Glucocorticoid pulsatility : implications for brain functioning

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Chapter 1

General introduction

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1.1 Pulsatile patterns of glucocorticoid release

The hypothalamic-pituitary-adrenal (HPA) axis is one of the major neuroendocrine axes of the body as it controls a plethora of functions in the brain and periphery via its end secretory product, the glucocorticoids [Box 1; (Fig. 1)]. Stress, either physical or psychological, rapidly triggers alterations in physiological states. Consequently, an acute elevation in glucocorticoids (cortisol in humans, corticosterone in rodents) facilitates prompt restoration of homeostasis in anticipation of new events. This occurs in part via negative feedback regulation of glucocorticoids indicating that these hormones are essential in the adaptation to stress (de Kloet et al. 2005, McEwen 2007).

The actions of glucocorticoids are mediated via two steroid hormone receptors, the mineralocorticoid and glucocorticoid receptor (MR and GR) that together maintain HPA axis activity [Box 2; (Reul & de Kloet 1985)]. While MR is considered to regulate tonic HPA axis activity and the threshold of the system to stress ('proactive' mode), GR promotes recovery from stress ['reactive' mode; (De Kloet et al. 1998)]. The receptor mechanisms and their relation to glucocorticoid pulsatility are extensively discussed later in this chapter. In short, glucocorticoids are considered to enable an individual to respond and adapt to a certain stressor and prepare for a subsequent event via permissive, suppressive and preparative steroid actions (Sapolsky et al. 2000).

Circadian rhythms in HPA axis activity

The circadian (or diurnal) rhythm (from the Latin circa diem, 'around a day'), is a roughly 24 hour-cycle in all bodily processes (i.e. biochemical, physiological, endocrine or behavioural) in all organisms including plants, animals, fungi and cyanobacteria (Panda et al. 2002, Reppert & Weaver 2002). These rhythms allow organisms to anticipate and prepare for precise and regular environmental changes (i.e. light/dark cycle, seasonal changes) thereby increasing the efficiency in metabolic demand.

Under basal conditions, there is a pronounced circadian rhythm in HPA axis activity (Veldhuis et al. 1990, Young et al. 2004). In man, peak levels of cortisol are observed at the end of the resting period in preparation of the increased metabolic demands of the active phase. Nocturnal animals such as rodents, peak in corticosterone levels towards the end of the afternoon when the dark cycle begins (Fig. 2). Besides many other processes, lack of diurnal corticosterone variation not only attenuates stress responsiveness (Akana et al. 1992), it also changes serotonin receptor 1a functioning (Leitch et al. 2003) and attenuates neurogenesis (Huang & Herbert 2006). As such, daily cyclic variations in glucocorticoid hormone

concentrations are thought to be fundamental for the maintenance of physiology and well being, as deviations from the normal release pattern are considered to enhance vulnerability to stress-related disease (Young et al. 2004, de Kloet et al. 2005, Herbert et al. 2006).

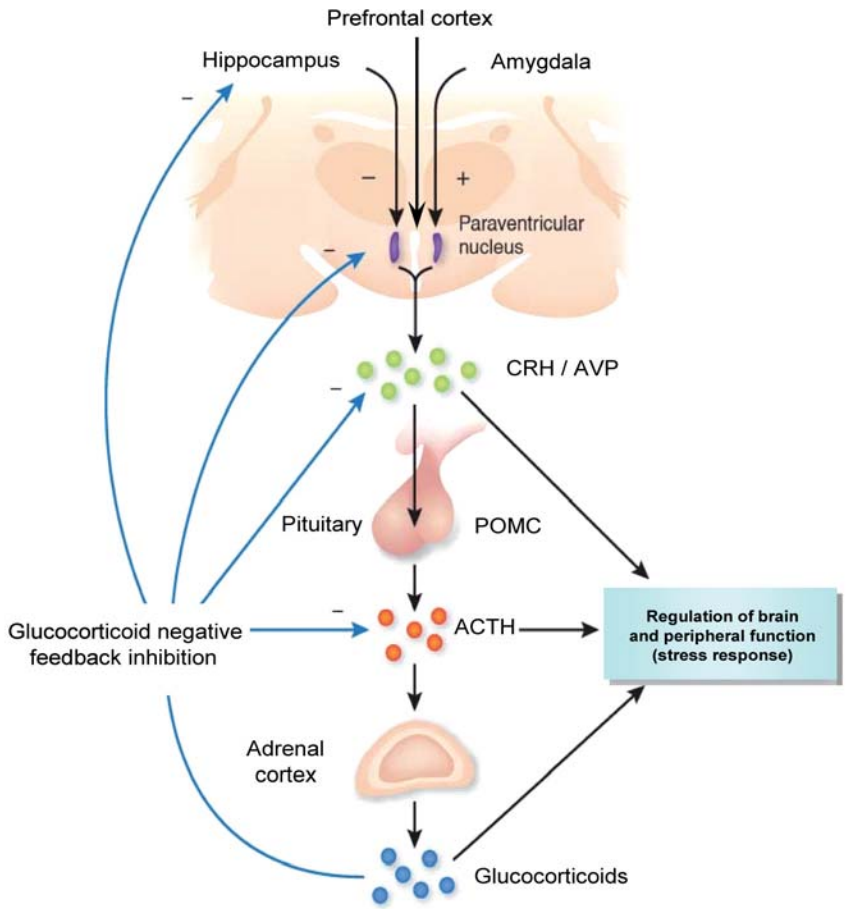

Figure 1 | Schematic representation of the different components of the HPA axis. Adapted and modified with permission from (Hyman 2009).

Box 1. The stress response

Stress is broadly defined as an actual or anticipated (physical or psychological) disruption of homeostasis or threat to well-being. It provokes rapid alterations in physiological states and the activation of two systems that together function in a controlled manner to rapidly restore homeostasis and balance (de Kloet et al. 2005, McEwen 2007). First, the autonomic nervous system (ANS) responds within seconds to disturbances in the body via sympathetic and parasympathetic innervations throughout the body. The sympathetic activation primarily increases circulating levels of adrenalin and noradrenalin in blood plasma but also increases heart rate and force of contraction, peripheral vasoconstriction and mobilises energy preparing the body for an adequate response to the stressor, hence the 'fight-or-flight' response. A few minutes later, activation of the secondary, slightly slower, HPA axis results in acute elevation of circulating glucocorticoid levels by the adrenal cortex (Fig. 1). This occurs via activation of the parvocellular region of the paraventricular nucleus (PVN) of the hypothalamus which is a major relay centre for processing cognitive and emotional information from limbic areas, but also physical processes such as inflammation (Ulrich-Lai & Herman 2009). The neuroendocrine cells in the PVN synthesise and secrete the hormones corticotrophin-releasing hormone (CRH) and arginine vasopressin (AVP). These peptides are secreted into the hypophysial portal vasculature in the external zone of the median eminence from where they travel through the portal system to induce release of adrenocorticotropic hormone (ACTH) from the anterior pituitary gland. Stress-induced ACTH release is primarily triggered by CRH but is known to be potentiated by AVP (Gillies et al. 1982, Rivier & Vale 1983). ACTH in turn acts on the inner adrenal cortex (zona fasciculata) to initiate the synthesis and release of glucocorticoid hormones.

Glucocorticoids have many important functions including regulation of glucose, fat and protein metabolism, anti-inflammatory actions and can affect mood and cognitive functions (De Kloet et al. 1998, Dallman et al. 2003). As mentioned above, these hormones are also implicated in the regulation of the primary response to stress. Accordingly during stress, glucocorticoids feedback to different areas in the brain and pituitary via an inhibitory negative feedback loop (Fig. 1). This rapidly shuts down the stress response by tightly controlling the degree and duration of stress-induced ACTH and corticosterone responses thereby restoring homeostasis (Dallman et al. 1987, Dallman 2005). Via this mechanism, glucocorticoids also maintain physiology under normal basal conditions by feedback on the pituitary gland to inhibit further hormone release (Jones et al. 1977, Dallman et al. 1987, Watts 2005). Negative feedback inhibition is therefore suggested to be an essential adaptive mechanism in physiology and the response to stress (Keller-Wood & Dallman 1984).

Ultradian corticosterone rhythms

The development of high frequency (automated) sampling paradigms, intra-tissue microdialysis in non-stressed conditions and statistical algorhythms has greatly facilitated the knowledge on glucocorticoid rhythms. It especially led to the discovery that circadian release of glucocorticoids in fact consists of more frequently released hormone bursts reflected in blood plasma as a distinct ultradian rhythm (< 24 hours), previously regarded as 'experimental noise' (Fig. 2). Ultradian glucocorticoid hormone pulses are characteristically released with a periodicity of approximately 60 min in blood plasma and have been described in numerous species including rat (Jasper & Engeland 1991, Windle et al. 1998b), rhesus monkey (Holaday et al. 1977, Tapp et al. 1984), sheep (Cook 2001) and humans (Weitzman et al. 1971, Lewis et al. 2005, Henley et al. 2009).

The secretory pattern of glucocorticoids is also maintained across the blood-brain-barrier in the extracellular fluid suggesting that target tissues such as the brain (but also the receptors located there) are exposed to rapidly fluctuating steroid levels (Cook 2001, Droste et al. 2008). Ultradian pulses increase in amplitude and to a lesser extent in frequency, according the time of day due to increased adrenal sensitivity to ACTH (Ulrich-Lai et al. 2006) and increased CRH drive (Walker et al. 2010), resulting in a circadian profile of hormone release (Fig. 2). Superimposed on these rhythms is the central nervous system (CNS) mediated glucocorticoid response to a stressor.

Ultradian rhythms in other hormonal systems

Rapid oscillations in hormone levels seem to be a ubiquitous phenomenon in hormonal systems and are not restricted to glucocorticoids. For instance, highly dynamic secretory patterns have been described for gonadotrophin-releasing hormone [GnRH; (Belchetz et al. 1978, Rothman & Wierman 2007)], luteinising hormone [LH; (Knobil et al. 1980)], growth hormone [GH; (Waxman et al. 1995)], noradrenalin (Tapp et al. 1981) and insulin (Matthews et al. 1983). These pulsatile patterns are considered biologically significant: they are highly conserved during evolution and across species and are required for appropriate actions of receptors and target tissues (Hauffa 2001). For instance, rapidly fluctuating levels of GH elicit significant sexual dimorphic effects on gene expression (Waxman et al. 1995). Similarly, modulation of episodic release of GnRH influences the secretory patterns of LH and FSH and prevents receptor desensitisation (Belchetz et al. 1978, Wildt et al. 1981). Furthermore, intermittent, in contrast to continuous administration of parathyroid hormone is beneficial for bone formation (Schmitt et al. 2000), while continuous GH administration attenuates growth (Gevers et al. 1996). Also, insulin in men is more efficient when delivered in a phasic, rather than tonic fashion (Matthews et al. 1983). Therefore, it is generally believed that rapid oscillations in hormone presentation not only exist to maintain receptor responsiveness in target tissues as it will otherwise desensitise/down-regulate with tonic exposure, hormonal

actions also seem more efficient when delivered in a pulsatile manner (Hauffa 2001). With respect to glucocorticoid biology it is however currently not known what (and how) ultradian hormone signalling contributes to physiology and nuclear receptor functioning.

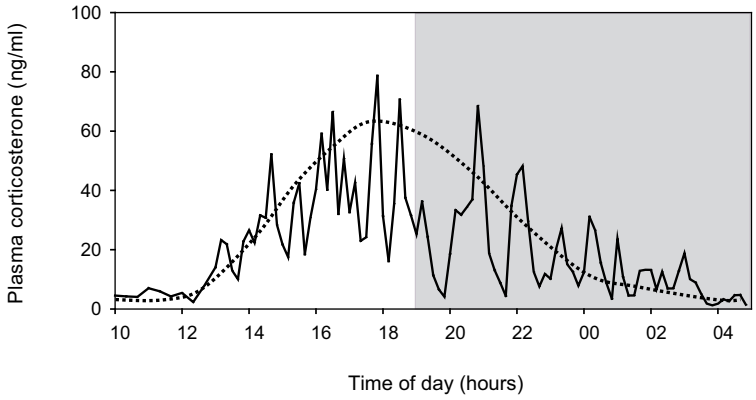

Figure 2 | Ultradian (smooth line) and circadian rhythm (dotted line) of corticosterone under basal conditions in a male Sprague-Dawley rat. Representative individual 19-h profile of corticosterone in blood plasma automatically collected every 10 minutes between 13.00 and 05.00 and every 20 minutes between 10.00 – 13.00. The grey area indicates the dark period of the light/dark cycle.

Box 2. Mineralocorticoid and glucocorticoid receptors

The actions of glucocorticoids are exerted by the corticosteroid receptors that reside in target tissues: mineralocorticoid and glucocorticoid receptors, MR and GR respectively. GR is ubiquitously expressed in the brain and periphery while MR localisation is more restricted to the hippocampus, prefrontal cortex and amygdala, structures essential for learning, memory formation and emotional behaviour (van Eekelen et al. 1987, Arriza et al. 1988, Ahima & Harlan 1990). Both receptors colocalise in neurons from several areas including the hippocampus (Han et al. 2005). MR has a 10-fold higher affinity for corticosterone (Kd: 0.5 nM) than GR (Kd: 5 nM) which renders the hippocampus responsive to both basal and stress-induced elevations of corticosterone. MR is thus extensively occupied under basal conditions regulating tonic HPA activity while GR is additionally recruited when corticosterone levels rise, such as during stress, the circadian rise in corticosterone (Reul & de Kloet 1985, Spencer et al. 1993, De Kloet et al. 1998, Kitchener et al. 2004) and the peak of hourly ultradian pulses (Conway-Campbell et al. 2007, Stavreva et al. 2009).

MR and GR belong to the superfamily of nuclear receptors (de Kloet 1995, Mangelsdorf et al. 1995). It is currently believed that in their inactive state the receptors reside in the cytoplasm, stabilised by chaperone molecules [i.e. heat shock proteins and immunophilins (Wochnik et al. 2005, Nishi & Kawata 2006, Picard 2006)]. Upon glucocorticoid binding, the transport machinery (i.e. importins) is recruited which actively translocates the entire complex to the nucleus using the microtubule network as a guiding scaffold (Pratt et al. 2004, Fitzsimons et al. 2008). In the nucleus, the receptors function as a ligand-activated transcription factors to modulate genomic events via transcription of glucocorticoid target genes. This occurs via transactivation by interacting directly with the chromatin of glucocorticoid-responsive promoter regions and subsequent recruitment of coactivators or via transrepression by protein-protein interactions with other transcription factors and corepressors (Datson et al. 2008, van der Laan & Meijer 2008).

Besides the classical nuclear receptors, accumulating anatomical and electrophysiological data have described membrane-bound variants of MR and GR (Orchinik et al. 1991, Di et al. 2003, Johnson et al. 2005, Karst et al. 2005), suggested to mediate rapid non-genomic glucocorticoids effects via second messenger pathways. Recently, it was discovered that non-genomic MR in limbic structures enhances the presynaptic glutamate release probability and reduces postsynaptic hyperpolarisation via the ERK1/2 pathway and K+-conductance with the net result to enhance excitatory transmission (Karst et al. 2005, Olijslagers et al. 2008). On the other hand in the PVN, non-genomic GR reduces net neuronal excitatory transmission via endocannabinoids and nitric oxide (Di et al. 2003, Di et al. 2009). At least for membrane MR it is known that it requires stress-induced levels of corticosterone as its affinity is much lower than that of the nuclear MR. This receptor is thus implicated in the onset of stress while the reaction is contained by genomic GR–mediated events. It is therefore currently believed that the nuclear and membrane-bound variants of MR and GR act together to maintain basal and stress-induced HPA axis activity.

1.2 Glucocorticoid pulsatile patterns and stress responsiveness

Abolishing diurnal corticosterone rhythms alters HPA axis responsiveness to stress (Dallman et al. 1987, Jacobson et al. 1988, Akana et al. 1992). The interaction of glucocorticoid ultradian patterns and stress responsiveness is however not very clear. The application of high frequency blood sampling has greatly added to the understanding of this relationship. Post-hoc analysis of hormone profiles of rats exposed to noise stress indicated that animals only responded with increased corticosterone when the stress coincided with an ascending phase of an ultradian pulse (Fig. 3). Stress during a falling phase, however, did not result in a significant corticosterone response [hyporesponsiveness; (Windle et al. 1998b)]. The underlying mechanism is not yet clear and either a facilitated stress response during the ascending phase and/or an inhibitory effect during the descending phase have been suggested. Irrespectively, it is evident that the onset of a stressor in relation to the phase of an ultradian pulse can determine the physiological response to stress. Similarly, the propensity to behave aggressively is increased during the ascending phase of an ultradian corticosterone pulse (Haller et al. 2000b). Furthermore, pulse amplitude and frequency seem to be major determinants in the outcome of acute HPA axis reactivity with reduced corticosterone responses with increased pulse amplitude and frequency (Windle et al. 2001, Atkinson et al. 2006). These observations explain for a part the inter-individual variation in responsiveness to a stimulus that is normally observed in stress experiments but also emphasises the fact that acute HPA axis reactivity heavily depends on the individual pulse characteristics that make up the ultradian pattern.

The concept of ultradian pulses being associated with stress responsiveness is reminiscent of the earlier concept of rate sensitive feedback. In this respect, several studies showed fast negative feedback inhibition only to be present during the brief interval (i.e. first 2-5 min) when plasma corticosterone levels are rapidly rising (e.g. as occurs during stress) and disappears when it has reached a plateau or when infusion is stopped (Dallman & Yates 1969, Jones & Neame 1971). It has been hypothesised that in the context of ultradian glucocorticoid pulses, the secretory phase of each ultradian surge could also induce rapid negative feedback inhibition generated by the acute rise in glucocorticoid levels consequently resulting in inhibition during the descending phase. Hence, the concept of rapidly alternating phases of HPA axis activation and inhibition which could determine the outcome of stress responsiveness depending on the 'state' of the HPA axis (Windle et al. 1998b, Lightman et al. 2008). In addition, the magnitude of the stress effect is not only influenced by the phase of ultradian pulses, but also seems to depend on the rate of rise of hormone concentration (i.e. for a peak of given duration: amplitude height), with increasing negative feedback inhibition with increasing corticosterone infusion rate (Dallman & Yates 1969, Kaneko & Hiroshige 1978). Even though the underlying mechanism is still unknown, it is therefore hypothesised

that in both basal and stress-induced circumstances, negative feedback interacts in a rate-sensitive manner with the different phases and the amplitude of individual ultradian pulses thereby determining the outcome of stress responsiveness.

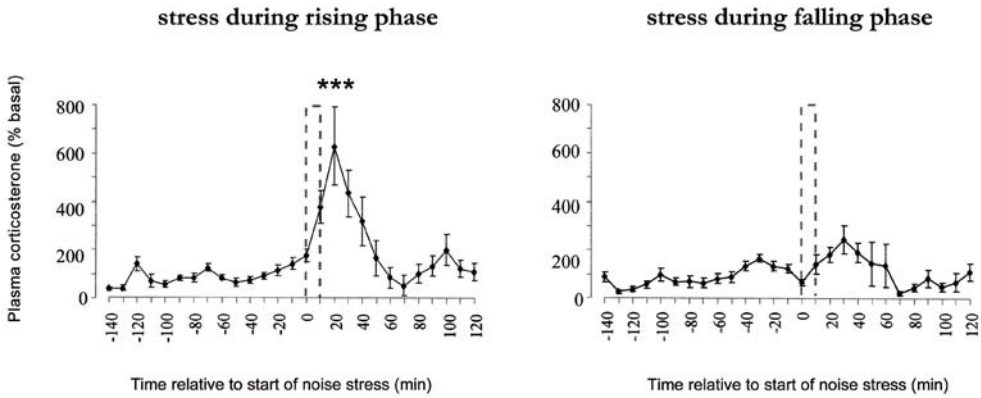

Figure 3 | The effect of noise stress (10 min of 114 dB, broken line) on plasma corticosterone concentrations in female Sprague Dawley rats when the stress coincided with a rising (left) or falling phase (right) of a basal corticosterone pulse. *** $p < 0.05$ vs falling group. Adapted and modified from (Windle et al. 1998b).

1.3 Changes in glucocorticoid pulsatile patterns

While the generation and coordination of ultradian glucocorticoid pulses is an ongoing discussion, it is clear that secretory corticosteroid patterns can be highly variable between and within individuals. For instance in rodents, changes in the pattern of ultradian pulsatility are often seen in physiology during normal transitions throughout the lifespan (Fig. 4). Lactation increases pulse amplitude while during ageing the hourly pattern becomes disorganised (Lightman 1992, Young et al. 2004, Lightman 2008). Sexual diergism in corticosterone pulsatile characteristics is also evident (Fig. 4A), and is mainly attributed to gonadal steroids as hormone replacement after gonadectomy reverses hypersecretion in males and hyposecretion in females (Seale et al. 2004a, Seale et al. 2004b). Also, clear genetic influences in pulse characteristics are reflected in different strains of rat (Lewis and Fischer rats) that differ in stress responsiveness and their susceptibility to autoimmune disease (Windle et al. 1998a). Overall, the studies described above strongly suggest that ultradian glucocorticoid patterns can be remarkably variable depending on the physiological state or genetic background.

Alterations in glucocorticoid pulsatile patterns are, however, also associated with stress-related disorders. In humans, depression is associated with enhanced pulse magnitude thereby abolishing circadian variation in pulse amplitude, resulting in dramatically changed patterns of hormone exposure to tissue (Deuschle et al. 1997, Holsboer 2000, Young et al. 2004). In Cushing's syndrome, the normal variation of cortisol is dampened or completely abolished due to elevations in cortisol levels in the quiescent period [Fig. 4C; (Boyar et al. 1979, van Aken et al. 2005)]. Other disorders like Parkinson's and Huntington's disease but also panic disorders in humans (Abelson & Curtis 1996, Hartmann et al. 1997, Aziz et al. 2009) and inflammation and early life stress in rodents (Harbuz et al. 1999, Shanks et al. 2000, Windle et al. 2001) are characterised by alterations in pulse characteristics (Fig. 4B). Currently it is not known whether the disorganisation in pulsatile patterns is causal to the disorder or vice versa. It may however be hypothesised that deviations from the optimal ultradian pattern could precipitate disease as daily variations in glucocorticoid hormone concentrations are thought to be fundamental for the maintenance of physiology, and overall metabolic, cognitive and behavioural well being (Dallman et al. 2003, Young et al. 2004, de Kloet et al. 2005, Herbert et al. 2006). Even so and as described in this chapter, the functional contribution of ultradian glucocorticoid pulses to HPA axis activity, stress responsiveness and the consequences of changes in pulse characteristics for physiology and brain functioning are largely unknown.

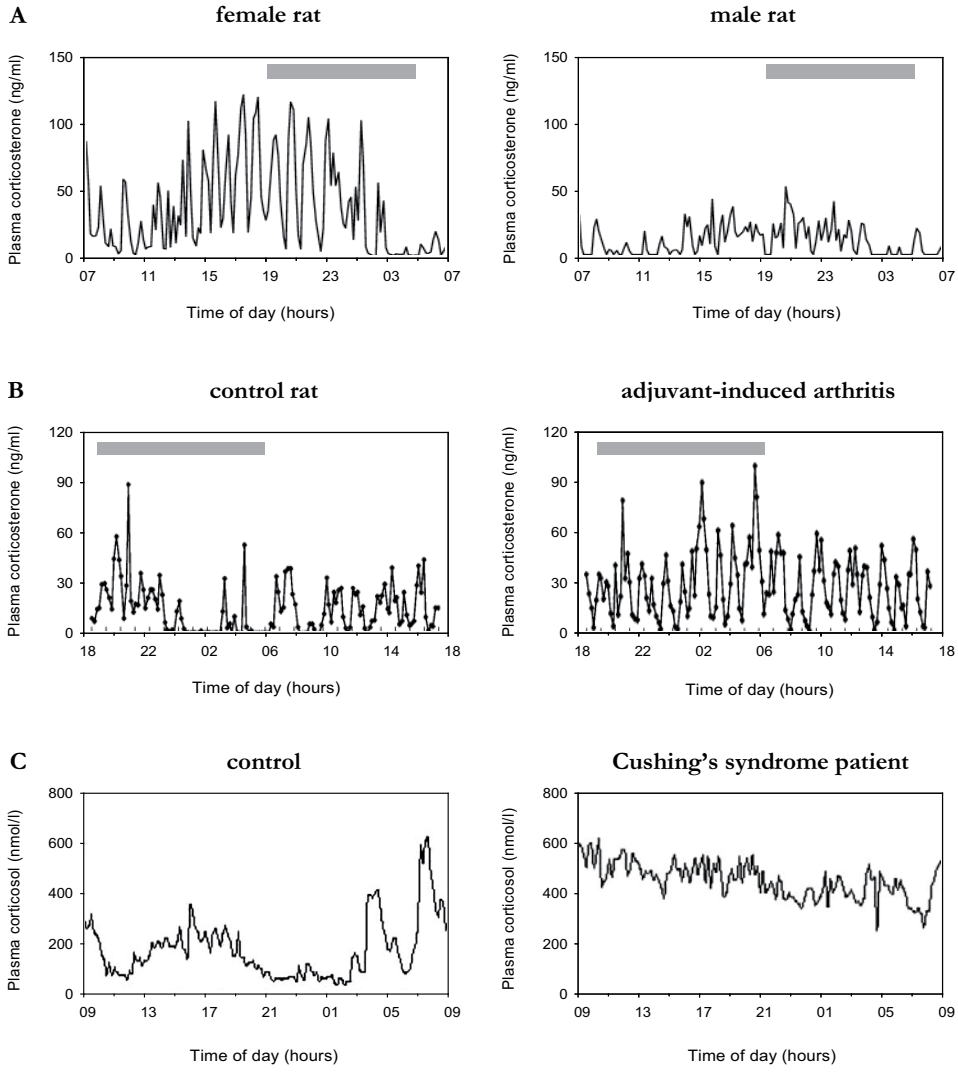

Figure 4 | Examples of differences in ultradian corticosteroid rhythms in A | female and male Wistar rats, B | adjuvant-induced arthritis after 13 days in rats compared to controls and C | unilateral adenoma in Cushing's syndrome. Data taken from (Windle et al. 2001, Seale et al. 2004a, van Aken et al. 2005). The dark phase of the cycle is shown by the filled bar.

1.4 Coordination of glucocorticoid pulsatile patterns

The suprachiasmatic nucleus

In mammals, the suprachiasmatic nucleus (SCN; also known as the biological clock) is the principal pacemaker in the coordination and generation of physiological circadian rhythms. The rodent SCN consists of approximately 16.000 neurons located in two clusters in the anterior hypothalamus in the brain (Van den Pol 1980). It is entrained by light as it receives input from the retina via the retinohypothalamic tract consisting of photoreceptive ganglion cells specialised in luminance coding. Photic cues conveyed to the SCN by this tract ensure that internal circadian time is entrained by solar time as it temporally adapts cycles in physiology and metabolism (Berson et al. 2002). Internal time sometimes gets disorganised when it loses synchronisation with external day/night cycles as for instance occurs with a jet-lag or during sleep disorders (Hastings et al. 2003, Maywood et al. 2006).

Circadian timing is an intrinsic property of individual SCN cells as isolated SCN cells in culture continue to exhibit spontaneous circadian electric and metabolic rhythms (Welsh et al. 1995). GABA acts as one of the primary synchronising signals among SCN neurons but other signals (i.e. AVP and vasoactive intestinal polypeptide) have also been described (Liu & Reppert 2000, Maywood et al. 2006). Knowledge about the genes that encode the intracellular mechanism that underlies circadian timing comes mostly from genetic screening studies in Drosophila while mammalian homologues have been found and cloned recently (Reppert & Weaver 2002). Progress in this field has been exponential, but simplistically, the core elements are timekeeping genes which function as transcription factors such as Period, Clock and Crytochrome. The expression of these genes are periodically switched on and off by posttranslational modifications in interacting autoregulatory positive and negative feedback loops with a periodicity of approximately 24 hours. Accordingly, waves of transcription and translation of key clock components ultimately drive and determine rhythmic properties of SCN neurons (i.e. firing rate, neurosecretion and membrane potential; (Panda et al. 2002, Hastings et al. 2003).

The SCN carries circadian outflow to local clocks in neural or peripheral targets via light entrainment, sympathetic nervous system projections, food intake, body temperature but also chemical and hormonal cues (Hastings et al. 2003, Cuninkova & Brown 2008). The SCN thus functions as the master clock and coordinates a plethora of peripheral 'slave' oscillators. Though some intrinsic autonomous components exist, this result in local rhythmic expression of clock genes in almost all bodily tissues, ensuring temporal integration and regulation of local physiological rhythms across an organism (Cuninkova & Brown 2008). How molecular transcriptional oscillations exactly control rhythmic neurotransmitter and peptide secretion and hence, endocrine, metabolic and behavioural circuits is increasingly

becoming clear (Vansteensel et al. 2008, Houben et al. 2009). A salient detail is that glucocorticoids and the GR have been implicated in the rhythmic control of some clock genes such as Per1 and Per2 and in fact seem selectively required for some glucocorticoid functions such as glucose homeostasis (Balsalobre et al. 2000, So et al. 2009).

Generation of glucocorticoid circadian rhythms

The HPA axis is one of the major input targets of the SCN, regulated via a network of complex neuronal pathways which has been subject to many anatomical studies (Swanson & Cowan 1975, Van den Pol 1980, Kalsbeek & Buijs 2002). The majority of projections remain confined within the boundaries of the medial hypothalamus targeting the medial preoptic area, the PVN, the subPVN and the dorsomedial nucleus of the hypothalamus. Increasing glucocorticoid levels during the circadian peak are thought to arise from increased drive from the SCN, most likely due to a number of different mechanisms. One major group of SCN target neurons comprise the CRH (but also TRH and GnRH) containing endocrine cells. Basal activity in corticosterone during the circadian trough is maintained by constitutive autonomous secretion of CRH while increased release in the evening requires active stimulation of afferents that project from the SCN to the PVN and median eminence (Kaneko et al. 1980, Engeland & Arnhold 2005). Circadian variation in corticosterone is considered to be driven almost exclusively by rhythmic secretion of CRH, but not so much ACTH as corticosterone rhythms persist in absence of ACTH cycling (Watts 2005, Ulrich-Lai et al. 2006). Alternative pathways implicate the autonomous nervous system in circadian control of corticosterone via 1) SCN-mediated activation of excitatory splanchnic nerve innervation and 2) adrenal gland sensitivity to ACTH (Jasper & Engeland 1994, Oster et al. 2006, Ulrich-Lai et al. 2006, Dickmeis 2009). Both consequently result in larger corticosterone bursts in the evening in rodents.

Generation of glucocorticoid ultradian rhythms

The mechanism and location of the ultradian pulse generator are however less clear. From lesion studies it is known that the SCN does not control ultradian corticosterone release as these rhythms persist in blood plasma after ablation of the nucleus (Watanabe & Hiroshige 1981, Buijs et al. 1993). In that respect, ultradian CRH continues to be rhythmically released from isolated macaque hypothalami and rat median eminence, suggesting that generation of rapid phasic CRH pulses is an intrinsic property of the hypothalamus (Ixart et al. 1987, Mershon et al. 1992). However, the underlying mechanisms are not yet clear. Oscillators in the adrenal gland itself have also been identified recently suggesting intrinsic generation and/or modification of pulsatile corticosterone patterns (Oster et al. 2006, Son et al. 2008). In addition, the adrenal gland is extensively innervated by autonomous nerve fibers such as the splanchnic nerve exerting inhibitory control resulting in very low circulating corticoste-

rone levels during the circadian trough (Jasper & Engeland 1994). Alternatively, inhibitory ultra short feedback is suggested to control the rapid increase and decrease in corticosterone levels thereby generating oscillations in steroid levels (Windle et al. 1998b, Lightman et al. 2008). In fact, theoretical modelling has recently demonstrated that a combination of delay and feedforward and feedback loops in HPA axis activity creates episodic feedback signals at the level of the pituitary gland that is necessary to maintain feedforward-feedback oscillatory activity between the pituitary and adrenal gland, hence creating self-sustaining ultradian oscillations in glucocorticoid secretion (Walker et al. 2010).

1.5 Glucocorticoid pulsatile patterns and corticosteroid receptors

As outlined in the previous paragraphs, the contribution of ultradian glucocorticoid pulses to physiology but also nuclear receptor functioning is not well understood and must therefore be thoroughly addressed. It is known that MR in the brain is activated throughout the entire 24-h cycle (Reul & de Kloet 1985, Spencer et al. 1993, Bradbury et al. 1994). GR, on the other hand, is additionally recruited when corticosterone levels rise as its nuclear retention varies according to circadian fluctuations in corticosterone levels, but also during stress (Reul & de Kloet 1985, Kitchener et al. 2004). Moreover, repeated rapid nuclear translocation of hippocampal GR following intravenous corticosterone injections mimicking ultradian pulses was demonstrated in rats while MR was continuously retained in the nucleus (Conway-Campbell et al. 2007). More importantly, these studies imply that long- term disturbances in ultradian glucocorticoid pulsatility would thus mainly affect GR, rather than MR. Therefore, the classical nuclear variants of MR and GR are distinctively differently occupied and activated depending on the pattern of glucocorticoid exposure which hypothetically could lead to differential regulation of glucocorticoid target genes. Currently it is not known how ultradian glucocorticoid fluctuations affect the membrane-bound variants of MR and GR. It may be hypothesised however that in view of the rapid non-genomic actions of glucocorticoids together with the slower genomic effects, the relative balance of glucocorticoid action via membrane-bound and nuclear variants of MR and GR in the brain could rapidly change over the duration of a single ultradian pulse due to fast changes in concentrations of available ligand (Young et al. 2004, Joels et al. 2008a).

Intracellular dynamics of corticosteroids receptors

While most studies have looked at gene regulation after long term stimulation by glucocorticoids, accumulating evidence indicates that the nuclear receptor mechanism is not the static process as previously considered but is in fact highly dynamic. Intra-tissue microdialysis demonstrated that glucocorticoid target tissues are exposed to rapidly fluctuating hormone

levels resulting in a highly dynamic environment for nuclear receptors (Cook 2001, Droste et al. 2008). Using photobleaching techniques in living cells it was first discovered that GR rapidly exchanges at regulatory sites in the genome in a ligand and ATP-dependent manner (McNally et al. 2000, Stavreva et al. 2004). More fundamentally, GR only responds in an ultradian manner to natural glucocorticoids and not synthetic ligands such as dexamethasone. This results in consecutive ultradian waves of receptor nuclear translocation and GR occupancy of GREs [Fig. 5, (Stavreva et al. 2009)]. Moreover, the dynamic promoter occupancy of glucocorticoid target genes coincides with oscillations in the 'chaperone protein cycle', as well as RNA Polymerase II loading and exchange, which fluctuate according to the changes in the extracellular hormone concentrations. Consequently, this results in 'gene-pulsing' of transcriptional patterns of nascent RNA [Fig 5, (Stavreva et al. 2009)].

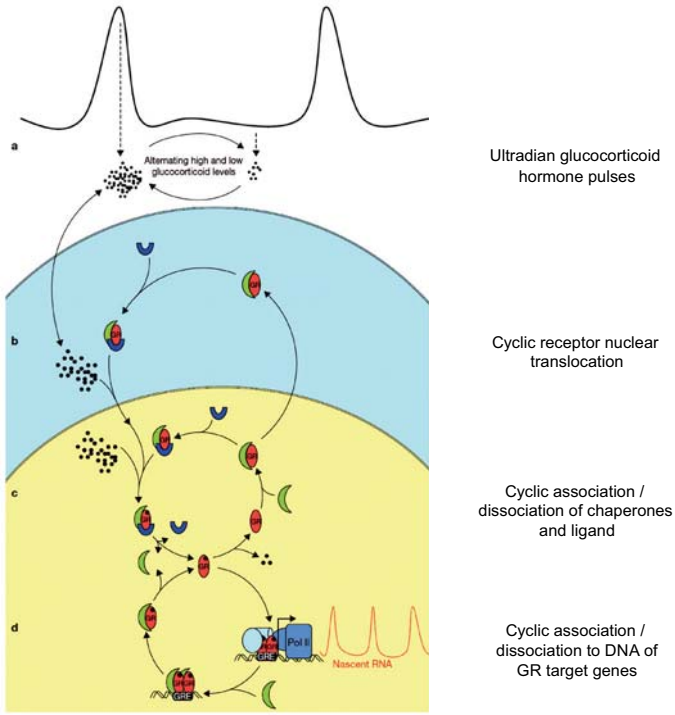

Figure 5 | Simplified coupling of ultradian hormone secretion to GR-driven transcriptional cycling. A | Ultradian hormone pulsing by the adrenal glands generates successive high and low levels of circulating glucocorticoids (black dots) which enter the cells. B | In the presence of hormone, the GR with its chaperones (green crescent, HSP90/p23 complexes; blue half-ring, HSP70 complex) binds the hormone and translocates into the nucleus. C | Chaperones cycle within the nucleus, which accompanies the fast dissociation and reassociation of hormone with GR. D | The association and dissociation of the hormone-bound GR with DNA, to which chaperones also contribute. The transcriptionally active cycle of DNA-bound GR is permitted by the recruitment of cofactors (light blue boxes) driving the activity of the RNA Polymerase II (Pol II, dark blue box). Hormone pulses result in similar pulses in transcriptional activity, reflected by bursts of nascent mRNAs. Adapted and modified from (Desvergne & Heligon 2009).

Cyclic activity of other nuclear receptors (i.e. androgen and estrogen receptors, AR and ER respectively) has been described before (Kang et al. 2002, Metivier et al. 2003, Reid et al. 2003). Interestingly, GR cycling is attenuated during constant presence of steroid hormone while AR and ER continue to oscillate. This suggests that receptor cycling is an intrinsic property of AR and ER while GR oscillatory action is actively externally driven by hormone bursts of the adrenal gland (Jasper & Engeland 1991). At the moment, there is relatively little information available on how physiological relevant levels of glucocorticoid pulses affect MR and GR in target tissues in vivo and specifically in the brain. This not only has important implications for glucocorticoid biology but could also add to our understanding of nuclear receptor functioning. Furthermore as described in the previous paragraphs, episodic release of for instance GnRH and GH prevent receptor desensitisation and maintains target tissue responsiveness. In conclusion, it has been proposed that pulsatile glucocorticoid release coordinates dynamic receptor interactions necessary for transcriptional programming (Lightman et al. 2008, Desvergne & Heligon 2009, Stavreva et al. 2009). By rapidly sensing changes in the local ligand environment, GR is crucial in propagating biologically appropriate actions.

1.6 Significance of glucocorticoid pulsatility

The clinical relevance of the secretory hormone pattern of for instance GnRH and GH is well recognised (Hauffa 2001). These hormones have proven to be successful targets for therapeutic purposes. The pulsatile nature of glucocorticoids is however poorly understood or even regarded as not important. As describe in the previous paragraphs, disturbances in glucocorticoid rhythms and aberrant HPA axis functioning are well recognised signs in patients suffering from stress-related disorders. Furthermore, it has been hypothesised that deviations from the optimal glucocorticoid pattern may precipitate disease as daily variations in glucocorticoids are fundamental for physiology, but also emotional and cognitive well being (Young et al. 2004, de Kloet et al. 2005, Herbert et al. 2006). New insights in glucocorticoid pulsatility could also have important implications for the therapeutic application of glucocorticoids based on the temporal aspect of hormone action. For instance, Addison's patients suffer from sever hypocortisolimia and could greatly benefit from pulsatile administration of these hormones. Transient release patterns of other hormones are considered crucial for physiology and well being (Belchetz et al. 1978, Thompson et al. 2003, Rothman & Wierman 2007). Manipulation of the temporal aspect is already a successfully used approach in clinical therapy for instance for GH (Amato et al. 2000) and estrogen-replacement therapy in post-menopausal women (Shoupe 2001).

However, in relation to glucocorticoids, no such administration protocols have been designed yet. Better understanding of pulsatile glucocorticoid release and the underlying nuclear receptor mechanism in the brain may therefore greatly contribute to the prognosis and treatment of disease, irrespective of the currently unknown specific causes of rhythmic corticosteroid dysregulation.

1.7 Scope of the thesis

As outlined in this chapter, the significance of glucocorticoid pulsatility and its importance for physiology and brain functioning is not yet clear. However, changes in glucocorticoid pulsatile rhythms are thought to compromise 'resilience' to stress and may be an important factor in the aetiology of stress-related diseases. The overall objective of this thesis is therefore to assess the role of glucocorticoid pulsatile patterns in brain and pituitary responsiveness to stressful challenges.

Ultradian corticosterone pulsatility is well characterised in the rat and is highly similar to human ultradian rhythmicity. Therefore in this thesis, we use the rat as a model system to study the consequences of glucocorticoid pulsatility on different levels of brain functioning. Due to the high frequency of the endogenous ultradian pulses and the lack of rhythmic synchronisation between animals, it is necessary to control the pattern of hormone release. Hereto, we have surgically or pharmacologically modulated pulsatile variations in corticosterone hormone levels. The endogenous corticosterone pulsatile patterns are effectively eliminated, flattened or mimicked by removal of the adrenal glands [adrenalectomy (ADX)] and/or corticosterone replacement in the form of subcutaneous pellet implantation or automated intravenous infusion. In addition, the knowledge of pulsatile corticosterone secretion is increasing with the use of high-frequency automated blood sampling (ABS) techniques in free-running animals (Windle et al. 1998a, Droste et al. 2008) in conjunction with the development of sophisticated statistical algorithms (Merriam & Wachter 1982). Recently, an automated steroid infusion system together with high frequency ABS was developed (Lightman et al. 2008). The use of this highly sophisticated model system puts us in the unique position to tightly control corticosterone infusion in either pulsatile or constant pattern under different experimental conditions.

The specific aims addressed in this thesis are:

1) to determine the effect of disturbances in pulsatile patterns of corticoste rone on glucocorticoid signalling in the rat hippocampus.

2) to investigate the implications of recovery from disturbed corticosterone pulsatility for behavioural and neuroendocrine stress responsiveness.

3) to study the stress-induced neuroendocrine and behavioural response to as stressor as a function of the pattern, amplitude and phase of ultradian glucocorticoid pulses.

Outline of the thesis and experimental approach

In order to study receptor nuclear translocation as a marker for glucocorticoid signalling, we need to find the most suitable antibody for further studies. In **chapter 2** commercially available GR primary antibodies are tested in parallel. Here, GR nuclear translocation patterns after glucocorticoid treatment are compared between cultured cells and CA1 pyramidal and dentate gyrus granule cells of the rat hippocampus visualised by immunohistochemistry and confocal imaging.

The outcome of chapter 2 is used in **chapter 3** to further characterise receptor translocation in vivo. We test the hypothesis that glucocorticoid hormone stimulation indeed results in MR and GR translocation in the rat hippocampus and that this may depend on the hormonal background of the animal. In this study, both intact and ADX rats are injected with a dose of corticosterone mimicking the stress response. Changes in subcellular distribution patterns of MR and GR at different time points after glucocorticoid injection are measured in single cells in the different hippocampal subfields visualised by immunohistochemistry and confocal imaging.

In **chapter 4** studies are reported exploring long-term disturbances in endogenous corticosterone pulsatile patterns for target tissue sensitivity to an additional glucocorticoid challenge that mimics the stress response. Diurnal and ultradian corticosterone fluctuations are clamped at constant levels by administering corticosterone exogenously by means of subcutaneous corticosterone pellet implantation in intact animals. Additionally, recovery from constant exposure is studied in groups that had the pellet removed 24 hours prior to the challenge. Molecular markers for MR and GR responsiveness to this acute challenge were visualised in the rat hippocampal area by in situ hybridisation and immunohistochemistry. Additionally, molecular approaches such as Western Blot and chromatin immunoprecipitation (ChIP) are used to study other aspects of the receptors.

Extending chapter 4 by using the same experimental paradigm, we investigate and validate in **chapter 5** the consequences of subcutaneous corticosterone pellet implantation on endogenous diurnal and ultradian corticosterone rhythmicity. High frequency ABS is used to study the effect on corticosterone levels before and after removal of corticosterone pellet implantation. Behavioural and neuroendocrine responsiveness to noise stress is assessed as a functional parameter.

In **chapter 6** studies are described investigating the significance of glucocorticoid pulsatility for negative feedback, behavioural stress responsiveness and markers for neuronal activation after stress. In this study, we specifically discriminate between the pattern, phase and amplitude of ultradian pulses in relation to stress. For this purpose ADX rats are automatically infused with either constant or pulsatile corticosterone patterns. Noise stress is applied to study stress-induced ACTH responsiveness in automatically collected blood samples via the ABS. Expression levels of CRH, POMC, MR and GR are used as parameters to characterise long-term changes in HPA axis activity in different areas of the brain. In addition, c-fos mRNA expression and home cage behaviour are analysed as a measure of acute stress responsiveness.

A general discussion of the data is presented in **chapter 7** and a synopsis of the major findings of this thesis is presented in **chapter 8**.