL/100g tissue/min in cortical areas) (85). Prior research has shown an improved accuracy of ASL-derived human cerebral blood perfusion measurements after accounting for capillary water permeability (86). Post-quantification processing involved B_0 unwarping with assistance from the B_0 fieldmap images for subsequent co-registration of

perfusion images with corresponding high-resolution anatomical images and the latter with MNI152 standard space.

Regional perfusion values were extracted from these ROIs, which were contained in the family wise error-corrected clusters, exhibiting statistically significant variance of their %BOLD signal responses in the IFEPT fMRI experiment between the treatment groups. The influence of the different cortisol rhythms on the absolute perfusion of these ROIs was evaluated with a paired-samples t-test. Tests for detecting outliers and normality in the distribution of data have been used and taken into consideration. One or two genuinely unusual values were detected in some ASL datasets (values of studentized residuals higher or lower than 3); they were retained in the datasets, but a Wilcoxon signed-rank test was performed in addition to the corresponding paired-samples t-test (and the outcome of both tests is provided in Table S8). This test can be considered as the nonparametric equivalent to the paired-samples t-test. Two-tailed tests were performed for all analyses and p was set to 0.05.

LSEQ. Subjective sleep quality was assessed as proposed by Parrott and Hindmarch (87), where 10 self-rating questions examine 4 domains of sleeping behaviour: (i) getting to sleep, (ii) quality of sleep, (iii) awakening from sleep and (iv) behaviour following waking. Rating takes place on a 100-mm VAS (a higher score indicating poorer sleep). The score for each of the 4 domains (per study arm and participant) was derived from the average of the scores in the corresponding questions.

FERT. Two hundred and fifty images of artificially created human faces were displayed (one at a time) on the computer screen for 500 ms and each time replaced by a blank screen until the examinee gave his response. The facial characteristics of each image were developed based on the Pictures of Affect Series (88), but morphed between each prototype emotion and neutral in a way to produce images depicting the full emotion (100%) and images presenting the emotion with gradually increasing degrees of ambiguity. For each emotion, 40 images were displayed (equally weighted for other non-emotional features like gender, skin colour, eye colour etc.) containing an equal number of 10 different intensity levels of the emotion (starting at 10% and increasing by 10). Additionally, 10 faces with a neutral expression have been included. Images were presented in a random order. Each participant was asked to respond to each face by pressing the button he thought was fitting the best to the facial expression, as quickly and as accurately as possible. %Accuracy ($[\text{Number of correct responses for each emotion}] \times 100 / 40$) and reaction time for correct choices (in ms), were among the dependent variables recorded in this task.

Other dependent variables created from the data were the %destination of misclassifications (for each given facial emotional valence category, being the number of responses of all other facial expressions wrongly classified as this given category divided 2.1 and normalized to account for the unequal number between the different kinds of positive and negative facial valences presented), the %origin of misclassifications; the latter refers to the total number of times that each kind of emotional face was misclassified to each other kind of emotional or neutral face per treatment arm per participant %normalized by the maximum possible number of such misclassifications that could have taken place (see also Fig. S18).

FDOT. One hundred and ninety-two pairs of images depicting facial expressions were displayed on the screen (one pair at a time) for 100 ms. The images used in this task were taken from the JACFEE/ JACNeuF sets of facial expressions (89). Each pair of faces comprised one emotional and one neutral expression of the same individual (in 128 of the trials) or two neutral expressions of the same individual (in 64 of the trials). Half of the

emotional faces were fearful and half were happy. In the fearful–neutral and happy–neutral face trials, the emotional faces were appearing above and below the central fixation position with equal frequency. In half cases of each type of trials (neutral-neutral, happy-neutral and fearful-neutral), faces were masked; instead of being displayed for 100 ms (like in the unmasked condition), each pair of faces was displayed for only 16 ms and followed by a mask (constructed from a jumbled face), for the remaining 84 ms. Moreover, on half of the happy–neutral and fearful-neutral face trials, the probe was appearing in the same position as the emotional face, and on the other half, in the same position as the neutral face. This task design involved congruent trials (dots replace an emotional face) and incongruent trials (dots replace a neutral face while an emotional face is present). The 192 trials were divided in 8 blocks of unmasked trials (12 trials per block) and 8 blocks of masked trials (12 trials per block), which were presented in an alternating order. Incorrect trials were excluded from the data analysis. Attentional vigilance scores were calculated for each participant by subtracting the mean reaction time of congruent trials from incongruent trials (incongruent trials minus congruent trials) (77, 90).

N-back. This test comprised of 16 sessions of letter presentation (one letter at a time during each session) and four levels of difficulty (types of n-back). Subjects were asked to make a response to the letters they see based on the type of the n-back; for this purpose, they were instructed to use two buttons, one labelled as SAME and one as DIFFERENT. In the “zero-back” type, the subjects were instructed to press SAME every time the predefined target letter (X) was appearing on the screen, while for all other letters they should respond by pressing DIFFERENT. In the “one-back” type of the test, participants needed to pay attention to the letters as they change and, starting from the second letter, press SAME if the letter on the screen was the same as the exact previous one. If not they needed to press DIFFERENT. In the “two-back” type of the test, participants needed press SAME if the letter on the screen was the same as the letter they saw two trials ago and DIFFERENT for all other letters. Lastly, in the “three-back” type of the test, participants needed to press SAME if the letter on the screen was the same as the letter they saw 3 trials ago and the DIFFERENT for all other letters.

The 16 n-back sessions were displayed in a pseudo-random order (four of each kind), and participants were informed at the beginning of each session about the kind of n-back session to follow and given time to review the instructions and remember what they needed to do. Each session contained 10 trials (letters), each of which was presented for 500 ms. The inter-stimulus interval was 1517 ms. In the whole n-back test, the target stimuli were presented with a probability of 30%.

Performance in all types of n-back sessions was measured as %accuracy ($[1 - ((\text{No of wrong} + \text{No of non-responses}) / \text{Total No of trials})] * 100$) and response time (across correct trials) for each of the task conditions (91, 92). The accuracy of responses in the cognitively very low-demanding zero-back and one-back sessions were used to assess the presence of any systematic bias in the performance of the subjects, since they primarily rely on the clear understanding of the task instructions, sustained attention to viewing the continuously changing letters and sustained motivation in trying to perform as good as possible. Thus, too many incorrect responses is a serious indication that their overall performance in the n-back test has been systematically biased by parameters other than different cortisol dynamics (for example confusion with the task instructions, or loss of attention during engagement in the task or loss of motivation in trying to achieve the best possible performance). The accuracy of responses and reaction times in the cognitively high-demanding 2-back and 3-back sessions were used as indicators of the working memory performance, according to latest psychometric theoretical schemes and practices (93).

Statistical analysis of the behavioural data. Statistical analysis was performed using IBM® SPSS Version 23, and corresponding graphs were created in GraphPad Prism Version 5.03. The influence of the different cortisol rhythms on subjects' responses as assessed by the EMA, LSEQ, FERT, FDOT and n-back was evaluated with repeated measures mixed model ANOVA, either three-way (FDOT), or two-way (FERT, n-back), or one-way (EMA, LSEQ) depending on the psychological test. In all cases, one of the within-subject factors was the treatment group (three levels: SCC, SCP, PO). Tests for detecting outliers, normality in the distribution of data (Shapiro-Wilk's test) and sphericity have been used and taken into consideration for the data analysis. Two genuinely unusual values were detected in the FERT dataset referring to reaction time; in this case, rmANOVA was performed with and without the outliers, and in both cases no significant differences were found between the treatment groups. Two subjects were excluded in advance from the working memory performance analysis, because they showed extremely low accuracy scores in the control tasks (0- and 1-back datasets), which indicated the presence of a systematic bias in those subjects' performance. The datasets derived from FERT and the negative affect scores were not normally distributed. Nevertheless, it is widely accepted that ANOVA is robust to deviations from normality. In addition, non-normality does not substantially affect Type I error rate. Although the assumption of sphericity (Mauchly's test) was not violated in any case, we adopted the most conservative statistical suggestions (94) and used Greenhouse-Geisser correction regardless. Two-tailed tests were performed for all analyses and p was set to 0.05. All results shown in the corresponding Table are means \pm S.D. Confidence intervals (CI) refer to \pm 2 S.D. As a measure of the effect size, ω^2 has been used in the case of the ANOVAs and Cohen's d in the case of the student's t-test. Pairwise comparisons with Bonferroni adjustment have been performed to investigate any (simple) main effects of treatment between the 3 study groups in cases where statistically significant two-way interactions involving the factor of treatment were specified. In the case of the FERT, two post-hoc two-way repeated measures mixed model ANOVAs were performed to investigate the impact of the different glucocorticoid dynamics on the %destination and %origin of misclassifications. Within each ANOVA, each group/factorial level contained data independent from all other groups/factorial levels. In the case of FDOT, two-way 2x3 rmANOVA have been further performed to investigate the effect of [treatment x valence] and [treatment x masking] on the emotion-related attentional bias. Moreover, one-sample t-tests were used to compare attentional bias scores to zero within each group to clarify where an absolute bias was present. Moreover, Pearson's product-moment correlation has been used to investigate any possible relationships between the self-perceived quality of sleep and the negative affect scores of the individuals with the output from the rest of the behavioural measures (Tables S10 and S19). If datasets with a significant association were not normally distributed (like in the case of the FERT and the negative affect scores), the strength of association was also assessed by the Spearman's rank-order correlation test, which is the non-parametric equivalent of Pearson's product-moment correlation. The relationship would be considered strong if both tests show a similar outcome. Since data deriving from BOLD signal responses are known not to meet the several assumptions of parametric testing, particularly with respect to homoscedasticity and homogeneity of the sample (95), nonparametric methods for performing correlation analyses are preferred; therefore, only Spearman's rank-order correlation test has been used for all post-hoc correlation analyses involving such data.

Authors' statement. All study-related procedures not directly available in this paper, are provided in the supplementary materials. Moreover, the methodological presentation of this study, a CONSORT statement, trial flow diagram and checklist, detailed information about the inclusion & exclusion criteria, the recruitment process, the medical issues related to this study, quality control, bioethics and trial monitoring, the optimization and validation processes of the pharmacological interventions, the precise doses of the drugs, the routes and rates of administration, a detailed description of the 2 fMRI paradigms used (IFEPT and the visual

stimulation task), as well as the technical parameters of the sequences used for the fMRI and ASL experiments, and our ROIs, are available in Kalafatakis et al. (57).

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