Kalafatakis, K., Russell, G. M., & Lightman, S. L. (2019). Mechanisms in endocrinology: Does circadian and ultradian glucocorticoid exposure affect the brain? *European Journal of Endocrinology*, *180*(2), R73-R89. https://doi.org/10.1530/EJE-18-0853

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Journal:	*European Journal of Endocrinology*
Manuscript ID	Draft
mstype:	Invited Review
Date Submitted by the Author:	n/a
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Keywords:	glucocorticoid rhythmicity, brain function, circadian, ultradian

REVIEW

Does circadian and ultradian glucocorticoid exposure affect the brain?

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Short title: Glucocorticoid rhythmicity and the brain

Keywords: glucocorticoid rhythmicity, brain function, circadian, ultradian

Word count: 6383 (including abstract 233 words, declaration of interest and funding)

Abstract

Glucocorticoids are a class of systematically secreted hormones, vital for mammalian life, which are intensively investigated for more than 80 years. They regulate multiple body processes like metabolism, fluid homeostasis, immune and stress system responsivity, as well as brain function. Glucocorticoids have a complex rhythm by which they are released to circulation from the adrenal cortex. The hormone exhibits a circadian variation, with high hormonal levels being secreted just prior and during the active part of the day, and progressively lower and lower amounts being released during the inactive part of it. Underlying this diurnal variation there is a more dynamic, ultradian rhythm composed of frequent episodes of glucocorticoid secretion (hormonal pulses). Accumulating evidence from observational, *in silico*, *in vitro* and *in vivo*, preclinical and clinical studies suggest that both aspects of glucocorticoid rhythmicity are preserved among mammalian species and are important for brain function. The central nervous system is exposed to both aspects of the hormonal rhythm, and has

developed mechanisms able to perceive them, and translate them to differential cellular events, genomic and non-genomic. Thus, glucocorticoid rhythmicity regulates various physiological neural and glial processes, under baseline and stressful conditions, and hormonal dysrhythmicity has been associated with cognitive and behavioural defects. This raises a number of clinical implications concerning (i) glucocorticoid involvement in neuropsychiatric disease, and (ii) improving the therapeutic efficacy or expanding the role of glucocorticoid-based treatments in such conditions.

Introduction

Glucocorticoids (GCs, corticosterone in rodents and predominantly cortisol in human) are a class of steroid hormones, vital for mammalian life, which are synthesized by the adrenal glands, secreted into the systematic circulation and travel throughout the body to exert their pleiotropic effects on cellular function, primarily affecting metabolism, the immune system and cognitive and emotional function. The complexity of their biology is illustrated by the fact that after almost 80 years of intensively investigating these molecules, we still have only superficial understanding of their molecular effects and the system level homeostatic functions they control.

Despite these caveats, almost all clinical specialties use natural or synthetic GCs to treat multiple conditions, primarily exploiting their immunomodulatory actions on high doses; from gastroenterologists (inflammatory bowel disease), dermatologists (serious allergies, psoriasis), rheumatologists (rheumatoid arthritis, systemic lupus erythematosus and other autoimmune disorders) and pulmonologists (asthma) to surgeons (serious bacterial infections and shock), oncologists (in combination with first line anti-neoplastic drugs under multi-drug schemes), nephrologists (some forms of glomerulonephritis), anesthesiologists (in combination with first line pain killers under multi-drug schemes), neurologists (multiple sclerosis, other inflammatory or traumatic encephalopathies, myelopathies and neuropathies) and endocrinologists (mainly for replacement therapy in adrenal insufficiency) (1, 2, 3).

The need to fully elucidate GCs' biological relevance is crucial since GCs are a fundamental aspect of the non-specific neuroendocrine response of the mammalian body to multiple internal and external stressors. One of the major systems affected in these states is the central nervous and it is well recognized that long term or high dose GC therapy is associated with neuropsychiatric disorders.

Two of the most characteristic features of GC physiology, well explained in relevant medical textbooks, are their circadian variation and their central role in stress responses. Indeed, GCs are a paradigm for the role of internal biological clocks, regulating the variations in biological needs across the 24-hour day. A few hours before awakening (morning in human, night in rodents), the hypothalamic suprachiasmatic nucleus (SCN) reduces its inhibitory input to the paraventricular nucleus (PVN) and median eminence (4), which in turn allows an increase in the secretion of corticotropin-releasing hormone (CRH) into the hypophyseal portal circulation. Consequently, CRH

upregulates corticotrophin (ACTH) secretion by corticotropic cells of the anterior pituitary, which travel via the systemic circulation to adrenal glands and stimulate GC biosynthesis/ release. This results to the natural circadian peak of GCs, followed by a gradual fall to reach nadir levels during the inactive part of the day. The circadian characteristics of GC secretion may vary both within and between individuals. They depend on genetic, epigenetic, age- and gender-related variables (5, 6, 7), intrinsic environmental factors and long-term neurocognitive adaptations to perceived stress, as well as the integrity of the corresponding anatomical structures involved in the feedforward-feedback circuits (8), and the mode of function of peripheral clocks, regulating for instance the circadian variation of the adrenal sensitivity to ACTH stimulation (9).

Responses to external as well as internal stressful stimuli also elicit a dramatic increase in GC secretion. Both brainstem and limbic structures are important in these responses. The hippocampus for example exerts an inhibitory effect over HPA activity at the onset and termination of the stress response (10), while the amygdala enhances the stress-related GC secretion in a region-specific manner; with central and medial amygdaloidal nuclei being responsive to different stressful stimuli (intrinsic-inflammatory and extrinsic-environmental respectively) and subsequently contributing to the acute stress responses. On the other hand, the basolateral amygdala has a role in the chronic stress integration. Parts of the prefrontal cortex also regulate HPA activity, and consequently GC secretion. All these brain structures project via the bed nucleus of stria terminalis to subcortical, hypothalamic and brainstem regions that in turn innervate the medial parvocellular part of PVN (11). This implies that, in the context of stress responses, multiple steps are involved in the chain of regulatory control initiated by central stimuli,

with the final message though eventually translated into changes in hypothalamic CRH secretion (consequently leading to changes in ACTH secretion and thus changes to GC secretion).

What has been much less clear in textbooks on medical physiology, is the fact that under baseline conditions the GC circadian variation is actually made up from an underlying, more dynamic rhythm; oscillatory pulses of ACTH and GCs. This is the ultradian rhythm of the hormone. Where does this ultradian rhythm derive from? And is it biologically significant, especially for brain function? And if yes, are there any clinical implications concerning GC involvement in neuropsychiatric disease or improving the therapeutic efficacy of GC-based treatments or even expanding their role in neuropsychiatric conditions? This review will try to answer some of these question by providing a summary of the relevant scientific evidence.

Is there an ultradian rhythm? Observational and *in silico* studies on GC pulsatility

GC pulsatility is a conserved mechanism in mammalian species

Surprisingly, despite the fact that GC pulsatility had been observed as early as the 1970's, there has been little or no investigation of its biological importance until the last decade. There are no mammalian species studied which lack GC pulsatility and this includes rodents, sheep, deer and cows (12, 13) as well as horses and monkeys. The baseline frequency of this ultradian rhythm may alter with the size and the developmental stage of the animal, being less than 60 minutes for rodents and late-gestation fetal horses (14), more than 60 for rhesus macaques (15, 16) and deer (17), and 90 minutes in sheep (18). All these studies have also demonstrated the existence of a strong correlation between

ACTH and GC ultradian rhythms (14, 16, 19). In this context, a more recent study on rodents provided strong evidence that ACTH pulsatility is necessary for GC pulsatile biosynthesis and secretion, and indeed the exposure of adrenal glands to non-pulsatile ACTH abolished their capacity to produce a pulsatile transcriptional activity of genes involved in steroidogenesis, leading to a loss of adrenal corticosterone secretion (20). Moreover, the experimental disruption of circadian inputs to the HPA activity (for instance lesioning hypothalamic nuclei or exposing animals to constant light conditions) did not interfere with the ultradian component of GC rhythmicity (21).

Multiple clinical observational studies have also confirmed the presence of the ultradian GC rhythm in man, under healthy conditions (19, 22, 23, 24, 25), as well as under pathological conditions related to chronic stress system activation, including neurodegenerative disorders (26), depression (27), fibromyalgia and chronic fatigue syndrome (28) or obstructive sleep apnea (29). The GC pulses vary in amplitude and duration throughout the day due to variable input from hypothalamic nuclei, and a typical human 24-hour profile, under healthy and non-stressful conditions, contains approximately 8-16 glucocorticoid pulses (occurring every 60-180 min) (23, 30). The ultradian rhythm of GC secretion is also preserved across gender (31) and, despite changes in pulse amplitude and duration, even during acute stress responses (32, 33). But where does this ultradian rhythm come from?

Origin of GC pulsatility

Since the ultradian rhythm of GC secretion is not abolished by the removal of hypothalamic CRH circadian cues, we focused on the characteristics of the interplay between the anterior pituitary and adrenal glands. As mentioned earlier, ACTH plays a

key role on this: after reaching the steroidogenic cells of the *zona fasciculata*, it binds to its specific receptor melanocortin type-2 (MC2R), causing an increase to the intracellular levels of cAMP, which in turn activate the protein kinase A pathway, leading to post-translational modifications (mainly phosphorylation/ activation) of proteins involved in cholesterol metabolism like the hormone-sensitive lipase (HSL) and the steroidogenic acute regulatory protein (StAR), which regulate the levels of intracellular cholesterol and its transport within the mitochondrial matrix to initiate the steroidogenic process (34). Therefore, ACTH exerts a positive feedforward regulation on GC biosynthesis.

After release into the systemic circulation, GCs feedback on corticotropic cells of the anterior pituitary to inhibit the release of ACTH. This results in a negative (self)regulation on GC biosynthesis. This positive feedforward – negative feedback loop is characterized by built-in delays (i.e. there is an inherent temporal distance between each positive feedforward activation of MC2Rs by ACTH and the subsequent release of GCs due to the need for *de novo* GC biosynthesis). By using mathematical biomodelling approaches, accommodating the previously mentioned dynamics between ACTH and GC secretion with the inherent delays, as well as other parameters related to GC clearance through liver (bile acids) and kidneys (urine) (35), we were able to demonstrate that the interplay between pituitary and adrenals creates a system that leads its components (ACTH, GCs) to a self-sustaining oscillatory activity (21, 36, 37), independent of any other cues. What we have described is in effect a sub-hypothalamic pulse generator (Figure 1).

This leads us to the key question: as the brain is naturally exposed to these GC pulses, how are brain cells able to perceive GC pulsatility and translate for appropriate

signaling events? Furthermore, what are the implication of this for therapeutics- both replacement therapy and synthetic corticosteroid treatment?

The neurobiological significance of the GC circadian rhythm

Before focusing on the ultradian rhythm of GCs, we should not underestimate the significance of their diurnal variation for brain function. GC circadian rhythmicity is an integral feature of the regulation of glucose homeostasis, impacting directly on neuronal and glial homeostasis (38). The GC circadian rhythm is synchronized with the rhythm of other major, brain-specific stimuli such as brain-derived neurotrophic factor, which has a direct interaction with GCs regulating fundamental neural and circuital processes like neurogenesis, dendritic remodeling and synaptic plasticity (39). The GC surge of the diurnal peak also modulates the rhythmic expression of various GC-sensitive genes in a brain-region specific manner, like tryptophan hydroxylase-2 in the raphe neurons (40) or period-2 in the central nucleus of the amygdala (41), and promotes stimulus-driven, non-genomic events, like the postsynaptic dendritic spine formation in the cortex after motor skill learning. At the same time, GC circadian troughs are required for stabilizing newly formed spines crucial for long-term memory retention. Conversely, chronic and excessive exposure to GCs eliminates learning-associated new spines and disrupts previously acquired memories (42).

In addition, the circadian rhythm of GCs has enormous, multi-level effects on behaviour, psychophysiology and -pathology: (i) changes in the characteristics of the diurnal variation (steeper peaks or flatter slopes) have been linked to an increased self-reported negative affect (43), and an inverse relationship has been reported between

the diurnal rhythms of cortisol and positive affect (44). (ii) The diurnal cortisol profile has been also associated with the neural activity in parts of the medial prefrontal cortex (ventromedial and orbitofrontal), an association that is lost in anhedonic subjects (45). (iii) Enhancement of the diurnal peak of GCs (without changing the overall amount of daily GC exposure or any other aspects of the HPA activity) may exert anxiolytic effects (46). (iv) Elimination of the GC circadian peak leads to a significant reduction in locomotor activity during the active periods of the day, comparable to the inactive parts of it (47). (v) Circadian misalignment due to GC circadian rhythm phase shifts has been linked to acute episodes (mania or depression) in the context of bipolar disorder (48). (vi) The diurnal variation in circulating GCs modulates the analgesic effect of morphine by regulating the expression of the μ-opioid receptors in brainstem (49).

It is clear that the GC circadian rhythm provides a strong chronobiological signal controlling the daily homeostasis of energy balance in brain cells, as well as fundamental aspects of neural survivability, plasticity and multi-neuronal network characteristics. These effects are linked to both genomic and non-genomic cellular events, and eventually contribute to the circadian variability of mood and behaviour, whose disruption is linked to psychiatric symptomatology (Figure 2). Thus, over a period of 24 hours, the alternation of the circulating GC levels between a state a high abundance and a state of low bioavailability seems to be crucial for brain physiology. The next question is whether the ultradian pattern of GC rhythm could be of similar neurobiological significance. Is it possible that the circadian variation of the hormone can only be optimally translated into its neurobiological effects if delivered in a pulsatile manner?

Preclinical studies on the neurobiological significance of GC pulsatility

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235 Does the brain perceive GC circadian and ultradian rhythms?

236 The debate around the significance of GC rhythmicity on brain function would be
237 pointless if the nervous system was not exposed to oscillating signals of extracellular GCs.
238 In the systematic circulation GCs are bound to GC-carrier proteins and albumin and it is
239 only the free fraction of cortisol that is active and available to diffuse into the central
240 nervous system. And even then, this active fraction of GCs can get excreted at the site
241 of the blood-brain barrier (due to the activity of the P-glycoprotein) and locally, in the
242 microenvironment of neurons and glia, be converted to inactive forms (50). *In vivo* micro-
243 dialysis studies in rodents have demonstrated, though, that both the circadian and
244 ultradian rhythms of free GCs are maintained in the systemic circulation, the nervous
245 system and the subcutaneous tissue (51). These observations are gender-independent
246 (52). It is worth noting though, that this synchronicity between plasma and brain free GC
247 oscillations might be modified under conditions of acute changes in the mode of the GC
248 rhythm, as in the context of an acute stress response (53). These results have partially also
249 been confirmed in man (54).

250

251 Is the brain able to translate GC pulsatility into cellular events?

252 The debate around the significance of GC pulsatility on brain function would also be
253 pointless if the brain cells didn't possess the means to translate dynamic hormonal
254 oscillations into differential signaling events. Neurons and glial cells have developed ways
255 to sense GC pulsatility. The basis of this sensation lies into the properties of the two classes
256 of GC-sensitive receptors, the mineralocorticoid receptors (MRs) and the glucocorticoid
257 receptors (GRs), found in the central nervous system. Since many areas of the brain lack

the enzyme 11β-hydroxysteroid dehydrogenase isoform II, cortisol and corticosterone can activate both GRs and MRs in these areas. The most prominent sites of MR expression in the central nervous system include hippocampus, lateral septum, amygdala, and to a lesser extent cerebral cortex, cerebellum, caudate-putamen complex, and hypothalamus, while areas of GR expression include cingulate cortex, hippocampus, PVN and supraoptic nucleus, lateral geniculate, lateral and medial amygdala, thalamus, cerebellum and cerebral cortex (55, 56, 57, 58).

MRs have a much higher affinity for binding with GCs compared to GRs; consequently, MRs remain occupied even during low GC levels, while GR binding requires higher GC concentrations, like those during the peak of individual pulses or following acute stress (59). Moreover, over the last two decades, it has been gradually realised that these classes of receptors, although considered as transcription factors (i.e. regulators of gene expression) with delayed effects, also possess rapid, non-genomic effects in brain cells; these effects have been attributed to non-nuclear variants of these receptors, and for those effects higher GC levels are required as well. Thus, depending on the GC levels, a different combination of MRs and GRs get activated, resulting in a different set of rapid and delayed effects (60).

The ultradian GC rhythm determines the cyclical shift in the location of GRs and to a lesser extent MRs. At the peak of an endogenous pulse GRs translocate to the nucleus and bind to glucocorticoid response elements (GREs) on the DNA, initiating chromatin modifications including histone acetylation and docking of RNA polymerase 2 to initiate gene transcription. At the trough of each pulse, GRs will come off the DNA and either remain in the nucleus bound to chaperone proteins or be ubiquitinated and enter the nuclear proteasome for degradation (61, 62, 63). Duration of GC exposure also

differentially regulates GR and MR expression, as well as determining the binding properties of MR- and GR-related coactivators and corepressors, and the formation of MR-GR heterodimeric complexes (64, 65, 66, 67, 68, 69, 70).

The overall result of this is that corticolimbic regions of the brain -in particular- are equipped with the molecular machinery to sense GC pulsatility; the next question arising therefore is where do all these events lead to? What aspects of neural and brain function are regulated by GC pulsatility?

Which aspects of neural and brain physiology are modulated by GC pulsatility?

Over the last decade, research efforts exploring the neurobiological significance of GC ultradian rhythmicity have intensified. A variety of neural processes seem to be sensitive to GC pulsatility ranging from genomic events to rapid modifications in synaptic plasticity, hippocampal neurogenesis (71) and, eventually, behavioural phenotypes.

GC-dependent genomic events are sensitive to the dynamic pattern of the hormonal oscillations and form transcriptional patterns that respond differentially to specific aspects of GC rhythmicity in a brain region-specific manner. The latter has been shown by both, *in vivo* and *in vitro* experimentation. For instance, hourly corticosterone pulses in rodents induced episodic bursts of transcription of the gene period-1 in the hippocampus. This lead to a plateau in the accumulative mature transcript throughout the time course of the pulsatile exposure, indicating that GC pulsatility works optimally for steady state period-1 expression. The plateau dropped to baseline within 2 hours of the final pulse, indicating that any perturbation to the pulse frequency or duration would have rapid quantitative effects on the levels of the gene products (72). A similar pulsatile motif, following *in vitro* exposure to a pulsatile GC treatment, on the transcription of GR-

regulated genes has been reported for sulfite oxidase, a mitochondrial enzyme involved in cellular energy production, GC-induced leucine zipper, a transcription factor, tissue transglutaminase, a protein regulating cytoskeletal properties and involved in neurodegenerative processes, and melatonin receptor 1B. That pulsatile motif of gene expression is lost if the GC rhythm switches from pulsatile to non-pulsatile, or if natural GCs are replaced with synthetic ones with a huge potency for GRs, like dexamethasone (73). Increased sensitivity to GC pulsatility has been also observed for serum/GC regulated kinase 1, implicated in the regulation of ion channels, cell survivability and long-term memory formation, and pro-opiomelanocortin, the ACTH precursor, in pituitary but not in prefrontal cortex of rodents (74). Finally, gene ontology analysis of the transcriptome of HeLa cells contrasting *in vitro* pulsatile versus continuous cortisol exposure revealed expression differences in genes involved in cytoskeletal homeostasis and cell adhesion (75).

Aside the delayed, genomic events synchronized with the dynamic hormonal oscillations, rapid, non-nuclear events have been also described, indicating that spikes in GC concentrations can very quickly regulate neural processes, like neurotransmission and synaptic plasticity in a brain region-specific manner. For instance, GCs enhance transiently the frequency of miniature excitatory postsynaptic potentials in CA1 (hippocampal) pyramidal neurons, pointing to a hormone-dependent enhancement of glutamate release probability via a pathway involving membrane-located MRs (76). A similar phenomenon has been observed in the basolateral amygdala; contrary to the hippocampus, though, the upregulation in glutamatergic neurotransmission is long-lasting and greatly affects the responsiveness to subsequent surges of GCs in a GR-dependent manner (77). More recent studies additionally showed that the frequency of

the hormonal pulses differentially regulate the frequency of miniature excitatory postsynaptic currents, AMPA receptor trafficking and the induction of long-term potentiation in cultures of hippocampal neurons and dorsal hippocampal slices from rodent brains (78, 79). Related to this, GC-activated membrane-associated GRs promote the interaction between phospho-CREB and CREB-binding proteins, leading to epigenomic events (histone acetylation) in both the hippocampus and insular cortex, following training on object recognition, associated with memory consolidation (80). Finally, it has been illustrated that acute psychological stress resulted in the upregulation of the neuroplasticity-associated immediate-early genes c-Fos and Egr-1 in granule neurons of the dentate gyrus (hippocampus), following the serine-10 phosphorylation and lysine-14 acetylation in histone H3, which were induced by the activation of the nuclear kinases MSK1 and Elk-1. The latter required a rapid protein-protein interaction between the phosphorylated ERK1/2 and GC-activated GRs, linked to long-lasting behavioral responses to stress (81).

Eventually, GC pulsatility affects behavioural responses (82) and the readiness of the stress system for an effective mobilization. Emotional and motor responses to external stressors or aggressive challenges are more prominent when the cue coincides with the rising phase of the ultradian GC pulse compared to the falling phase (83). Moreover, disruption of the normal ultradian GC rhythm has been associated with changes in the stress responsiveness and a dissociation between hormonal and behavioural responses to stress (84). Furthermore, *in silico* approaches also strongly suggest that the presence of pulsatility in homeostatic HPA function confers the potential for increased acute stress responsiveness (85).

How does brain physiology incorporate the different aspects of GC rhythmicity?

In parallel to findings in peripheral tissues (9, 38, 86), which possess local circadian clocks regulating the diurnal variation in GC sensitivity, similar mechanisms occur in different brain regions, that could modulate fundamental circadian processes, like metabolism, oxidative stress response, DNA repair and autophagy (at a cellular level), or memory, sleep-awake cycles, mood, and eventually behaviour (at a systems level) (87). Subject to brain region-specific and (in some cases possibly) temporally-varying hormonal sensitivity, GC pulsatility optimizes the circadian sustainability of GC stimulation, applies a temporal filter on GC effects (especially those mediated by GRs and non-nuclear MRs), as well as keeps the nervous system competent for properly integrating external stimuli or changes in internal states.

A typical example on the sustainability of GC stimulation is the fact that GC pulsatility preserves the stock of available mature transcript of the period-1 gene in hippocampal cells (72), as we mentioned earlier. Perhaps though, the most crucial aspect of GC pulsatility is that it offers the brain an extended temporal window (on a daily basis) for effective, immediate responses to internal or external challenges (83), as well as successful, long-term adaptation. Pulsatility enables the maintenance of a reactive and responsive signaling system which is not downregulated by constant receptor activation. Moreover, in the context of confronting a challenging situation, the subsequent activation of such a range of different types of GC-sensitive receptors contributes to an ability to have temporally specific responses to a stressor: non-nuclear MRs seem to be necessary for coordinating the initial brain response to stress (in accordance with their fast, nongenomic actions), while at a later stage, GRs initiate the processes responsible for reestablishing homeostasis and mediating the successful

neurobehavioral adaptations to increase effectiveness towards confronting future incidences (60). Furthermore, outside the context of stress induction, the frequent GC surges increase the probability of GC stimulation coinciding with (or dissociating from) activation by other, interacting biomolecules, with which GCs have additive or nullifying effects. A prominent example is brain-derived neurotrophic factor (88).

Finally, it is worth mentioning that body states accompanied by disruptions of GC pulsatility, leading to a prolonged exposure to high GC levels, have been linked to a weakened GR activation. For instance, rapid GR-dependent negative feedback regulation of ACTH release under basal conditions or acute stress (24) is reduced in major depression, a condition accompanied with an overactive HPA axis (89). Other examples involve the reduction of immune system's sensitivity to GCs' immunosuppressive effects during chronic psychological stress (90), or the selective down-regulation of hippocampal GRs under sustained stress in rodents and non-human primates (91) or after the experimental induction of viral encephalitis in rats (92).

The GC ultradian rhythm appears to provide a very important neurobiological signal which differentially regulates the gene expression profile and various second messenger systems of intracellular signal transduction of brain cells and, eventually, impacts cognition, behaviour and stress responsiveness (Figure 2). Similar to the hormone's circadian rhythm, disruption of the normal characteristics of the ultradian rhythm have, very recently, been linked to animal models of neuropsychiatric disease (93). But what are the clinical implications of all these? Does GC rhythmicity have a similar significance for human brain function?

Clinical studies: is GC rhythmicity important for the human brain?

Effects of oral GC administration on the human brain

Before focusing on the relevance of GC rhythmicity for the human brain function, we need to establish which domains of human cognition are influenced by GC input. A number of clinical trials in healthy subjects, using functional neuroimaging (fMRI) techniques and psychological experiments, have added valuable insights. In these studies, participants were receiving one dose of hydrocortisone or placebo, usually orally, and subsequently underwent some form of a cognitive or psychological task, measuring an aspect of human brain function, with or without the concurrent application of an fMRI protocol. The timepoints for applying the outcome measures after hydrocortisone administration were either 60-120 minutes, reflecting the rapid effects of the hormone, and/ or 180-240 minutes, reflecting the delayed effects of the hormone.

Under such experimental settings, it has been shown that GCs interfere with various systems of memory processing. For instance, it has been shown that (i) intravenous 100mg hydrocortisone infusion acutely increases the involvement of the prefrontal and parietal cortex, while reducing the involvement of the hippocampus, in a working memory task (n-back) (94), (ii) 10mg of hydrocortisone improves working memory performance in the same kind of task (n-back) 240 minutes after their *per os* administration, an effect related to increased neuronal activity in the dorsolateral prefrontal cortex (95), (iii) 20mg of hydrocortisone reduce prefrontal and hippocampal responses during memory encoding sessions 180 minutes after their *per os* administration (96), (iv) 10 mg of oral hydrocortisone uptake increase the neural processing of the anteromedial prefrontal cortex during sessions of autobiographical memory retrieval 60 minutes post-administration (97).

Under such experimental settings, it has been also illustrated that GCs facilitate the neurocognitive transitions between the unstressed brain, its stressed and its post-stress state. In particular, data suggest that (i) cortisol levels are positively correlated with a functional coupling between amygdala and medial prefrontal cortex under relatively non-stressful conditions (98), but negatively correlated with a sustained functional connectivity between amygdala and hippocampus during the post-stress period (99), (ii) 10 mg of hydrocortisone reduce the interaction of amygdala with areas responsible for initiating and preserving a stress response (locus coeruleus, hypothalamus, and hippocampus), while they increase the interaction of amygdala with areas associated with executive functions (middle frontal and temporal gyrus) 105 minutes after their *per os* administration (100), (iii) a stress-induced increase in GC levels augments the functional coupling between amygdala and dorsal striatum (101), but reduces the learning-related hippocampal processing in an MR-dependent manner, during a combined trace and delay fear conditioning paradigm (102, 103).

Finally, under such experimental settings, it has been shown that GCs interfere with emotional processing. Thus (i) 10 mg of hydrocortisone reduces amygdala responsivity to emotional faces 75 and 285 minutes after their *per os* administration, while slowly strengthening the functional coupling between amygdala and medial prefrontal cortex, leading to a normalised response to the negative emotional stimuli (104), and (ii) 10 mg of hydrocortisone modulates the impact of emotional distraction of attentive processing in a time-specific manner; 60 minutes after *per os* administration, there is increased emotional interference (associated with reduced amygdala inhibition to aversive words and enhanced amygdala connectivity with fronto-parietal brain regions), but later on (270 minutes after their *per os* administration) decreased overall activity in the cuneus,

possibly indicating reduced bottom-up attentional processing, and disrupted amygdala connectivity to the insula, potentially reducing emotional interference (105).

Given these findings on GC involvement in memory, emotional processing and stress-related neural processing, it is of no surprise that one of the most well-described effects of GCs on the human brain (supported by integrative research, combining preclinical experimentation and clinical studies) involves the modulation of the mnemonic processing of emotionally arousing experiences (106). In the context of a stress response, GCs enhance memory consolidation and impair memory retrieval. This phenomenon is associated with a shift from a hippocampus- controlled to a dorsal striatum-controlled cognitive processing. This shift requires the involvement of the amygdala, and GCs enhance in a rapid, GR-dependent manner the noradrenaline-induced rise in intraneuronal cAMP levels in the basolateral amygdala, which upregulate the protein kinase A-dependent downstream pathways, involving among others the endocannabinoid system (107).

GCs clearly exert important effects over the human brain function, as anticipated by the strong preclinical evidence presented before. But is their rhythmicity so important from a clinical point of view as well?

Observational studies on the relationship between GC dysrhythmicity and brain pathology

The most obvious sources of GC dysrhythmicity are conditions directly impacting GC biosynthesis; either adrenal insufficiency (for instance Addison's disease, AD, or congenital adrenal hyperplasia, CAH), leading to hypocortisolism, or Cushing's syndrome, leading to hypercortisolism. In the former cases, GCs are replaced orally, in a

manner that doesn't replicate neither the circadian nor the ultradian rhythm of the hormone (3). Cushing's disease has been correlated with brain atrophy, memory impairment, and depression, while correction of hypercortisolism (but not the optimal daily GC rhythm), though attenuating brain atrophy, does not successfully reverse cognitive deficits (108, 109). In relation to these results, a recent study highlighted the presence of functional alterations in emotional processing of amygdala and hippocampus in adolescents with chronic endogenous hypercortisolemia due to Cushing's disease, that are not associated with affective or memory symptoms (110). On the other hand, hypocortisolism also exerts damaging effects centered on the corticolimbic areas of the brain, and age seems to be reversely associated with the degree of brains' susceptibility to absence of GCs; there is some evidence that CAH is correlated with decreased growth, development and dysregulated function of the amygdala (111, 112), disrupted white matter integrity (113), bilateral periventricular white matter hyperintensities and cortico-subcortical atrophy (114, 115), as well as cognitive deficits (116, 117).

Brain pathology however that is totally separate from the circuits regulating HPA activity, can also lead to GC dysrhythmicity. In a study of stroke patients with right-sided infarction (118), researchers observed an altered tonic and phasic cortisol secretion and a damaged stress response compared to stroke patients with left-sided infarction or healthy age-matched controls.

Where things become more complicated are in various neuropsychiatric disorders, where it is difficult to establish whether GC dysrhythmicity has a causal relationship with the neuropathological sequel or whether it is the result of the neuropathological process. In such cases, a vicious cycle develops between these two

variables. Such conditions include patients with Alzheimer's disease, Parkinson's disease and post-traumatic stress disorder, which show disruptions in the circadian and ultradian GC rhythmicity (26, 119, 120), subgroups of patients with major depression, fibromyalgia and chronic fatigue syndrome, with the HPA being overactive in the former (121) and malfunctioning in the two latter cases (122, 123, 124). Very recently, Vargas et al. (125) proposed that a disrupted ultradian cortisol rhythm could be a potential neurobiological substrate for chronic insomnia.

In addition to these endogenous perturbations the most frequent clinical causes of GC dysrhythmicity is the exogenous, systemic administration of synthetic GCs. These interfere with GC signaling cascades, as well as disrupt both the physiological feedforward-feedback interplay between adrenal glands and pituitary, which gives rise to the ultradian rhythm, and the negative feedback effect of natural GCs on the hypothalamus, which modulates the circadian properties of the hormonal rhythm. Are GC-based therapies then linked to neuropsychiatric symptomatology?

<u>Is there a relationship between GC-based therapeutics and neuropsychiatric symptomatology?</u>

The prolonged use of GC-based regimes and/or their administration in high doses is accompanied by numerous adverse effects, including neuropsychiatric (126). The list of symptoms spans almost every kind of cognitive or emotional disturbance: memory impairments (declarative memory, working memory and explicit memory), agitation, anxiety, fear, hypomania, irritability, lethargy, mood lability, psychosis. Individuals who develop psychiatric manifestations on short courses of GCs most commonly report

euphoria, while those on long-term therapy tend to develop depressive symptoms. The timing of GC administration has been strongly linked to sleep disturbances as well.

The most striking finding , however, is the poor clinical outcome in the simplest therapeutic situation when GCs are prescribed as replacement therapy in primary adrenocortical insufficiency (127), even when the daily amount of GCs administered does not differ from that produced by the human body under physiological conditions. In 2002, Løvås K et al. (128) reported that Addisonian patients receiving substitution therapy (cortisone acetate and fludrocortisone) had reduced general health perception and vitality, and increased fatigue, as assessed by psychological self-evaluation scales (Short Form 36 and the Fatigue questionnaires). Recent studies, over a decade later, confirmed these observations, that health-related quality of life is significantly impaired in Addisonian patients compared with the age-matched, and gender-matched general population, despite the proper use of the recommended oral hydrocortisone doses (129, 130). The mental fatigue, accounting for a significant portion of these patient's poorer quality of life, is characterized by higher prevalence of mood disorders (mainly depression), memory impairment and sleep disorders (131). Therefore, the fact that restoration of the physiological GC levels might not be sufficient for them to exert their normal neurobiological effects, provides support for the idea that the pattern of GC rhythmicity may be crucial even for basic mood regulation.

<u>What do GC replacement therapies tell us about the significance of GC rhythmicity for the human brain?</u>

Current protocols on GC replacement in states of adrenal insufficiency recommend the oral administration of hydrocortisone 2-3 times daily (or longer acting synthetic

prednisolone once daily in the morning), with the morning dose being at least 50% of the total daily GC dose. Such a pattern of GC administration cannot replicate neither the circadian nor the ultradian rhythm of the hormone. For example, the natural circadian peak of GCs in human anticipates the need for morning activities by commencing secretion several hours prior the morning awaking whereas the morning dose of oral replacement therapy (which is responsible of creating the diurnal peak in patients with adrenal insufficiency) is taken post-awaking resulting in a hormonal peak about one hour later. Furthermore, 3 doses of oral GC replacement create a form of hormonal ultradian rhythm characterised by a much smaller number of daily pulses, with a much longer duration and inter-pulse intervals than normally present. This raises the question whether an improvement in the pharmacological replication of the circadian and ultradian rhythm of GC substitution could be also followed by an improvement in clinical markers of brain function in patients with adrenal insufficiency, which would be also a strong indication of the neurobiological significance of GC rhythmicity for the human brain physiology.

Five clinical studies and three case reports have been published over the last decade, comparing the administration of hydrocortisone by continuous smooth subcutaneous infusion mimicking the diurnal but not the ultradian pattern of plasma cortisol (SCHI), with currently considered optimal oral therapy (OT) (132) in patients with AD or CAH. The main focus of these studies was markers on the endocrine and metabolic state of the patients (133) together with other questions related to personalised medicine (134, 135, 136). Compared to OT, the SCHI was found to improve the self-perceived mood, feelings of fatigue, vitality and physical function in Addisonian patients, while not affecting subjective or objective measures of sleep behaviour at 12 weeks (137). These

568 favorable effects developed over a period of many weeks both in this and a similar
569 concurrent clinical trial (138). A similar, favorable effect in markers of fatigue, mood and
570 vitality has been observed in SCHI over OT in CAH patients at 6 months (139), which were
571 maintained at 18 months (140).

572 More direct evidence on the neurobiological importance of GC rhythmicity for
573 the human brain has been published recently. We created a human model of adrenal
574 insufficiency by pharmacologically blocking GC biosynthesis (oral administration of
575 metyrapone 3 times daily) and replacing the hormone in three different modes; using
576 either (i) oral treatment (OT), (ii) constant subcutaneous infusion (SCHI), or (iii) a novel,
577 subcutaneous, pump-based method, delivering different size pulses of hydrocortisone
578 every 3 hours, that reproduced both the natural circadian and ultradian patterns of
579 cortisol. We then examined the neurocognitive effects of these different GC rhythms
580 using functional neuroimaging techniques and a set of cognitive and behavioural tests,
581 markers of sleep behaviour, working memory and emotional processing, in a randomised,
582 double-blind, placebo-controlled, crossover study (141). We were able to demonstrate
583 that non-pulsatile GC exposure (i.e. SCHI) correlates with poorer quality of sleep and that
584 both SCHI and OT were associated with poorer working memory performance under
585 increased cognitive demands. Moreover, we were able to illustrate that different
586 patterns of plasma GC oscillations have a differential impact on the participation and
587 functional connectivity of brain regions underlying emotional processing (amygdala,
588 dorsal striatum, insula, orbitofrontal cortex) affecting attentional bias to and recognition
589 accuracy of emotional cues (142). These data support the notion that changes in GC
590 rhythmicity can modulate the neural dynamics regulating mood and anxiety in man
591 (Figure 3).

Future studies should systematically explore the clinical utility of manipulating features of GC rhythmicity both to improve personalized treatment strategies and neuropsychiatric disease subclassification. We believe a better chronobiological approach to GC therapeutics is urgently needed.

Declaration of interest

The authors declare no conflict of interest.

Funding

The study on glucocorticoids has been supported by Research Grants from Wellcome Trust (Grant No 089647/Z/09/Z) and Medical Research Council (DCS Grant No MR/J0125481/1).

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Legend to Figure 1

Regulation of glucocorticoid (GC) circadian and ultradian oscillations. In hypothalamus, the suprachiasmatic nucleus regulates the circadian changes in secretion of the corticotropin-releasing hormone (CRH) from the neighboring paraventricular nucleus. This in turn provides the diurnal pattern of activation of the pituitary corticotropes (green arrow) which secrete corticotropin (ACTH) into the circulation and thence the adrenal cortex where it initiates a feedforward activation of GC biosynthesis (green arrow).This

necessity for de novo GC biosynthesis (which cannot be stored in vesicles due to its lipophilic nature) results in a built-in delay before the metabolic product can be released, and feedback at the level of the pituitary to suppress ACTH (red arrow). Mathematical biomodelling suggests that such a positive feedforward – negative feedback system with built-in delays leads to a self-sustaining oscillatory activity and is the basis for ultradian GC pulsatility. Changes in hypothalamic drive can superimpose on this rhythm, by modifying the amplitude and magnitude of each ACTH pulse, and thus establishing the well recognised diurnal rhythm. This is itself modified by feedback inhibition from the circulating levels of GCs (red arrow). The adrenal cortex itself has a local clock mechanism that also contributes to circadian variation by altering adrenal sensitivity to ACTH stimulation across the circadian cycle. The activity of corticolimbic brain regions (in response to external cues or internal states), brainstem (responding to inflammatory stimuli or pain) as well as other peripheral stimuli (for instance inflammatory cues or stressors) may affect the downstream pathways either controlling the secretion of CRH or the tissues' sensitivity to the ACTH or GC stimulation.

Legend to Figure 2

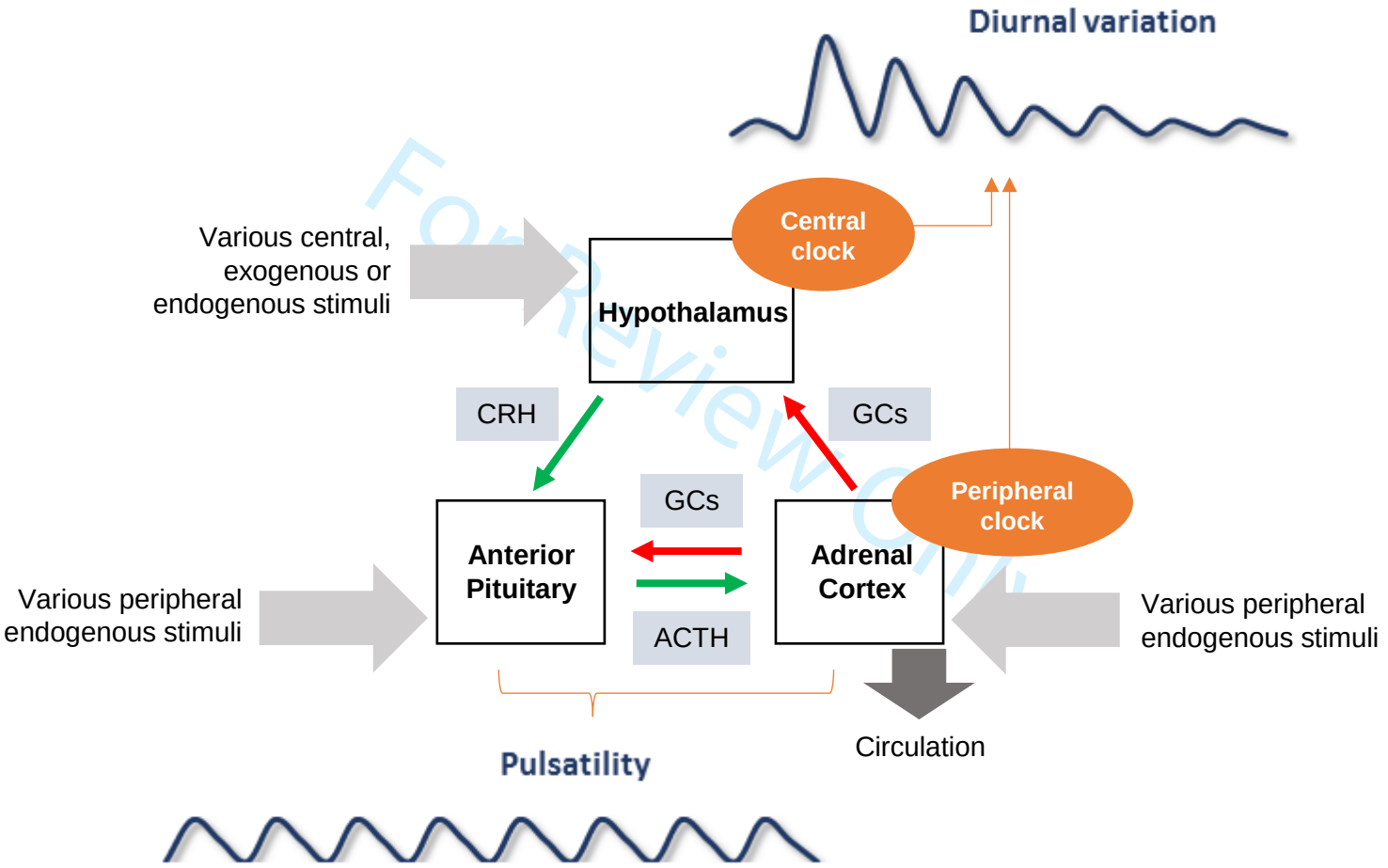
The complex rhythm of glucocorticoid (GC) synthesis has major neurobiological significance. The GC diurnal peak promotes stimuli-driven, postsynaptic dendritic spine formation in the cerebral cortex, facilitating the learning process. At the same time, GC circadian troughs are required for stabilizing these newly formed spines, and thus achieving long-term memory retention. Loss of the diurnal variation in GC levels eliminates learning-associated new spines and disrupts previously acquired memories. Other examples on the significance of the circadian GC rhythm include the time-of-day-

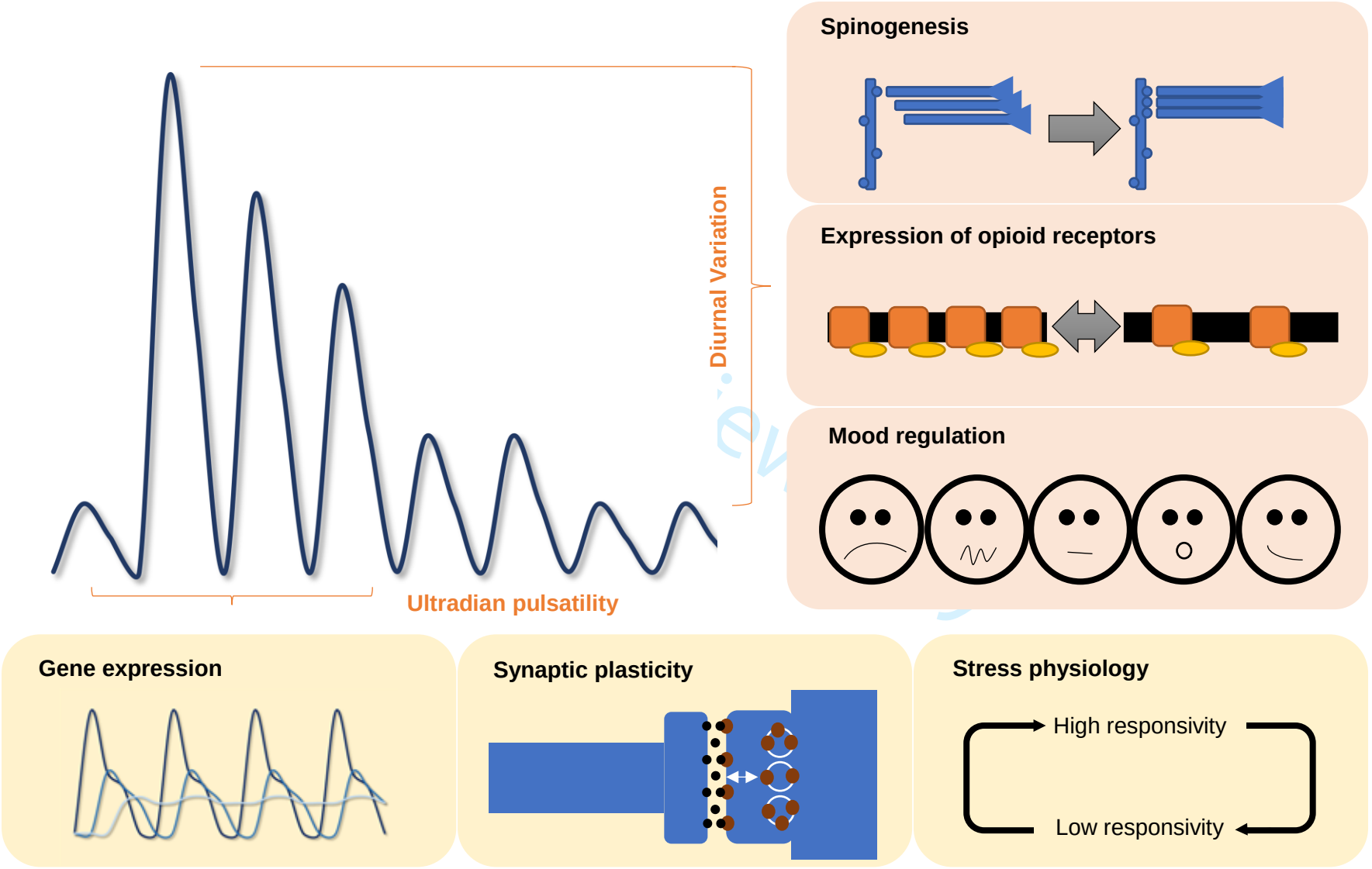
dependent analgesic effect of morphine (due to the GC-dependent circadian variability in the expression of μ-opioid receptors in the brainstem) and the association of changes in GC diurnal variation in humans with self-perceived positive and negative affect. Ultradian GC pulsatility also has neurobiological consequences. Each pulse is translated into a GC receptor – DNA binding event (left bottom yellow frame, dark blue oscillations), subsequently translated into a pulsatile biosynthesis of hnRNA (left bottom yellow frame, blue oscillations), which regulates mature transcript of GC-sensitive genes (left bottom yellow frame, light blue curve). Furthermore, the frequency of GC pulses differentially regulates processes crucial for synaptic plasticity, including release of glutamate from presynaptic terminals and glutamate receptor trafficking of postsynaptic neurons. Finally, GC pulsatility enables the rapid alternation between periods with reduced and periods with increased responsivity to stressful insults across the day.

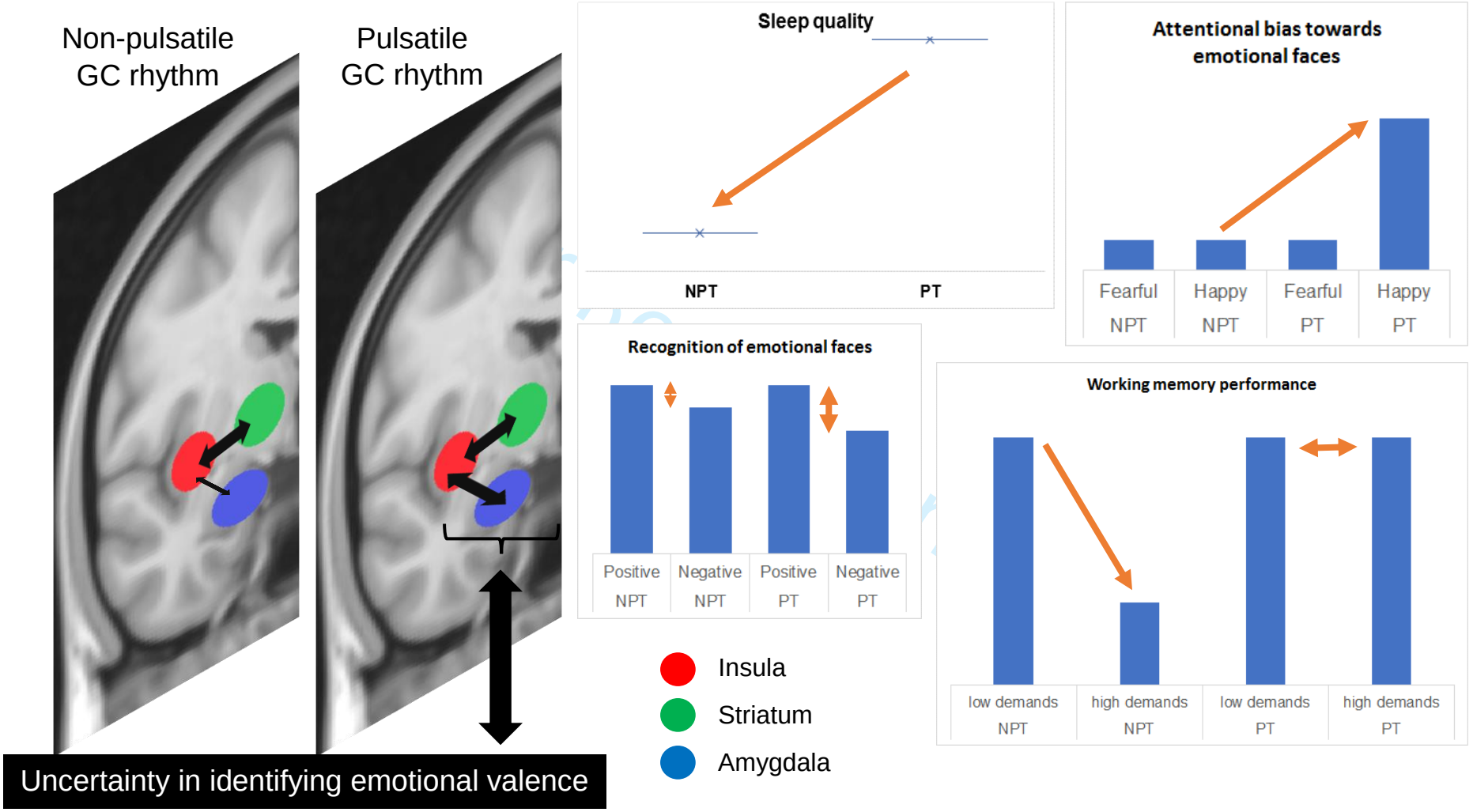
Legend to Figure 3

The importance of glucocorticoid (GC) pulsatility for the human brain. Comparing circadian patterns of cortisol infused in physiological pulses (PT) with the same dose of circadian cortisol infused as a smooth infusion (non-pulsatile infusion, NPT), brain function was investigated by neuroimaging and psychological measures, focusing on three domains: sleep behaviour, working memory and, primarily, emotional processing. Subjects on the NPT experienced poorer quality of sleep and working memory performance compared to the PT arm of the study. Moreover, subjects on PT preferentially engaged with positively valenced facial expressions and showed a reduced accuracy in correctly discriminating between negatively valenced human faces (i.e. increased ambiguity in perceiving negative emotional stimuli), a response

similar to that seen in healthy subjects and depressed patients receiving antidepressants. The between-treatment group changes in emotional ambiguity were linked to changes in the underlying role and functional connectivity among corticolimbic regions, mediating emotional processing. While in PT the functional connectivity between amygdala and insula, and striatum and insula, during encoding of emotional cues is strong, and the intensity of the neural processing in all these structures (especially for the amygdala) is associated with the degree of uncertainty in discriminating between emotional valences, this association is lost in NPT, combined with a reduction in the functional connectivity between amygdala and insula. Collectively, these data support the notion that GC pulsatility may facilitate the optimal functioning of neural mechanisms underlying emotional processing, and perhaps a protective mechanism against susceptibility to depression.




Diurnal Variation
Ultradian pulsatility
Spinogenesis
Expression of opioid receptors
Mood regulation
Gene expression
Synaptic plasticity
Stress physiology
High responsivity
Low responsivity


Non-pulsatile GC rhythm
Pulsatile GC rhythm
Uncertainty in identifying emotional valence
Sleep quality
NPT
PT
Attentional bias towards emotional faces
Fearful NPT
Happy NPT
Fearful PT
Happy PT
Recognition of emotional faces
Positive NPT
Negative NPT
Positive PT
Negative PT
Working memory performance
low demands NPT
high demands NPT
low demands PT
high demands PT
Insula
Striatum
Amygdala