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Glucocorticoid pulsatility : implications for brain functioning

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Glucocorticoid pulsatility

Implications for brain functioning

Ratna Angela Sarabdjitsingh

Angela Sarabdjitsingh

Glucocorticoid pulsatility: implications for brain functioning

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Glucocorticoid pulsatility

Implications for brain functioning

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List of abbreviations

ABS	automated blood sampling
ACTH	adrenocorticotropic hormone
ADX	adrenalectomy
ANS	autonomous nervous system
AVP	arginine vasopressin
ChIP	chromatin immunoprecipitation
CNS	central nervous system
CORT	corticosterone
CRH	corticotrophin releasing hormone
Dex	dexamethasone
DG	dentate gyrus
GH	growth hormone
GnRH	gonadotrophin-releasing hormone
GR	glucocorticoid receptor
GRE	glucocorticoid response element
HPA axis	hypothalamic-pituitary-adrenal axis
IHC	immunohistochemistry
i.p.	intraperitoneal
IR	immunoreactivity
ISH	in situ hybridisation
MR	mineralocorticoid receptor
PVN	paraventricular nucleus
WO	washout

Preface

Everything has rhythm, hormones are no exception. Rapid oscillations in steroid levels are a ubiquitous phenomenon in hormonal systems and are not restricted to glucocorticoids. For instance, highly fluctuating levels of gonadotrophin-releasing hormone, growth hormone and insulin have been described previously. Furthermore, frequency encoding via circulating hormones as intracellular signals is a well accepted method of communication within mammalian systems. Accordingly, these ultradian hormone patterns are tightly controlled and are consequently required for appropriate action of receptors and target tissue sensitivity. However, dysregulation of the secretory pattern of single hormones in disease states and subsequently the underlying signalling mechanism, is well defined in most cases.

Pronounced ultradian and circadian rhythms in the hormones of the hypothalamic-pituitary-adrenal (HPA) axis (i.e. glucocorticoids), one of the body's major neuroendocrine axes, were already demonstrated several decades ago. Until now, the clinical relevance of the pulsatile nature of glucocorticoids was poorly understood or sometimes even regarded as not important. Its evolutionary conservation across many species however implies biological significance. Indeed, glucocorticoids have been proven to be crucial for a plethora of bodily functions, for example emotion, cognition and the central mechanism underlying the adaptation to stress. Furthermore, disturbances in the characteristic temporal pattern of glucocorticoid exposure have often been described in stress-related pathology. However, the significance of glucocorticoids secretory patterns for physiology, stress responsiveness and nuclear receptor signalling is still largely unexplored. As such, this thesis will discuss glucocorticoid pulsatile patterns and the implications for HPA axis activity and brain functioning.

Chapter 1

General introduction

Outline

1.1 Pulsatile patterns of glucocorticoid release
1.2 Glucocorticoid pulsatile patterns and stress responsiveness
1.3 Changes in glucocorticoid pulsatile patterns
1.4 Coordination of glucocorticoid pulsatile patterns
1.5 Glucocorticoid pulsatile patterns and corticosteroid receptors
1.6 Significance of glucocorticoid pulsatility
1.7 Scope of the thesis

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).

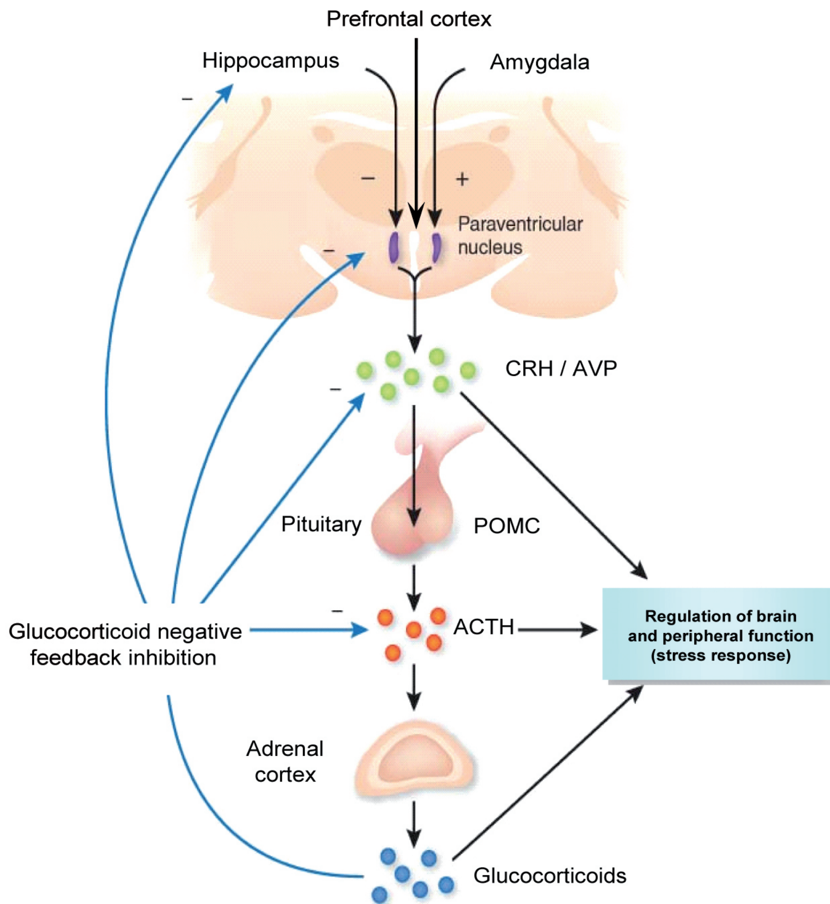

Figure 1 | Schematic representation of the different components of the HPA axis. Adapted and modified with permission from (Hyman 2009).

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.

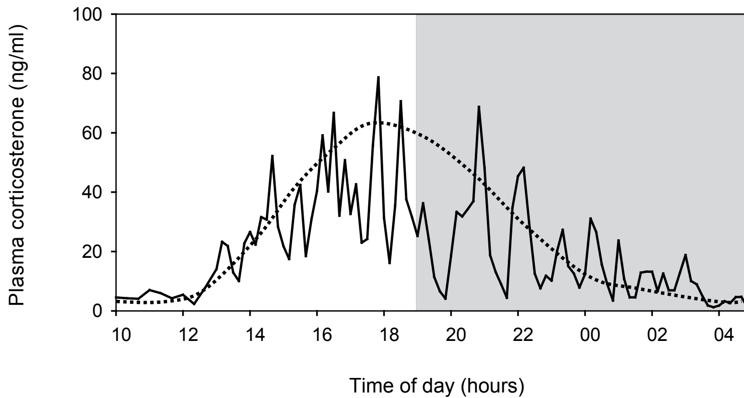

Figure 2 | Ultradian (smooth line) and circadian rhythm (dotted line) of corticosterone under basal conditions in a male Sprague-Dawley rat. Representative individual 19-h profile of corticosterone in blood plasma automatically collected every 10 minutes between 13.00 and 05.00 and every 20 minutes between 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.

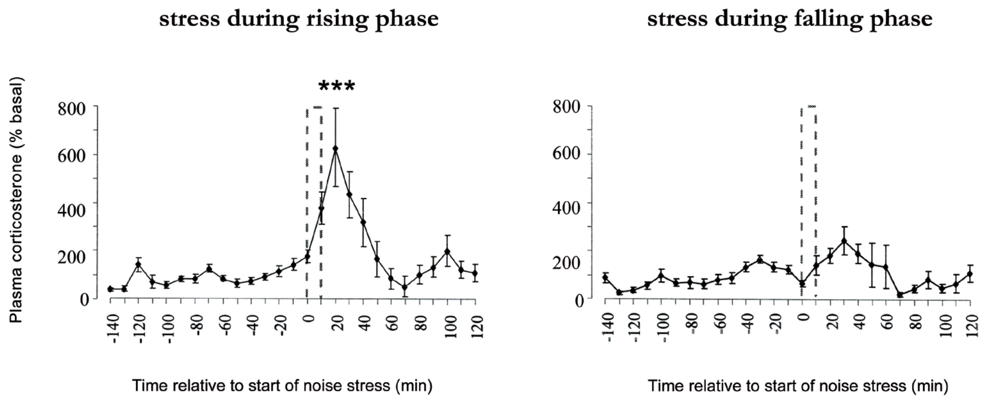

Figure 3 | The effect of noise stress (10 min of 114 dB, broken line) on plasma corticosterone concentrations in female Sprague Dawley rats when the stress coincided with a rising (left) or falling phase (right) of a basal corticosterone pulse. *** $p < 0.05$ vs falling group. Adapted and modified from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

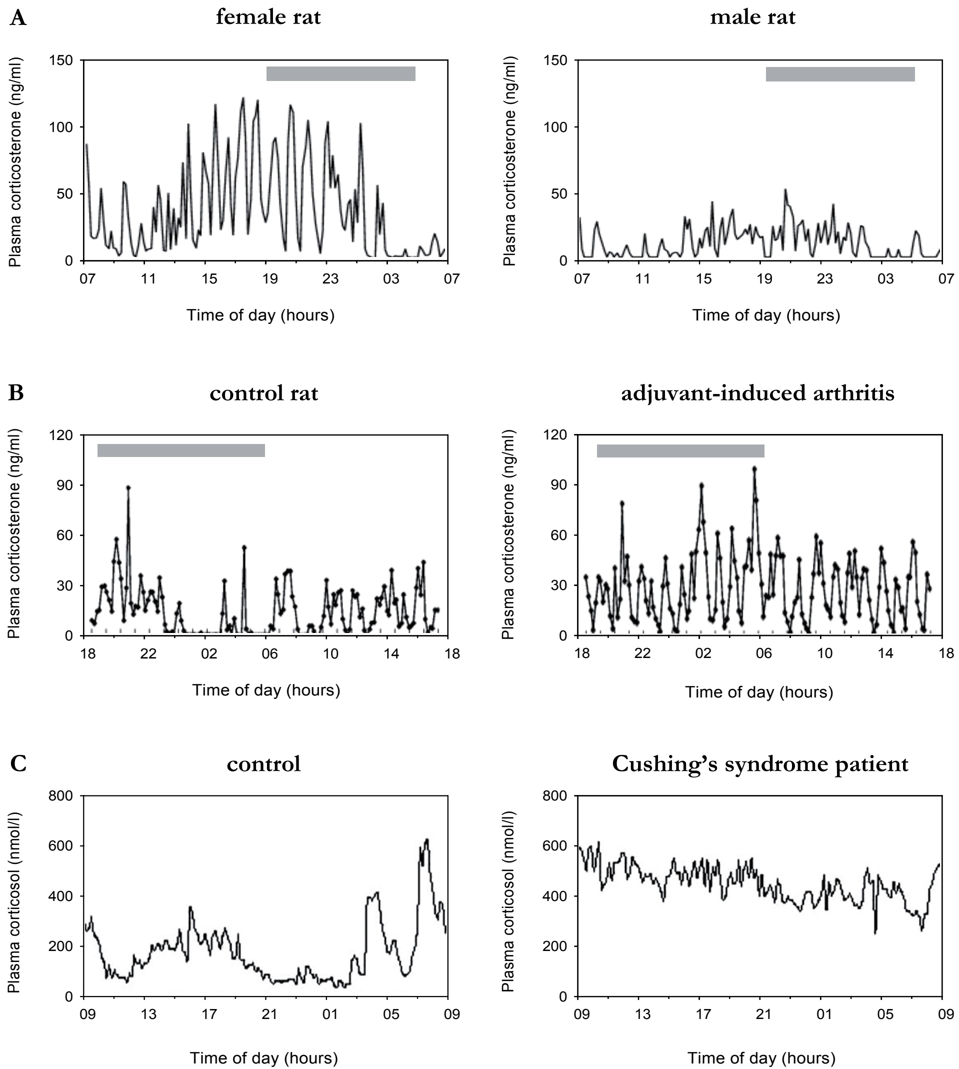

Figure 4 | Examples of differences in ultradian corticosteroid rhythms in A | female and male Wistar rats, B | adjuvant-induced arthritis after 13 days in rats compared to controls and C | unilateral adenoma in Cushing's syndrome. Data taken from (Windle et al. 2001, Seale et al. 2004a, van Aken et al.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

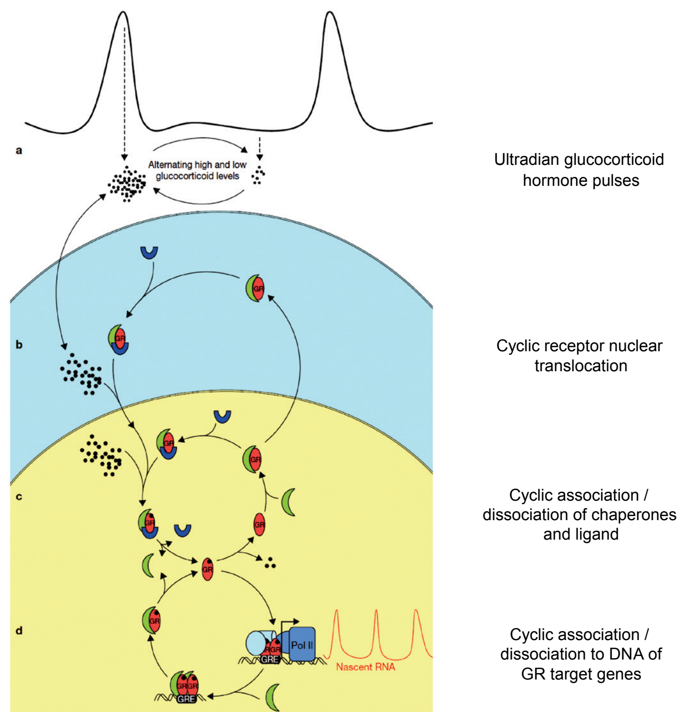

Figure 5 | Simplified coupling of ultradian hormone secretion to GR-driven transcriptional cycling. A | Ultradian hormone pulsing by the adrenal glands generates successive high and low levels of circulating glucocorticoids (black dots) which enter the cells. B | In the presence of hormone, the GR with 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**.

Chapter 2

Specificity of glucocorticoid receptor primary antibodies for analysis of receptor localisation patterns in cultured cells and rat hippocampus

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Brain Research, in press

Abstract

After glucocorticoid stimulation, glucocorticoid receptors (GRs) are translocated to the nucleus to modulate transcription of glucocorticoid target genes. The subcellular distribution and trafficking of GR in cultured cells has been studied quite intensively using several techniques. However, the intracellular localisation of nuclear receptors in ligand-free and stimulated conditions in vivo is still controversial, in part because of inconsistent results with different antibodies. Knowledge of trafficking of GR in vivo could greatly contribute to understanding nuclear receptor signalling.

Therefore, in this study we systematically compared a panel of different primary GR antibodies using immunohistochemistry and confocal imaging. Nuclear translocation patterns at different time points after glucocorticoid stimulation were compared in cultured AtT20 cells and rat hippocampal CA1 and dentate gyrus cells. The BuGR2 antibody consistently detected GR nuclear translocation patterns between in vivo and in vitro settings, but the other GR primary antibodies provided contradictory results. While GR H300 and P20 strongly detected nuclear GR immunoreactivity after glucocorticoid stimulation in both CA1 and dentate gyrus cells, the same antibodies provided poor results in cultured cells. The opposite was found for the primary GR M20 antibody.

These data indicate that with a particular glucocorticoid receptor antibody the findings in cell culture studies cannot always be extrapolated to in vivo situations. Moreover, different antibodies disclose different features of the glucocorticoid receptor translocation process.

Introduction

Glucocorticoids play a pivotal role in the homeostasis of many biological systems including the stress response, energy metabolism and the immune and inflammatory response (de Kloet et al. 2005). Their effects are mediated by two members of the nuclear receptor family, the mineralocorticoid and glucocorticoid receptor [MR and GR; (Reul & de Kloet 1985, Mangelsdorf et al. 1995)]. MR has a high affinity for naturally occurring glucocorticoids resulting in extensive receptor occupancy, whereas GR has a tenfold lower affinity for corticosterone and is therefore only activated during stress and the circadian peak (Reul & de Kloet 1985, Spencer et al. 1993, Kitchener et al. 2004, Furay et al. 2008).

The most common view on GR activation is that the unliganded receptor is part of a chaperone complex consisting of several proteins such as heat shock protein 90 and immunophilins which stabilise the receptor in the cytosol (DeFranco et al. 1998, Wochnik et al. 2005, Nishi & Kawata 2006, Picard 2006). Upon glucocorticoid binding, the ligand binding domain (LBD) of GR undergoes a major conformational change and dissociates from the chaperone proteins. Exposure of the nuclear localisation signal triggers the recruitment of the transport machinery (i.e. importins) which actively translocate the entire complex to the nucleus via a microtubule network (Harrell et al. 2004, Pratt et al. 2004, Fitzsimons et al. 2008). In the nucleus, GR functions as a ligand-activated transcription factor and mediates genomic events by modulating the transcription of glucocorticoid target genes via transactivation and transrepression (Datson et al. 2008, van der Laan & Meijer 2008).

The subcellular distribution and trafficking of GR in cultured cells has been studied quite intensively using several techniques such as green fluorescent protein (GFP)-tagged receptors, fluorescence after photobleaching (FRAP) and immunohistochemistry which have proven to be powerful tools for mechanistic assessment of nuclear receptor signalling (Htun et al. 1996, Nishi & Kawata 2006, Kawata et al. 2008, Stavreva et al. 2009). In such studies, MR and the progesterone receptor are found in both cellular compartments, while estrogen receptor-α and -β are thought to be localised predominantly in the nucleus (Fejes-Toth et al. 1998, Htun et al. 1999, Lim et al. 1999). On the other hand, GR and the androgen receptor are considered to reside in the cytoplasm (Htun et al. 1996, Georget et al. 1997), but also this has been disputed (Brink et al. 1992). However, there is much controversy about the subcellular distribution patterns of nuclear receptors in ligand-free conditions in vivo, as practical limitations still restrict the use of GFP-tagged receptors and FRAP in most animal models and thus favour the use of antibodies.

Knowledge of trafficking of GR in vivo could greatly contribute to understanding nuclear receptor signalling in the context of target tissues. During the past decades, several

GR-specific primary antibodies have become available for immunohistochemical studies. However, inconsistency in expression patterns among antibodies is not uncommon and has led to controversial results. Only rarely have different primary antibodies been tested in single experiments without any other variable parameters. These studies altogether indicate the need for more systematic comparison of different primary GR antibodies.

In this study we used immunohistochemistry and confocal imaging to screen a panel of different GR-specific primary antibodies in cultured cells and in rat hippocampus to compare nuclear translocation patterns. We found contradictory translocation pattern between the in vitro culture and the living hippocampus. The results indicate that findings from cell culture studies cannot always be extrapolated to in vivo situations and that the outcome of a study may depend on the choice of the antibody.

Experimental procedures

Cell culture

AtT-20/D-16V mouse tumour cells were grown and maintained in Dulbecco's modified Eagle's medium (4500 mg/L glucose; Invitrogen, USA) supplemented with 0.5% penicillin/streptomycin, 10% horse serum, and 10% fetal bovine serum (Invitrogen Life Technologies, The Netherlands) in a humidified atmosphere of 5% CO2 at 37 ºC as described previously (van der Laan et al. 2008a). A day before stimulation, 1 x 105 cells per chamber were plated and maintained in Lab-TekTM Permanox Chamber Slides (Nalge Nunc International, Belgium) in steroid-free medium. This medium is devoid of lipophilic components such as hormones and consists of 4500 mg/L glucose (Invitrogen, USA) supplemented with 0.5% penicillin/streptomycin and 1% stripped foetal bovine serum. The foetal bovine serum was stripped by incubating overnight at 4 ˚C with 2% of dextran-coated charcoal (Sigma-Aldrich, The Netherlands), spinning down for 5 min at 3000 rpm and subsequent filtering with autoclaved filter tops. The entire procedure was repeated after which the serum was aliquoted and stored at -20 ˚C until use.

Animals

Adult male Sprague-Dawley rats (± 250 grams) were purchased from Harlan (Leiden, The Netherlands) and group housed (4 animals/cage) in rooms on a 12h/12h light/dark cycle (lights on at 07:00 h). Food pellets and drinking water were available ad libitum. Experiments were approved by the Local Committee for Animal Health, Ethics and Research of the University of Leiden (DEC nr. 05080). Animal care was conducted in accordance with the EC Council Directive of November 1986 (86/609/EEC).

Surgery

To exclude interference from endogenously circulating corticosteroids, animals underwent bilateral adrenalectomy (ADX) performed under isoflurane anaesthesia. Animals were let to recover for three days while their drinking water was supplemented with 0.9% saline. In order to study GR translocation patterns in CA1 and dentate gyrus cells of the hippocampus, rats (n = 3-4) were injected intraperitoneally (i.p.) with 3 mg/kg corticosterone (CORT-HBC; Sigma-Aldrich, USA) as described previously (van Steensel et al. 1996, Conway-Campbell et al. 2007). Animals were sacrificed at different time points after injection, respectively 0, 30, 60, or 120 minutes. Blood plasma was collected to verify that the adrenal glands were completely removed and to monitor corticosterone levels in blood after injection (Fig. 4E). Samples were processed using a corticosterone radioimmunoassay kit according to the manufacturer's instructions (MP Biomedicals Inc., CA., USA).

Tissue processing

Transcardial perfusion-fixation was performed to process the brains for tissue sectioning and immunohistochemistry as described previously (Heine et al. 2004). Briefly, animals were deeply anaesthetised in the morning by i.p. injection of pentobarbital sodium salt (Nembutal 1 ml/kg bodyweight; A.U.V., Cuijk, The Netherlands) and then perfused intracardially with ice-cold 0.9% saline followed by 4% PFA in 0.1 M phosphate buffer, pH 7.4. Following perfusion the brains were removed, cryoprotected by complete saturation in a solution of 30% sucrose in 4% PFA, pH 7.4, and snap-frozen, Coronal section were cut at 30 μm using a Leica 1900 cryostat, and stored in an antifreeze solution (30% ethylene glycol, 20% glycerol, 0.02 M Na2HPO4, 6.6 mM NaH2PO4) at -20 ˚C until further use.

GR immunohistochemistry in cultured cells

To study receptor translocation patterns after glucocorticoid treatment, cells were stimulated with either vehicle or 10-7 M dexamethasone (Sigma-Aldrich, The Netherlands) known to induce significant GR translocation in cultured cells (Nishi et al. 1999, Fitzsimons et al. 2008). Respectively 0, 5, 15 and 30 min after stimulation, cells were processed for immunofluorescence to visualise subcellular distribution patterns of GR as described previously (Morsink et al. 2006). Briefly, cells were fixed with 4% paraformaldehyde (PFA) in 0.1M phosphate buffered saline (PBS), pH 7.4. After blocking in PBS supplemented with 5% normal goat serum and 0.3% TX-100, a panel of different GR-specific primary antibodies was used to detect GR immunoreactivity (IR). The subsequent primary antibodies were used: rabbit polyclonals raised against human GR, M-20 (aa 5-20), H300 (aa 121-420) and P20 (aa 750-769) from Santa Cruz Biotechnology Inc, Germany, or mouse monoclonal BuGR2 (aa 407-423 of rat GR) from Abcam, UK and rabbit polyclonal GR-Kawata (N-terminal domain of rat GR) kindly provided by prof. dr. M. Kawata (Kyoto, Japan). Antibodies were

diluted (1:500 or 1:2000 for GR-Kawata) in PBS containing 1 % BSA and 0.1 % TX-100 for 60 min at room temperature. As a control, the primary antibody was omitted or substituted with equal amounts of normal rabbit or mouse IgG. After washing, cells were incubated with AlexaFluor-488 labelled goat-anti-rabbit IgG or AlexaFluor-A488 goat anti-mouse IgG (1:1000, Molecular Probes, USA) in PBS containing 0.1 % TX-100 and 1 % BSA for 60 min at room temperature. Finally, cells were washed and nuclei were visualised with Hoechst 33258 (1:10000, Molecular Probes, USA). Slides were mounted with Aqua Polymount (Polysciences Inc, USA) and stored in the dark until further analysis.

GR immunohistochemistry in rat brain slices

To study changes in subcellular distribution pattern of GR-IR in the rat hippocampus as a consequence of glucocorticoid treatment, free-floating immunohistochemistry was performed on brain slices as described previously (Sarabdjitsingh et al. 2009). Briefly, non-specific binding was blocked by incubating the sections in 2% BSA, 0.3% TX-100 in 0.1M PBS. After rinsing, sections were incubated with different GR-specific primary antibodies as described above [P20, M20 and H300 (1: 500), Santa Cruz Biotechnology, Germany and BuGR2 (1:500) from Abcam, UK] in 0.3% TX-100 in PBS for 72 hours at 4 °C. Serial sections of the same animals were used for the different antibodies. At the time of the current experiment the GR antibody from Kawata was no longer available and therefore not included in this experiment. After washing, sections were incubated with AlexaFluor-488 labelled goat-anti-rabbit IgG or goat-anti-mouse IgG (1:1000; Molecular Probes, USA) in 0.3% TX-100, 2% BSA in PBS for 2 hours at room temperature. Finally, nuclei were visualised with Hoechst 33258 (1:10000, Molecular Probes, USA) after which sections were mounted with Aqua Polymount (Polysciences, Inc), and stored in the dark until further analysis.

Control sections were incubated with equal amounts of normal rabbit IgG (Santa Cruz Biotechnology, USA), which were used as substitute for the primary antibody. Additionally, sections were incubated without any primary antibodies to check for any aspecific binding of the secondary antibodies.

Confocal imaging and quantification

Subcellular distribution patterns of GR-IR in both cultured cells and brain slices was examined by a Leica Q550IW confocal microscope. Images were acquired at 630x magnification (155 x 155 µm, 1 µm focal plane). All settings for filters, lasers and images were left unchanged during imaging in order to relate changes in nuclear and cytoplasmic GR-IR due to experimental conditions and not due to microscope parameters. For the AtT20 cells, a group size of approximately 30 cells per time points was examined, taken from three individual wells. Per animal, two frames of the CA1 pyramidal cell layer or dentate gyrus were imaged with 30-40 cells on average.

Fluorescence intensity values of nuclear and cytoplasmic GR-IR were quantified by measuring optical density (ImageJ 1.32j analysis software; NIH, USA; http://rsb.info.nih.gov/ij/) similarly as described before (van der Laan et al. 2005, Morsink et al. 2006, Sarabdjitsingh et al. 2009). Briefly, Hoechst staining was used to identify the boundaries between the nuclear surface and cytoplasm of individual cells and was circled with the analysis software. These circles served as a template and were pasted onto the corresponding GR images to measure the optical density within and outside the nucleus. Background levels, indicated by sections and cells that were incubated with equal amounts of normal IgG, were also measured and subtracted from the total signal to obtain the specific signal.

Statistical analysis

Data are presented as mean ± SEM. Differences in mean optical density were examined by a one-way ANOVA. Tukey's post-hoc testing was applied to compare individual groups where applicable. Statistical significance was accepted at p-value < 0.05.

Results

Visualisation of GR nuclear translocation patterns in AtT20 cells

GR subcellular distribution patterns in AtT20 cells visualised with different GR-specific antibodies at different time points after dexamethasone (dex) treatment are illustrated in Fig. 1. Before stimulation (0 min), GR immunoreactivity (IR) was evenly distributed over the nuclear and cytoplasmic compartment with all five antibodies (Fig. 1 A1-E1). Stimulation with dex resulted in a significant accumulation in nuclear GR-IR observed with the BuGR2 (Fig. 1 A1-A4), M20 (Fig. 1 C1-C4) and GR-Kawata antibody (Fig. 1 E1-E4). This effect was readily observed 5 min after treatment (Fig. 1 A2, C2 and E2) while complete nuclear translocation was reached after 30 min (Fig. 1 A4, C4 and E4). In comparison, dex stimulation resulted in very moderate to no nuclear translocation when visualised with the H300 (Fig.1 B1-B4) and P20 antibody (Fig. 1 D1-D4).

Quantification of GR nuclear translocation patterns in AtT20 cells

Fluorescence intensity values were measured in both the nuclear and cytoplasmic compartment of cells stimulated with dex. Fig. 2 depicts the fold increase in those compartments as visualised with the different GR primary antibodies. Nuclear intensity increased approximately 3-fold after 30 min of dex stimulation observed with the BuGR2, M20 antibody and GR-Kawata (Fig. 2A, C, E, $p < 0.001$). A more modest increase was observed with the H300

(Fig. 2B, $p < 0.001$), while GR-IR with P20 remained unchanged after dex treatment (Fig. 2D, $p = 0.60$). A small decrease in cytoplasmic fluorescent intensity was observed with the M20 antibody, P20 and GR-Kawata (Fig. 2C-E, $p < 0.01$).

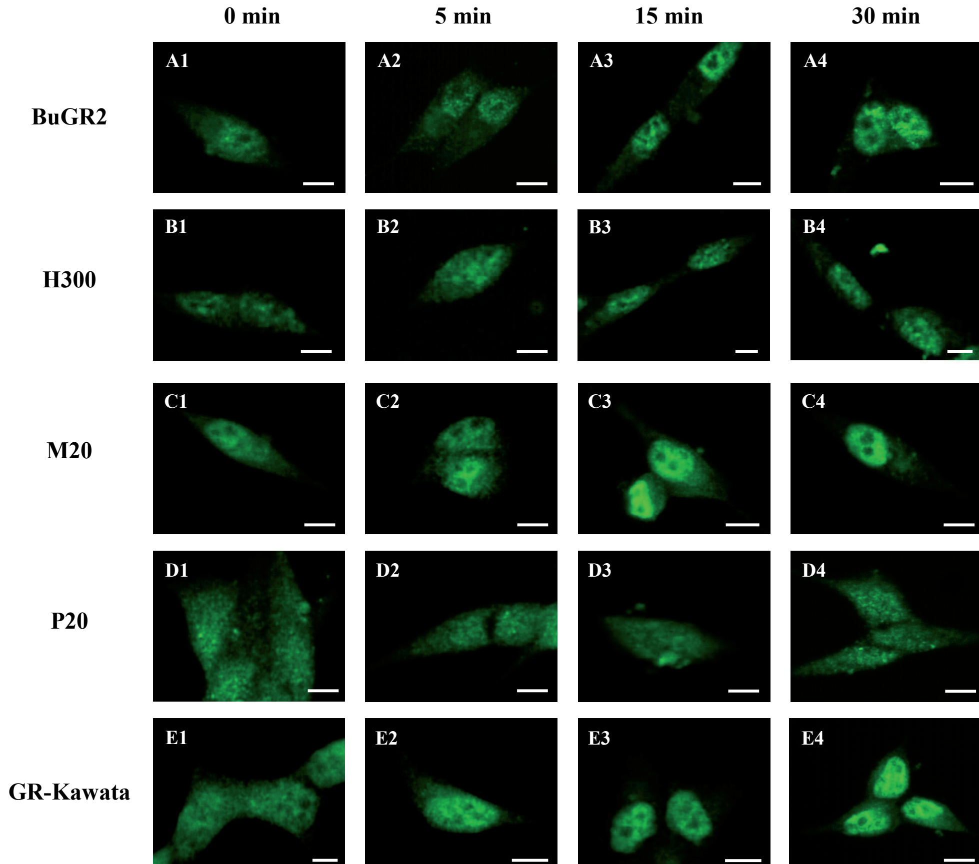


Figure 1 | GR nuclear translocation patterns in cultured Att20 cells. Time curve analysis of subcellular distribution of GR IR in AtT20 cells 0, 5, 15 and 30 min after 10-7 M dexamethasone stimulation using confocal imaging. A1-E1 | No differences in distribution pattern before stimulation (0 min) between the different antibodies M20, GR-Kawata, BuGR2, H300 and P20 were observed. Clear nuclear GR-IR was observed in cells visualised with the A1-A4 | BuGR2, C1-C4 | M20 and E1-E4 | GR-Kawata antibody already 5 min after stimulation, while moderate to no nuclear localisation was observed with the B1-B4 | H300 and D1-D4 | P20 antibody. Scale bar = 10 µm.

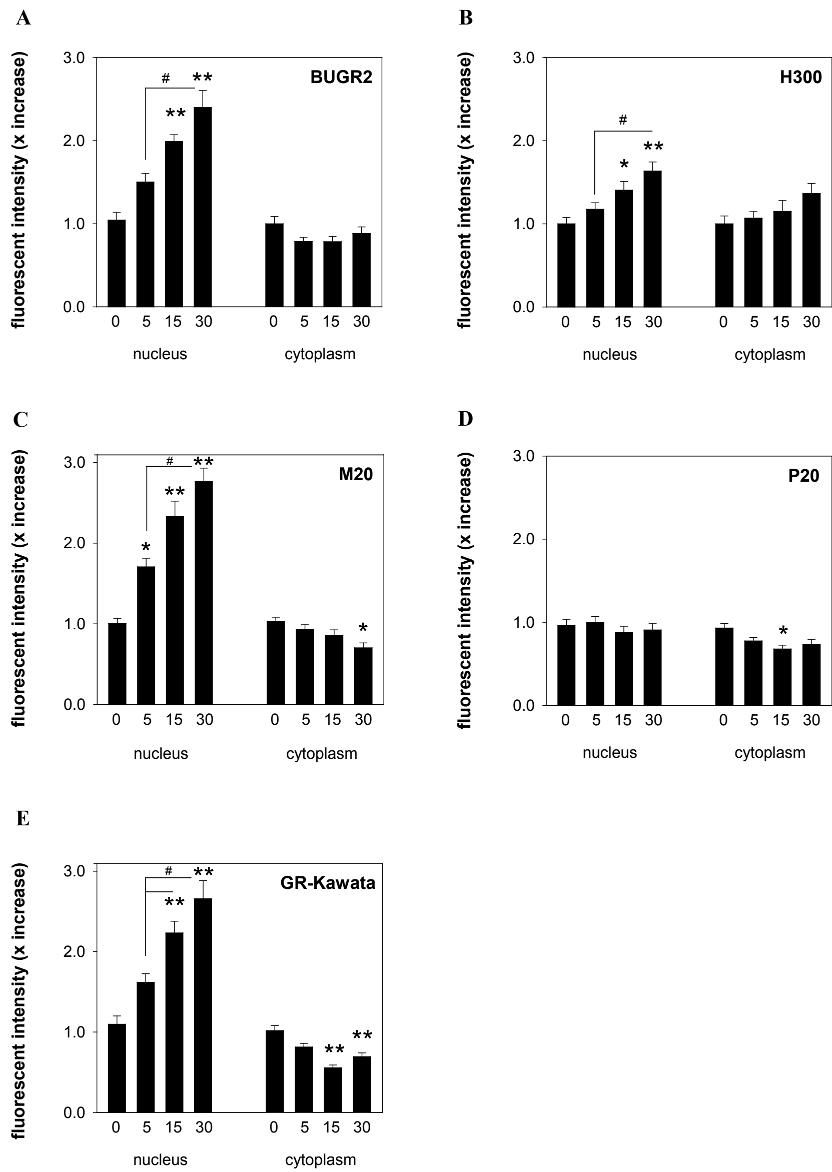


Figure 2 | Quantification of GR nuclear translocation patterns in cultured Att20 cells. Fluorescent intensity values of GR-IR in the nuclear and cytoplasmic compartment are shown at 0, 5, 15 and 30 min after 10-7 M dex stimulation visualised with different GR primary antibodies. Dex stimulation resulted in approximately 3-fold increase in fluorescent intensity in the nuclear compartment visualised with A | BuGR2 ($F(3, 108) = 18.20$, $p < 0.001$), C | M20 ($F(3, 99) = p < 0.001$) and E | GR-Kawata ($F(3, 105) = 22.01$, $p < 0.001$). Cells visualised with B | H300 ($F(3, 112) = 10.37$, $p < 0.001$) and D | P20 ($F(3,99)$, $p = 0.63$) showed modest to no increase in the nuclear compartment after stimulation. Interestingly, a small but significant decrease in cytoplasmic IR was observed with C | M20 ($F(3, 101) = 4.54$, $p < 0.01$), D | P20 ($F(3, 99) = 4.08$, $p < 0.01$ and E | GR-Kawata ($F(3, 120) = 16.05$, $p < 0.001$). Data is expressed as mean ± SEM. One-way ANOVA and Tukey's post-hoc test, ** $p < 0.001$ and * $p < 0.01$ vs veh, # $p < 0.05$ 5 vs 15 and 30 min, n = ± 30 cells per time point.

Visualisation of GR nuclear translocation patterns in rat hippocampal CA1 cells

Before stimulation (0 min), clear GR-IR was detected in the cytoplasmic compartment of CA1 cells with the primary GR BuGR2, H300, M20 and the P20 antibodies (Fig. 3 A1-D1). No nuclear GR IR was observed at this time point with any of the antibodies. After glucocorticoid stimulation strong accumulation of nuclear GR-IR was observed with the BuGR2 antibody, similar to the observations in cultured cells (Fig. 3 A1-A4). However with the other GR primary antibodies striking differences were observed in nuclear translocation patterns compared to cultured cells. While in AtT20 cells the M20 antibody nicely visualised nuclear localisation of GR, in CA1 cells the same antibody was not capable in recognising nuclear GR-IR molecules at any of the time points after injection (Fig. 3 C1-C4). Only an increase in cytoplasmic GR-IR was detected with this antibody. In contrast, while the H300 and P20 antibodies poorly detected nuclear GR in the AtT20 cells, in CA1 cells strong nuclear GR accumulation, especially with the H300 antibody, was observed already from 60 minutes after glucocorticoid treatment (Fig. 3 B1-B4 and D1-D4).

Quantification of GR nuclear translocation patterns in rat hippocampal CA1 cells

Fluorescence intensity values were measured in both the nuclear and cytoplasmic compartment of CA1 cells after corticosterone injection. Fig. 4 depicts the fold increase in those compartments as visualised with the different GR primary antibodies. In agreement with the confocal images, we observed increased GR-IR in the nuclear compartment after glucocorticoid treatment with some of the antibodies. Nuclear intensity increased (more than) approximately 4-fold 120 min after corticosterone injection observed with the BuGR2, H300 (Fig. 4A and B, $p < 0.001$) and P20 antibody (Fig. 4D, $p < 0.01$) while no increase was observed with the M20 antibody (Fig. 4C, $p = 0.42$). In contrast, GR-IR in the cytoplasmic compartment as visualised with the M20 antibody increased significantly to approximately 2-fold (Fig. 4C, $p < 0.01$).

Quantification of GR nuclear translocation patterns in rat dentate gyrus cells

To generalise the in vivo findings in CA1 cells, another hippocampal cell population, granule dentate gyrus cells was analysed (Fig. 5). For this purpose, we compared the H300 and M20 primary antibodies which provided distinctly different results in CA1 cells. In agreement with the results from CA1 cells, we observed increased GR-IR in the nuclear compartment after glucocorticoid treatment visualised with H300 (Fig. 5A, $p < 0.01$), but not with M20 (Fig. 5B, $p = 0.62$). Similarly, an approximately 2-fold increase was observed in cytoplasmic GR-IR intensity with the latter antibody (Fig. 5B, $p = 0.04$).

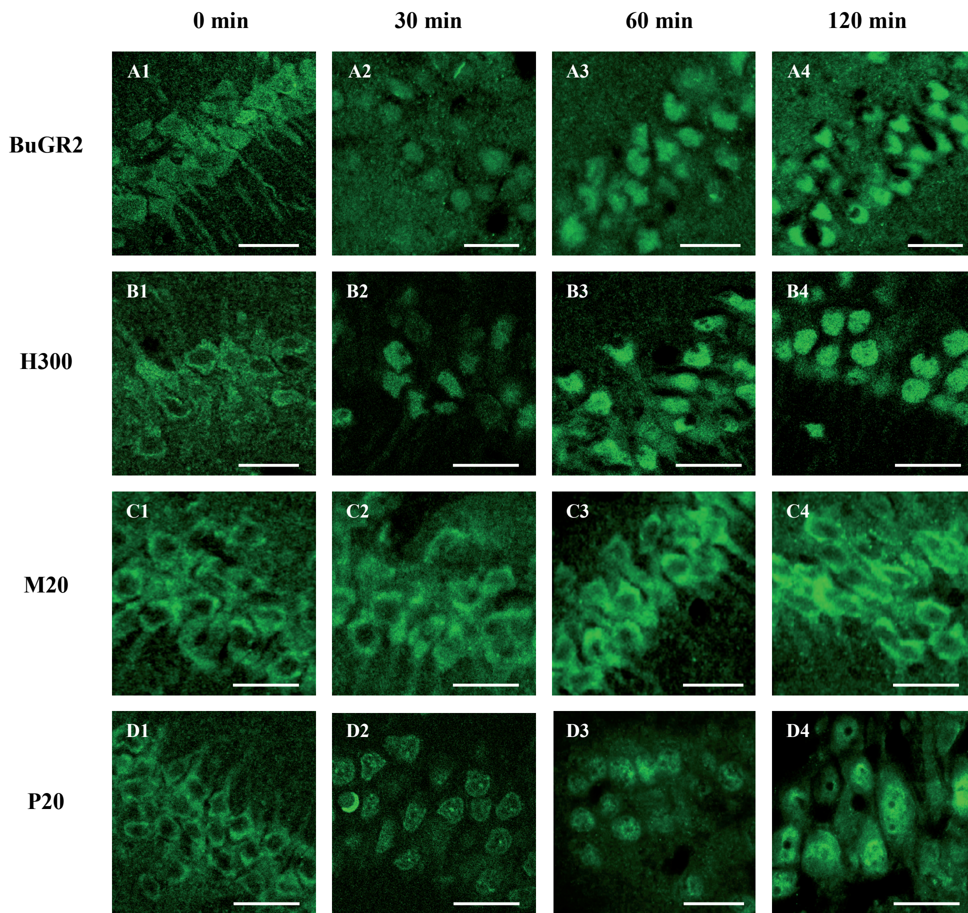
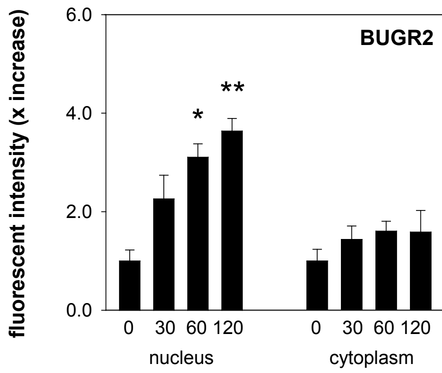


Figure 3 | GR nuclear translocation patterns in rat CA1 cells. Time curve analysis of subcellular distribution of GR-IR in CA1 cells of ADX rat hippocampus at 0, 30, 60 and 120 min after 3 mg/kg corticosterone injection. A1-D1 | Before stimulation (0 min), GR-IR visualised with all GR antibodies was mostly localised in the cytoplasmic compartment. In contrast to the AtT20 cells, no increase in nuclear GR-IR was visible after glucocorticoid stimulation with the A1-A4 | BuGR2 and C1-C4 | M20 antibody while the B1-B4 | H300 and D1-D4 | P20 antibodies clearly visualised nuclear GR distribution. Scale bar = 20 μm.

A



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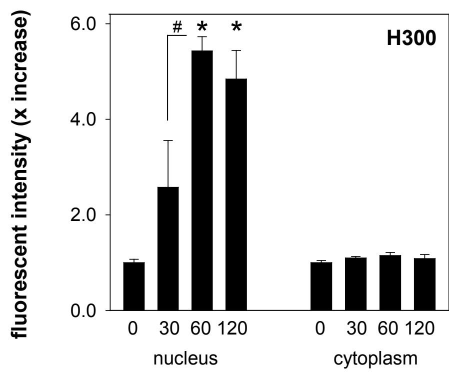

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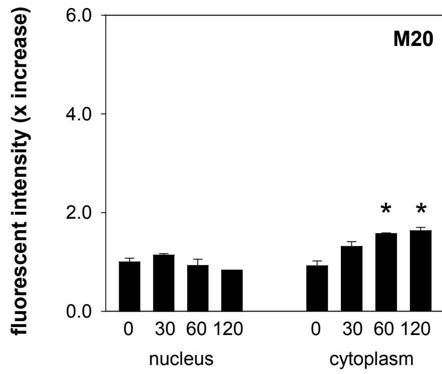

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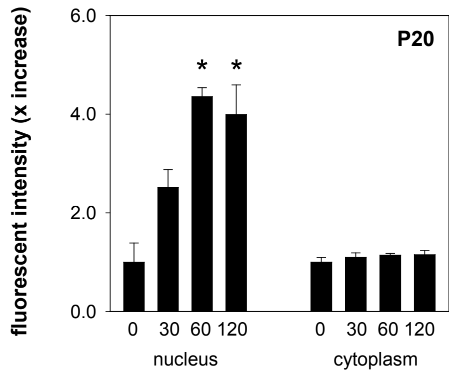

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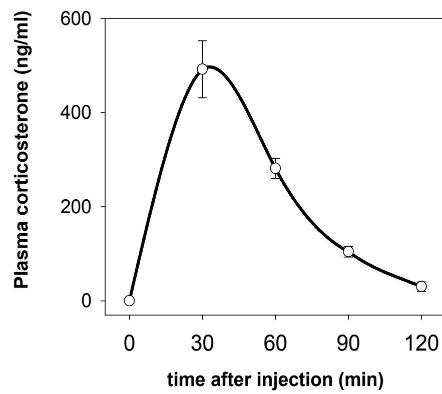

< Figure 4 | Quantification of GR nuclear translocation patterns in rat CA1 cells. Fluorescent intensity values of GR-IR in the nuclear and cytoplasmic compartment of ADX rat CA1 cells are shown at 0, 30, 60 and 120 min after 3 mg/kg ip corticosterone injection visualised with different GR primary antibodies. Corticosterone injection resulted in increased fluorescent intensity in the nuclear compartment visualized with A | BuGR2 ($F(3,10) = 14.77$, $p < 0.001$), B | H300 ($F(3, 9) = 14.13$, $p < 0.001$) and D | P20 ($F(3, 10) = p < 0.01$). Interestingly, cells visualised with C | M20 ($F(3, 8) = 1.06$, $p = 0.42$) showed no increase in the nuclear compartment after glucocorticoid stimulation. The opposite was found for the cytoplasmic compartment in which corticosterone injection resulted in increased GR-IR visualised with C | M20 ($F(3, 6) = 12.85$, $p < 0.01$ but not with A | BuGR2 ($F(3, 10) = 1.11$, $p = 0.39$), B | H300 ($F(3, 10) = 1.24$, $p = 0.34$) and D | P20 ($F(3, 11) = 0.78$, $p = 0.53$). E | plasma corticosterone levels were monitored before and at different time points after injection in ADX rats. Before injection (t = 0 min), corticosterone levels were indeed very low or non detectable. Data is expressed as mean ± SEM. One-way ANOVA and Tukey's post-hoc test, ** $p < 0.001$ and * $p < 0.01$ vs veh, # $p < 0.05$ 5 vs 15 and 30 min, n = 3-4 rats per time point.

Chapter 2

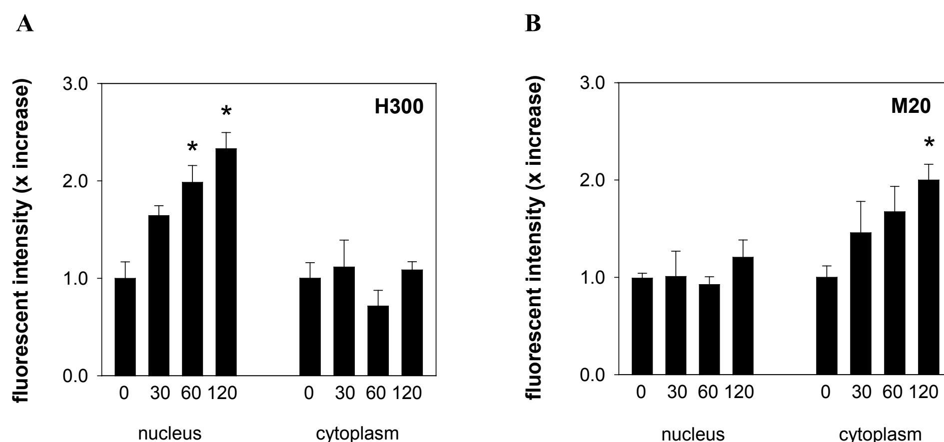

Figure 5 | Quantification of GR nuclear translocation patterns in rat dentate gyrus cells. Fluorescent intensity values of GR-IR in the nuclear and cytoplasmic compartment of ADX rat dentate gyrus granule cells are shown at 0, 30, 60 and 120 min after 3 mg/kg ip corticosterone injection visualised with different GR primary antibodies. Corticosterone injection resulted in increased fluorescent intensity in the nuclear compartment visualised with A | H300 ($F(3,10) = 5.88$, $p = 0.014$) but not with B | M20 ($F(3, 9) = 0.62$, $p < 0.62$). However, cytoplasmic GR-IR increased significantly after glucocorticoid stimulation ($F(3, 7) = 3.60$, $p = 0.04$). Data is expressed as mean ± SEM. One-way ANOVA and Tukey's post-hoc test, * $p < 0.01$ vs veh, n = 3-4 rats per time point.

Discussion

In this study we have investigated the differences between antibodies in determination of GR-IR subcellular distribution patterns after glucocorticoid stimulation in cultured cells and in hippocampus. To prevent confounding differential dose-dependent steroid effects on GR nuclear translocation (Nishi et al. 1999, Spiga & Lightman 2009), and to only study the consequences of the different GR-specific primary antibodies, we choose to expose both cultured cells and rats to very high concentrations of steroid thereby saturating most GR molecules. While some of the antibodies provided consistent cell specific results in GR nuclear translocation patterns between cultured cells and tissue, the data showed for others distinctly different results. The findings demonstrate that the outcome of ligand-induced GR nuclear translocation depends on the particular antibody and cannot always be extrapolated between experimental conditions.

We observed contradictory results in GR nuclear translocation patterns between cultured AtT20 cells and rat CA1 cells with the same primary antibodies (i.e. M20, H300, P20). This may reflect either cell type-specificity or procedural differences, and both may be related to in vitro versus in vivo conditions. While we have no in vivo data for pituitary cells, another cell population in the brain, the granule dentate gyrus cells, showed very similar results as were found in CA1. However, we did find two studies where hippocampal GR immunoreactivity was successfully detected using the M20 antibody (Sheng et al. 2003, Furay et al. 2008). Yet these studies used slightly different protocols optimised for DAB staining. There are also known biological differences in GR between cultured cells and actual tissue. For example, complete redistribution of nuclear GR over the cytoplasm after steroid withdrawal may last more than 24 hours (Hache et al. 1999), while in vivo GR is rapidly shuttled in and out of the nucleus on an hourly time-scale (Conway-Campbell et al. 2007, Sarabdjitsingh et al. 2010). We favour the interpretation that the pattern of nuclear interactions of GR differs in a systematic way between cell lines and tissues, depending on the conditions.

The various stages in the receptor life cycle may also provide differences in the availability of specific epitopes (i.e. steric hindrance due to conformation changes). The antibodies used in this study are all raised against the N-terminal domain of the GR, except the P20 antibody. This antibody is raised against the end of the C-terminus (aa 750-769) that harbours the ligand binding domain. In AtT20 cells, the latter antibody did not detect nuclear GR after glucocorticoid stimulation suggesting that this part of the C-terminus is hidden from the antibody. In rat CA1 cells, the N-terminal raised M20 antibody (aa 5-20) failed to detect changes in nuclear GR-IR. This suggests that in this condition, the immunogenicity of the N-terminal domain rather than the C-terminus is affected by ligand-induced conformational changes. Similarly, it is possible that the counter intuitive increase in cytoplasmic

immunoreactivity after glucocorticoid treatment, in an antibody-specific manner, is due to increased epitope accessibility after stimulation with ligand. Typically, this would not be observed with other antibody based techniques that use denaturing methods (e.g. Western Blot).

It is also possible that the observed differences between antibodies could be explained by the use of different ligands in the two conditions, dexamethasone and corticosterone. The ligand induced conformational change of the receptor is crucial for the distinction between agonists and antagonists, and may also differ to some extent for these two agonists. Functionally, differences between full agonists have been observed for intranuclear mobility, but not for translocation at saturating concentrations (Schaaf & Cidlowski 2003, Schaaf et al. 2005). This in itself does not exclude the agonist dependence of the immunogenicity of GR. Although not all GR antibodies were included then, we have previously used corticosterone in cultured cells and indeed have reported similar GR nuclear translocation with the M20 antibody as described here for dexamethasone (Morsink et al. 2006). Because changes in conformation induced by different agonists primarily take place in the LBD, they probably have little effect on the accessibility of antibodies raised against the N-terminal domain (e.g. H300, M20, BuGR2 and GR-Kawata). The consequences for C-terminally raised antibodies such as the P20, are however unknown, and we have to be aware that particular ligands may cause antibody-dependent outcomes in translocation studies.

Epitope accessibility is also determined by the local cellular context. Conformation changes of GR are partly determined by dissociation of chaperones upon ligand binding, binding for active transport to the nucleus or to other nuclear proteins and is therefore considered a key determinant in receptor function (Pratt et al. 2004, Meijer et al. 2006, Nishi & Kawata 2006, Picard 2006). Differential expression and regulation of molecules that make up GR complexes have not been systematically compared between cultured cells and tissue yet and could therefore contribute to the differences between experimental conditions.

Furthermore, the intracellular localisation of GR may also depend on the composition of the fixative. For instance, addition of gluteraldehyde minimises potential redistribution of the receptor during the fixation procedure (Brink et al. 1992). Taking these methodological aspects into account, some have described a relative absence of GR in the primate hippocampus (Sanchez et al. 2000), while others have shown prominent expression levels of GR in the human hippocampus (Wang 2009).

Although cross-linking fixation methods are regarded as effective means to preserve protein in tissue, it is possible that the different methods of fixative delivery in our study (i.e. direct incubation or transcardial perfusion fixation) result in different fixation strengths [albeit with the same fixative (4% paraformaldehyde, pH 7.4)], and contribute to differences in

epitope accessibility of the antibody. In that respect, antigen retrieval techniques such as heating by microwave or phosphatase pretreatment which aim to reverse deleterious effects of fixation (Shi et al. 1996, Pileri et al. 1997, MacIntyre 2001, Shi et al. 2001, Fritschy 2008), could be used to study the degree of epitope masking in settings like these. However, antigen retrieval techniques which allow renaturation of the structure of fixed protein have been proven beneficial only in particular circumstances and in some cases tend to increase aspecific binding (Lucassen et al. 1995). Though antigen retrieval methods are being widely addressed, more detailed studies with different types and durations of fixatives with ranging pH values on receptor distribution are suggested to elaborate on the consequences on receptor distribution.

In the current study we chose to investigate GR nuclear translocation patterns under steroid-free conditions (e.g. adrenalectomy and stripped serum media). Previously we have shown that GR, but also MR, nuclear translocation indeed depends on the physiological state of an animal but may also interfere with the basal conditions as low levels of both receptors may be retained in the nucleus before glucocorticoid treatment (Sarabdjitsingh et al. 2009, Sarabdjitsingh et al. 2010). However, our approach prevented interference of endogenously circulating glucocorticoids and differences in basal steroid conditions between the in vivo and in vitro situation.

Even though the recent generation of transgenic GR-GFP knock-in mice provide promising animal models for future research (Brewer et al. 2002, Usuku et al. 2005, Nishi et al. 2007), the current ongoing work still heavily depends on the use of primary antibodies.
Besides immunohistochemistry, other antibody-based techniques that study GR in its 'natural' folded conformation such as chromatin immunoprecipitation (as opposed to denaturation with i.e. Western Blotting) may very well deal with similar epitope specificities as describe here. Our data make the point that inconsistency in (subcellular) protein expression patterns and controversy in published results could be attributed to a combination of the type of antibody, epitope accessibility and fixation procedure / composition as well as the local cellular context.

In conclusion, we report that the choice of the primary antibody can have important implications for the interpretation of glucocorticoid action on the identification of receptor subcellular distribution patterns and cannot be extrapolated between different conditions. We propose the use of different GR primary antibodies for different purposes. For direct comparisons between cultured cells and tissue, the BuGR2 and H300 primary antibodies are recommended for visualisation of GR localisation. For comparisons within cultured cells, we recommend the use of BuGR2, M20 and/or GR-Kawata antibody, while we suggest that BuGR2, H300 and P20 are more suitable for brain tissue.

Acknowledgements
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Chapter 3

Subregion-specific differences in translocation patterns of mineralocorticoid and glucocorticoid receptors in rat hippocampus

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Brain Res. 2009 Jan 16; 1249:43-53

Abstract

Corticosteroids exert important effects on brain function via glucocorticoid (GRs) and mineralocorticoid receptors (MRs) by inducing receptor translocation to the nucleus, where the receptor-ligand complexes modulate transcription of target genes. Based on studies describing uneven receptor expression patterns, regionally different corticosterone action, and the importance of timing of corticosteroid effects, we hypothesised that differential patterns of MR and GR translocation exist in the rat hippocampus in response to a single glucocorticoid stimulus.

Temporal patterns of receptor translocation were investigated in both intact and adrenalectomised (ADX) male Sprague-Dawley rats. Animals were sacrificed at different time points after intraperitoneal administration of 3 mg/kg corticosterone. Specific MR and GR primary antibodies were used for immunohistochemistry and confocal microscopy to visualise and quantify receptor subcellular localisation in the different subregions of the hippocampus.

Results indicate (1) significant subregion-specific differences in translocation patterns for both MR and GR, with respect to the extent and timing of nuclear translocation and (2) specific differences between ADX and intact animals that are most prominent in the dentate gyrus: while corticosterone given to intact animals induced a marked nuclear increase in both MR and GR, in ADX animals nuclear MR signal was already elevated in the absence of hormone and did not change after steroid treatment.

We conclude that in response to a single stress-like increase in corticosterone distinct region-specific MR- and GR-dependent translocation patterns exist in the rat hippocampus, which may underlie region-specific effects relevant to homeostatic control.

Introduction

Adrenal corticosteroid hormones are important for the adequate onset and termination of the stress response, and as such, are essential factors in the maintenance of homeostasis. Their actions include numerous regulatory effects on brain function such as modulation of neurogenesis and memory formation (Herbert et al. 2006), behavioural and neuroendocrine responses (Oitzl et al. 1997), but also neuronal excitability and neurotransmitter release (Joels 2008). Therefore, excessive corticosteroid levels or insufficient adaptation to stress may impair homeostasis and brain function severely and are thus considered major risk factors for the onset of stress-related disorders like depression and post-traumatic stress disorder (de Kloet et al. 2005).

One essential issue concerning the functioning of the stress system is to understand how the effects of corticosteroids are differently mediated by the two types of corticosteroid receptors, the mineralocorticoid and glucocorticoid receptor [MR and GR; (Reul & de Kloet 1985)]. MR and GR are members of the nuclear receptor superfamily known to act as ligand-activated transcription factors (Mangelsdorf et al. 1995) and are widely present in brain. Both receptors are colocalised in high abundance in the hippocampus (Van Eekelen & De Kloet 1992, Han et al. 2005), a brain structure important for learning and memory and particularly susceptible to the effects of corticosteroids. Currently, it is widely accepted that in absence of the ligand, the receptors are part of a large protein heterocomplex containing a number of chaperone proteins (i.e. Hsp90 and p23), stabilising the complex in the cytoplasm. Activation of MR and GR by ligand binding results in dynamic conformational changes thereby rapidly remodelling the chaperone complex and exposing the nuclear localisation signal. As a consequence, the receptor-ligand complex is actively translocated to the nucleus using the microtubule cytoskeleton as a guiding scaffold. In the nucleus, it associates with glucocorticoid response elements (GREs) in the promoter regions of corticosteroid target genes in order to modulate their expression (Pratt et al. 2004, Nishi & Kawata 2006, Picard 2006). Importantly, corticosteroids differentially affect different neuronal cell populations underscoring the relevance of cell specific mechanisms of signalling (Joels 2006, Fitzsimons et al. 2008).

GRE occupancy by GR is a primary determinant of glucocorticoid responsiveness in cultured cells (So et al. 2007), and is also essential in spatial memory formation (Oitzl et al. 2001). Since the import and export of the receptor from the nucleus are obviously necessary steps in the signalling mechanism, receptor translocation could potentially be a more upstream rate limiting step in the transduction of the steroid signal to nerve cells in the brain. However, most knowledge concerning the dynamics of the corticosteroid receptors is based on cell culture models (Nishi & Kawata 2006) and the exact mechanism through which these

receptors act in brain is still unclear emphasising the need for more in vivo studies. For instance, upon hormone withdrawal in cell lines, GR reassociates rapidly into a cochaperone complex, but only slowly redistributes to the cytoplasm over periods that can extend over 12–24 h (Hache et al. 1999), while a recent study using rat hippocampal homogenate demonstrated more rapid GR translocation to and from the nucleus after administration of hormone pulses (Conway-Campbell et al. 2007).

Previous in vivo studies have focused more on the distribution of MR and GR in the rat hippocampus at limited time points (Ahima & Harlan 1990, Ahima et al. 1991, Cintra et al. 1994, van Steensel et al. 1996, Han et al. 2005), providing more time-restricted information. Others have used hippocampal homogenate to study receptor translocation (Kitchener et al. 2004, Conway-Campbell et al. 2007), thereby losing detailed information of receptor kinetics in different hippocampal subregions. The contribution of the different hippocampal subregions on specific processes like neuronal excitability (Joels 2006), but also on learning and memory formation (Kubik et al. 2007), has been described previously emphasising their individual importance. In line with this concept, it has been suggested that hippocampal cells may have unique transporting mechanisms for translocating corticosteroid receptors to the nucleus (Nishi & Kawata 2006). Indeed, differential expression of components of the chaperone complex in the hippocampus like cytoskeletal proteins (Arnold & Trojanowski 1996) and hsp90 (Patchev et al. 1994) may suggest region-specific translocation patterns of the receptors and consequently may explain region-specific regulatory effects of glucocorticoids.

Therefore, we assessed the dynamic response of MR and GR in different subregions of the rat hippocampus after corticosteroid treatment providing high resolution and detailed information with the use of immunohistochemistry and confocal imaging. We quantified MR and GR translocation patterns in intact and adrenalectomised rats after glucocorticoid treatment at different time points after injection and found that subcellular (changes in) localisation patterns differ between hippocampal regions.

Experimental procedures

Animals

Adult male Sprague-Dawley rats (mean weight ± 250 grams) were purchased from Harlan (Leiden, The Netherlands) and group housed in rooms on a 12h/12h light/dark cycle (lights on at 07:00 AM). Food pellets and drinking water were available ad libitum. Animals were handled daily for a week before the start of the experiment. Animals that underwent bilateral adrenalectomy, performed under isoflurane anaesthesia, were let to recover for

three days while their drinking water was supplemented with 0.9% saline. Experiments were approved by the Local Committee for Animal Health, Ethics and Research of the University of Leiden (DEC nr. 05080). Animal care was conducted in accordance with the EC Council Directive of November 1986 (86/609/EEC).

Experimental design

In order to study MR and GR translocation patterns in the rat hippocampus, both intact (n = 4 per group) and adrenalectomised rats (ADX; n = 3 per group) were injected intraperitoneally (i.p.) with 3 mg/kg corticosterone (CORT-HBC; Sigma, St. Louis, USA) and sacrificed at different time points thereafter. Previous studies have shown that this concentration is sufficient to induce hippocampal GR translocation to the nucleus as detected by Western blot (Kitchener et al. 2004, Conway-Campbell et al. 2007) and immunofluorescence (van Steensel et al. 1996). ADX animals were sacrificed respectively 0, 30, 60 and 120 min after injection while intact animals were sacrificed at 0, 60, 120 and 180 minutes. As a control, intact animals were injected with vehicle (0.9% NaCl) and sacrificed at 0 and 60 min after injection. Though recently it was demonstrated that there is no difference in nuclear AM and PM hippocampal protein levels, at least for GR (Furay et al. 2006), the study was performed in the morning between 08.00-12.00 AM. All animals were sacrificed before the circadian rise in corticosterone levels to prevent interference of endogenously circulating corticosterone levels. In all groups, plasma corticosterone concentrations were measured to verify that the adrenal glands were removed completely and to monitor corticosterone levels in blood at multiple time points after injection. This was done using a corticosterone radioimmunoassay kit (MP Biomedicals Inc., CA., USA) according to the manufacturer's instructions.

Tissue preparation and processing

Transcardial perfusion-fixation was performed to process the brains for tissue sectioning and immunohistochemistry as described previously (Heine et al. 2004). Briefly, animals were deeply anaesthetised in the morning by i.p. injection of pentobarbital sodium salt (Nembutal 1 ml/kg bodyweight; A.U.V., Cuijk, The Netherlands) and then perfused intracardially with ice-cold 0.9% saline, pH 7.4 followed by 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB), pH 7.4. Afterwards, brains were removed and cryoprotected by placing them in a solution of 30% sucrose in 4% PFA, pH 7.4 until complete saturation. Brains were snap-frozen and using a Leica 1900 cryostat sectioned coronally at 30 μm and stored in an antifreeze solution at -20 °C until further use.

Free-floating immunohistochemistry

To study changes in subcellular distribution pattern of MR and GR immunoreactivity (IR) in the rat hippocampus as a consequence of corticosterone administration, free-floating immunohistochemistry was performed on brain slices. First, sections were rinsed thoroughly with 0.1M PBS, pH 7.4. To block non-specific binding, slices were incubated with 0.1M PB, pH 7.4 containing 2% bovine serum albumine (BSA) and 0.3% TX-100 for two hours. After rinsing, sections were incubated with rabbit polyclonal GR (1:500, H300, Santa Cruz Biotechnology, USA) and mouse monoclonal MR antibodies [1:500, MR1-18 1D5; (Gomez-Sanchez et al. 2006)] diluted in PBS containing 0.3% TX-100 for 72 hours at 4 ˚C. After washing, sections were incubated with AlexaFluor-488 labelled goat-anti-rabbit IgG and AlexaFluor-A594 goat anti-mouse IgG (1:1000; Molecular Probes, USA) in PBS containing 0.3% TX-100 and 2% BSA for two hours. Finally, sections were washed and nuclei were visualised with Hoechst 33242 (Molecular Probes, USA) in PBS after which sections were mounted with Aqua Polymount (Polysciences, Inc) and stored in the dark until further analysis. Controls sections were incubated with equal amounts of normal rabbit and mouse IgG (Santa Cruz Biotechnology, USA) which were used as substitute for the primary antibody. Additionally, secondary fluorescent labels were swapped to check cross-reactivity and sections were incubated without any primary antibodies to check for any aspecific binding of the secondary antibodies.

Confocal microscopy and image analysis

To examine the subcellular distribution pattern of MR and GR IR in the different subfields of the rat hippocampus, a Leica Q550IW confocal microscope was used. Images (630x magnification, 155 x 155 μm, 1 μm focal plane) were acquired from the pyramidal cell fields CA1, CA2 and CA3 and dentate gyrus (DG). To relate changes in nuclear IR due to experimental conditions and not due to microscopical parameters, all settings for filters, lasers and images were left unchanged during imaging. Per animal, two frames per hippocampal subfield were imaged with 30-40 cells on average.

To assess the percentage of IR cells in each hippocampal subfield, MR and GR positive cells with a signal above background were included as indicated by aspecific antibodies (normal IgGs). Background levels were indicated by sections that were incubated with equal amounts of normal IgG. Per animal (ADX n = 12, intact n = 14), all cells from two confocal frames for each hippocampal subregion were counted. The multiple sections that were imaged per animal all had a different plane of focus. The amount of positive cells as percentage of the total amount of cells as indicated by the nuclear Hoechst stain is expressed as mean percentage (± SEM). No statistical difference was found between the percentages in ADX and intact animals.

To quantify differences in MR and GR subcellular distribution patterns, changes in fluorescence intensity values of nuclear immunoreactivity were measured using ImageJ 1.32j analysis software (NIH, USA; http://rsb.info.nih.gov/ij/) similarly as described previously (van der Laan et al. 2005, Morsink et al. 2006). Briefly, Hoechst staining was used to identify the boundaries between the nuclear surface and cytoplasm of individual cells and was circled with the analysis software. These circles served as a template and were pasted onto the corresponding MR and GR images to measure the optical density (mean grey levels) within the nucleus. Non-specific binding (normal mouse and rabbit IgG) and background staining of the sections were also measured and subtracted from the total signal to obtain the specific signal. Only cells that had a clear ovally-shaped nucleus with a diameter of approximately 5-7 μm and showed IR clearly above background were included for analysis thereby excluding cells that were not in the plane of focus.

Statistical analysis

Data are presented as mean ± SEM. Differences in mean optical density were examined by a one-way ANOVA. Tukey's post-hoc testing was applied to compare individual groups where applicable. Statistical significance was accepted at p-value < 0.05.

Results

Distribution of MR and GR immunoreactivity in rat hippocampus

Dual labelling immunohistochemistry and confocal imaging using specific MR and GR primary antibodies were used to study subcellular distribution patterns in the rat hippocampus. The specificity of these antibodies for MR and GR was shown earlier by others (Ko et al. 2002, Wochnik et al. 2005, Gomez-Sanchez et al. 2006), and was here again verified for immunohistochemistry by comparing the distribution pattern of the antibodies to previous studies. Results demonstrate a classic distribution pattern for both MR and GR expression in the rat hippocampus (Fig. 1A). As expected, high immunoreactivity (IR) for both receptor types was found in CA1, CA2 and DG while in CA3, very low levels of GR and high levels of MR IR were detected. On basis of these results, it was concluded that the antibodies were suitable for detection of MR and GR and therefore appropriate for detecting changes in subcellular distribution patterns in the rat hippocampus.

Before measuring translocation patterns of MR and GR in all subregions of the hippocampus, the fraction of cells that contributed to the observed effect was assessed. Therefore, we quantified the amount of MR and GR IR cells as a percentage of the total cell population

in each of the individual subregions labelled by the nuclear Hoechst staining (Fig. 1B). No significant differences were found between ADX and intact animals. On average, the fraction of cells that expressed MR and GR were approximately 95% in CA1, CA2 and DG. As expected, in CA3 the percentage of GR IR cells was much lower (44 ± 4 %; average ± SEM)) while MR was expressed in more cells (83 ± 2 %). The degree of colocalisation was also assessed in CA1 cells and in percentages were found to be 92.1 ± 2.4 and 91.9 ± 2.0 for GR+/MR+ and MR+/GR+ positive cells respectively (data not shown; average ± SEM). These number are highly similar to those reported in a recent study that thoroughly described the degree of colocalisation between MR and GR IR cells (Han et al. 2005).

Confocal imaging of MR & GR translocation patterns in hippocampal subregions

To study changes in subcellular localisation of MR and GR as a consequence of corticosteroid treatment, we injected rats with a high dose of corticosteroids (3 mg/kg i.p.) and sacrificed them at different time points thereafter. As a control, intact animals were injected with vehicle and sacrificed either at 0 or 60 min after injection. Confocal imaging and quantification of MR and GR IR in CA1 cells showed no change in nuclear intensity as a consequence of vehicle injection (Fig. 2A).

Initially, to avoid interference of endogenous corticosteroids, adrenalectomised animals were used as a model system to investigate MR and GR translocation as a direct consequence of corticosteroid treatment. In this first study, using ADX rats, predominant nuclear localisation of the signal was still observed 120 minutes after the injection. To also get information on the termination of the response to a single injection, in the second experiment with intact animals, we adjusted the time course by omitting an earlier time point (30 min) and included a later time point (180 min after injection). Corticosterone levels before and after injection were monitored in vehicle injected intact rats and corticosterone injected ADX and intact rats (Fig. 2B). As expected, no significant increase in corticosterone level was observed in vehicle injected intact rats. Before injection in ADX rats (0 min), no endogenously circulating corticosterone was detectable, while in intact animals basal levels were 36 ± 9 ng/ml. A bolus injection of 3 mg/kg corticosterone resulted in similar peak levels of 492 ± 61 ng/ml in ADX and 410 ± 56 ng/ml corticosterone 30 min after injection which then gradually returned to baseline.

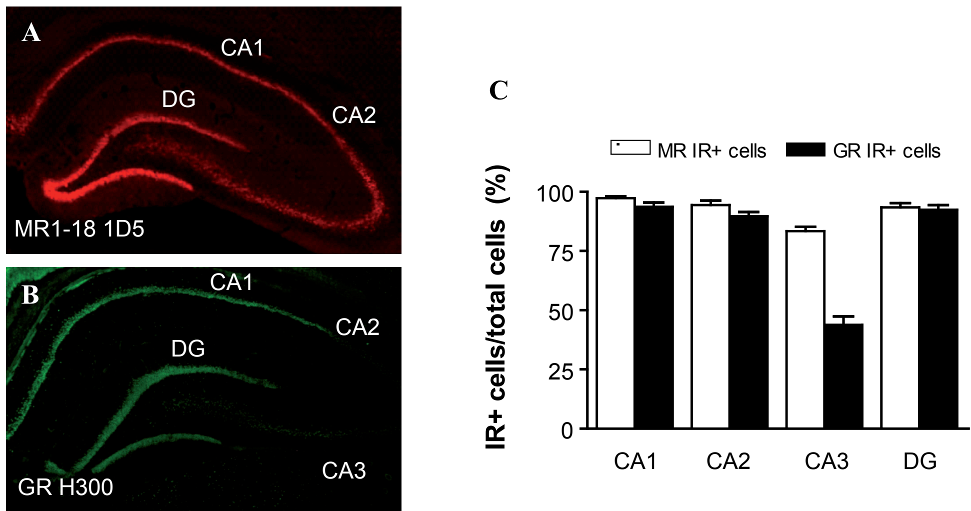

Figure 1 | Distribution and quantification of MR and GR immunoreactive (IR) cells in the rat hippocampus. A and B| Confocal imaging of MR and GR IR using specific MR (upper panel) and GR (lower panel) antibodies in the rat hippocampus demonstrate a classic staining pattern. Typically, MR is distributed in the cells of pyramidal cell layers CA1-3 and dentate gyrus. GR is also expressed in these hippocampal subfields but at very low levels in CA3 subfield. C | Quantification of the percentage of MR (open bars) and GR (filled bars) IR cells in different subfields of the rat hippocampus. IR cells that were clearly above background were included in the analysis. Background levels were indicated by expression levels of non-specific antibodies. In CA1, CA2 and CA3 almost all cells expressed MR and GR. In the CA3 field, GR is expressed at low levels in approximately 44 ± 4 % of the cell population. Bars indicate mean ± SEM (n=26).

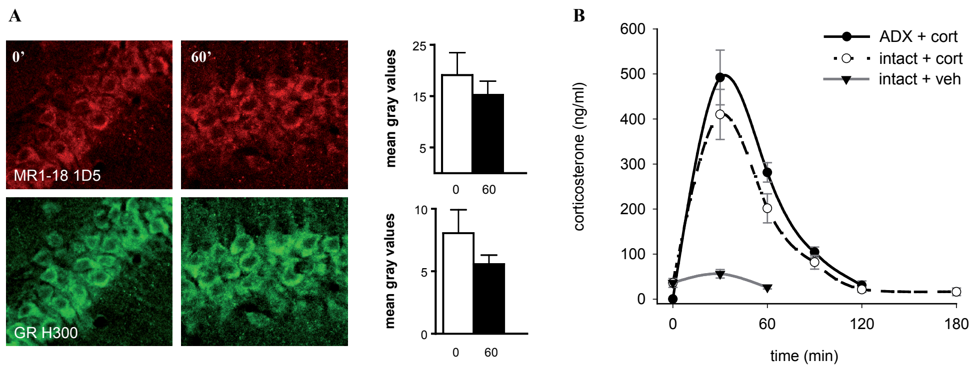

Figure 2 | Representative confocal imaging and quantification of MR and GR subcellular distribution in CA1 cells of vehicle injected intact animals and plasma corticosterone levels. A | As demonstrated by confocal imaging and quantification, no translocation of either MR nor GR is observed 60 min after vehicle injection in intact animals in CA1 cells. B | Plasma corticosterone levels in vehicle injected intact rats and corticosterone injected vehicle and ADX rats at multiple time points after an i.p. injection of 3 mg/kg corticosterone. Values represent mean ± SEM.

Confocal imaging of MR and GR IR at different time points after corticosteroid treatment in hippocampus of ADX rats demonstrated subregion-specific differences in MR and GR translocation patterns as illustrated for CA1 and DG in Figure 3. In CA1 (Fig. 3A), we observed rather uniform translocation patterns for MR and GR. For both receptors, IR was found to be localised predominantly in the cytoplasmic compartment at time point 0 min. Localisation relative to the nuclear compartment was verified using Hoechst as a nuclear marker. After corticosterone injection, increasing levels of nuclear IR for both receptors were observed with a maximum at 60 min after injection, demonstrating a classic translocation pattern from the cytoplasmic to the nuclear compartment. Surprisingly, in DG (Fig. 3B), and to a lesser extent in CA2 and CA3 (data not shown), a considerable amount of MR and GR IR was already present in the nucleus at time point 0 min, when no endogenously circulating hormone was present. After injection with corticosterone, an additional increase in nuclear MR and GR IR was observed which was more evident for GR than MR. Quantification of the translocation patterns of all the subregions is shown in Figure 4-7.

Quantification of MR and GR translocation patterns in hippocampal subregions

Changes in MR and GR subcellular distribution were quantified by measuring fluorescence intensity values in the nucleus of MR and GR positive cells in each of the hippocampal subfields of ADX and intact rats (Fig. 4-7). A significant increase in nuclear IR for both MR and GR was observed in CA1 in ADX rats with maximal levels at 60 minutes after corticosterone injection (Fig. 4A). In intact animals, a similar response was observed and at 180 min nuclear levels of MR and GR IR were back to basal again (Fig. 4B). A notable difference between intact and ADX rats in MR and GR translocation was found in the DG (Fig. 5). While the pattern of translocation of MR and GR in DG of intact rats highly resembled CA1 with increased levels at 60 and 120 min and basal levels again at 180 min after injection (Fig. 5B), the pattern in ADX rats was quite different (Fig. 5A). Already at 0 min, nuclear localisation of MR and GR was observed in the DG. Corticosterone injection increased nuclear levels of GR significantly 120 min after injection whilst surprisingly, no additional increase in nuclear MR IR was observed.

In CA2 (Fig. 6), similar translocation patterns for both intact and ADX rats were observed with increased nuclear levels for both MR and GR after 60 min. Though a slight drop in nuclear receptor levels was observed at 180 min, levels remained significantly higher than the control (0 min) for all the time points measured for GR but not for MR. In CA3 in both intact and ADX animals (Fig. 7), a slight but statistically not significant increase in nuclear GR levels was observed while MR nuclear signal did increase significantly after 60 min in ADX and 120 min in intact animals.

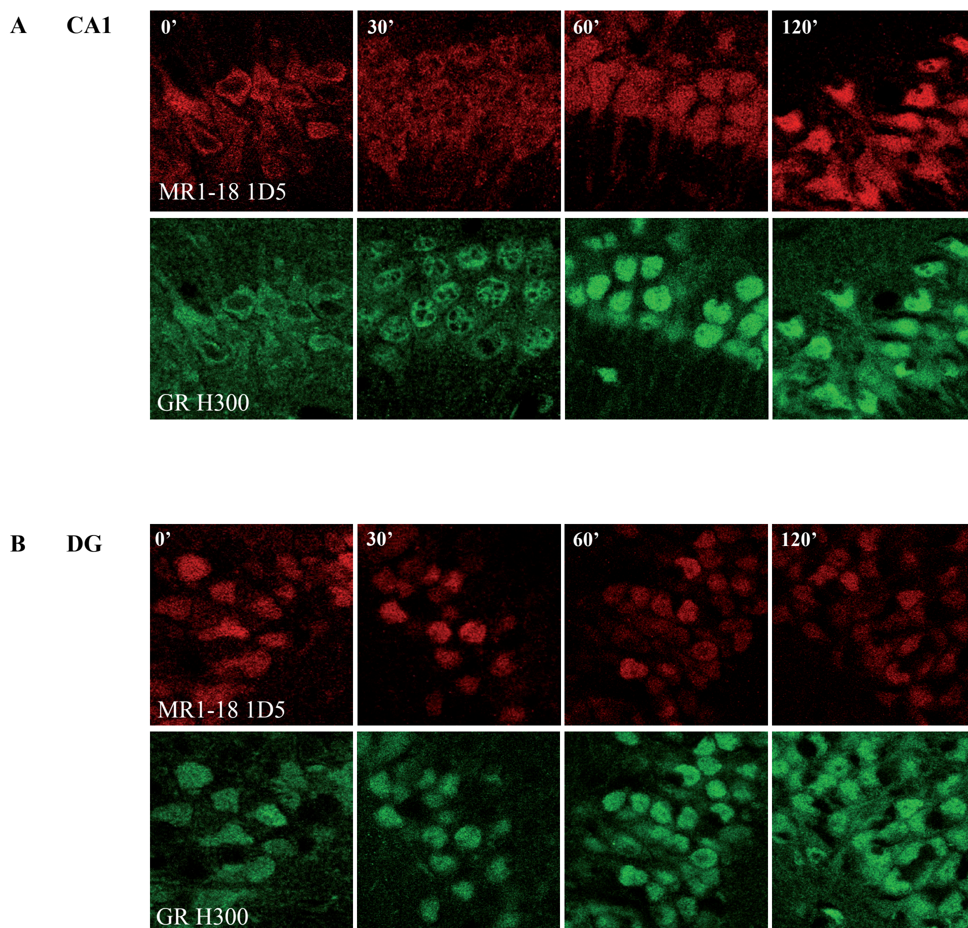

Figure 3 | Representative confocal imaging of MR (red) and GR (green) translocation patterns in hippocampus of ADX rats after corticosterone treatment. A | Classic translocation patterns for both MR and GR are observed in CA1. At t=0 min, MR and GR IR is localised mainly in the cytoplasm. After injection, MR and GR IR starts to accumulate in the nucleus and is completely nuclear at t =60 min. B | In the DG, nuclear IR of MR and GR is already present at t=0. After injection, an additional increase in nuclear IR for both receptors is observed, though to a lesser extent than in CA1.

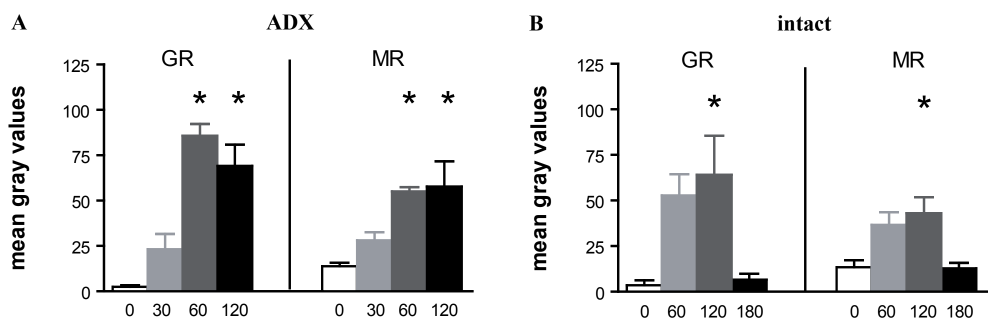

Figure 4 | Quantification of MR and GR nuclear translocation in CA1 of ADX and intact rats at different time points after corticosterone treatment. Nuclear localisation of MR and GR in CA1 at different time points after corticosterone injection is rather similar between A | ADX and B | intact animals for the time points measured. Bars represent corrected mean grey levels (mean ± SEM). Asterisks indicate significant differences at a p-value smaller than 0.05 compared to control (0 min).

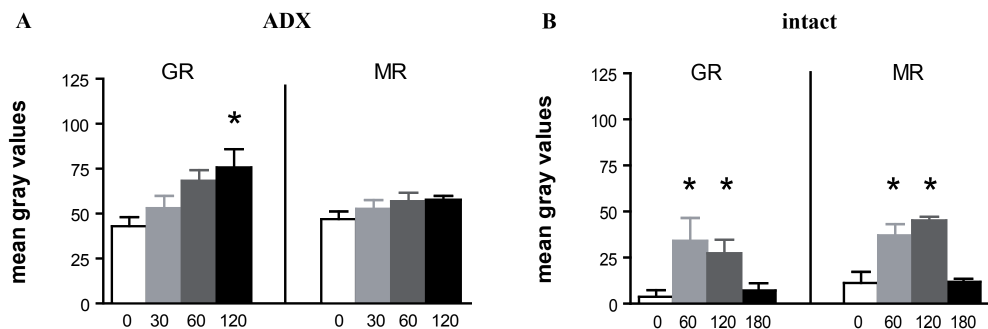

Figure 5 | Quantification of MR and GR nuclear translocation in DG of ADX and intact rats at different time points after corticosterone treatment. Nuclear translocation of MR and GR in B | intact animals resembles translocation patterns in CA1. A | ADX animals show higher nuclear localisation of MR and GR in controls (0 min) compared to intact animals but no significant increase in MR translocation is observed. Bars represent corrected mean grey levels (mean ± SEM). Asterisks indicate significant differences at a p-value smaller than 0.05 compared to control (0 min).

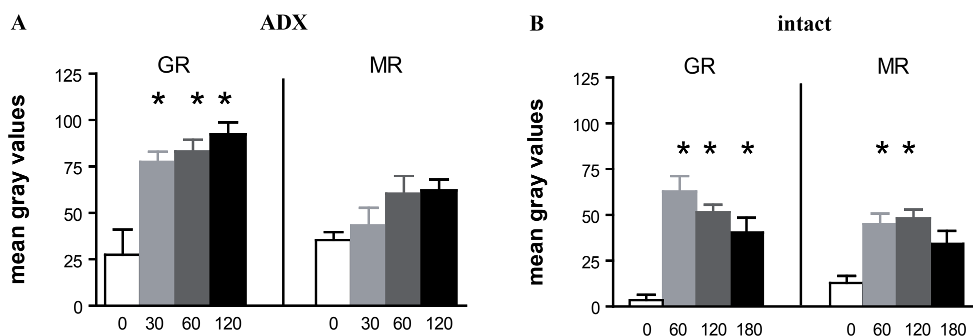

Figure 6 | Quantification of MR and GR nuclear translocation in CA2 of ADX and intact rats at different time points after corticosterone treatment. Nuclear localisation of MR and GR in CA2 cells demonstrate similar translocation patterns between A | ADX and B | intact animals at different time points after corticosterone injection. Bars represent corrected mean grey levels (mean ± SEM). Asterisks indicate significant differences at a p-value smaller than 0.05 compared to control (0 min).

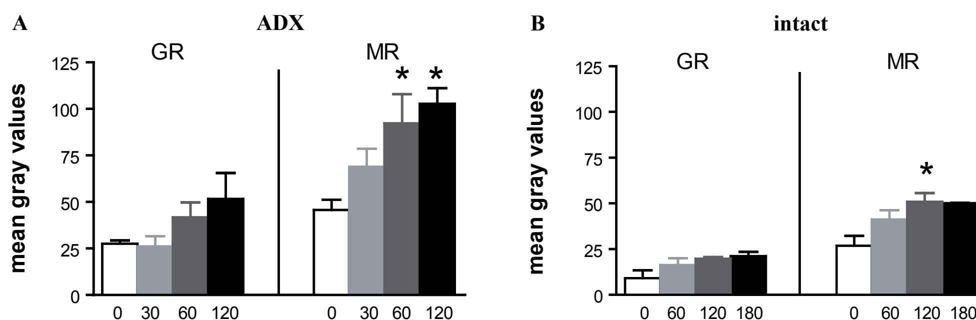

Figure 7 | Quantification of MR and GR nuclear translocation in CA3 of ADX and intact rats at different time points after corticosterone treatment. Though the extent of MR nuclear translocation is larger in A | ADX compared to B | intact animals, the patterns are quite similar. No significant increase in nuclear translocation of GR is observed in this area. Bars represent corrected mean grey levels (mean ± SEM). Asterisks indicate significant differences at a p-value smaller than 0.05 compared to control (0 min).

Discussion

Over the last decades, the distribution patterns of MR and GR in rodent brain have been investigated intensively (Ahima & Harlan 1990, Ahima et al. 1991, Van Eekelen & De Kloet 1992, Cintra et al. 1994, Han et al. 2005). While these studies have proven to be fundamental for the current knowledge of MR and GR localisation in the brain, they did not investigate the temporal aspects of MR and GR dynamics as a consequence of acute corticosteroid treatment in such detail. In this study we investigated MR and GR translocation at different time points after acute treatment using GR rabbit polyclonal antibody (H300; Santa Cruz Biotechnology) and MR mouse monoclonal antibody (1D5 1-18) both of which have been described and used in literature previously (Ko et al. 2002, Wochnik et al. 2005, Gomez-Sanchez et al. 2006, Conway-Campbell et al. 2007).

The expression patterns we obtained for MR and GR are in agreement with previously published papers as mentioned above. As expected, both antibodies demonstrated a typical distribution pattern with clear immunoreactivity in hippocampal subregions CA1, CA2, CA3 and DG while GR was expressed at very low expression levels in a subset of the CA3 cell population. Combined with information from previously published papers (Ko et al. 2002, Wochnik et al. 2005, Gomez-Sanchez et al. 2006), we therefore assumed that these antibodies indeed were highly capable in specifically recognising MR and GR protein in the rat hippocampus and thus can contribute to investigating tissue distribution and the detailed changes in subcellular localisation of MR and GR which can be detected by immunohistochemistry and confocal imaging.

Earlier studies reported on the effects of a single dose of corticosterone or aldosterone on MR and GR translocation in different regions of the hippocampus 5 min and 2 hours after injection in both ADX and intact rats (Ahima & Harlan 1991, Ahima et al. 1991). Using the high spatial resolution that fluorescent immunohistochemistry provides combined with the temporal aspects of the experimental design, the present study provides new and more detailed information simultaneously describing the translocation patterns of MR and GR in the different subfields of the rat hippocampus at multiple time points between 0 and 180 min in both ADX and intact animals. We show that differences exist in translocation speed and extent between various subfields after corticosteroid treatment with CA1 and DG as most interesting examples.

The phenomenon of hippocampal subregion-specific differences has been described previously in the literature, though not yet with respect to MR and GR dynamics. It is known that specific hippocampal subregions contribute uniquely to specific behavioural processes like learning and memory formation (Gilbert et al. 2001, Kubik et al. 2007). In relation to

corticosteroids, differential effects on different cell populations have been described (Joels 2006, Kavushansky et al. 2006), implying region-specific signalling mechanism. For instance, differences in corticosterone-induced effects on voltage-dependent calcium currents and the expression of calcium channel subunits between CA1 and DG have been observed (Joels et al. 2008b) but also subregion-specific effects on serotonin-1A receptor mRNA expression down regulation under control of MR and GR (Meijer & de Kloet 1994, Meijer & de Kloet 1995). Furthermore, in CA1, a U-shaped dose-dependency of corticosterone on certain responses like serotonin-1A receptor hyperpolarisation is observed but not in other regions like DG (Joels 2006). The differences in translocation and thus participation of both MR and GR between the respective hippocampal subregions may therefore contribute to this phenomenon.

The differential regulatory effects of glucocorticoids on the subregions of the hippocampus could therefore already start at the level of receptor translocation and thus be a necessary step in the proper transduction of the steroid signal in nerve cells, converging into higher functional levels.

An intriguing question left to be answered is what could be the underlying cause for these differences in translocation between hippocampal subregions and conditions. Still very little is known about region-specific factors that determine steroid receptor translocation rate and extent. Differential expression of genes in the hippocampus that are influencing nuclear translocation provide more insight. Unequal distribution patterns of microtubule-associated proteins [MAPs; (Arnold & Trojanowski 1996)] and chaperone proteins (Patchev et al. 1994) in the hippocampus have been described previously but large-scale expression profiling studies on laser-microdissected hippocampal subregions in combination with GeneChip technology revealed more detail in specific differences in cellular context between hippocampal subregions (Datson et al. 2004). Interestingly, Datson and colleagues found many genes to be differentially expressed between CA3 and DG that belong to the receptor transport complex including several MAPs, chaperone proteins like FKbp1a and parts of the dynein transport complex. Though the exact functional contribution of many proteins of the translocation mechanism have yet to be fully elucidated, research concentrating largely on GR dynamics have produced promising models in which MAPs are heavily involved in the transport of GR in a cell type-specific manner (Fitzsimons et al. 2008).

The local concentration of corticosterone, or hormonal bioavailability, is another factor that could contribute to the subregion-specific differences in translocation patterns, determining the exact amount of corticosterone that is sensed by the cells of the different subregions. One factor could be the energy-dependent efflux transporter P-glycoprotein (Pgp) mdr1b, capable of transporting corticosterone. Specific mdr1b mRNA expression was found in all divisions of the hippocampus although part of the signal seems to be present outside

the principal layers (Volk et al. 2004). Dentate granule cells express relatively high levels of mdr1b mRNA as detected by in situ hybridisation (Karssen et al. 2004) which could contribute to the lesser extent of nuclear translocation we observed in this area. Another factor is the local enzymatic activity of 11β-HSD1 that catalyses the accumulation of active corticosteroids, thereby controlling the concentration of corticosterone available for corticosteroid receptor binding in brain (Seckl 1997). However, studies on regional differences in the tissue distribution of 11β-HSD1 in the hippocampus have not been conclusive. Jamieson and colleagues described a more homogenous distribution with higher expression levels in CA3 (Jamieson et al. 1999), while a more recent paper described regional differences with high expression levels in CA1 and DG (Buren et al. 2007). To what extent 11β-HSD1 and Pgp mdr1b contribute to the subregional specificity in translocation patterns remains to be established.

Though we observed rather similar translocation patterns of MR and GR between intact and ADX rats, the DG provided an interesting exception. In DG, and to a lesser extent in CA2 and CA3, even before corticosteroid treatment, a substantial amount of MR and GR is located in the nucleus of ADX animals. The receptors therefore seem to be retained in the nucleus specifically in these regions after removal of the adrenals, or lack of glucocorticoids. Such retention of unliganded receptor has been noted before (Brink et al. 1992, Pekki et al. 1992), but remains unexplained. At least for MR it is known that the receptor can act promiscuous. For instance, not only corticosterone and aldosterone are naturally occurring ligands but cross-talk with other steroids, like progesterone, also occurs and could therefore influence MR localisation in absence of glucocorticoids (Rupprecht et al. 1993). These results raise the question whether corticosteroid receptors dynamics may also be regulated in a cell-specific manner by other ligands which in vivo only become evident after ADX and may also apply for GR. Additionally, nuclear localisation of MR and GR after ADX is also known to depend on the time after adrenalectomy (Ahima & Harlan 1991, Ahima et al. 1991). Subregional expression profiling of glucocorticoid effects has not yet been done and the possible changes in the local cellular context due to changes in glucocorticoid levels have yet to be described and could provide more insight in these DG-specific effects.

MR and GR differ in their affinity for several ligands. While GR is also selective for naturally occurring and synthetic glucocorticoids; MR binds corticosterone and aldosterone with a very high affinity, i.e. about 10-fold higher than GR (de Kloet et al. 2005). Given that endogenously circulating levels of glucocorticoids should be enough to extensively occupy MR (Reul & de Kloet 1985) and the lack of MR nuclear localisation in CA1 of basal adrenally intact animals is striking (Fig. 4B). Possibly, the differences in strain of rats, experimental design, fixation of the tissue and the choice of different methodological approaches (i.e. the use of antibodies) could explain these discrepancies with earlier studies. A partial explanation may involve the very low levels of corticosterone secretion in the morning observed

in Sprague-Dawley rats, which may preclude extensive MR occupation (Spiga et al. 2007). Based on the higher affinity of MR for corticosterone, one mightalso expect to see differences in translocation kinetics of MR and GR, e.g. a more rapid translocation of MR. However, we observed rather similar translocation rates of MR and GR to the nucleus, with the exception of the DG after ADX. This uniform behaviour is most likely explained by the dose of corticosterone that was used in this study. Peak levels of approximately 410-490 ng/ml (30 min after injection; Fig. 2B) saturate both MR and GR thereby masking the outcome of differences in receptor affinities for the extent of receptor translocation to the nucleus. The expected differences in translocation rate and onset may probably only be evident when lower, more physiological doses of corticosterone levels are administered. Additional information about the relationship between hormone plasma concentrations and receptor translocation could come from correlations in individual animals. Unfortunately, the statistical power of such correlations in the present experiment is very weak, and no meaningful relationships resulted from such an analysis (data not shown).

In summary, the results of this study suggest a revision of the view that MR and GR translocate uniformly in the rat hippocampus since we have demonstrated that they are transported in a subregion-specific manner. Even though these data indicate the need for further studies on the underlying mechanism of this differential translocation in the hippocampus, they could help to explain the cell type-specific effects of corticosteroids in the hippocampus. Also, the data show that in CA1, the translocation of MR to the nucleus occurs in response to a rise in ligand concentrations; hence the ligand concentration is rate limiting for at least nuclear localisation. In other subfields, the nuclear localisation in absence of ligand is not further increased when ligand concentrations rise, hence the receptor seems to be the rate limiting factor. Furthermore, our results illustrate that immunohistochemical studies can provide detailed information about the localisation of the receptors in vivo, which can assist in the evaluation of receptor activation during pharmacological treatments and physiological conditions.

Acknowledgements

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Chapter 4

Disrupted corticosterone pulsatile patterns attenuate responsiveness to glucocorticoid signalling in rat brain

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Abstract

Chronically elevated circulating glucocorticoid levels are thought to enhance vulnerability to psychopathology. Here we hypothesised that such sustained glucocorticoid levels, disturbing corticosterone pulsatility, attenuate glucocorticoid receptor signalling and target gene responsiveness to an acute challenge in the rat brain.

Rats were implanted with vehicle, 40% or 100% corticosterone pellets known to flatten ultradian and circadian rhythmicity while maintaining daily average levels or mimic pathological conditions. Additionally, recovery from constant exposure was studied in groups that had the pellet removed 24 hours prior to the challenge. Molecular markers for receptor responsiveness (receptor levels, nuclear translocation, promoter occupancy and target gene expression) to an acute challenge mimicking the stress response (3 mg/kg ip) were studied in the hippocampal area.

Implantation of 40% and 100% corticosterone pellets dose-dependently down-regulated GR and attenuated MR and GR translocation to the acute challenge. Interestingly, while target gene Gilz expression to the challenge was already attenuated by tonic daily average levels (40%), Sgk-1 was only affected after constant high corticosterone exposure (100%) indicating altered receptor responsiveness due to treatment. Washout of 100% cort recovered all molecular markers (partial) while removal of the 40% cort pellet still attenuated responsiveness to the challenge.

We propose that corticosteroid pulsatility is crucial in maintaining normal responsiveness to glucocorticoids. While the results with 100% corticosterone are likely attributed to receptor saturation, subtle changes in the pattern of exposure (40%) induces changes at least as severe for glucocorticoid signalling as overt hypercorticism suggesting an underlying mechanism sensitive to the pattern of hormone exposure.

Introduction

Rhythmic secretion of glucocorticoids is one of the major characteristics of the hypothalamic-pituitary-adrenal (HPA) axis, which plays a pivotal role in maintenance of homeostasis (de Kloet et al. 2005, Herbert et al. 2006). This rhythmicity is based on approximately hourly ultradian bursts of corticosterone into the blood stream showing circadian variation in amplitude (Tapp et al. 1984, Jasper & Engeland 1991, Windle et al. 1998a) and also exists at the level of target tissues such as the brain (Droste et al. 2008). Pulse amplitude and frequency can be remarkably variable in different physiological states, (i.e. lactation and ageing (Lightman et al. 2000)). However, dysregulation of ultradian rhythmicity is also apparent in stress-related disease (Deuschle et al. 1997, Hartmann et al. 1997), though its precise function still remains unknown (Young et al. 2004, de Kloet & Sarabdjitsingh 2008, Lightman et al. 2008).

The actions of glucocorticoids are mediated by the high affinity mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR); the latter receptor has a ten-fold lower affinity for corticosterone (Reul & de Kloet 1985). Both nuclear receptors are abundantly expressed in the brain, especially in limbic areas such as the hippocampus (Herman 1993, De Kloet et al. 1998). MR and GR act as ligand-activated transcription factors that translocate to the nucleus upon ligand binding. In the nucleus the receptors modulate target gene expression via interactions with specific glucocorticoid responsive elements (GREs) in the regulatory region of glucocorticoid target genes or via protein-protein interactions with other transcription factors (Mangelsdorf et al. 1995, Datson et al. 2008). Nuclear translocation of GR is known to vary according to circadian fluctuations in corticosterone levels (Kitchener et al. 2004). Moreover, repeated rapid nuclear translocation of GR following intravenous corticosterone injections that mimic ultradian pulses was demonstrated in rats (Stavreva et al. 2009) while MR was continuously retained in the nucleus (Conway-Campbell et al. 2007), implying that disturbances in glucocorticoid pulsatility would mainly affect GR.

It is now being recognised that access and binding of steroid receptors to regulatory elements in the genome (Metivier et al. 2003), including GR, is highly dynamic (George et al. 2009, Stavreva et al. 2009). The individual steps of the receptor signalling cascade (i.e. nuclear translocation, chromatin binding and modulation of target gene expression) together determine cellular and/or tissue sensitivity to glucocorticoids and efficacy (Kino 2007). Still, little information is available on how rapidly fluctuating glucocorticoid levels in vivo affect receptor signalling, transcriptional output and hence target tissue sensitivity thereby determining the functional effects of glucocorticoid action. Changes in pattern of glucocorticoid exposure could possibly contribute to dysregulated glucocorticoid receptor signalling. Understanding the interplay between rapidly fluctuating glucocorticoid levels and

receptor signalling therefore could greatly contribute to our knowledge of cellular and tissue responses.

In the present study, we aimed to investigate the link between glucocorticoid pulsatility and target tissue sensitivity in response to an acute increase in glucocorticoids such as occurs during acute stress. Evidence from other hormonal systems suggests that the function of rapid ligand bursts is to maintain tissue sensitivity and receptor responsiveness (Hauffa 2001). Therefore we hypothesised that constant levels of glucocorticoids, thus loss of pulsatility, will similarly affect glucocorticoid signalling by changing the responsiveness of the glucocorticoid receptor and its proximal targets.

Glucocorticoid pulsatility was abolished by subcutaneous corticosterone pellet implantation thereby exogenously inducing constant levels of glucocorticoids (Akana et al. 1985, Akana et al. 1992, Meijer et al. 1997). Consequently, we studied molecular markers for glucocorticoid signalling in the rat hippocampal area in response to an acute corticosterone challenge, superimposed on the chronically changed pattern of exposure. We also investigated whether these changes can be normalised after reinstatement of glucocorticoid pulsatility. Here we find that the transient response in glucocorticoid signalling is attenuated during exposure to constant corticosterone levels, but that this apparent resistance is reversible.

Experimental procedures

Animals

Male Sprague-Dawley rats (Harlan, Leiden, The Netherlands) weighing approximately 250 grams on the day of surgery, were group housed on a 12 h/12 h light/dark cycle (lights on at 07:00 AM) in a temperature controlled facility. Animals were handled daily for a week before the start of the experiment. Food and water were provided ad libitum. All experimental manipulations were done in the morning. Experiments were approved by the Local Committee for Animal Health, Ethics and Research of the University of Leiden (DEC nr. 07166). Animal care was conducted in accordance with the EC Council Directive of November 1986 (86/609/EEC).

Experimental design

Hormone levels were clamped at different constant levels using 40% and 100% corticosterone (cort) pellets (40 or 100 mg corticosterone; MP Biomedicals, Ohio, USA), and compared to 100 mg vehicle (cholesterol, Sigma-Aldrich, St. Louis, USA). Using isoflurane anaesthesia, pellets were subcutaneously implanted in intact animals randomly assigned to either

of the five treatment groups (n = 24): vehicle, 40%, 100%, 40% WO (washout) and 100% WO cort. Animals were weighed daily during the seven days after pellet implantation. On experimental day 7, blood samples were collected via the tail vein in the morning (08.00) and afternoon (17.00) to verify flattening of circadian cort levels in plasma without anaesthesia (Fluttert et al. 2000). Pilot studies indicated initial enhanced release of corticosterone from the pellet on the first two days after implantation and steady-state levels thereafter (Meijer et al. 1997). As described in the results section, corticosterone levels were indeed constant the day before the corticosterone challenge. In addition, high frequency blood sampling was used to verify flattened corticosterone rhythms with higher resolution [10 min intervals; (RA Sarabdjitsingh 2009)].

Early in the morning on post-surgery day 8 (08.00-09.00), animals from the WO groups were briefly anaesthetised during which the pellet was rapidly removed (washout) while the animals from the other groups were sham-operated. To ascertain normalisation in responsiveness of most of the available GR molecules, the washout period was decided to be 24 hours, according the half life time of GR redistribution to the cytoplasm following hormone withdrawal [t1/2 = 8-9 hours (Hache et al. 1999)]. After 24 hours, animals from all five groups were challenged with a high dose of corticosterone (3 mg/kg ip corticosterone-HBC; Sigma-Aldrich, USA). Tail blood samples were taken before and during the challenge to monitor corticosterone levels in blood. Animals were decapitated (n = 8 per time point per treatment group) 0, 60 and 180 min after injection. Brain tissue was collected, snap-frozen in isopentane on dry ice and stored at -80 ºC until further processing. Of each animal, one hemisphere of the brain was used for immunohistochemistry and in situ hybridisation while the hippocampus of the other hemisphere was isolated for Western blot analysis or chromatin immunoprecipitation (ChIP). Additionally, the thymus and adrenal glands were dissected, cleaned and weighed.

Corticosterone measurements

Blood samples were centrifuged for 15 min at 4000 rpm at 4 ˚C. Plasma was stored at -80 ˚C until processed using a commercially available radio immuno assay (RIA; MP Biomedicals Inc., CA., USA) according to the manufacturer's instructions.

Protein sample preparation

Hippocampal total protein samples (n = 12) were obtained by homogenising in ice-cold lysis buffer containing 1% NP-40, 1% sodium deoxycholate, 0,1% SDS, 0.15 M NaCl, 50mM Tris, pH 7.5 and protease inhibitors (Complete Mini cocktail tablets, Roche, The Netherlands). Protein concentrations were determined by a BCA protein assay (Pierce, Rockford, IL, USA).

Western blotting and analysis

Western blot was performed as described before (Conway-Campbell et al. 2007). Twenty μg protein samples were loaded on 5-8% polyacrylamide gels and transferred to PVDF membranes (Amersham Biosciences, Uppsala, Sweden). Membranes were probed with either primary anti-GR antibody (M20, Santa Cruz Biotechnology) or anti-MR antibody [MR1-18 1D5, kindly supplied by Dr. Gomez-Sanchez (Gomez-Sanchez et al. 2006)] and subsequently with anti-rabbit IgG-HRP (Santa Cruz Biotechnology) or anti-mouse IgG-HRP (Amersham Biosciences). Signal was detected using enhanced chemiluminescence reagent (ECLplus, Amersham Biosciences) and enhanced chemoluminescence hyperfilm (Amersham Biosciences). Membranes were also probed for α-tubulin as a loading control (Sigma Aldrich, USA).

Western blot bands were quantified by densitometry using an Epson perfection scanner (Epson Europe, NL) and ImageJ analysis software (NIH, USA; http://rsb.info.nih.gov/ij/). Optical density of GR and MR bands were corrected for film background and normalised for α-tubulin. Samples were compared within an individual blot, calculating fold induction from the control group loaded on each blot (vehicle t = 0). Fold inductions from all data sets were pooled to obtain group means ± SEM.

Immunohistochemistry

To visualise the subcellular distribution pattern of MR and GR immunoreactivity (IR) in hippocampal CA1, immunohistochemistry was performed on 20 μm slices as described previously (Sarabdjitsingh et al. 2009). Sections were incubated with primary GR (H300 Santa Cruz Biotechnology, USA) and MR antibodies (MR1-18 1D5). After washing, sections were incubated with secondary antibodies AlexaFluor- A488 and AlexaFluor-A594 IgG (Molecular Probes, USA). Nuclei were visualised with Hoechst 33258 (Molecular Probes, USA) and slides were mounted with Aqua Polymount (Polysciences, Inc). Controls included incubation of slides with equal amounts of normal rabbit and mouse IgG (Santa Cruz Biotechnology, USA), swapping of secondary fluorescent labels and incubation without any primary antibodies.

Confocal microscopy and image analysis

Nuclear translocation patterns were measured as previously described (van der Laan et al. 2005, Sarabdjitsingh et al. 2009). Briefly, the Hoechst stain was used as a template to identify the nucleus in sections. Fluorescence intensity values of nuclear IR were measured using ImageJ 1.32j analysis software (NIH, USA) on images (630x magnification, 155 x 155 μm) acquired with a Nikon confocal microscope (Nikon TE 2000-e). Per animal (n = 8 per time point), two frames with 30-40 cells on average were acquired. Non-specific binding (normal mouse and rabbit IgG) and background staining of the sections were also measured and

subtracted from the total signal to obtain the specific signal. Cells that had a clear ovally-shaped nucleus with a diameter of approximately 5-7 μm and showed IR clearly above background were included for analysis thereby excluding cells that were not in the plane of focus.

In situ hybridisation

In situ hybridisation was used to study expression patterns of glucocorticoid target genes Sgk-1 and Gilz in brain. In situ hybridisation was performed on 20 μm slices as previously published (Meijer et al. 2000). 33Phosporus end-labelled oligonucleotide probes (2 x 106 dpm) for Gilz (van der Laan et al. 2008b) and Sgk-1 (van Gemert et al. 2006) were used. The signal was quantified from films exposed 6-8 days (X-OMAT AR, Kodak, Rochester, NY) using ImageJ 1.32j analysis software. Relative expression levels were determined and related to standard curves of 14C (RPA 504 microscales, Amersham, UK), included to ensure that grey values were within the linear range between 0 and 255. Three hippocampal sections per animal (n =8 per time point) were analysed.

Chromatin immunoprecipitation and RT-qPCR

Chromatin immunoprecipitation to study Sgk-1 promoter occupancy by GR was adapted and modified from Van der Laan et al. (van der Laan et al. 2008b). Fixed chromatin derived from the hippocampi of three animals was pooled and sheared, yielding fragments of 100-500 bp (20 pulses of 30 seconds; Bioruptor, Diagenode). Immunoprecipitation was performed with either 6 μg of GR-specific H300 or normal rabbit IgG (Santa Cruz Biotechnology) overnight at 4 °C. After DNA recovery (Nucleospin, Macherey-Nagel), RT-qPCR was performed to study enrichment of Sgk-1 promoter fragments in the different treatment groups (LightCycler FastStart DNA Master PLUS SYBR Green I, Roche), according to manufacturer's instructions. Primers were designed around the glucocorticoid response element (GRE) of the rat Sgk-1 promoter (GGCTCAAAATTTATGCGGAA (Sgk-1 forward); CGGAAATAAGTCTCTGCGCT (Sgk-1 reverse). Myoglobulin was used a negative control for GR chromatin occupancy: CCTCACATGGGCAGCTATTT (myoglobin forward); GCTTGTGCAAGTCCAGACAG (myoglobulin reverse). PCR-products were analysed on an agarose-gel to check the length of the amplicon. Immunoprecipitation with a non-specific antibody (normal IgG) did not result in increased DNA recovery and was used to correct the GR immunoprecipitated samples (Wang et al. 2004, van der Laan et al. 2008b) .

Statistical analysis

Data are presented as mean ± SEM. Differences between groups were examined by one-way or two-way ANOVA. Tukey's post-hoc testing was applied to compare individual groups where applicable. Statistical significance was accepted at a p-value < 0.05.

Results

Controls for subcutaneous corticosterone pellet treatment

To validate that subcutaneous corticosterone pellet implantation effectively clamped circadian fluctuations at expected concentrations, morning and evening corticosterone samples were taken seven days after pellet implantation (n = 24). Vehicle animals showed typical circadian variation in corticosterone levels (21.4 ± 2.7 and 171.7 ± 20.1 ng/ml; morning and evening levels, respectively). No circadian variation in morning and evening corticosterone levels were found in the 40% (85.4 ± 6.6 and 90.0 ± 16.4 ng/ml) or 100% cort groups (254.8 ± 15.0 and 233.5 ± 14.5 ng/ml) indicating constant exposure around either daily average levels or at levels seen during stress-related disorders, respectively. Pellet removal in the morning of post-surgery day 8 resulted in rapid decrease in corticosterone levels (morning corticosterone levels the day after pellet removal: 12.5 ± 3.9 and 33.3 ± 6.9 ng/ml, 40% and 100% cort WO respectively). These values did not differ from vehicle animals (22.5 ± 3.0 ng/ml).

Corticosterone pellet implantation led to a dose-dependent decrease in body and thymus weight (Fig. 1A-B; $F(4, 115) = 158.9$; $p < 0.05$ and $F(4, 115) = 208.4$; $p < 0.001$) and decreased adrenal weight (Fig. 1C; $F(4, 114) = 21.1$; $p < 0.01$), indicating that corticosterone pellet implantation was effective. Pellet removal and 24 hours of washout of exogenous corticosterone did not result in recovery of any of the parameters measured (Fig. 1; dashed bars).

Cort pellet implantation down-regulates MR and GR protein in hippocampus

Western blot analysis for MR and GR was performed on hippocampal homogenate (Fig. 2). MR protein showed a trend towards reduction in the 100% cort group (Fig. 2A; $p = 0.07$). Pellet removal reversed any trend towards decreased MR protein levels in the 100% WO cort group. MR protein levels were not affected by 40% cort pellet implantation or washout.

Cort pellet implantation reduced GR protein levels in the 40% and significantly in the 100% cort pellets groups to 0.76 ± 0.07 and 0.65 ± 0.08, respectively compared to vehicle ($F(2, 31) = 5.1$; $p < 0.01$; Fig. 2B). GR protein levels in the 40% WO cort group were still reduced after washout (0.74 ± 0.1), but in the 100% WO cort group a recovery was noted as values were not different from vehicle treated animals (0.88 ± 0.13; $F(2, 27) = 1.2$; $p = 0.33$).

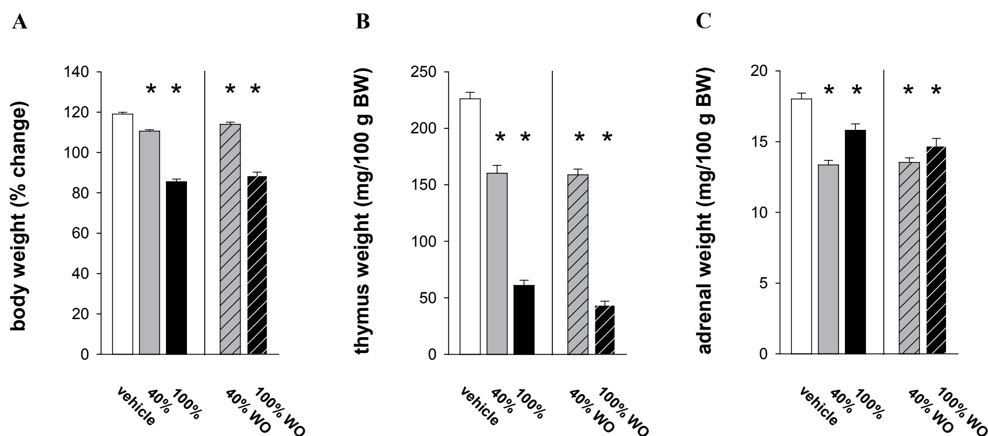

Figure 1 | Corticosterone pellet implantation affects glucocorticoid sensitive parameters. Animals implanted for 7 days with vehicle, 40% or 100% cort pellets (n=24). The dashed bars depict animals in which the cort pellet was removed (40% WO and 100% WO) and show dose-dependent decreases in A | relative body weight gain, B | thymus and C | adrenal weight as indicated with one-way ANOVA (* $p < 0.05$ compared to vehicle; Tukey's post-hoc test).

Next to the effect of cort pellet implantation, we studied whether there was an acute effect of the corticosterone injection (cort challenge) on MR and GR protein levels. Western blot analysis of hippocampal homogenate of rats from the time groups 0, 60 and 180 min after the cort challenge of all treatment groups, showed no effect of time on MR ($p = 0.35$) or GR protein levels ($p = 0.49$; two-way ANOVA; data not shown) indicating that acute changes in gene expression were not complicated by concomitant changes in receptor expression.

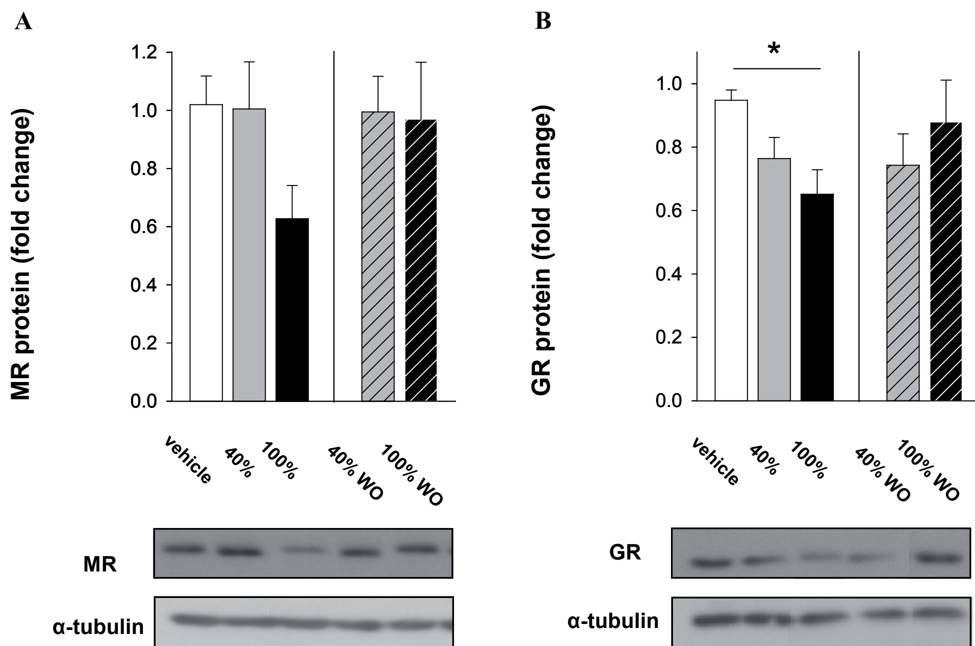

Figure 2 | Corticosterone pellet implantation decreases MR and GR protein levels. Western blot analysis of receptor protein levels in hippocampal homogenate (n = 12) after 7 days of vehicle, 40%, and 100% cort pellet implantation shows no significant effect on A | MR protein levels. B | A dose-dependent decrease was observed for GR protein levels in the 100% cort group. Interestingly, after washout (dashed bars), GR levels recovered in the 100% WO group, while the 40% cort group was still affected. Receptor protein levels were normalized against α-tubulin. * $p < 0.01$ compared to vehicle; one-way ANOVA and Tukey's post-hoc test.

Corticosterone pellet implantation attenuates GR translocation in CA1 cells

To study a different aspect of the receptor protein, we visualised corticosterone-induced MR and GR translocation patterns to the nucleus, rather than total protein hippocampal levels (Fig. 2), using immunohistochemistry and confocal imaging. MR immunoreactivity (IR) in CA1 pyramidal cells of the rat hippocampus was mostly localised in the nucleus before the cort challenge in all conditions (Fig. 3A and C, left panel). No difference in baseline levels of the different treatment groups was detected ($F(2, 19) = 1.5$; $p = 0.24$). Nuclear MR IR showed a very modest but significant increase after the challenge only in vehicle pellet rats at 60 minutes (Fig. 3A; $F(2, 16) = 3.6$; $p = 0.049$); no MR translocation was observed in the other treatment groups. Washout of corticosterone levels did not result in recovery of MR nuclear translocation in the 40% and 100% WO groups.

In contrast, low levels of nuclear GR IR were observed before the challenge in the vehicle group (Fig. 3B and C, right panel). After the challenge, a strong transient increase in nuclear

GR IR was observed ($F(2, 18) = 8.8$; $p < 0.01$) which returned back to baseline levels after 180 min, clearly indicating nuclear translocation of GR in CA1 cells. Implantation of the 40% cort pellet did not affect basal levels of nuclear GR IR, but completely abolished the increase in nuclear GR signal at 60 minutes after the acute injection (Fig. 3B). The 100% cort pellet increased 'basal' levels of nuclear GR significantly ($F(2, 18) = 8.7$; $p < 0.01$), but no additional nuclear GR localisation was observed after the cort challenge. Washout of corticosterone levels did not result in recovery of GR nuclear translocation in the 40% WO group. However, a trend towards recovery in GR translocation ($p = 0.1$) was found in the 100% WO group (Fig. 3B).

Corticosterone pellet implantation affects glucocorticoid target gene expression

To monitor primary receptor responsiveness we studied the expression profiles of two primary glucocorticoid-responsive genes: Gilz (Yachi et al. 2007, van der Laan et al. 2008b) and Sgk-1 (van Gemert et al. 2006) in the rat hippocampal area.

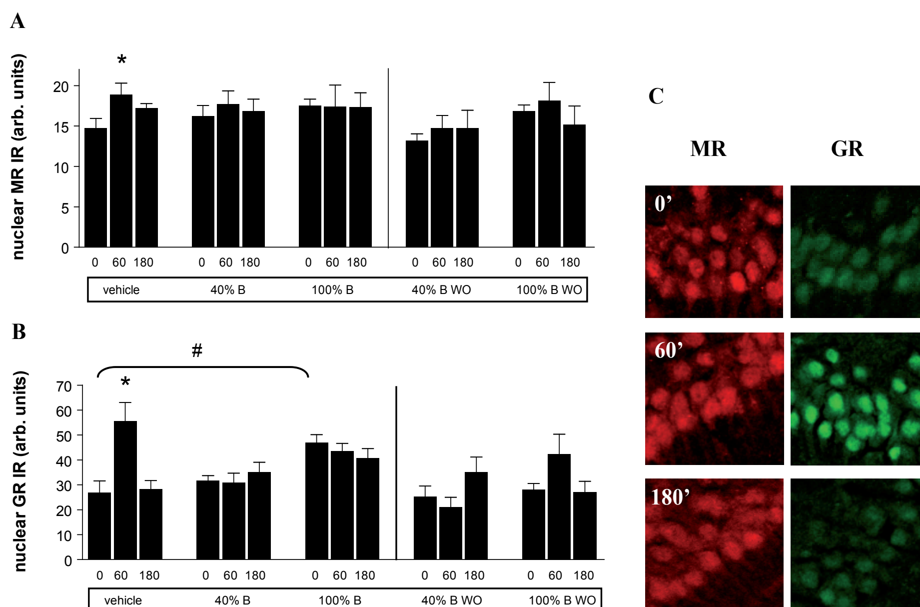

Figure 3 | Subcellular localisation of MR and GR in pyramidal CA1 cells is affected by cort pellet implantation. A | Quantification of nuclear intensity visualised by C | immunohistochemistry and confocal imaging only showed an effect of the cort challenge on MR nuclear localisation in the vehicle group (* $p < 0.05$). B | A clear transient increase in nuclear GR IR was observed in vehicle animals after the challenge (* $p < 0.01$). This effect was abolished in the 40% and 100% cort groups while basal levels were increased in the 100% cort group (# $p < 0.01$). Removal of 100% cort pellets resulted in a trend ($p = 0.1$) towards recovery in GR translocation. N = 8 animals per time point.

Gilz expression in rat hippocampus

In vehicle pellet animals, low basal expression of Gilz was observed in the CA1 pyramidal cell layer, while relatively high expression was found in CA3, dentate gyrus and cortex (Fig. 4A). In CA1 in vehicle animals, the cort challenge led to increased expression levels at 60 min ($F(2, 19) = 3.6$; $p < 0.05$). Gilz mRNA expression in CA1 in the 40% and 100% cort pellet groups resembled the GR translocation data. In the 100% cort, but not the 40% group, pellet implantation resulted in increased 'basal' expression levels ($F(2, 20) = 6.4$; $p < 0.05$) but neither group responded to the additional corticosterone challenge (Fig. 4B). Though pellet removal and washout of exogenous corticosterone decreased baseline levels back to vehicle, the pattern of gene expression did not fully recover while the kinetics of the response in the 100% WO group seemed changed: a trend towards recovery was found for the 100% cort WO group at 60 and 180 minutes after injection ($p = 0.07$). In CA3 (Fig. 4C), expression levels did not change as a consequence of time or treatment.

Sgk-1 expression in rat hippocampal area

Under basal conditions, Sgk-1 mRNA was expressed in all hippocampal regions, but highest in CA3. The corticosterone challenge led to a marked increase in the CA1 hippocampal subregion but also in the white matter surrounding the hippocampus which dropped back to baseline after 180 min (Fig. 5A). This increase has been reported before and probably reflects regulation in oligodendrocytes (van Gemert et al. 2006). Sgk-1 expression in white matter (Fig. 5B; corpus callosum) was maximally sustained in the 100% cort group but expression in response to constant and acute changes in corticosterone in the 40% cort pellet implantation group ($F(2,19) = 46.9$; $p < 0.001$) was identical to the vehicle pellet group ($F(2,20) = 185.5$; $p < 0.001$). Washout of exogenous corticosterone recovered the transient response in Sgk-1 expression in the 100% WO group (Fig. 5B; $F(2,20) = 130.1$; $p < 0.001$). Similar results were observed for CA1, but in the 100% cort group, induction of 'basal' Sgk-1 mRNA levels was absent. In contrast to the data for Gilz mRNA, responsiveness remained present in the 40% cort pellet group (Fig. 5C). Similar to Gilz mRNA, in CA3 no effect of constant and acute cort exposure was observed for Sgk-1 mRNA (Fig. 5D).

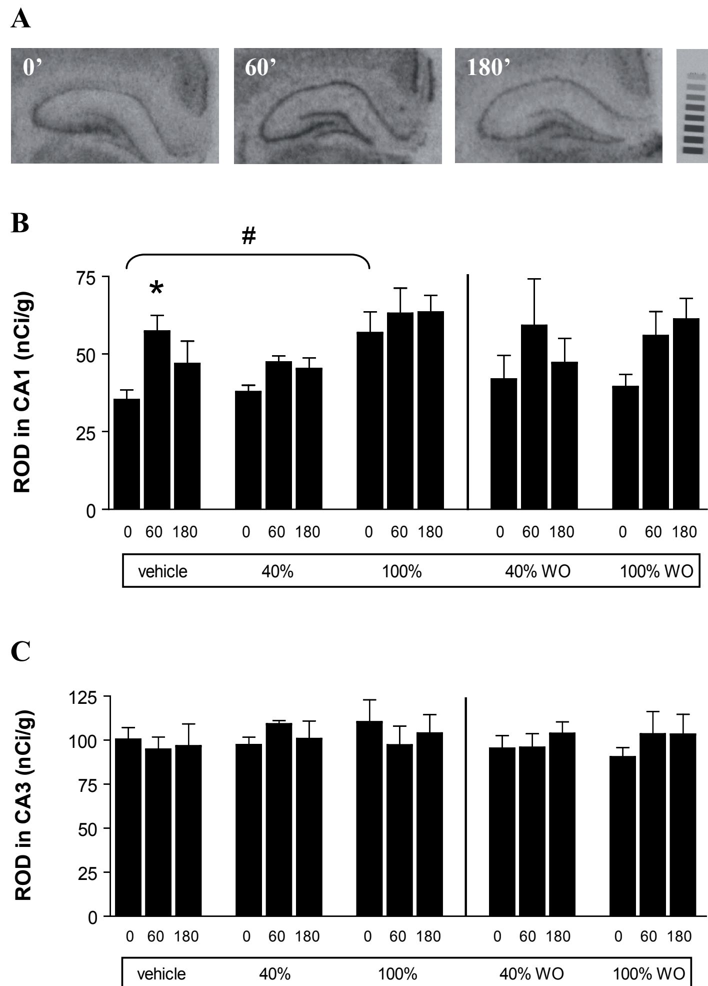

Figure 4 | Gilz mRNA expression profiles in rat hippocampus. A | Gilz expression was visualised in vehicle animals 0, 60 and 180 min after the cort challenge (n = 8 per time point) using ISH. Values were calibrated against a C14 grey-scale ladder. Quantification of relative optical density (ROD) in B | CA1 pyramidal cells show transient responses in vehicle animals (* $p < 0.05$) but sustained responses after 40% and 100% cort pellet implantation. Cort pellet implantation increased 'basal' levels significantly in the 100% cort group (# $p < 0.05$). C | No significant effect of time or treatment was found in the CA3 pyramidal cell layer.

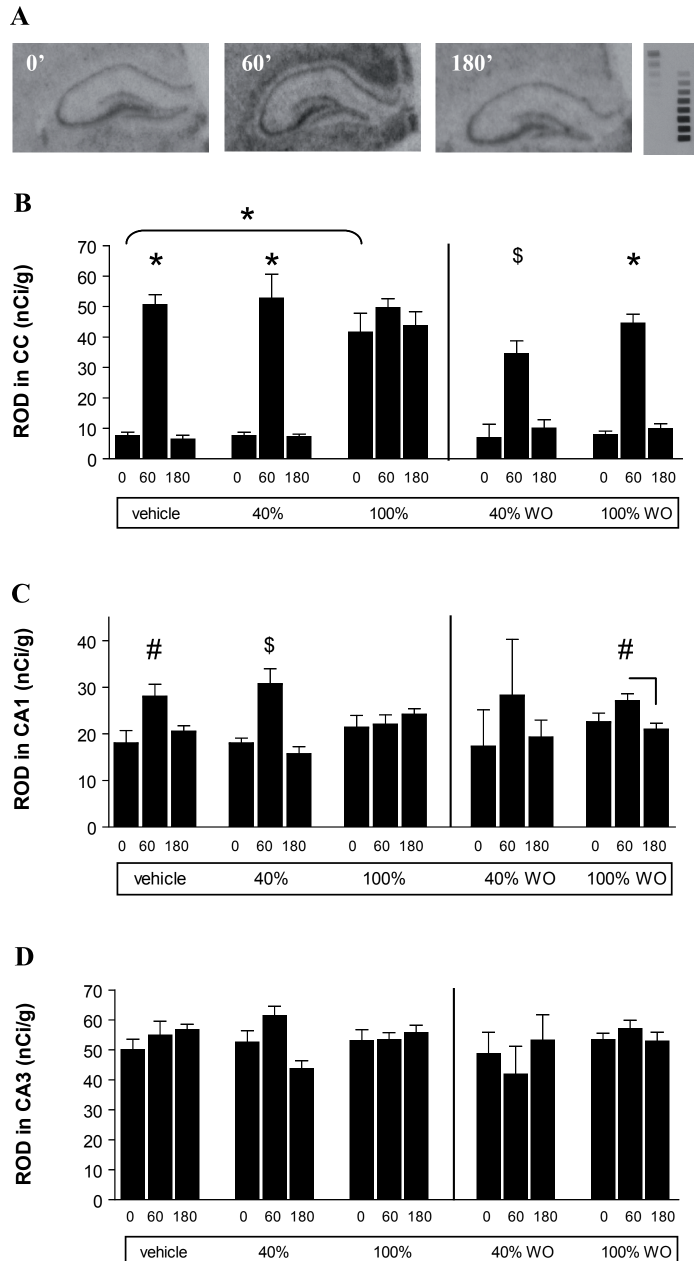

Figure 5 | Sgk-1 mRNA expression profiles in rat hippocampus. A | ISH was used to visualise Sgk-1 expression in vehicle animals 0, 60 and 180 min after the cort challenge (n = 8 per time point). Values were calibrated against a C14 grey-scale ladder. Quantification ROD in B | white matter corpus callosum and C | CA1 pyramidal cells show transient increases in response to the challenge in vehicle and 40% treated animals, but not in 100% cort animals. D | No significant effect of time or treatment was found in the CA3 (* $p < 0.001$, \$ $p < 0.01$, # $p < 0.05$).

Sgk-1 promoter occupancy by GR correlates with expression profiles

Because the gene expression profiles were different between the treatment groups, we determined whether the altered profiles were also accompanied by changes at the level of GR chromatin occupancy using chromatin immunoprecipitation (ChIP) for the Sgk-1 gene. Since no significant difference in Sgk-1 expression profiles between vehicle and 40% cort animals was observed, we chose to compare vehicle and 100% cort treated animals. In vehicle pellet animals, GR occupancy increased 3.6 ± 0.2 fold at 60 min, and returned back to baseline 180 min after the challenge ($p < 0.001$; Fig. 6), indicating that GR binding at response elements correlates with the response profile of this target gene. Challenging the 100% cort group did not result in increased DNA recovery in the GR-specific groups since levels were already increased before injection. Again, we observed elevated basal levels compared to vehicle ($p < 0.01$) which was still slightly elevated after washout. Washout of corticosterone resulted in recovery of the transient increase in Sgk-1 promoter occupancy by GR by 3.1 ± 0.1 fold 60 min after the challenge ($p < 0.001$; Fig. 6). Control measurements for the negative control locus of the myoglobulin gene showed no enrichment of GR in the vehicle group after the challenge ($p = 0.95$; data not shown), indicating that the observed increase in promoter occupancy of glucocorticoid target gene Sgk-1 is specific.

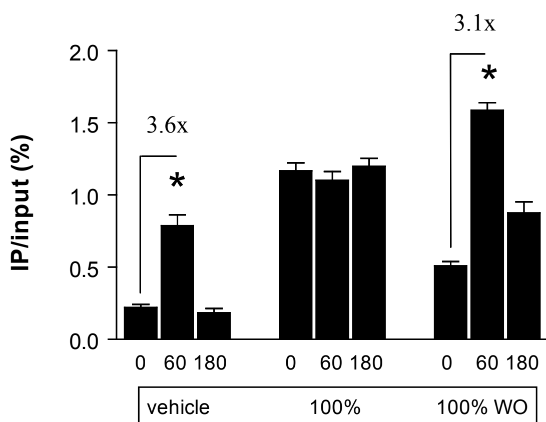

Figure 6 | Sgk-1 promoter occupancy in rat hippocampus. Chromatin immunoprecipitation and RT-qPCR to represent Sgk-1 promoter occupancy by GR for vehicle and 100% cort pellet animals (3 hippocampi pooled per time point). In the vehicle animals the cort challenge resulted in a transient increase in promoter occupancy by GR 60 min after while this response was attenuated in the 100% cort animals. Basal levels were increased compared to vehicle ($p < 0.01$). Washout of corticosterone shows a recovery in the transient response to the challenge. (* $p < 0.001$).

Discussion

In the present study we used two different concentrations of corticosterone pellets that abolish normal corticosterone pulsatility by clamping hormone levels around daily average (40% cort pellet) or at supra-physiological levels as may be seen during pathology (100% cort pellet). We have shown considerable changes in molecular markers for both chronic and acute glucocorticoid action in the rat hippocampus. Moreover, we demonstrate that the consequences of subtle variations in glucocorticoid pattern are at least as severe as those with overt hypercorticism. We therefore propose that pulsatile glucocorticoid levels are essential in maintaining receptor responsiveness, particularly of the GR, and prevent desensitisation of some targets. Consequently, we suggest that the pattern of hormone exposure is a major determinant in the adaptive capacity of target tissues in the face of acute stress.

Validation of experimental design

Subcutaneous corticosterone pellet implantation, providing a constant signal in blood plasma, mimics some of the risk factors for stress-related disease by disrupting normal glucocorticoid variation (Young et al. 2004, de Kloet et al. 2005, Herbert et al. 2006, Lightman et al. 2008). In line with previous studies that have shown flattening of circadian corticosterone rhythms (Akana et al. 1985, Akana et al. 1992, Meijer et al. 1997), we effectively clamped corticosterone levels around the daily average or at levels seen during chronic stress [40% and 100% cort pellet, respectively (RA Sarabdjitsingh 2009)]. This allowed us to directly compare the impact of a corticosterone challenge on molecular targets in the context of either constant of fluctuating hormone patterns.

Typically, subcutaneous corticosterone pellet implantation results in dose-dependent decreases in body, thymus and adrenal gland weight (Spencer et al. 1991, Akana et al. 1992). This decrease was also present in the 40% cort group, though to a lesser extent than the 100% cort group, suggesting mild hypercorticism. However, we cannot exclude that the observed decreases in the 40% group are due to alterations in the pattern of plasma corticosterone since we assume that changed pulsatility may affect glucocorticoid sensitive parameters. We interpret our groups as the 40% cort lacking pulsatility with daily cort exposure in the normal range and the 100% group lacking pulsatility while having overt hypercorticism.

100% corticosterone pellet treatment

MR and GR are both regulated by glucocorticoids (De Kloet et al. 1998). In the present data, the dose-dependent down-regulation of hippocampal GR rather than MR protein (Spencer et al. 1991), was more pronounced after constant corticosterone exposure. Additionally GR nuclear translocation patterns were clearly more attenuated after the challenge.

We assume that MR is already fully saturated under the conditions of 40% cort treatment (Reul & de Kloet 1985, Meijer et al. 1997) as no additional increase in nuclear MR translocation was observed in our study. Also, we did not specifically address MR and MR-targets as the dose of corticosterone (3 mg/kg i.p.) that was used to challenge the adrenally intact animals is more suitable to study GR-dependent effects. This is supported by the lack of glucocorticoid regulation in the CA3 pyramidal cell layer of the hippocampus. This region has very low levels of GR but expresses MR (Arriza et al. 1988, Ahima & Harlan 1990, Sarabdjitsingh et al. 2009). Even though the target genes Gilz and Sgk-1 that were used in this study are both expressed in this area under basal conditions, and are regulated by glucocorticoids in adrenalectomised animals (van der Laan et al. 2008b), we did not observe any change in transcriptional output after constant or acute corticosterone treatment. Thus, our data show that both the 40% and 100% pellets in particular affected GR signalling.

GR homologous down-regulation after chronic high glucocorticoid treatment has been observed before (Sapolsky et al. 1984, Reul et al. 1989, Spencer et al. 1991). In spite of down-regulation, GR still exerted tonic effects, e.g. as is seen from the constant high occupancy of the Sgk-1 promoter. Many studies described such sustained effects of chronic high corticosterone levels on the brain mediated via GR. For instance, feedback sensitivity of the HPA axis after stress is attenuated after chronic treatment with corticosterone through occupancy of GR (Akana et al. 1992, Bradbury et al. 1994). Also, CA1 pyramidal cells seem to lose their potential to normalise enhanced activity after stress (Joels et al. 2007), e.g. as seen from the risk of calcium overloading (Karst & Joels 2007) and attenuation of functional 5-HT responses by developing resistance to GR-mediated enhancement of membrane hyperpolarisation (Karten et al. 1999). We observed sustained effects of GR on expression of target genes Sgk-1 and Gilz in a gene and cell type dependent manner. This is in line with recent data that show complex kinetic gene response profiles under constant glucocorticoid conditions which can be transient but also tonic (John et al. 2009).

The acute effects of corticosterone that are superimposed on the 100% corticosterone treatment were attenuated, e.g. GR nuclear translocation and Sgk-1 promoter occupancy after the cort challenge. The very high circulating levels of corticosterone are very likely to saturate GR and render it non-responsive to additional hormone. This likely creates a non-favourable situation in which the normal flexibility in glucocorticoid signalling is lost.

40% corticosterone pellet treatment

The results from the present study show that 40% cort pellet treatment, which results in much lower, physiologically relevant constant corticosterone levels, also has tonic effects. Previous studies have shown attenuated stress responsiveness (Akana et al. 1992, Meijer et al. 1997) and suppressed expression levels of hippocampal 5-HT1A receptor (Meijer et al.

1997). These effects were interpreted as mediated via MR (Bradbury et al. 1994, Meijer et al. 2000). In our study some additional GR mediated effects occurred, e.g. thymus involution and a trend towards down-regulation of hippocampal GR protein levels. However, 40% cort treatment did not change baseline expression of our rather sensitive expression markers. A lack of GR responsiveness became evident after the acute challenge as no additional increase in GR nuclear translocation and target gene expression was observed. This suggests that the functionality of glucocorticoid pulsatility becomes apparent when the system is challenged and requires a rapid onset and termination of tissue responses. In the 40% cort group the pattern, but not so much the total amount, of corticosterone exposure differs from vehicle animals (RA Sarabdjitsingh 2009). We propose that the pattern of corticosterone exposure is therefore a major determinant in glucocorticoid signalling and that changes in this pattern have at least as many consequences for cellular responses than overt hypercorticism (100% cort). This is also strengthened by the normalisation in GR responsiveness after washout of constant 100% corticosterone levels but not in the 40% cort group. This suggests that tonic effects of glucocorticoids can be reversible and are modulated by an underlying mechanism that is sensitive to changes in the normal pulsatile pattern.

The use of 40% cort pellet resulted in aberrant GR translocation while the transcriptional profiles of target genes were affected gene-specifically. While induction of Gilz expression was already attenuated by 40% cort pellet implantation, Sgk-1 expression in the hippocampal area was only sustained after exposure to high levels of corticosterone (100% cort). We therefore conclude that receptor translocation is certainly not rate-limiting for all signalling pathways. The molecular response to glucocorticoids via GR involves many complex regulatory actions and cannot therefore simply be explained by the duration and presence of the hormone. These gene-specific effects may be related to differences in the potency of GR at target genes, resulting in different transcriptional output patterns (Meijer 2006, John et al. 2009). In addition, rapid receptor dynamics together with the repeated cycling of chaperones and cofactors continuously change the local environment to which promoters are exposed and underline the time and region -dependency of receptor effectiveness (Biddie & Hager 2009).

Conclusion

Frequency encoding of intercellular signals is a well accepted mode of communication in mammalian systems including the endocrine system. For instance, rapidly fluctuating levels of growth hormone elicits 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). These studies imply that ultradian hormone signalling enables systems to maintain responsiveness and prevent receptor desensitisation and is supported by our

findings. Manipulation of the temporal aspect is already a successfully used approach in clinical therapy for instance for growth hormones (Amato et al. 2000) and estrogens (Shoupe 2001). Linking the knowledge about ultradian glucocorticoid pulsatility and receptor signalling could therefore greatly contribute to clinical therapy.

In the present study, we provide evidence that frequency encoding by rapid glucocorticoid signalling is critical in functionally maintaining receptor responsiveness and preventing desensitisation of some targets. We clearly demonstrate that the ability of the receptor to respond to glucocorticoids as defined by receptor translocation and binding to its proximal targets is attenuated when pulsatile corticosterone signalling is lost. We therefore propose that the molecular response of target tissues induced by a corticosterone challenge is a resultant of the dynamic interplay between the corticosterone exposure regime (pulsatile or continuous) and steroid receptor signalling. The findings of this study suggest that pulsatile glucocorticoid release is required to maintain the normal 'resilience' in glucocorticoid responsiveness.

Acknowledgements

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Chapter 5

Recovery from disrupted ultradian glucocorticoid rhythmicity reveals a dissociation between hormonal and behavioural stress responsiveness

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Abstract

Ultradian release of glucocorticoids is thought to be essential for homeostasis and health. Deviation from this pulsatile release pattern is considered to compromise resilience to stress-related disease, even after hormone levels have normalised. Here, we investigate how constant exposure to different concentrations of corticosterone affects diurnal and ultradian pulsatility. The rate of recovery in pulsatile HPA activity after withdrawal of exogenous corticosterone is also examined. Finally, the behavioural and neuroendocrine responsiveness to an audiogenic stressor is studied.

Adrenally intact male rats were subcutaneously implanted with vehicle, 40% or 100% corticosterone pellets for 7 days. The continuous release of corticosterone from these implants abolished diurnal and ultradian corticosterone variation as measured with high frequency automated blood sampling. Pellet removal on post-surgery day 8 allowed rapid recovery of endogenous rhythms in animals previously exposed to daily average concentrations (40%) but not after exposure to high concentrations (100%) of corticosterone. Behavioural and neuroendocrine responsiveness to stress was distinctly different between the treatment groups. Audiogenic stimulation one day after pellet removal resulted in a similar corticosterone response in animals previously exposed to 40% corticosterone or vehicle. The 40% pellet group showed, however, less and shorter behavioural activity (i.e. locomotion, risk assessment) to noise stress as compared to 100% corticosterone and vehicle treated animals.

In conclusion, unlike the animals impanted with 100% corticosterone, we find that basal HPA axis activity in the 40% group which had mean daily levels of circulating corticosterone in the physiological range, rapidly reverts to the characteristic pulsatile pattern of corticosterone secretion. Upon reinstatement of the ultradian rhythm, despite the fact that these animals did not differ from controls in their response to noise stress, they did show substantial changes in their behavioural response to stress.

Introduction

The diurnal variation in the secretion of glucocorticoids is principally controlled by the hypothalamic suprachiasmatic nucleus and is fine-tuned by autonomic nervous system activity and afferents of the central hypothalamic-pituitary-adrenal (HPA) axis (Kalsbeek & Buijs 2002, Dickmeis 2009). Daily 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). These diurnal fluctuations consist of approximately hourly, ultradian glucocorticoid hormone bursts that change in amplitude according the time of day (Jasper & Engeland 1991, Windle et al. 1998b, Lewis et al. 2005). Superimposed on these rhythms is the HPA-driven glucocorticoid response to a stressor.

Alterations in glucocorticoid pulsatile patterns can occur and are associated with normal transitions in physiological states but also stress-related disorders. For instance in rodents, lactation suppresses pulsatility, while during ageing the hourly pattern becomes disorganised (Lightman et al. 2000). In humans, depression is associated with enhanced pulse magnitude thereby abolishing circadian variation in pulse amplitude, resulting in dramatically changed patterns of tissue exposure to hormone (Holsboer 2000, Young et al. 2004, de Kloet & Sarabdjitsingh 2008, Lightman et al. 2008). However, the functional contribution of ultradian glucocorticoid pulses to HPA axis reactivity and stress responsiveness, and the consequences of changes in pulse characteristics for physiology are largely unknown.

Diurnal rhythmicity can experimentally be abolished by subcutaneous implantation of corticosterone releasing pellets that elevate circadian trough concentrations. Consequently via negative feedback mechanisms this leads to a compensatory lower output of corticosterone from the adrenal cortex at the time of the circadian peak (Akana et al. 1985, Akana et al. 1992, Meijer et al. 1997). Previously, we have demonstrated that such flattening of the diurnal corticosterone patterns has substantial effects on glucocorticoid receptor (GR) signalling in the rat hippocampus: while tonic actions of glucocorticoids are apparent (Akana et al. 1992, Meijer et al. 1997), we found that the response in expression of glucocorticoid marker genes to acute elevations superimposed on the constant hormone signal is attenuated. Surprisingly, constant exposure to moderate concentrations of corticosterone in some aspects had greater consequences for GR responsiveness than overt hypercorticism (Sarabdjitsingh et al. 2010). These data indicate that the tissue responsiveness to glucocorticoids depends on the pattern of ligand exposure and that a pulsatile pattern is necessary to prevent receptor desensitisation.

However, a number of issues are still unresolved. It remains to be proven that ultradian variations are abolished in the pellet model. Also, it is unknown how fast the negative feedback action abates after cessation of constant exogenous corticosterone and when the endogenous pulses re-emerge. Observations in rodents and humans with a history of changes in corticosteroid exposure suggest that even after normalisation of hormone levels, there are residual disturbances in the brain (Ratka et al. 1988, Tiemensma et al. 2010). In that respect, while it is known that neuroendocrine stress responsiveness is clearly affected under conditions of flattened corticosterone (Akana et al. 1985, Akana et al. 1992, Dallman et al. 2004, Joels et al. 2007), very little data is available on the behavioural response to stress after changes in pulse characteristics.

To this end, adrenally intact male rats were subcutaneously implanted with vehicle, 40% or 100% corticosterone pellets, respectively. As such animals were exposed to either high, pathological concentrations of corticosterone (100% pellet) or on average, normal daily concentrations but in a continuous (40% pellet) rather than pulsatile manner (vehicle pellet). After seven days of pellet implantation corticosterone pulsatile profiles were measured in individual animals using high frequency automated blood sampling (Windle et al. 1998b, Atkinson et al. 2006). Pellets were rapidly removed on day 8 to assess how fast ultradian rhythms recover after cessation of constant corticosterone exposure (washout). As functional readout we measured hormonal and behavioural responsiveness to white noise stress.

We validate that different concentrations of constant corticosterone exposure in adrenally intact rats indeed abolish both diurnal and ultradian rhythmicity. We also find that HPA axis activity flexibly recovers from long term exposure to 40% but not 100% corticosterone, but that in contrast the behavioural stress responsiveness is affected after washout in the 40% corticosterone group, compared to vehicle.

Experimental procedures

Subjects

Experiments were conducted on male Sprague-Dawley rats (Harlan, UK) weighing approximately 250 g at the time of surgery. After arrival, animals were group housed (4 animals per cage) under standard environmental conditions and a 14 : 10 h light/dark cycle (lights on 05.15 h) and were allowed to acclimatise for a week. Food and water were provided ad libitum throughout the experiment. Animal procedures were approved by the University of Bristol Ethical Review Group. Animal care was conducted in accordance with Home Office guidelines, the UK Animals (Scientific Procedures) Act 1986 and the EC Council Directive of November 1986 (86/609/EEC).

Surgery

Surgery was performed essentially as described previously (Spiga et al. 2007). Briefly, animals were anaesthetised with a combination of Hypnorm (0.32 mg/kg fentanyl citrate and 10 mg/kg fluanisone, i.m.; Janssen Pharmaceuticals, Oxford, UK) and diazepam (2.6 mg/kg i.p.; Phoenix Pharmaceuticals, Gloucester, UK). The right jugular vein was cannulated by inserting a polythene cannula (Portex, Hythe, UK) into the vessel. The free end of the cannula was exteriorised through a scalp incision and tunnelled through a protective spring. During the same surgery, 100 mg pellets containing either 40% or 100% corticosterone (40% and 100% corticosterone respectively; ICN Biomedicals, Irvine, USA) or 100% cholesterol (vehicle, Sigma-Aldrich, St. Louis, USA), were subcutaneously implanted between the shoulder blades to obtain constant concentrations of corticosterone in blood (Akana et al. 1985, Akana et al. 1992, Meijer et al. 1997). Following surgery, animals were individually housed and kept in the automated blood sampling room. The cannula was attached to a mechanical swivel that rotated through 360° in a horizontal plane and 180° through a vertical plane, allowing the rats to maximise freedom of movement. The cannulae were flushed daily with heparinised saline to maintain patency.

Experimental design

One cohort of animals was used which were subjected to different phases of the experimental design.

Phase 1: *basal diurnal and ultradian corticosterone rhythms*

The effect of different concentrations of exogenous corticosterone on basal diurnal and ultradian corticosterone rhythms was studied. Six days after pellet implantation, the cannula of each animal was connected to an automated blood sampling (ABS) system as previously described in detail (Windle et al. 1998b, Atkinson et al. 2006). On post-surgery day 7, blood samples (37 μl aliquots) were automatically collected with 10 min intervals at a dilution of 1:5 in heparinised saline. Due to practical limitations in the number of samples that can be collected with the ABS in the same rat it was not possible to obtain a full corticosterone profile on this experimental day. Therefore, samples were collected during the trough (07.00 and 10.00 hours) and peak (18.00 and 21.00 hours) of hormonal release, the times during which strong differences in diurnal and ultradian concentrations have been described extensively (Windle et al. 1998b, Atkinson et al. 2006, Spiga et al. 2007). Plasma was separated by centrifugation and then stored at −80 °C until processed for corticosterone measurements as described below. Due to practical reasons in blood sampling we had dropouts in the vehicle group for the endocrine studies (phase 1 and 2). The obtained corticosterone values in this group are however consistent between the experiments and with previously published data (Atkinson et al. 2006, Spiga et al. 2007). Fortunately, these animals were still available for the behavioural studies (phase 3).

Phase 2: *basal diurnal and ultradian corticosterone rhythms after pellet removal*

To investigate whether corticosterone rhythmicity would recover within one day of termination of constant corticosterone administration, on day 8 post-surgery the implanted pellets were removed (washout). Between 08.00-09.00 the pellets were removed under brief isoflurane anaesthesia (2-3 min) and animals were reconnected to the ABS at 09.30. Automated blood sampling started at 10.00 and samples were collected every 20 min (10:00-13:00) or 10 min (13:00- 05:00). The impact of exogenous corticosterone replacement on body weight, thymus weight and adrenal gland weight has been reported previously (Sarabdjitsingh et al. 2010).

Phase 3: *Stress-induced behavioural and corticosterone responses*

Stress-induced corticosterone release and behavioural responsiveness were studied after cessation of constant corticosterone concentrations. Animals were then exposed to white noise stress (99 dB for 10 min) in the morning of day 9 post-surgery (06.00). Blood samples were collected every 20 min using the ABS between 05:00 and 07:20. During the same time animal home cage behaviour was recorded using cameras mounted above the cage of each animal. Behavioural analysis was performed 10 min before, during and after relative to the onset of the stressor.

Corticosterone measurements

Blood samples were centrifuged for 15 min at 4000 rpm at 4 °C. Plasma was stored at -80 °C until processed using a commercially available radio immuno assay (RIA; MP Biomedicals Inc., CA., USA) according to the manufacturer's instructions. Inter-assay and intra-assay variance was 6.9% and 7.3%, respectively.

Behavioural analysis

The following behaviours were analysed with a semiautomatic scoring system (The Observer Mobile 4.1, Noldus Information Technology, The Netherlands): i) locomotion (walking, front and back paws change position), ii) risk assessment ('seeking' behaviour while the body is still or stretched), iii) face washing (grooming of face using front paws), iv) body grooming (grooming using front and back paws, i.e. scratching), and v) sitting (e.g. sitting or sleeping). Total activity was calculated by adding the scores of behaviours i – iv. In addition, the number of vi) rearing (raising of the rat and then lowering both front paws) and vii) digging events (moving around of cage bedding with front paws) was counted. The data is presented in either 10 min bins (Table 3) or plotted per min to study changes in behavioural patterns over time (Fig. 4).

Statistical analysis

Statistical analyses were performed using SPSS 16.0 (SPSS Inc., Chicago, IL, USA). Data are expressed as the mean ± SEM except for individual corticosterone profiles. For phase 1, two-way ANOVA was used to study the diurnal differences in mean corticosterone and AUC concentrations between treatment groups. Due to limited number of samples collected for analysis PULSAR analysis may not be reliable on short time intervals. Therefore, as an indication of ultradian pulsatility, repeated measures-ANOVA was used to analyse the effect of time on corticosterone concentrations in the 3 hours time frames in morning and evening. For phase 2, the hormone profile for each individual animal was analysed using the PULSAR algorithm (Merriam & Wachter 1982) with previously published G-values (Spiga et al. 2007). The following pulse characteristics were analysed: area under the curve (AUC), mean daily corticosterone concentration, number, height (absolute increase from zero baseline), amplitude (absolute increase from respective baseline) and frequency of corticosterone pulses. One-way ANOVA and independent Student's t-tests were used to test statistical differences in pulse characteristics between corticosterone- and vehicle- treated rats. For phase 3, repeated measures ANOVA was used to indicate time and treatment effects in stress-induced corticosterone profiles. Behavioural data were analysed using one-way ANOVA or two-way repeated measures ANOVA with time and treatment as factors. Where applicable, post-hoc tests were used as indicated in the figure legends. With respect to repeated measures ANOVA, sphericity (e.g. comparable to homogeinity of variance) was tested with Mauchly's test. In case the conditions were not met, the rather conservative Greenhouse-Geisser correction was used to in order to have a valid F-ratio. Statistical significance was accepted at a p-value < 0.05.

Results

Effect of corticosterone pellet implantation on basal corticosterone rhythms

Figure 1 depicts mean (Fig. 1A) and individual morning and evening plasma corticosterone profiles (Fig. 1B-D) of animals from each treatment group measured on day 7 of pellet implantation. Mean corticosterone concentrations and AUC are shown in Table 1. In agreement with previous studies (Atkinson et al. 2006, Spiga et al. 2007), we observed in vehicle-treated animals very low to non-detectable concentrations in the morning and distinctively higher fluctuating hormone concentrations in the evening (Fig. 1A and B), indicating significant diurnal differences in mean corticosterone concentrations (Table 1; $p = 0.03$) and AUC ($p = 0.04$). In contrast, exogenous corticosterone exposure induced clear effects on corticosterone rhythms: both 40% and 100% corticosterone pellet implantation effectively flattened diurnal corticosterone rhythmicity while elevating basal concentrations in after-

noon and/or in the morning (Fig. 1A, C-D). No statistical differences between morning and evening mean corticosterone concentrations or AUC were found within these groups, while mean corticosterone concentrations in the 100% group were significantly higher compared to vehicle-treated animals (Table 1; $p < 0.05$).

As expected, ultradian pulses were only detected in the evening in vehicle-treated animals (effect of time: $p = 0.02$) while the other treatment groups did not show statistically significant differences in the morning or evening (Fig. 1A-B). These results show that both 40% and 100% corticosterone pellet implantation successfully abolished both diurnal and ultradian corticosterone rhythms.

Table 1 | Morning and evening AUC and mean corticosterone concentrations in rats implanted with vehicle, 40% or 100% corticosterone pellets

	Time of day	AUC	Mean corticosterone (ng/ml)
Vehicle	07.00-10.00	164 ± 44	9.2 ± 3.4
	18.00-21.00	621 ± 102 *	36.5 ± 13.7 *
40% cort	07.00-10.00	342 ± 77	21.6 ± 5.0
	18.00-21.00	323 ± 49	19.1 ± 3.8
100% cort	07.00-10.00	3269 ± 570 #	180.2 ± 41.0 #
	18.00-21.00	3182 ± 501 #	170.3 ± 37.6 #

Values represent mean ± SEM of AUC and mean corticosterone concentrations in rats exposed to vehicle (n=4), 40% (n=7) and 100% corticosterone pellet (n=7) for 7 days measured in the morning (07.00 – 10.00) and in the evening (18.00 – 21.00). Two-way ANOVA (Games-Howell post-hoc test) was used to analyse diurnal differences in AUC ($F(5, 12) = 13.1$; $p < 0.001$) and mean corticosterone concentrations ($F(5, 13) = 11.9$; $p < 0.001$). Vehicle animals showed normal diurnal variation in mean corticosterone concentrations and AUC (* $p < 0.05$). No diurnal differences were detected in the 40% and 100% corticosterone pellet groups. Corticosterone concentrations and AUC were significantly higher in the 100% cort treated animals compared to morning and evening concentrations of vehicle animals (# $p < 0.05$).

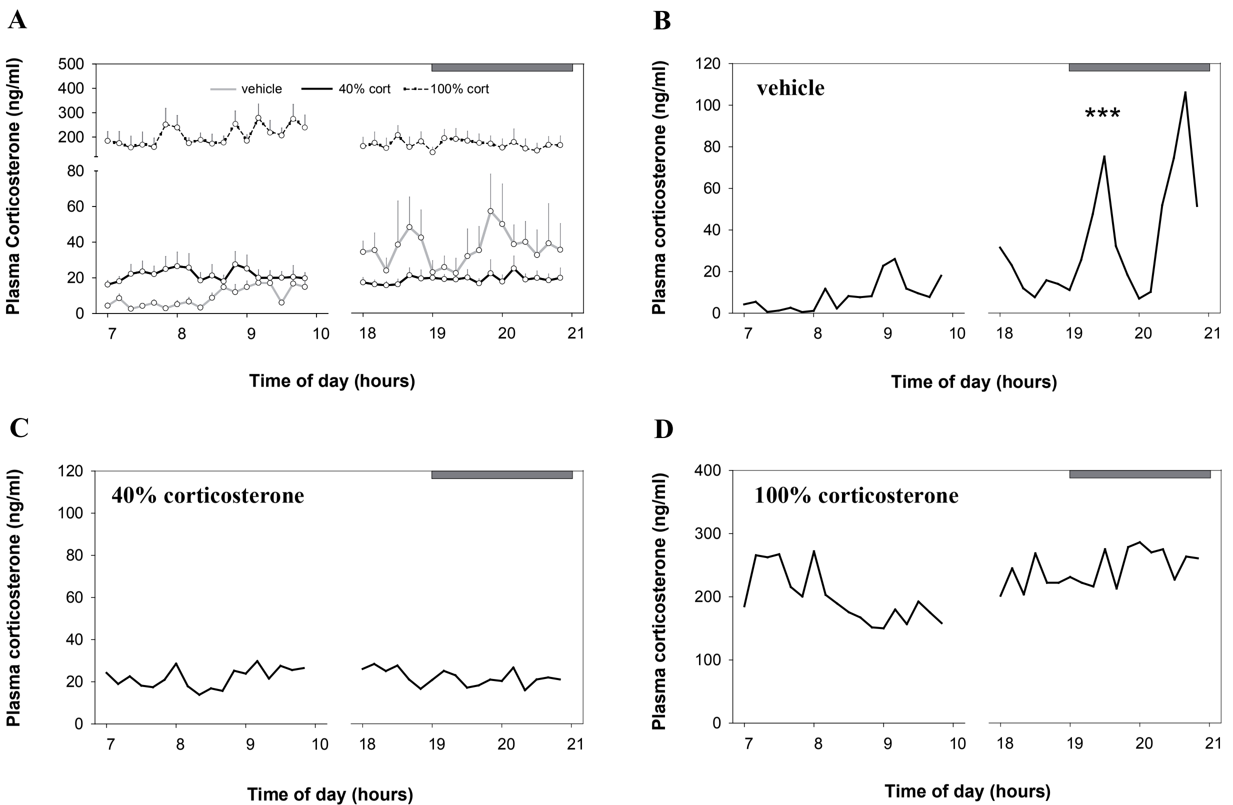

Figure 1 | Effect of subcutaneous corticosterone pellet implantation on diurnal and ultradian plasma corticosterone rhythms. Data represent A | group averages (mean + SEM) and representative individual plasma corticosterone profiles of rats implanted with B | vehicle (n=4), C | 40% (n=7), and D | 100% corticosterone pellets (n=7). Blood samples were collected at 10-min intervals from 07.00 - 10.00 and 18.00-21.00 h on day 7 post-surgery. Significant ultradian fluctuations in corticosterone concentrations were only detected in the evening in vehicle treated animals (repeated-measures- ANOVA) indicating successful flattening of ultradian pulses in 40% and 100% corticosterone pellet animals. Effect of time: *** $F(1.9, 5.8) = 9.1$; $p = 0.02$ with Bonferroni's post-hoc test. Grey bar indicates the dark phase.

Effect of pellet removal on basal diurnal and ultradian corticosterone rhythms

To determine whether endogenous corticosterone pulsatility recovers within one day from constant exogenous corticosterone administration, on day 8 post-surgery, pellets were removed early in the morning. Figure 2 shows group averaged and individual plasma corticosterone profiles over 19 hours after removal of pellets and reconnection to the ABS system. In vehicle-treated animals, typical diurnal and ultradian variation was observed with increasing mean corticosterone concentrations and ultradian pulses towards the dark phase (Fig. 2A-B). In 40% corticosterone-treated animals, pellet removal resulted in a rapid fall of corticosterone to very low concentrations and a recovery of ultradian and diurnal rhythms (see below). The onset of detectable ultradian pulses was observed around 16.00 suggesting a delay in reoccurance of pulsatility compared to vehicle animals (Fig. 2A and C).

Though in general a tendency towards decreased corticosterone concentrations was observed, in the 100% corticosterone group no clear effect of rapid reinstatement of ultradian corticosterone pulsatility was observed (Fig. 2A and D).

To study ultradian pulsatility in more detail, the PULSAR algorithm was used to identify pulse characteristics in individual animals (Table 2). The persistence of very high corticosterone concentrations in the 100% corticosterone group resulted in very high values for the different pulse parameters. To prevent these data from skewing the results and to compare the ultradian pattern of the 40% group to vehicle in more detail, pulse characteristics were statistically compared between vehicle and the 40% corticosterone group using independent Student's t-tests. PULSAR analysis revealed a decrease in mean corticosterone concentrations ($p = 0.04$) and pulse height ($p = 0.04$) in 40% cort-treated group, compared to vehicle. Furthermore, a trend toward a significant decrease was observed on AUC ($p = 0.06$) and pulse amplitude ($p = 0.05$). No significant difference in the number of pulses and pulse frequency ($p = 0.14$) was observed indicating very similar pulse characteristics of the 40% corticosterone group compared to vehicle.

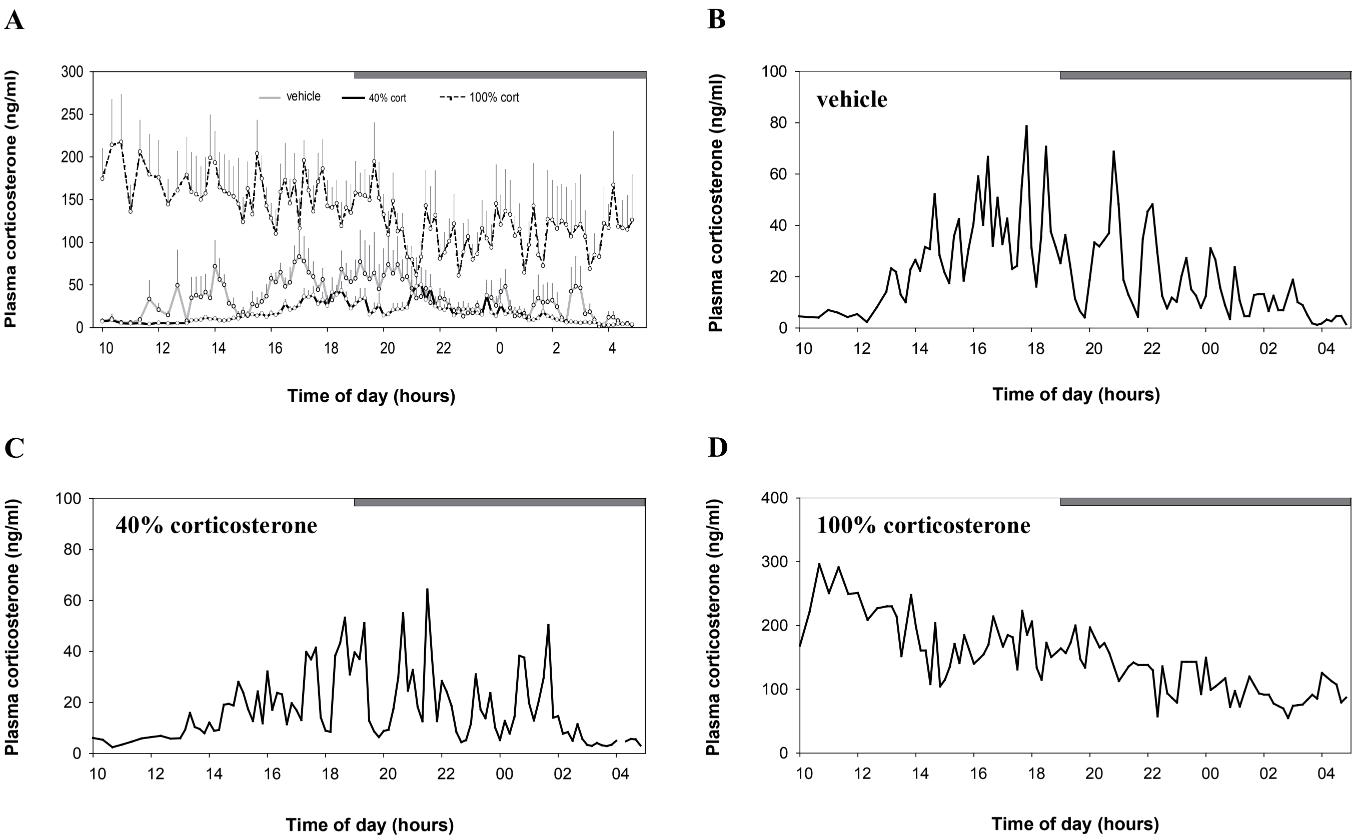

Figure 2 | Effect of corticosterone pellet removal (washout) on diurnal and ultradian plasma corticosterone rhythms. Data represent A | group averages (mean + SEM) and individual plasma corticosterone profiles measured after pellet removal in rats previously implanted with B | vehicle (n=4), C | 40% (n=6), or D | 100% corticosterone pellets (n=6). Pellets were removed at 08.00 of day 8 post-surgery; rats were reconnected to the ABS at 09.30 and samples were collected every 20 min (10:00-12:40) or 10 min (13:00-5:00). Grey bar indicates the dark phase.

Table 2 | Mean ± SEM of PULSAR parameters measurements of treated rats during 13.00 – 05.00 of the sampling period

	Vehicle	40% corticosterone	100% corticosterone
AUC	3491 ± 1124	1747 ± 317	13016 ± 2858
Mean corticosterone (ng/ml)	47.2 ± 14.0	22.4 ± 4.4 *	240.8 ± 65.2
Pulse number	5.8 ± 0.6	3.7 ± 1.4	8.5 ± 1.4
Pulse amplitude (ng/ml)	74.7 ± 19.2	35.6 ± 11.1	165.5 ± 51.0
Pulse height (ng/ml)	91.3 ± 20.1	49.1 ± 11.3 *	337.0 ± 78.3
Pulse frequency (pulse/h)	0.59 ± 0.06	0.39 ± 0.13	0.92 ± 0.11

Pellets from rats previously treated for 7 days with vehicle (n=4), 40% (n=6) or 100% corticosterone pellets (n=6) were removed in the morning of post-surgery day 8. PULSAR analysis only demonstrates significantly lower mean corticosterone concentrations and pulse height (* $p < 0.05$) in animals treated with 40% corticosterone pellets compared to vehicle as indicated by independent Student's t-tests.

Effect of previous constant corticosterone exposure on stress responsiveness

To study functional consequences of previous exposure to constant corticosterone concentrations, animals were exposed to noise stress (99 dB for 10 min) while blood samples were collected every 20 min using the ABS system. Figure 3 depicts the stress-induced increase in corticosterone concentrations in each treatment group. As expected, vehicle animals demonstrate an increase in corticosterone concentrations with hormone peak concentrations of 103 ± 11.9 ng/ml, 20 min after onset of the stress (effect of time: $p < 0.001$). Interestingly, animals that were previously implanted with 40% corticosterone pellets did not differ in their stress response compared to vehicle animals and had similar corticosterone peak concentrations (82.6 ± 22.1 ng/ml). Noise stress did not affect plasma corticosterone concentrations in the 100% corticosterone pellet group. At the time of stress basal corticosterone concentrations were increased compared to the other groups ($p < 0.01$).

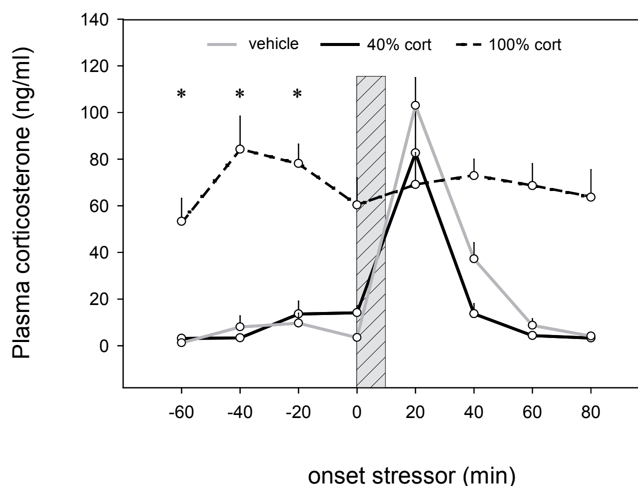

Figure 3 | Effect of previous constant corticosterone exposure on stress-induced plasma corticosterone release. Values indicate mean + SEM of plasma corticosterone measured in rats one day after removal of vehicle (n=4), 40% (n=6) or 100% corticosterone pellets (n=5). Blood samples were automatically collected every 20-min. Rats were exposed to 10 min of 99 dB (hatched bar) which increased corticosterone levels in vehicle and 40% corticosterone animals (effect of time: $F(7, 96) = 15.7$; $p < 0.001$). No difference in stress-induced corticosterone levels was observed between vehicle and 40% corticosterone animals. Animals previously treated with 100% cort show no significant response to the stressor as basal concentrations were elevated (* $p<0.01$; two-way repeated measures ANOVA and Bonferroni's post-hoc test).

Effect of previous constant cort exposure on stress-induced behavioural responses

Behavioural responsiveness to noise stress was assessed in 10 min intervals before, during and after the stress. Complete analysis of the data is reported in Table 3. Before exposure to the stressor, there was no difference between the treatments groups in any of the behaviours analysed, as shown in Fig. 4 and Table 3.

During the stressor, significant effects and interactions of time and treatment were found by two-way repeated measures and one-way ANOVA. Noise stress significantly induced changes in locomotion (Fig. 4A; $p < 0.001$), sitting (Fig 4B; $p < 0.001$) and risk assessment (Fig 4C; $p < 0.001$), respectively, indicating changes in the behavioural pattern over time. Analysis of interaction effect showed that in the 40% animals, the stressor induced significantly less locomotion ($p < 0.001$) and risk assessment ($p = 0.04$) compared to vehicle, indicating less total activity ($p < 0.05$); Table 3). Indeed, the duration of those behaviours after the onset of the stressor was shorter in 40% corticosterone animals compared to vehicle and 100% corticosterone animals as the percentage of time spent dropped almost to zero 2-3 min after onset of the stressor (Fig. 4A and C). In contrast, in the 100% group there was an increase in risk assessment ($p = 0.03$), number of digging events ($p < 0.01$) and a trend of

increased rearing events (p =0.1). However, in these animals there was no difference in the total activity compared to vehicle animals (p > 0.05; Table 3) indicating differences in the make up of the activity pattern compared to vehicle animals (Fig. 4 and Table 3). Furthermore for both corticosterone-treated groups, there was a decrease in the percentage of time spent face and body grooming during the stressor (Table 3; p < 0.05).

After cessation of the stressor, both 40% and 100% corticosterone animals continued to show decreased face grooming (Table 3; p < 0.01). No statistical significant differences were found in any of the other behaviours analysed.

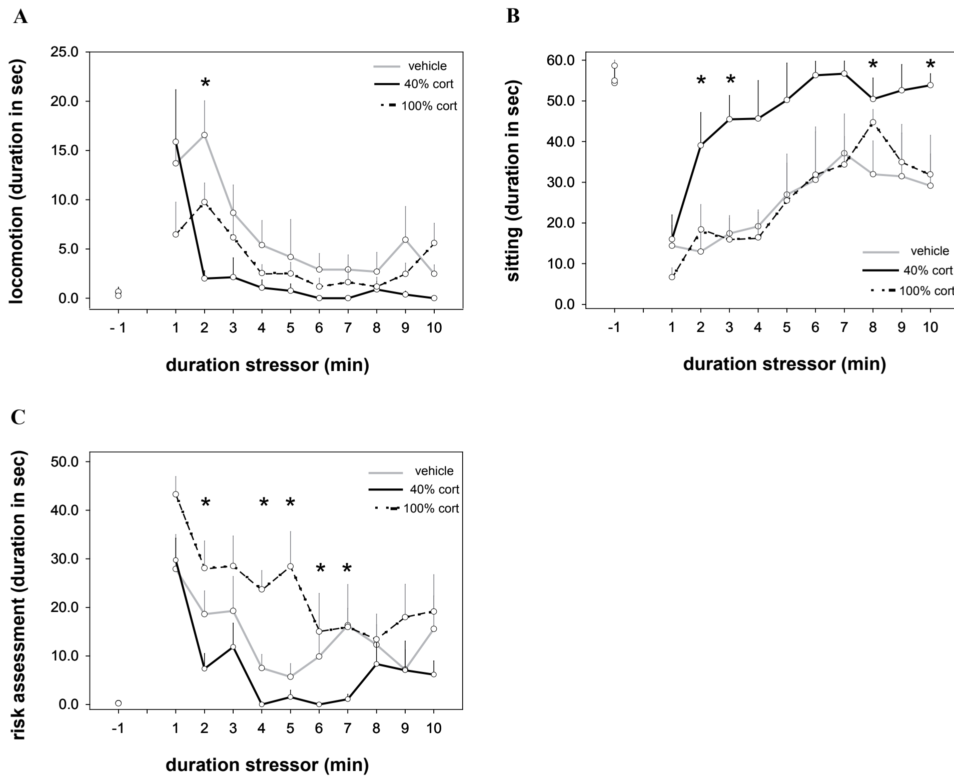

Figure 4 | Behavioural responses to 10 min of noise stress (99 dB). Behavioural changes to noise stress are presented in 1 min bins over time in A | locomotion ($F(10, 60) = 13.4$; $p < 0.001$), B | sitting ($F(10,60) = 12.3$; $p < 0.001$) and C | risk assessment (($F(3.7, 22.0) = 17.9$; $p < 0.001$). Analysis of interaction effect showed that in the 40% animals, the stressor induced significantly less locomotion ($F(20, 120) = 2.8$; $p < 0.001$) and risk assessment ($F(20, 120) = 1.7$; $p = 0.04$) compared to vehicle, indicating less total activity. Data is presented as mean ± SEM duration in sec per 1 min bin for vehicle (n=6), 40% (n=7) and 100% corticosterone pellet animals (n=7). * p<0.05 compared to vehicle as tested by two-way repeated measures ANOVA and Bonferroni's post-hoc test.

Table 3 | Behavioural analysis before, during and after noise stress

	Before stress (10 min)			During stress (10 min)			After stress (10 min)		
	vehicle	40% cort	100% cort	vehicle	40% cort	100% cort	vehicle	40% cort	100% cort
Sitting (% time)	90.6 ± 4.1	91.6 ± 2.2	97.7 ± 1.8	41.9 ± 8.3	71.4 ± 8 *	43.3 ± 6.3	58.3 ± 7.0	77.7 ± 9.7	83.8 ± 5.6
Total activity (% time)	9.4 ± 4.1	8.4 ± 2.2	2.3 ± 1.8	58.1 ± 8.3	28.6 ± 9 *	56.7 ± 6.3	41.7 ± 7.0	22.2 ± 9.7	16.2 ± 5.6
Locomotion (% time)	1.0 ± 0.5	1.1 ± 0.5	0.4 ± 0.3	10.9 ± 2.4	3.8 ± 1.2*	7.1 ± 1.5	2.3 ± 0.7	0.7 ± 0.5	0.9 ± 0.4
Risk assessment (% time)	0.5 ± 0.3	0.4 ± 0.3	0.4 ± 0.4	27.9 ± 7.4	12 ± 3.1 *	42 ± 6.0 *	7 .3 ± 3.4	10.0 ± 5.7	6.3 ± 2.9
Face washing (% time)	1.7 ± 1.1	1.9 ± 0.7	0.2 ± 0.2	17 ± 2.6	3.2 ± 1.7#	6.6 ± 1.6*	17.6 ± 4.1	4.8 ± 1.8#	4.0 ± 1.9#
Body grooming (% time)	6.2 ± 3.0	3.8 ± 1.1	1.3 ± 1.0	6.0 ± 2.2	0.7 ± 0.7*	1.0 ± 0.4*	8.2 ± 2.0	5.2 ± 3.0	2.1 ± 1.3
Rearing (events)	0 ± 0	0 ± 0	0.6 ± 0.6	7.5 ± 2.1	3.7 ± 1.8	10.3 ± 2.4	0.5 ± 0.3	0.4 ± 0.3	0.3 ± 0.3
Digging (events)	0.3 ± 0.3	1.3 ± 1.0	0 ± 0	0.2 ± 0.2	2.0 ± 1.0	7.5 ± 2.1	4.5 ± 2.5	1.6 ± 1.4	1.1 ± 0.9

One-way ANOVA and Tukey's post-hoc test (* $p < 0.05$; # $p < 0.01$ compared to vehicle) indicate differences in stress responsiveness in both the 40% and 100% corticosterone animals compared to vehicle animals. Data represent mean ± SEM of behavioural parameters scored per 10 min bins before during and after the stressor of vehicle (n=4), 40% (n=6) or 100% corticosterone pellets (n=6).

Discussion

In this study we demonstrated that constant exposure to exogenous corticosterone by means of subcutaneous pellet implantation in adrenally intact rats not only abolishes diurnal corticosterone rhythmicity but also diminishes rapid ultradian fluctuations. Termination of the constant signal resulted in rapid recovery of ultradian patterns in animals exposed to daily average concentrations (40% corticosterone), but not in animals exposed to high concentrations of corticosterone (100% corticosterone). Paradoxically, while the endocrine response to noise stress in the 40% corticosterone group was also not different compared to vehicle, these animals showed the most substantial changes in the behavioural response to stress.

Validation of model system

Plasma corticosterone concentrations were determined during time frames in which marked differences in diurnal and ultradian corticosterone levels have been well described in normal intact animals (Jasper & Engeland 1991, Atkinson et al. 2006, Spiga et al. 2007). Irrespective of limitations in the number of blood samples collected, our approach using high frequency ABS led to the novel observation of disappearance of ultradian corticosterone pulses after constant glucocorticoid administration while maintaining basal concentrations at constant daily average (40% corticosterone) or pathological concentrations (100% corticosterone). The 40% corticosterone group is of particular interest given that these animals only differed in the pattern from vehicle (e.g. constant vs pulsatile) but not in the total amount of corticosterone exposure (e.g. AUC and mean corticosterone concentrations). Furtermore, this finding confirms the notion that experimentally clamped levels of glucocorticoids can be achieved without adrenalectomy [(Akana et al. 1992, Meijer et al. 1997); provided no stressors are applied]. We conclude that 40% corticosterone pellet implantation can be used to easily manipulate pulse characteristics in intact rats while maintaining physiological glucocorticoid concentrations, whilst 100% corticosterone rather resembles conditions of stress-related disease.

We and others have clamped circadian circulating corticosterone concentrations in this way before (Akana et al. 1985, Akana et al. 1992, Meijer et al. 1997). The HPA axis via a constant negative feedback signal disrupts corticosterone rhythms by adjusting circadian and ultradian trough and peak concentrations resulting in steady-state corticosterone concentrations. In fact, episodic feedback signals are crucial for maintaining ultradian feedforward-feedback oscillitatory activity between the pituitary and adrenal gland (Walker et al. 2010). We have now demonstrated that the current approach is also suitable to study this relationship in more detail.

Normalisation of basal HPA axis activity after corticosterone removal

Previous studies have shown that for some measures of HPA axis reactivity, normalisation of circadian corticosterone in adrenalectomised animals is sufficient (Akana et al. 1988, Jacobson et al. 1988). Even though the current study does not allow us to fully descriminate between ultradian and circadian corticosterone pulsatile patterns in relation to HPA axis reactivity, this study is the first to present data on basal ultradian corticosterone patterns in intact animals. PULSAR analysis indicated rapid progressive normalisation of ultradian pulse characteristics within approximately 6 hours of removal of the 40% corticosterone pellet. This is most likely due to a delayed feedback mechanism suppressing pulse amplitude at earlier time points (Walker et al. 2010), and could also potentially affect ACTH. Limitations in the volume of blood that can be collected via ABS preclude conclusions on basal and stress-induced ACTH levels. However, this issue has been previously adressed in the literature as blunting circadian variation in intact animals by exogenous corticosterone administration attenuates ACTH release in response to stress (Akana et al. 1992). Although we have no information on the stress response, we observed supressed basal ACTH levels in morning and evening before and during corticosterone washout in a separate cohort of animals (data not shown).

The rapid decline of plasma hormone concentrations after 40% corticosterone pellet removal is in line with the normal corticosterone half life of approximately 10 minutes [an important factor in allowing the existence of sharp ultradian peaks (Windle et al. 1998b)]. The results of the 100% corticosterone group are dramatically different. No recovery of corticosterone pulsatility was observed, moreover, a very slow decline in circulating hormone concentrations after removal of the pellet was noticed. A practical explanation for the lack of ultradian recovery could be the formation of a local corticosterone depot in this group of animals as the slow decline did not follow the half life time of corticosterone. Even though the pellets were easily removed at the time of surgery, we noticed a thickening of the skin around the pellet possibly indicating the formation of a depot due to the very high local steroid concentrations. This was not observed in the vehicle and 40% corticosterone groups. In previous studies we did notice a 'normal' decline in corticosterone levels (Sarabdjitsingh et al. 2010), however. Therefore, it seems very likely that the issue in the present study is merely technical and may be attributed to the different sampling methods (tail vein sampling vs automated blood sampling) and/or animal supplier and surroundings (Leiden vs Bristol). The continued high concentrations prevent conclusions on the recovery of corticosterone pulses after washout of constant high concentrations of corticosterone. In the context of sustained stress, or treatment with (synthetic) glucocorticoids, prolonged exposure to high concentrations may actually occur. It would be very interesting to further explore whether ultradian rhythmicity and behavioural responsiveness is eventually restored in the 100% corticosterone group after washout. The issue of normalisation after high

corticosterone exposure therefore merits thorough investigation in future studies. However, for now this group can still be used as a reference for the effects of the 40% pellet on stress responsiveness.

Dissocation in behavioural and neuroendocrine response to noise stress

In agreement with earlier studies (Campeau & Watson 1997, Windle et al. 1998b, Atkinson et al. 2006), noise stress evoked a transient increase in corticosterone release in vehicle treated animals. Alterations in the basal pulse frequency and amplitude are known to be a major factor influencing reactivity to acute stressors (Windle et al. 1998a, Windle et al. 2001, Atkinson et al. 2006). The normalisation of the HPA response to stress in the 40% corticosterone animals is in line with the return of basal pulsatile corticosterone secretion. Although adrenal weight takes longer than 24 hours to recover (Sarabdjitsingh et al. 2010), a finding in line with the decrease in mean corticosterone concentrations and pulse height, we conclude that the activity of the axis in the 40% corticosterone animals seems to be very close to normal at the moment we applied the noise stress. Thus, the half life time of the suppression of HPA axis reactivity that is brought about by 40% corticosterone pellet implantation (Akana et al. 1992), is significantly less than 24 hours. This is reminiscent of the acute effects of mineralocorticoid and glucocorticoid receptor (MR and GR) ligands on the axis, that last in the order of hours (Ratka et al. 1989, van Haarst et al. 1996, Atkinson et al. 2008, Spiga et al. 2008). The absence of a HPA response to stress in the 100% group may be attributed to any negative feedback mechanism, because of the high concentrations of circulating steroid at the time of the stressor.

Interestingly, during the stressor, a strong dissociation between hormonal and behavioural stress responsiveness was observed. Our data indicate longer lasting changes induced by glucocorticoids in the brain even after normalisation of basal HPA axis activity. Others have also demonstrated behavioural changes in aggression and social deficits in animals chronically exposed to low levels of glucocorticoids (Haller et al. 2004). Using the same experimental paradigm of corticosterone pellet implantation as here we recently showed that GR levels and molecular signalling in the brain remain significantly affected after removal of the corticosterone pellets (Sarabdjitsingh et al. 2010). However, our results may also pertain to (m)any neurotransmitter system(s) such as the serotonergic system (Bush et al. 2003), but only few data are available. Flattened corticosterone secretion induced by implanted corticosterone pellets was previously found to decrease 5-HT1A receptor expression exclusively in the rat dentate gyrus (Meijer et al. 1997) and somatodendritic 5HT1A autoreceptors function in the raphe nucleus (Leitch et al. 2003). Functionally, however chronic excess of corticosterone obtained by daily injections of the steroid, attenuated 5HT1A receptor induced membrane hyperpolarisation in hippocampal CA1 neurons without affecting the 5HT1A receptors (Karten et al. 1999). How persistent these effects are however is not known.

With respect to the behavioural performance of high corticosterone animals, which in our hands was remarkably similar to vehicle rats, most available data point to substantial changes in information processing after chronic high corticosterone and stress (Fuchs et al. 2006, Joels et al. 2007). Chronic stress modulates limbic regions such as the hippocampus and amygdala and influences learning and memory in both rodents and humans (Schwabe et al. 2008, Schwabe et al. 2009), most likely due to changes in the neuroarchitecture and plasticity (Bodnoff et al. 1995, Fuchs et al. 2006, Joels et al. 2007). Chronic stress and high glucocorticoid concentrations are known to potentiate emotionality and exploratory behaviour in rodents (Tejani-Butt et al. 1994, Schwabe et al. 2008). In this study, the 40% corticosterone group showed a modest response to audiogenic stimulation in terms of attenuated behavioural activity (i.e. increased sitting) and risk assessment (i.e. reduced exploration). In contrast, the 100% corticosterone group resembles vehicle treated animals more but showed higher emotionality to the stressor with increased activity (i.e. increased locomotion, risk assessment and rearing). The current design allows dissection of these different behavioural aspects of chronic corticosterone exposure and suggests that disrupted pulsatility (40%) is more linked to exploratory behaviour and risk assessment. How persistent these effects are however remains to be determined though previous studies have suggested long-term effects of glucocorticoids on behaviour in rodents and humans (Ratka et al. 1988, Haller et al. 2004, Tiemensma et al. 2010). The duration of disturbed stress responsiveness likely depends on the duration of aberrant exposure. A parametric study of development and duration of changed responsiveness is needed before the validity of our model in relation to long term changes in humans can be estimated.

Retrospectively, it would certainly have been interesting to study behavioural stress responsiveness after 7 days of pellet implantion. However, as we aimed to focus on longer lasting glucocorticoid effects on the brain and possible restoration of ultradian corticosterone patterns we chose to compare the experimental groups to vehicle. Whether the altered stress reactivity in the 40% group after pellet removal is in fact advantageous or detrimental is open to interpretation and may depend on the particular circumstances. Our results suggest however that the history of HPA axis pulsatility of the individual has consequences for the behavioural response to stress, underscoring the diversity in the modulatory actions of pulsatile glucocorticoid release.

Persistence of behavioural effects

In the context of differential responses to an acute stressor, either the history of corticosterone exposure may differ (De Kloet et al. 1998, Sapolsky et al. 2000) or rapid (non-)genomic effects may play a role (Joels et al. 2008a). Ultradian glucocorticoid pulses are translated into consecutive bursts of GR nuclear translocation, DNA binding of GR and transcriptional initiation resulting in 'gene-pulsing' of native transcripts (Conway-Campbell et al. 2007,

Stavreva et al. 2009). In addition to the classic intracellular receptors that mediate genomic responses, there is strong evidence for non-genomic (behavioural) actions of glucocorticoids (Sandi et al. 1996, Mikics et al. 2004, Schwabe et al. 2009), putatively via low affinity membrane-associated MR and GR (Di et al. 2003, Karst et al. 2005). In the 40% group, the profile of corticosterone secretion during the stressor did not differ from control animals, and an explanation involving non-genomic mechanisms therefore hinges on changed responsiveness of non-genomic effects.

While the role or significance of glucocorticoid fluctuations is largely unknown, the evolutionary conservation of pulsatile endocrine systems suggests important biological and clinical consequences. In stress-related disease there are persistent effects of changes in basal pulse characteristics (Deuschle et al. 1997, Hartmann et al. 1997, Young et al. 2004). Our results show a striking parallel with data in humans. Emotional and cognitive effects are still highly prevalent in patients with long-term cured Cushing's disease compared to matched controls indicating possible irreversible effects of prolonged previous glucocorticoid excess [(Tiemensma et al. 2010); submitted]. Washout after corticosterone pellet implantation may therefore constitute a model to study these clinically relevant processes.

In conclusion, our data suggest that HPA axis activity is remarkably sensitive to changes in the pattern of corticosterone exposure and adjusts in a reversible fashion to alterations in this pattern as long as there is no overt hypercorticism. However, the data also indicate that normalisation of HPA activity in response to an acute stressor does not necessarily indicate normal behavioural responses, which may need more time to adapt. We propose that it is the pulsatile pattern, rather than the absolute concentrations, of corticosterone exposure that determines subsequent responsiveness to stress, a notion with obvious implications for understanding the pathogenesis of stress related disease.

Acknowledgements

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Chapter 6

Stress responsiveness varies over the ultradian glucocorticoid cycle in a brain region specific manner

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Abstract

Ultradian oscillations of glucocorticoid hormone secretion are crucial for transcriptional regulation of glucocorticoid responsive genes. Furthermore, there is strong evidence for disruption of normal pulsatile patterns in stress-related clinical disease. It is however not clear how the pulsatile characteristics of this hormone affect physiological responses in the intact individual.

We have addressed this question by artificially creating different patterns of corticosterone in adrenalectomised rats using a recently developed automated infusion system. This allowed us to administer identical amounts of corticosterone either by constant infusion or in hourly pulses. We also varied the pulse amplitude without altering total administered dose by adjusting both the concentration and duration of infused corticosterone. At the end of infusion – and either during the rising or falling phase of our exogenous pulse – animals were exposed to 10 minutes of 99 dB noise stress.

Constant administration of corticosterone resulted in a blunted ACTH response to stress, while pulsatile administration facilitated a brisker response markedly greater in the rising than in the falling phase of a corticosterone pulse. This differential phase-dependent effect was also seen in the behavioural response to noise which was much greater in the rising phase. The c-fos response was both brain nucleus and glucocorticoid pulse amplitude and phase-dependent, clearly showing differential effects of pulsatility on stress-induced activation of different brain circuits.

These data provide strong evidence for a novel mechanism of pattern-dependent stress responsivity in which different brain circuits show differential sensitivity to the phase and amplitude of pulsatile corticosterone exposure.

Introduction

Glucocorticoid hormones, the final products of the hypothalamic-pituitary-adrenal (HPA) axis, are essential for the control of homeostasis and the adaptation to stress (de Kloet et al. 2005, McEwen 2007). In mammals including humans, glucocorticoids are characteristically released in approximately hourly hormone bursts by the adrenal gland (Weitzman et al. 1971, Jasper & Engeland 1991, Windle et al. 1998b, Henley et al. 2009). These rapid ultradian hormone pulses increase in amplitude in anticipation of the active phase providing a circadian release pattern. Superimposed upon these basal endogenous rhythms is the CNS mediated glucocorticoid response to a stressor.

The pulse characteristics of ultradian rhythmicity in glucocorticoids are remarkably plastic and change under different physiological conditions (Lightman et al. 2000). Disorganisation of ultradian rhythmicity has also been described in stress-related disease (Deuschle et al. 1997, Hartmann et al. 1997, Young et al. 2004, Lightman et al. 2008, Henley et al. 2009). It appears that pulse amplitude, frequency and phase are all relevant variables in modulating the acute response of the HPA axis (Windle et al. 1998b, Windle et al. 2001, Atkinson et al. 2006, Russell et al. 2010). Even at the level of the behavioural response there is an impact of changes in ultradian pulse characteristics (Sarabdjitsingh et al. 2009b). This suggests that brain targets and receptors are responsive to the pulsatile patterns that have been recorded in rats for free tissue concentrations of the glucocorticoid hormone corticosterone (Droste et al. 2008).

There is also good in vitro and in vivo evidence that repeated exposure to glucocorticoid pulses results in consecutive waves of glucocorticoid receptor (GR) signalling and eventually 'gene pulsing' of native transcripts (Conway-Campbell et al. 2007, Stavreva et al. 2009). Furthermore, disruption of pulsatile patterns by continuous corticosterone administration attenuates glucocorticoid target gene responses in the rat brain to a subsequent challenge, revealing the sensitivity of receptor-mediated mechanisms to the underlying pattern of hormone presentation (Sarabdjitsingh et al. 2010). A major area that has not been investigated however, is the interaction of glucocorticoid pulsatility with physiological responses to stress.

Recently, a post-hoc analysis during different phases of the endogenous ultradian cycle, has indicated a much greater HPA responsiveness when the stress was applied during the ascending phase of a corticosterostone pulse (Windle et al. 1998b), suggesting either a facilitated stress response during the ascending phase and/or an inhibitory effect during the falling phase. Here we aimed to clarify the importance of the corticosterone pulses themselves by

carrying out a prospective study in which we could control all the glucocorticoid variables, and assess both the neuroendocrine and the behavioural response to a stressor at different phases and amplitudes of a pulse.

In order to achieve this goal, we used our recently developed computer controlled system in which automated steroid infusion is combined with high frequency automated blood sampling in freely moving rats (Lightman et al. 2008). This allows a precise and systematic infusion of corticosterone in either ultradian or constant patterns in adrenalectomised rats without changing the total amount of corticosterone exposure. The current approach provides a unique opportunity to study the functionality of glucocorticoid pulsatility and the consequence of alterations in ultradian pulses for stress and tissue responsiveness in a tightly controlled experimental design.

Experimental procedures

Subjects

Experiments were conducted on male Sprague-Dawley rats (Harlan, UK; ± 250 g at the time of surgery). After arrival, animals were allowed a week acclimatisation to the housing facility at 4 per cage under standard environmental conditions (21 ± 1 °C) and a 14 : 10 h light/dark cycle (lights on 05.15 h). Food and water were provided ad libitum. Animal procedures were approved by the University of Bristol Ethical Review Group. Animal care was conducted in accordance with Home Office guidelines, the UK Animals (Scientific Procedures) Act 1986 and the EC Council Directive of November 1986 (86/609/EEC). All possible efforts were made to minimise the number of animals used and their suffering.

Surgery

Intravenous cannulation of the jugular vein was essentially performed as described before (Spiga et al. 2007). Briefly, animals were anaesthetised with a combination of Hypnorm (0.32 mg/kg fentanyl citrate and 10 mg/kg fluanisone, i.m.; Janssen Pharmaceuticals, UK) and diazepam (2.6 mg/kg i.p.; Phoenix Pharmaceuticals, UK). The right jugular vein was cannulated by inserting a polythene cannula (Portex, UK) modified to contain two thinner cannulae to connect to both the infusion and sampling system simultaneously. During the same surgery, adrenal glands were removed (ADX) by the dorsal approach. Following recovery, animals had free access to food and 0.9% saline supplemented with corticosterone (25 mg/l, Sigma-Aldrich, USA). Animals were individually housed and kept in the automated blood sampling room where the cannulae were attached to a mechanical swivel allowing maximal freedom of movement. The cannulae were flushed daily with heparinised saline to

maintain patency. Twelve hours prior to infusion the corticosterone drinking solution was replaced with 0.9% saline.

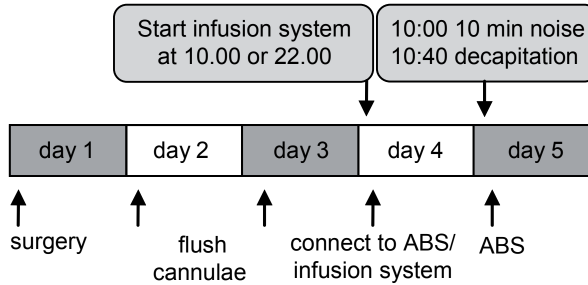
Automated corticosterone infusion and blood sampling

A schematic overview of the experimental design is depicted in Fig. 1. On post-surgery day 4, animals were connected to both the automated blood sampling (ABS) system and automated infusion system (Fig. 1A). One of the double cannulae was used to connect to the ABS system for sampling on day 5 as described in detail (Windle et al. 1997, Windle et al. 1998b). The other cannula was connected to a recently developed automated infusion system that allows ultradian or constant infusion of corticosterone (Lightman et al. 2008). Animals (n = 7 – 14) were infused for 12 hours with either 1) vehicle (3.75 mg/ml 2-hydroxypropyl-β-cyclodextrin (HBC) in 0.9% saline, Sigma-Aldrich, USA), 2) constant corticosterone clamped at 50 ng/ml (0.23 mg/ml corticosterone-HBC in 0.9% saline, Sigma-Aldrich, USA), or in ultradian pulses with an amplitude of 3) 50 ng/ml or 4) 100 ng/ml (0.12 or 0.23 mg/ml corticosterone-HBC, respectively, Fig. S1B). All groups were infused with the same amount of corticosterone to maintain equal exposure (area under the curve). Accordingly, infusion time was adjusted from 12 to 24 hours in the group receiving 50 ng cort pulses (Fig. 1B). Frequency of pulsatile infusion was 60 min with 20 min on-rate and 40 min off-rate. Infusion rate was 0.33 ml/hour for constant infusion and 1 ml/hour for pulsatile infusion during the on-rate. Animals remained undisturbed in their home cages during infusion, blood sampling and exposure to white noise.

Experimental design

Stress responsiveness in the different infusion groups (n = 7 – 14) was determined by activation of a white noise generator in the blood-sampling room. At 10.00 on post-surgery day 5, animals were exposed to 99 dB for a fixed period of 10 min. During the same time animal home cage behaviour was recorded using cameras mounted above each individual cage. Stress was applied 3 min after onset of either the rising or falling phase of the last ultradian pulse in both the 50 ng and 100 ng corticosterone groups or simultaneously in the constantly infused vehicle and corticosterone animals (Fig. 1B). Blood samples (25 µl aliquots) were collected remotely using the ABS system at a dilution of 1:8 in heparinised saline. Relative to the onset of the stress, samples were collected at time points -9, -2, 5, 12, 19, 26 and 33 min in chilled tubes containing EDTA and Trysalol inhibitors. Animals were sacrificed 30 min after termination of the stressor. Brains and pituitary glands were rapidly collected and frozen on dry ice.

A Schematic overview of experimental design



B Schematic overview of infusion protocols (day 4-5)

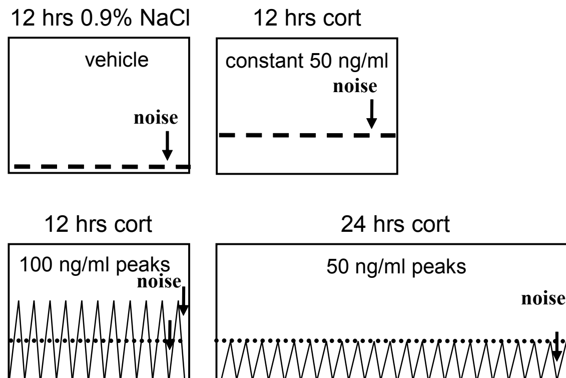


Figure 1 | Schematic representation of the experimental design. A | On day 4 infusion of corticosterone or vehicle started either 12 or 24 hours prior to stress. At 10.00 hr on day 5, animals were exposed to 10 min of noise stress (99 dB) and were decapitated 30 min after. Blood samples were automatically collected with the ABS before, during and after noise. B | Animals were infused for 12 hours with vehicle (0.9% saline), constant corticosterone clamped at 50 ng/ml, 100 ng/ml pulsatile infusion or 50 ng/ml pulsatile infusion. In the last group infusion time was increased to 24 hours to maintain an equal area under the curve between all treatment groups. Noise stress (indicated by the arrows) was applied 3 min after onset of a rising or falling phase in the pulsatile infused groups or simultaneously in the constantly infused groups.

Hormone measurements

Blood samples were centrifuged for 15 min at 4000 rpm at 4 °C. Plasma was stored at -80 °C until assayed with commercially available kits. ACTH levels were determined via immunoradiometric assay (IRMA; Diasorin Ltd, UK) and corticosterone via radioimmunoassay (RIA; MP Biomedicals Inc., USA) according to the manufacturer's instruction. All samples were processed in the same assay to exclude inter-assay variability. Intra-assay coefficients of variation were 2.8% and 7.3% for the ACTH and corticosterone assay, respectively.

Tissue preparation and in situ hybridisation

Coronal sections of PVN, hippocampus, amygdala (14 µm) and pituitary (8 µm) were mounted on Superfrost Plus slides (Menzel-Gläser, Germany). In situ hybridisation with 33Phosporus and 35Sulphur end-labelled oligonucleotide and riboprobes were used to visualise mRNA expression of c-fos, CRH, POMC, MR and GR as previously described (Rots et al. 1996, Karssen et al. 2005). The signal was quantified from films exposed 4 hr – 12 days (X-OMAT AR, Kodak, NY) using ImageJ 1.32j analysis software (NIH, USA; http://rsb.info.nih.gov/ij/). Relative expression levels were determined and related to standard curves of 14C (RPA 504 microscales, Amersham, UK).

Behavioural analysis

Home cage behaviour was analysed 10 min before, during and after stress. The following behaviours were analysed with a semiautomatic scoring system (The Observer Mobile 4.1, Noldus Information Technology, The Netherlands): i) risk assessment ('seeking' behaviour while the body is still or stretched), ii) face washing (grooming of face using front paws), iii) body grooming (grooming using front and back paws, i.e. scratching), iv) locomotion (walking, front and back paws move) and v) sitting (e.g. sitting or sleeping). Total activity was calculated by adding the scores of behaviours i-iv. In addition vi), the number of rearing events (raising of the rat and then lowering both front paws) was counted. Freezing was also included as behavioural variable. However, this type of behaviour was not observed in this study and therefore not included in further analysis.

Statistical analysis

Statistical analyses were performed using SPSS 16.0 (SPSS Inc., Chicago, IL, USA). All data are expressed as the mean ± SEM. For each study the appropriate tests and post-hoc tests, where applicable, were used as indicated in the corresponding figure legends. P-values < 0.05 were considered statistically significant.

Results

Effect of infused corticosterone on stress-induced ACTH release

To study the effect of stress on different patterns of infused corticosterone on ACTH release, animals were exposed to noise stress while blood samples were collected automatically (Fig. 2). Noise stress evoked a transient increase in ACTH release with hormone peaks 5 min after the onset of the stressor in all treatment groups (Fig. 2A; effect of time: $p < 0.001$). Corticosterone infusion attenuated basal as well as (absolute and relative) stress-induced ACTH levels to noise at all time points after onset of the stressor compared to vehicle infusion (Fig. 2A and B, $p < 0.001$). The response of ACTH, however, was much greater in the pulsatile infused animal compared to those receiving constant infusion (interaction effect: $p < 0.001$). Interestingly, basal ACTH levels were not affected by the pattern of corticosterone infusion itself (Fig. 2A).

In the animals receiving the pulsatile infusions, the ACTH response to stress depended both on the phase and the amplitude of the corticosterone pulses. Stressors applied during the falling phase of a corticosterone pulse resulted in significantly lower ACTH responses, both for the 50 ng and the 100 ng infused rats (Fig. 2C; $p < 0.001$). In addition, higher amplitude corticosterone peaks (100 vs. 50 ng infusion) led to a lower ACTH response ($p < 0.001$). Similar results were obtained by calculating the relative increase in ACTH from baseline (Fig. 2D).

Markers for central HPA axis activity

In order to determine whether there were basal hypothalamic or pituitary changes underlying the differences in stress responsiveness we measured the expression of a number of markers. Corticosterone infusion decreased CRH mRNA levels in PVN (Fig. S1A; $p = 0.02$) and POMC mRNA in pituitary (Fig. S1B; $p = 0.03$) relative to ADX vehicle infused rats, but this effect was independent of the pattern or phase of corticosterone infusion.

Both MR and GR mRNA expression in the hippocampal CA1 area were suppressed by corticosterone infusion relative to vehicle treated rats (Fig. S2A and B, $p < 0.01$). However, in comparison with constant cort and 100 ng cort infused animals, there was a slight but significant higher ($p < 0.05$) expression of MR and GR for both 50 ng infused groups. Similar patterns were found for MR in dentate gyrus ($p < 0.05$) but not in CA3 (Fig. S2C and E). GR mRNA expression levels were suppressed uniformly across corticosterone infusion groups in CA3 and dentate gyrus (Fig. S2D and F). In addition, there were no significant changes in GR expression in the PVN (Fig. S2G, $p = 0.7$) or pituitary (Fig. S2H, $p = 0.16$) related to the pattern or phase of corticosterone infusion.

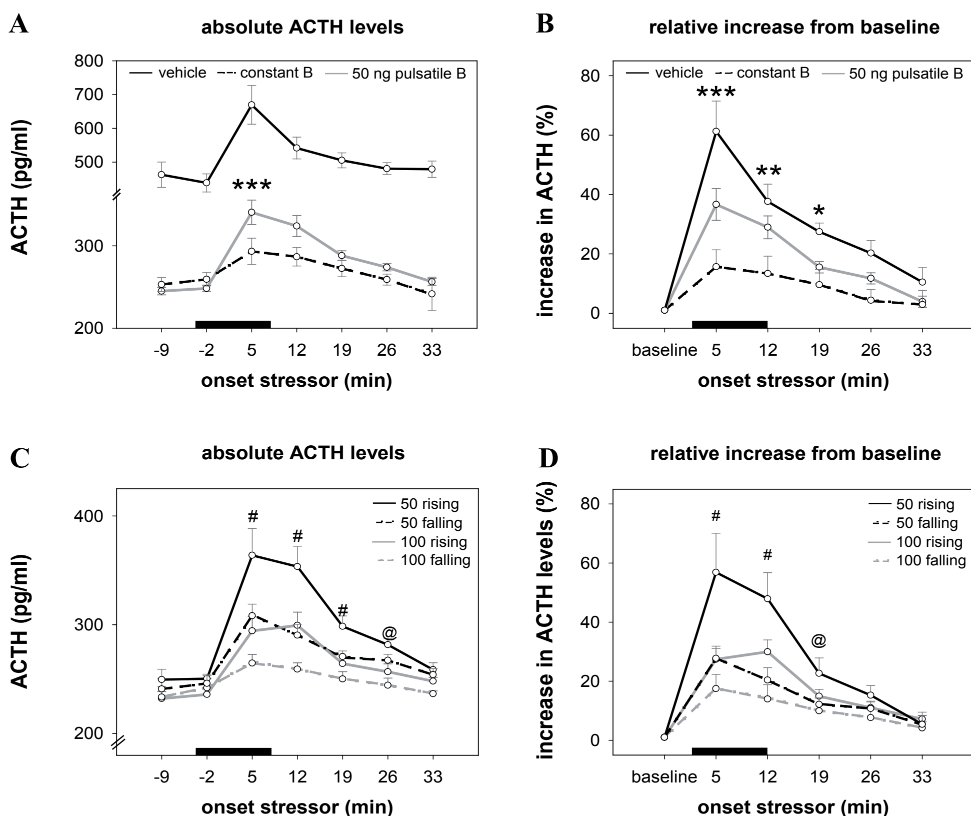
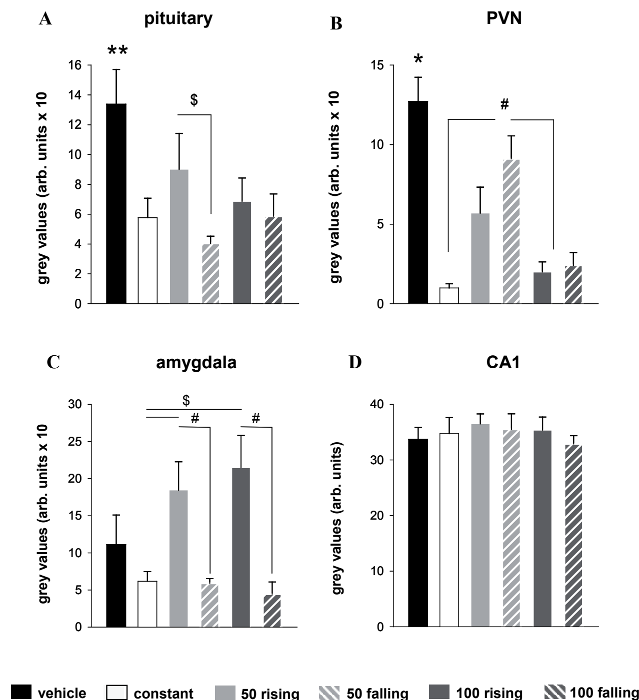


Figure 2 | Basal and noise stress-induced ACTH levels. A | 10 min noise stress evoked a transient increase in absolute ACTH levels in vehicle, constant or pulsatile corticosterone (50 ng cort pulses) infused animals (effect of time: $F(6, 180) = 12.93$, $p < 0.001$). Constant corticosterone infusion attenuates stress-induced ACTH compared to pulsatile infusion at 5 min after onset of the stressor (interaction effect: $F(10, 180) = 2.52$, $p < 0.01$). B | Similar but more pronounced differences were demonstrated by analysing the relative increase in ACTH from baseline (time: $F(5, 157) = 28.47$, $p < 0.001$; interaction effect: $F(10, 157) = 3.24$, $p < 0.001$). C | Stress applied 3 min after onset of the different phases of the last pulse resulted in increased ACTH release (time: $F(6, 183) = 226.5$, $p < 0.001$). However, stress-induced ACTH release in the falling phase was lower compared to the rising phase (interaction effect: $F(18, 183) = 18.25$, $p < 0.001$). D | Data plotted as relative increase from baseline showed similar results (time: $F(5, 158) = 241.2$, $p < 0.001$; interaction effect: $F(15, 158) = 21.46$, $p < 0.001$). Data is analysed by two-way RM ANOVA and Bonferonni's post-hoc test (*** $p <0.01$, ** $p < 0.05$ all groups; * $p < 0.05$ vehicle vs constant; # $p < 0.001$ 50 rising vs 50 ng falling, 50 ng rising vs 100 ng rising, 100 ng rising vs 100 ng falling; @ $p < 0.05$ 50 ng rising vs 50 ng falling, 50 ng rising vs 100 ng rising; n = 7-14).

Immediate early gene c-fos expression in different brain regions

To evaluate the effects of different pulse characteristics on neuronal responses to stress, c-fos mRNA was measured. Region-specific differences in c-fos expression were found in the pituitary gland (Fig 3A, $p < 0.01$), PVN (Fig. 3B, $p < 0.001$), amygdala (Fig. 3C, $p < 0.01$) and hippocampal subfield CA1 (Fig. 3D, $p = 0.54$). Representative images of c-fos mRNA expression in the corresponding areas are depicted in Fig. S3.

In the pituitary (Fig. 3A), c-fos expression in vehicle infused animals was significantly increased compared to all the corticosterone infused groups ($p < 0.01$), except the 50 ng rising group. In the 50 ng cort infused animals c-fos levels were markedly higher after stress exposure during the rising phase ($p = 0.03$). We observed the opposite in the PVN (Fig. 3B): with lower c-fos expression during the rising phase of 50 ng cort infused animals ($p < 0.05$). In both PVN and pituitary, we found decreased c-fos mRNA expression as a consequence of increased cort amplitude (veh vs 100 ng cort pulses, $p < 0.01$). c-Fos expression in the amygdala was markedly higher in rats that were stressed during the rising phase of the pulse, independent of its amplitude (Fig. 3C; $p < 0.01$), while no significant difference in c-fos expression between vehicle and constant cort infused was observed. No effect of corticosterone treatment or phase was observed in c-fos expression in the CA1 region (Fig. 3D, $p = 0.54$), hippocampal subregions CA3 (Fig. S4A, $p = 0.47$) and dentate gyrus (Fig. S4B $p = 0.56$).



< Figure 3 | C-fos mRNA expression in different brain regions. Differential c-fos expression patterns were found in A | pituitary gland ($F(5,31) = 3.76$; $p < 0.01$), B | PVN ($F(5,34) = 14.6$; $p < 0.001$) and C | amygdala ($F(5,33) = 6.0$; $p < 0.01$) but not in D | hippocampal CA1 cells ($F(5,38) = 0.30$; $p = 0.54$). Data represents mean ± SEM. One-way ANOVA and LSD or Tukey's post-hoc test, * $p < 0.05$, ** $p < 0.01$ veh vs other groups, # $p < 0.01$, $ $p < 0.05$ (n = 7-8).

Behavioural response to noise stress

Before and after the stressor, the animals did not differ in total activity (Fig. 4A and S5A). During stress, however, the behavioural response of the 50 ng corticosterone animals was phase-dependent as noise stress increased total activity but to a lesser extent in animals stressed during the falling phase (Fig. 4A; $p < 0.001$).

Detailed analysis of recordings during the stressor using interaction analysis of the individual behavioural parameters indicated significantly higher risk assessment in animals stressed during the rising phase of a 50 ng ultradian pulse, particularly in the initial minutes after onset of the stressor (Fig. 4B; $p < 0.01$). In contrast, animals stressed during the falling phase explored less throughout the stress period. Similar results were found for rearing, face and body grooming (Fig. S5B and C).

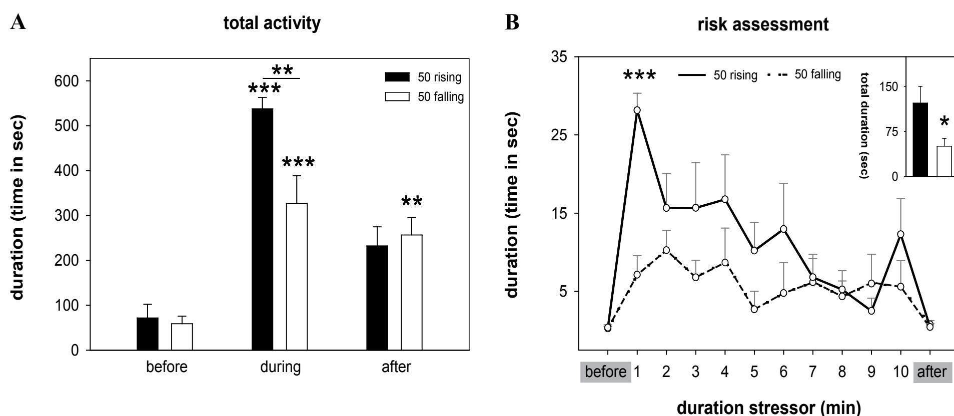


Figure 4 | Behavioural analysis to noise stress. Noise stress evoked a transient increase in A | total activity ($F(5, 38) = 21.3$; $p < 0.001$) but significantly more in 50 ng corticosterone infused animals stressed during the rising (black bars) compared to the falling phase (open bars). No difference was found before or after the stressor. Data is expressed in 10 min bins before, during and after the stressor (n = 7). B | Similarly, behavioural activity plotted in 1 min bins during the stressor shows increased risk assessment in 50 ng cort infused animals stressed on the rising (interaction effect: $F(11, 132) = 2.6$; $p < 0.01$). Inset indicates total duration per 10 min during the stressor. Two-way ANOVA and Tukey's post-hoc test and two-way RM ANOVA and Bonferroni's post-hoc test, n = 7, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

Discussion

The concept that rapid fluctuations in endocrine systems are major determinants for the outcome of target tissue responses and physiology has been well explored for GnRH and growth hormone (Belchetz et al. 1978, Hauffa 2001, Veldhuis et al. 2001). It is now increasingly being recognised that episodic, pulsatile glucocorticoid release is also crucial for hormone signalling in the brain and other tissues (Conway-Campbell et al. 2007, Stavreva et al. 2009, Sarabdjitsingh et al. 2010). However, practical limitations in regulating rapid ultradian pulses in living animals have, until now, hampered progress in obtaining evidence for the physiological importance of glucocorticoid pulsatility. The advanced automated steroid infusion system used in this study overcomes these limitations and provides the facility to ensure the tight control of infusion of different ultradian patterns of corticosterone (Lightman et al. 2008). This technical development has opened up the opportunity to examine how corticosterone pulsatility might interact with stress responsiveness. In the current study we have been able to investigate the contribution of individual ultradian glucocorticoid pulse characteristics such as pattern, phase and amplitude in a controlled fashion to stress responsivity with a dose that is similar to the normal physiological mean 24 hour corticosterone secretion rate. Without altering the total administered dose we either gave a constant infusion or varied the pulse amplitude by adjusting both the concentration and duration of infused corticosterone. At the end of infusion – and either during the rising or falling phase of our exogenous pulse – animals were exposed to 10 minutes of 99 dB noise stress.

This design revealed that the pattern of corticosterone exposure had a powerful effect on ACTH responsivity to the noise stressor while no differences in basal ACTH levels prior to stress were observed. Constant infusion of corticosterone resulted in a clear suppression of ACTH response, while pulsatile infusion of the same dose of glucocorticoid permitted a larger response. This sensitising effect of pulsatility was even more pronounced during the rising phase of an ultradian pulse than during the falling phase. In addition, animals infused with higher amplitude pulses showed smaller increments in stress-induced ACTH release. These data thus clearly show that the neuroendocrine response to stress depends on circulating glucocorticoids in both a phase and amplitude dependent manner.

Our data explain previous studies that retrospectively correlated higher corticosterone responses to a stressor with the rising phase of an endogenous pulse and lower mean corticosterone responses with increased pulse frequency (Windle et al. 1998b, Windle et al. 2001). Other data suggest however that negative feedback of glucocorticoids should be greatest when corticosterone levels are rapidly rising within physiological concentrations in a process termed rate sensitive feedback (Dallman & Yates 1969, Kaneko & Hiroshige 1978). Whether the observed phase-dependence of the ACTH response is due to a delayed

inhibition after the rapid increase of corticosterone or possibly the facilitation of ACTH responsiveness during the ascending phase, cannot be answered from the present studies as we could not explore the apparent rate sensitivity in our studies using multiple physiological pulses of corticosterone rather than single infusions. What is clear from our design however, is that the onset of a stressor in relation to the phase or amplitude in a regimen of repeated ultradian pulses, is a major determinant of acute ACTH responsiveness and negative feedback inhibition. As constant corticosterone administration attenuates stress-induced ACTH release, every surge in glucocorticoid level could serve to prepare and maintain flexibility in ACTH release for adequate responses to stress.

In the current study, c-fos immediate early gene expression was used as a stressor-specific marker for activation of pituitary and brain pathways involved in the stress response. Since the pattern of corticosterone infusion only had an effect on ACTH release during noise stress and not under basal conditions, we studied c-fos expression following this stressor and clearly show selective activity of the pituitary and different neuronal pathways which depend on temporal aspects of the ultradian cycle.

The c-fos data in the pituitary gland suggest an interaction of pulse phase and amplitude and resembles the pattern of stress-induced ACTH release. Further interpretation is precluded by the fact that ACTH release is not always reflected in c-fos expression, if only because of the different time domains in which the responses develop (Ginsberg et al. 2006, Weinberg et al. 2007). Despite this, corticosterone clearly feeds back on the HPA axis imposing similar phase and amplitude dependence of the ACTH and c-fos response to stress-induced signalling, probably via pituitary and supra-pituitary sites (Windle et al. 1998a, Russell et al. 2010).

Our observations in the PVN also demonstrate interaction of pulse phase and amplitude in c-fos expression. The role of the PVN as ultradian feedback integrator is complicated. CRH neurones are not only themselves glucocorticoid sensitive but are also heavily innervated by networks of both inhibitory GABAergic and excitatory glutamatergic synaptic inputs from different brain nuclei, such as the amygdala and prefrontal cortex, that are under the influence of corticosterone (Herman et al. 2003, Herman et al. 2004). These various stress pathways and the integrated feedback signal produced by ultradian exposure together determine PVN outflow and are likely to contribute to the different pattern of c-fos activation found in this nucleus. The hippocampus, which is also regarded as a major input and exerts a trans-synaptic, primarily inhibitory, influence on the PVN (Jankord & Herman 2008), is different again, and shows no differential c-fos response to either acoustic stimulation or corticosterone pulsatility. Acoustic stimulation typically results in a wide pattern of brain activation including specific brain circuits involved in the stress response to noise but is known induce low c-fos responses in the hippocampus (Campeau & Watson 1997, Burow et al. 2005).

The most fascinating c-fos response was seen in the amygdala. This nucleus is of prime importance in the response to emotionally salient stimuli, with arousing experiences able to modulate amygdala structure and function (McGaugh 2004, Roozendaal et al. 2009). Of particular relevance to the current studies is its involvement in the behavioural responses to noise (Campeau & Watson 1997, Burow et al. 2005). We have not only been able to demonstrate a correlation between behaviour response and c-fos activation, but have now clearly shown for the first time that this is tightly related to the phase of the ultradian corticosteroid cycle.

An important aspect of these studies is that in addition to c-fos and neuroendocrine responses, there are also behavioural correlates to the amplitude and phase of the ultradian corticosterone pulses. We find that behavioural activity was highest when the noise stress was experienced during the rising phase of corticosterone infusion. These data fit well with the work of, Haller et al. observed, following post hoc evaluation of their results, that the propensity of rats to behave aggressively was increased during the rising phase of an ultradian pulse (Haller et al. 2000a). These data strongly suggest differences in reactivity of emotional brain centres, such as the amygdala, and that the emotionality and exploratory response to stress could be modulated by rapid fluctuations in corticosterone. The temporal correlations of the stress induced behavioural pattern of ACTH release and c-fos expression in the amygdala, suggests some glucocorticoid mediated integration, although the detailed mechanism of the links needs further evaluation. What we can conclude however is that both the neuroendocrine and behavioural response to stress vary over the ultradian cycle with hourly surges in corticosterone resulting in a more intense response to stress.

The mechanism underlying HPA pulsatility is itself a fascinating question with recent evidence suggesting a pituitary-adrenal oscillatory mechanism dependent on hypothalamic gain (Walker et al. 2010). The current study provides a framework to understand the mechanism(s) underlying the ultradian phase dependent changes in sensitivity of stress responsiveness. Logically one can assume that this is either related to an increased break during the falling phase or increased drive during the rising phase or indeed a combination of both of these. With respect to feedback effects that might be part of the explanation of our data, recent evidence suggest that centrally anti-mineralocorticoids can interfere with the dynamic generation and regulation of basal ultradian HPA activity and fast feedback in the rat (Atkinson et al. 2008), GR however is involved at the pituitary level and is likely to be responsible for recent data demonstrating the rapid inhibition of ultradian pulsatility by glucocorticoids (de Kloet et al. 1974, De Kloet et al. 1975, Russell et al. 2010).

Which ever of these is the base, the time domain of the altered efficacy of pulsatile corticosterone in control of ACTH release and behavioural stress responsiveness, suggests that rapid non-genomic effects play a role, although there is also increasing evidence for rapid

genomic responses to glucocorticoids (Stavreva et al. 2009). These non-genomic effects are thought to be mediated via putative membrane-bound variants of MR and GR (Hinz & Hirschelmann 2000, Karst et al. 2005). The 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 (Olijslagers et al. 2008). On the other hand, non-genomic GR in PVN reduces net neuronal excitatory transmission via endocannabinoids and nitric oxide (Di et al. 2003, Di et al. 2009). The balance between rapid non-genomic and slower genomic effects via membrane-bound and nuclear variants of MR and GR (De Kloet et al. 1998) may rapidly change over the duration of a single ultradian pulse and mediate differential modulatory effects of corticosterone in different areas of the brain. Studies in modulating different aspects of the pulse frequency as well as pulse peak and nadir, should allow us to address such mechanistic issues in the future and in particular help explain the enhancement and suppression of c-fos responses during the ascending and descending phases of the corticosteroid pulse seen most markedly in the amygdala.

Although ultradian activity of the HPA axis has been recognised for many years, it is only recently that we have become aware of the critical importance of rapid ultradian oscillations in glucocorticoid hormone exposure for transcriptional control and target tissue responsiveness (Conway-Campbell et al. 2007, Stavreva et al. 2009, Sarabdjitsingh et al. 2010). We have been able to demonstrate in the ADX corticosterone infused rat that the cyclical alterations in HPA responses described by Windle et al in intact rats (Windle et al. 1998b), as well as cyclical behavioural responses, are actually a consequence of the changing levels of circulating glucocorticoid – and indeed this is the first evidence for functional consequences of ultradian glucocorticoid pulsatility. This will be important not only for our understanding of glucocorticoid biology and signalling but probably also for the elucidation of the pathophysiology and aetiology of stress-related disorders (Dallman et al. 1987, de Kloet et al. 2005, Herbert et al. 2006).

Furthermore, the reduced ACTH and behavioural responses following continuous administration of glucocorticoids, serves as a warning to both experimental neuroendocrinologists and clinicians that steady state glucocorticoid levels are likely to result in unphysiological effects. For optimal function it seems that ultradian activity “primes” the HPA axis and different brain circuits through rapidly alternating feed forward and feedback modes providing “resilience” to the fundamentally important stress response.

Acknowledgements
This research was supported by NWO Mozaïek, NWO-IRTG DN95-420 and the Royal Academy of Arts and Sciences (KNAW). We gratefully acknowledge Yvonne Kershaw, Merv McKenna, dr. Francesca Spiga, Servane Lachize and Ans Tijssen for technical assistance.

Supplemental data

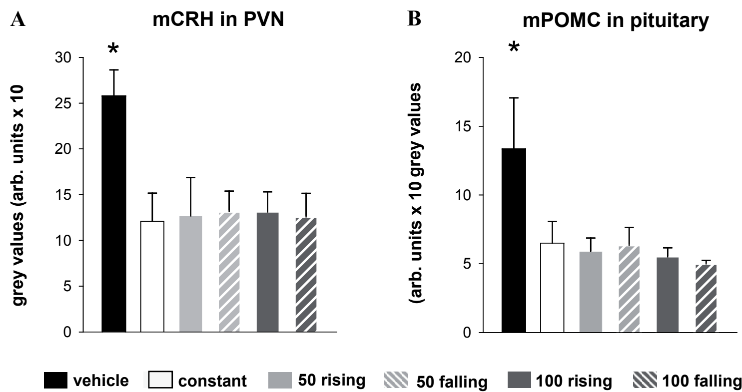


Figure S1 | mRNA expression of central HPA axis markers. Compared to vehicle, corticosterone infusion significantly lowered A | mCRH expression in PVN ($F(5,33) = 3.2$; $p = 0.02$) and B | mPOMC expression in the pituitary gland ($F(5,31) = 3.0$; $p = 0.03$). One-way ANOVA and Tukey's post-hoc test, * $p < 0.05$ veh vs other groups (n= 7-8).

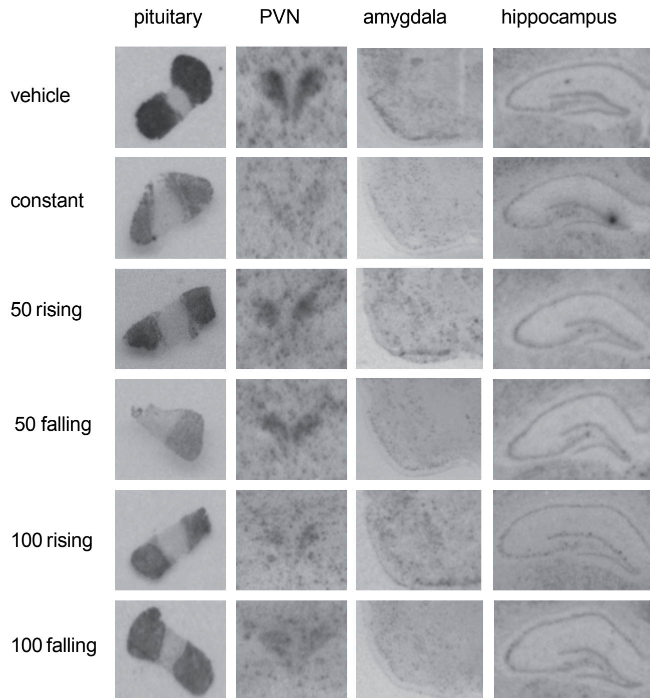


Figure S3 | C-fos mRNA expression in different brain regions. Representative images of c-fos in situ hybridisation per experimental group are depicted for pituitary gland, PVN, amygdala and hippocampus.

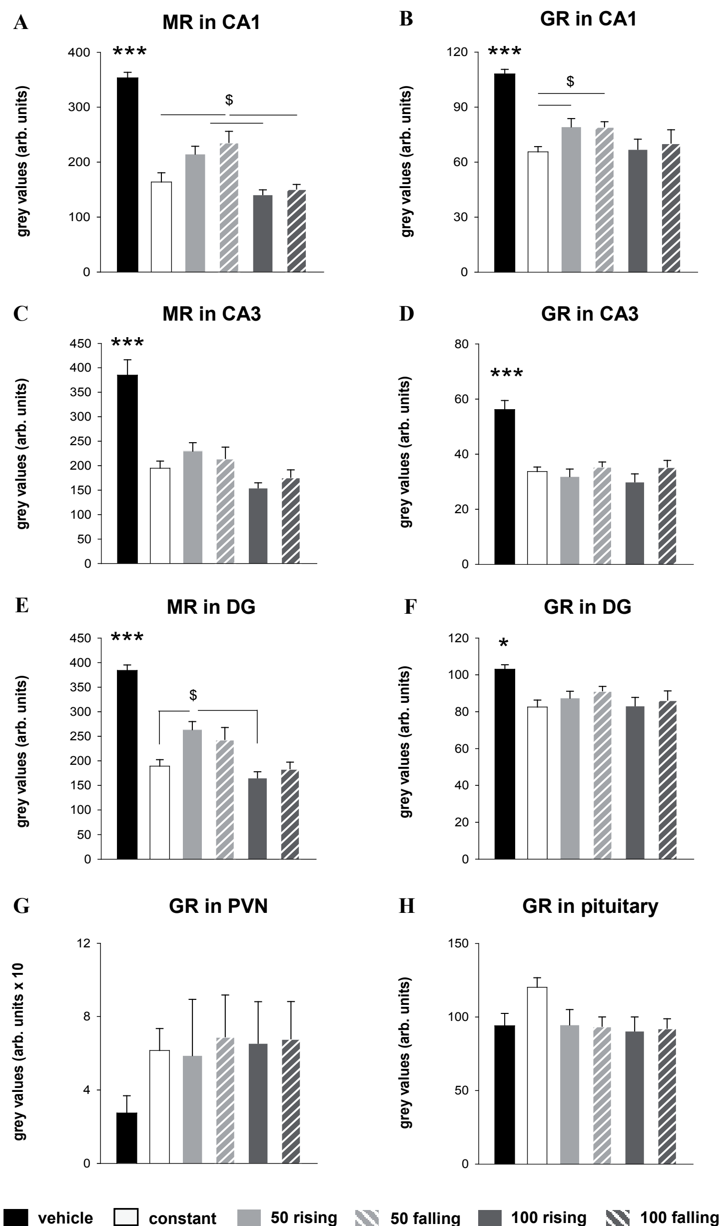


Figure S2 | MR and GR mRNA expression in different brain regions. Compared to vehicle infused animals, corticosterone infusion significantly lowered MR expression in A | hippocampal CA1($F(5,33) = 35.9$; $p < 0.001$), C | CA3 ($F(5,36) = 17.4$; $p < 0.001$) and E | DG ($F(5, 34) = 28.5$; $p < 0.001$. Similar results were found for GR expression in B | CA1 cells ($F(5,33) = 14.1$; $p < 0.001$), D | CA3 ($F(5,38) = 14.0$, $p < 0.001$) and F | DG ($F(5,34) = 4.30$; $p < 0.01$. Interestingly, MR and GR expression in CA1 and MR in DG was significantly increased in animals that were pulsatile infused with 50 ng corticosterone peaks. No difference in GR expression due to treatment or phase was observed in G | PVN ($F(5,30) = 0.60$; $p = 0.7$) or H | pituitary ($F(5,34) = 1.7$; $p = 0.16$). One-way ANOVA and Tukey's post-hoc test, *** $p < 0.001$ veh vs other groups, $ $p < 0.05$ vs 50 ng cort infusion (n= 7-8).

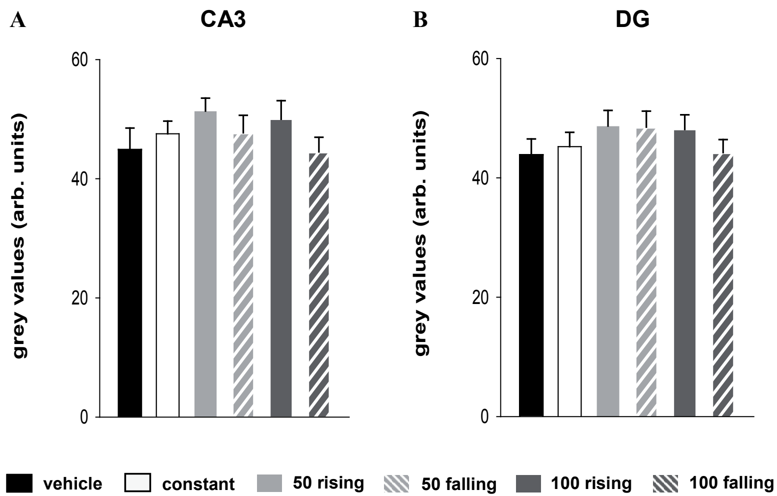


Figure S4 | C-fos mRNA expression in different regions of the brain. Infusion of vehicle or corticosterone did not differentially affect noise stress-induced c-fos expression in hippocampal subfields A | CA3 ($F(5, 37) = 0.94$; $p = 0.47$) or B | dentate gyrus ($F(5,37) = 0.80$; $p = 0.56$). Data was statistically analysed with one-way ANOVA (n = 7-8).

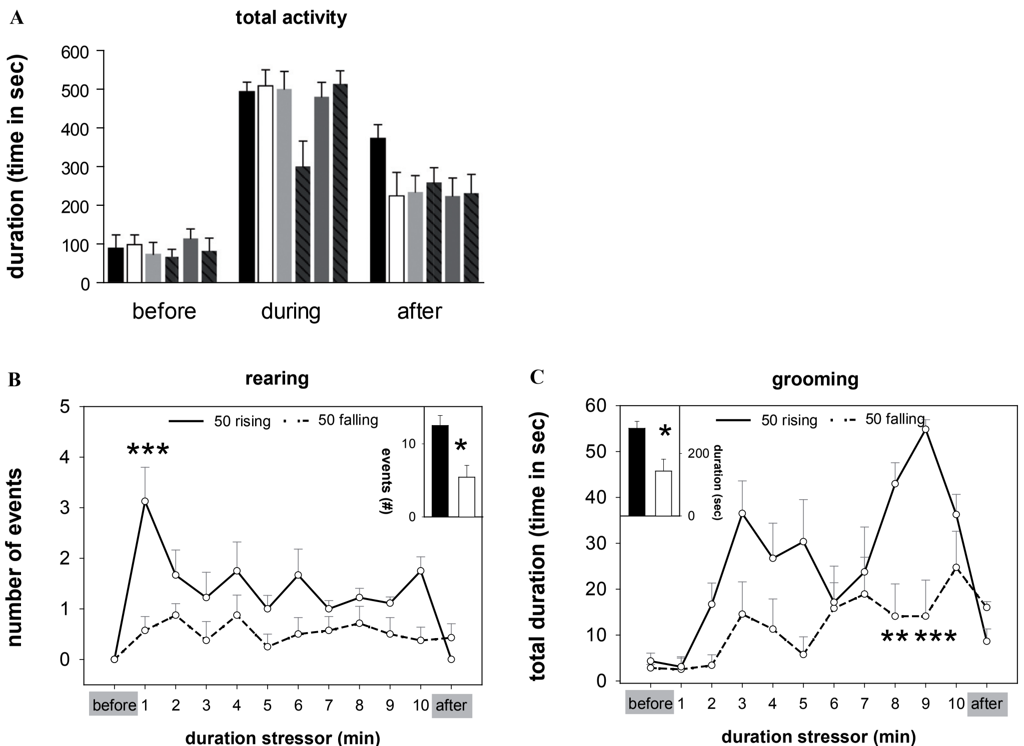


Figure S5 | Behavioural analysis to noise stress. Behavioural activity was examined in 10 min bins before, during and after the stressor. Noise stress evoked a transient increase in A| total activity in all treatment groups but significantly less in the 50 ng corticosterone infused animals stressed during the falling phase ($F(5, 45) = 2.95$; $p = 0.02$). No difference was found before or after the stressor between the different treatment groups. Similarly, behavioural activity plotted in 1 min bins during the stressor shows increased B | rearing (interaction effect: $F(11, 162) = 2.34$; $p = 0.011$) and C | grooming in 50 ng cort infused animals stressed on the rising (interaction effect: $F(11, 154) = 2.72$; $p < 0.01$). Inset indicates number of events or total duration per 10 min during the stressor. One-way ANOVA and Tukey's post-hoc test and two-way RM ANOVA and Bonferroni's post-hoc test, n = 7-8, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

Chapter 7

General discussion

Outline

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7.1 Introduction

As outlined in the general introduction, the significance of pulsatile glucocorticoid hormone secretion for physiology is not yet clear. Changes in these patterns are thought to compromise resilience and may thus be an important factor in the aetiology of stress-related diseases (Young et al. 2004, Lightman 2008). The overall objective of this thesis was therefore to assess the role of glucocorticoid pulsatile patterns in central glucocorticoid signalling and the neuroendocrine and behavioural responsiveness to stressful challenges.

Model system and experimental design

Practical limitations in the control of high frequency ultradian pulses in living animals have hampered progress in obtaining evidence for the functional consequences of glucocorticoid pulsatility. Furthermore, the lack of rhythmic synchronisation between animals necessitates control of the pattern of hormone release. In the current thesis we surgically or pharmacologically modulated pulsatile corticosterone hormone levels by exogenous steroid administration or automated steroid infusion either with or without automated blood sampling (Lightman et al. 2008). The technical development and availability of these methods has opened up the opportunity to examine how corticosterone pulsatility might interact with stress responsiveness and has indeed provided the first data beyond descriptive correlates as described in this thesis.

7.2 Glucocorticoid signalling

Glucocorticoid signalling mechanism

Accumulating evidence suggests that nuclear receptor access and binding to regulatory elements in the genome, but also methylation of DNA is highly dynamic (Metivier et al. 2003, Reid et al. 2003, Kangaspeska et al. 2008). With respect to GR, it is now being recognised that rapid ultradian oscillations in glucocorticoid hormone exposure are also crucial for transcriptional control (Desvergne & Heligon 2009, George et al. 2009, Stavreva et al. 2009). Upon glucocorticoid treatment, GR rapidly translocates to the nucleus in both cultured cells and hippocampal tissue (chapter 2 and 3). Irrespective of the administration paradigm, MR, however, is continuously retained in the nucleus of hippocampal cells (Conway-Campbell et al. 2007). Therefore we suggest that fluctuating levels of glucocorticoids which are conveyed into oscillatory activity of GR but not MR, result in differential recruitment of the receptors and coregulators which may involve different pathways in transrepression and transactivation of glucocorticoid target genes in the brain. This difference in ultradian re-

cruitment of MR and GR may simply reflect their different ligand binding affinities, kinetics and in vivo clearance rate (Reul & de Kloet 1985, Spencer et al. 1993). MR is extensively occupied at very low levels of circulating corticosterone and remains bound to ligand for much longer ($t_{1/2}$= 45 min) than GR ($t_{1/2}$= 5 min). GR thus rapidly associates and dissociates from its endogenous ligand and is recruited in an ultradian manner. Hence, we propose that disturbances in glucocorticoid pulsatility will therefore mainly affect this receptor rather than MR.

Glucocorticoid signalling in the rat brain

In relation to the brain, little information is available on how rapidly fluctuating glucocorticoid levels in vivo affect receptor signalling, transcriptional output and consequently target tissue sensitivity thereby determining the functional effects of glucocorticoid action and efficacy (Kino 2007). We have shown considerable changes in molecular markers for both chronic and acute glucocorticoid action in the rat hippocampus after disruption of endogenous pulsatile patterns (chapter 4). Overt hypercorticism (100% corticosterone) resulted in maximally sustained responses, most likely due to receptor saturation rendering GR non-responsive to additional hormone. This possibly creates a non-favourable situation in which the normal flexibility in glucocorticoid signalling is lost. Many studies described such sustained effects of chronic high corticosterone levels on the brain mediated via GR. For instance, feedback sensitivity of the HPA axis after stress is attenuated after chronic treatment with corticosterone (Akana et al. 1992), through occupancy of GR (Bradbury et al. 1994). Also, CA1 pyramidal cells seem to lose their potential to normalise enhanced activity after stress (Joels et al. 2007), e.g. as seen from the risk of calcium overloading (Karst & Joels 2007) and attenuation of functional 5-HT responses (Karten et al. 1999).

Clamping corticosterone levels at daily average levels (40% corticosterone), however, did not alter baseline expression of our rather sensitive expression markers. Remarkably, this treatment did still attenuate the transient response to a glucocorticoid challenge mimicking the stress response (chapter 4). The findings of this study suggest that pulsatile glucocorticoid release is required to maintain the normal 'resilience' in glucocorticoid target tissue responsiveness. Furthermore, subtle changes in the pattern of pulsatile exposure seem to induce changes at least as severe for glucocorticoid signalling as overt hypercorticism. This notion is strengthened by the normalisation in GR responsiveness after washout of constant 100% corticosterone levels but not in the 40% cort group. Using similar administration paradigms, previous studies have shown attenuated stress responsiveness (Akana et al. 1992) and suppressed levels of 5-HT1A receptor expression exclusively in the rat dentate gyrus (Meijer et al. 1997). These effects were interpreted as mediated via MR (Bradbury et al. 1994, Meijer et al. 2000). We and others now provide support for the additional involvement of GR in target tissue responses (Conway-Campbell et al. 2007, Stavreva et al. 2009),

as we show that particularly this receptor is sensitive to changes in the underlying pulsatile pattern of corticosterone.

Conclusion

Understanding the interplay between rapidly fluctuating glucocorticoid levels and receptor signalling could greatly contribute to our knowledge of cellular and tissue responses as changes in the pattern of glucocorticoid exposure could possibly contribute to dysregulated glucocorticoid receptor signalling (Kino 2007). We propose that the molecular response of target tissues induced by a corticosterone challenge is a resultant of the dynamic interplay between the corticosterone exposure regime (e.g. pulsatile or continuous) and steroid receptor signalling. The pattern of hormone exposure therefore seems a major factor in maintaining receptor responsiveness determining the adaptive capacity of target tissues in the face of acute stress.

7.3 HPA axis activity

Daily 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). However, the consequences of changes in pulse characteristics to HPA axis (re)activity are largely unknown. In the context of circadian rhythms, it has been shown that basal HPA axis activity and the neuroendocrine response to stress is clearly affected under conditions of flattened corticosterone (Akana et al. 1985, Akana et al. 1992, Dallman et al. 2004). However actual disruption of ultradian variation with respect to this pellet model remained to be proven. During chronic glucocorticoid exposure, the HPA axis via negative feedback adjusts circadian trough and peak concentrations resulting in steady-state corticosterone concentrations (Akana et al. 1985, Akana et al. 1992, Meijer et al. 1997). This consequently may disrupt episodic feedback signals crucial in maintaining ultradian feedforward-feedback oscillitatory activity between the pituitary and adrenal gland (Walker et al. 2010). We have now demonstrated that besides circadian variation, also rapid ultradian glucocorticoid pulses can be effectively flattened around the daily average by subcutaneous implantation of 40% corticosterone pellets (chapter 5). This not only mimics some of the risk factors for stress-related disease, it also provides a good and easy to manipulate model to study the consequent disruption in feedback activity.

Until now it was unknown how fast the negative feedback action abates after cessation of constant exogenous corticosterone and when the endogenous pulses re-emerge. Termina-

tion of constant exogenous corticosterone exposure resulted in rapid progressive normalisation of ultradian pulse characteristics within approximately 6 hours after removal of the pellet (chapter 5). This is most likely due to a delayed feedback mechanism suppressing pulse amplitude at earlier time points (Walker et al. 2010). Thus, the half life time of the suppression of HPA axis reactivity that is brought about by 40% corticosterone pellet implantation (Akana et al. 1992), is significantly less than 24 hours. This is reminiscent of the acute effects of MR and GR ligands on the axis, that last in the order of hours (Ratka et al. 1989, van Haarst et al. 1996, Atkinson et al. 2008, Spiga et al. 2008).

The endocrine response to noise stress also rapidly restored after reoccurrence of basal pulsatility. Paradoxically, we still observed substantial changes in the behavioural response to stress (chapter 5, discussed elsewhere in this chapter). These results show a striking parallel with observations in humans with a history of changes in cortisol exposure which suggest that even after normalisation of hormone levels there are residual disturbances in the brain (Tiemensma et al. 2010). Washout after continuous corticosterone administration may therefore constitute a model to study these clinically relevant processes.

Conclusion

We conclude that HPA axis activity is remarkably sensitive to changes in the characteristic pulsatile pattern of corticosterone exposure but adjusts in a reversible fashion to these alterations. We propose that it is the pulsatile pattern, rather than the absolute concentrations, of corticosterone exposure that determines subsequent responsiveness to stress, a notion with obvious implications for understanding the pathogenesis of stress related disease.

7.4 Neuroendocrine stress responsiveness in relation to pulse characteristics

Glucocorticoids play a major role in regulation of HPA axis activity through negative feedback inhibition (Keller-Wood & Dallman 1984, Dallman et al. 1987, Sapolsky et al. 1990), mediated via MR and GR (Ratka et al. 1989, van Haarst et al. 1996, Hinz & Hirschelmann 2000, Atkinson et al. 2008, Russell et al. 2010). The physiological importance of rapid and adequate feedback is directly underlined by many clinical studies that, for instance as seen during major depression, attribute hyperactivity of the HPA axis to defective negative feedback action of glucocorticoids (Dinan 1994). As a result, maladaptive feedback can have many adverse consequences including cognitive dysfunction and mood alterations, immunodeficiency, cardiovascular disorders and energy metabolism disorders (Dallman et al.

1987, De Kloet et al. 1998). It is however not clear how the feedback mechanism interacts with ultradian glucocorticoid rhythmicity.

The data presented in this thesis shows that glucocorticoid negative feedback inhibition brought about by corticosterone infusion, here in the context of stress, depends on the pattern, phase- and amplitude of corticosterone pulses (chapter 6). Constant infusion of corticosterone resulted in a clear suppression of ACTH responsiveness and has been described in literature before (Akana et al. 1992). Interestingly, pulsatile exposure of corticosterone resulted in a larger, more flexible response in ACTH responsivity to noise stress. This sensitising effect of pulsatility was even more pronounced during the rising phase of an ultradian pulse than during the falling phase. In addition, animals infused with higher amplitude pulses showed smaller increments in stress-induced ACTH release. These data thus clearly show that the neuroendocrine response to stress depends on circulating glucocorticoids in both a phase and amplitude dependent manner. This is in line with previous studies that retrospectively correlated higher corticosterone responses to a stressor with the rising phase of an endogenous pulse and lower mean corticosterone responses with increased pulse frequency (Windle et al. 1998b, Windle et al. 2001, Atkinson et al. 2006). However, we originally hypothesised that negative feedback of glucocorticoids should be greatest when corticosterone levels are rapidly rising within physiological concentrations in a process termed rate sensitive feedback (Dallman & Yates 1969, Kaneko & Hiroshige 1978). Although this apparent rate-sensitivity was not specifically addressed here, we found no evidence for this phenomenon. Comparisons between studies are complicated by the difference in duration of corticosterone exposure. In our study measurements were made after several pulses rather than the single infusions performed previously. The underlying explanation therefore most likely hinges on differential activation of genomic and non-genomic effects of corticosteroids and will be discussed later in this chapter.

Conclusion

It has recently been demonstrated that rapidly oscillating feedforward and feedback loops between the pituitary and adrenal gland give rise to ultradian corticosterone rhythms (Walker et al. 2010). Therefore, ultradian pulses may represent rapidly alternating phases of HPA axis activation and inhibition (Windle et al. 1998b, Lightman et al. 2008). In that respect, we suggest and clearly demonstrate differential HPA axis responsiveness over the ultradian cycle as the relation of the onset of a stressor to the phase or amplitude of ultradian pulses is a major determinant in acute HPA axis responsiveness. Continuous exposure to glucocorticoids attenuates stress-induced ACTH release. Therefore, we propose that transient surges in glucocorticoid level as achieved during the pulses serve to prepare and maintain flexibility in HPA axis activity for adequate responses to stress.

7.5 Behavioural responsiveness to stress

While alterations in the basal pulse frequency and amplitude are known to be a major factor influencing neurendocrine reactivity to acute stressors (Windle et al. 1998a, Windle et al. 2001, Atkinson et al. 2006), very little data is available on the behavioural response to stress. The results presented in chapter 5 demonstrate persistent changes in behavioural responsiveness after a history of flattened corticosterone rhythms, even after normalisation of basal HPA axis activity. Similar results in humans describe highly prevalent emotional and cognitive effects in patients with long-term cured Cushing's disease compared to matched controls (Tiemensma et al. 2010). These and our results suggest that changes in the history of HPA axis pulsatility of the individual has consequences for behaviour, emotion and cognition and may thus indicate possible irreversible effects of prolonged previous glucocorticoid excess on the brain. Future research should determine how persistent the consequences of a period of chronic glucocorticoid exposure are.

Besides the corticosteroid history of an animal (chapter 5), behavioural stress responsiveness also seems to depend acutely on the phase of ultradian pulses (chapter 6). In line with the previously described increased neuroendocrine response, we found that total activity and risk assessment were highest when the noise stressor was experienced during the rising phase of corticosterone infusion. Behavioural responsiveness has been correlated to ultradian corticosterone pulses before. In a model in which animals were divided post-hoc, others observed that the propensity to behave aggressively was increased during the rising phase of an ultradian pulse (Haller et al. 2000a). These data strongly suggest that brain centers such as the amygdala, normally activated and modulated by emotionally arousing experiences (McGaugh 2004, Roozendaal et al. 2009), may respond differently depending on the rapid fluctuations in corticosterone and thereby 'pulse-dependently' modulate the emotional and exploratory response to stress.

Though mostly examined under conditions of chronic exposure, glucocorticoids are indeed known to modulate, in different time domains, limbic regions such as the hippocampus and amygdala (Herman et al. 2003, Jankord & Herman 2008). This may influence learning and memory processes in both rodents and humans (Schwabe et al. 2008, Schwabe et al. 2009), most likely due to changes in the neuroarchitecture and plasticity (Bodnoff et al. 1995, Fuchs et al. 2006, Joels et al. 2007). Such changes may pertain to (m)any neurotransmitter system(s) and substantial changes in information processing after chronic corticosterone and stress (Fuchs et al. 2006, Joels et al. 2007). But we have also shown that genomic responses of particularly GR in the brain are sensitive to changes in hormone patterns and, in fact, remain significantly affected after removal of chronic corticosterone exposure (chapter 4). In addition, there is strong evidence for non-genomic (behavioural) actions of glucocor-

ticoids (Sandi et al. 1996, Mikics et al. 2004, Schwabe et al. 2009) via putative low affinity membrane-associated MR and GR (Di et al. 2003, Karst et al. 2005). Thus, an explanation for the variations in behavioural response to stress may involve non-genomic and genomic effects on multiple targets, including responsiveness of MR and GR themselves.

Conclusion

Our results suggest that the history as well as the actual phase of HPA axis pulsatility of the individual somehow are a determinant factor in the behavioural response to stress. We thus postulate that both the neuroendocrine and behavioural response to stress vary over the ultradian cycle as every hourly surge in corticosterone seem to open a window of enhanced susceptibility to stress. Understanding the significance of this hourly enhanced stressor susceptibility opens up interesting new perspectives for the role of ultradian rhythmicity in physiology and behaviour. Whether the reduced stress reactivity during a descending ultradian phase is in fact advantageous or detrimental is still open for investigation and may depend on the particular circumstances.

7.6 Differential activation of neuronal pathways

It is currently unclear to what extent central structures are sensitive to ultradian glucocorticoid regulation. The audiogenic stressor we used typically results in a wide pattern of brain activation (Campeau & Watson 1997, Burow et al. 2005). This includes many auditory structures but also specific brain circuits involved in the stress response to noise such as the PVN, amygdala, prefrontal cortex. We used c-fos immediate early gene expression as a marker for activation of neuronal pathways involved in the stress response in the context of ultradian pulses and is extensively discussed in chapter 6. As described previously in literature but also in our hands, the hippocampus does not seem to be responsive to audiogenic stimulation (Campeau & Watson 1997, Burow et al. 2005). Hence, this specific type of stressor may not be appropriate for analysis of the influence of the pattern, magnitude or phase of corticosterone pulses specifically in this area. On the other hand, we also clearly show that noise stress-induced c-fos expression in other brain circuits (i.e. PVN, pituitary and amygdala) are responsive to the magnitude and/or phase of ultradian pulses.

Conclusion

Glucocorticoid target tissues and the receptors located there are exposed to rapidly fluctuating levels of steroid hormone (Cook 2001, Droste et al. 2008). This, together with the impact of changes in ultradian pulse characteristics on behavioural stress responsiveness (chapter 5), suggests that the brain is indeed receptive to glucocorticoid pulsatility. We now

also demonstrate that neuronal pathways induced by noise stress respond selectively to different aspects of the ultradian cycle. A plausible explanation hinging on region-specific pulsatile effects of glucocorticoids most likely includes the local cellular receptor mechanism due to differential presence of MR and GR and (nuclear) cofactors in the different brain regions.

7.7 Possible mechanisms underlying glucocorticoid pulsatility

The present studies, together with recent literature on corticosteroid receptor action provides a framework for mechanistic understanding of the physiological effects of ultradian glucocorticoid pulses. MR and GR are involved themselves in the dynamic generation and maintenance of basal ultradian HPA axis activity (Spiga et al. 2007, Atkinson et al. 2008). As a consequence of rapidly changing ligand concentrations and differences in affinity, MR and GR may be differentialy recruited along the duration of a single ultradian pulse. The results described in this thesis suggest that MR and GR expression and function also seem to depend on the pattern and concentration of corticosterone administration and are differentially regulated in different brain areas (chapter 3, 4 and 6).

Both receptors are also involved in stress-induced HPA axis activity by mediating in complementary fashion the feedforward and feedback modes of glucocorticoid feedback operation (Ratka et al. 1989, Bradbury et al. 1994, van Haarst et al. 1996, Hinz & Hirschelmann 2000, Russell et al. 2010). The time domain of some parameters measured in the current thesis (i.e. ACTH release and behavioural stress responsiveness in chapter 6) suggests a role for rapid non-genomic, rather than slow genomic actions of glucocorticoids, via putative low affinity membrane-bound variants of MR and GR (Orchinik et al. 1991, Hinz & Hirschelmann 2000, Di et al. 2003, Karst et al. 2005). The 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, non-genomic GR reduces net neuronal excitatory transmission via endocannabinoids and nitric oxide (Di et al. 2003, Di et al. 2009). It is currently believed that the initial rise in corticosterone (e.g. during stress or the onset of an ultradian pulse) triggers non-genomic actions via the membrane-bound receptors which over time activate the slower, genomic GR. This has evolved into a new concept of corticosteroid action in the brain in which fast MR-triggered excitability via the membrane receptor interplay with the slow MR and GR-mediated genomic events (Joels et al. 2008a).

In relation to our ACTH data (chapter 6), a 'brake' during the falling phase is suggested by decreased ACTH release, i.e. development of a classical genomic feedback signal during rising phase that becomes apparent within 20 minutes and is predicted to maintain reciprocity until the next pulse. Alternatively increased 'drive' during the rising phase could result in stimulation of ACTH release or behaviour through activation of a rapid feedforward signal. The latter would likely depend on non-genomic mechanisms. Similar mechanisms may also underlie the translation of ultradian corticosterone oscillations into adequate behavioural responses but also more fundamental processes such as synaptic transmission and neuronal excitability. The apparent pure 'ascending phase'-dependent effect on c-fos expression in the amygdala in particular seems to call for an explanation involving a rapid, non-genomic component.

Conclusion

It seems that the mechanism underlying responsiveness to ultradian glucocorticoid pulsatility involves a balance between rapid non-genomic and slower genomic effects via membrane-bound and nuclear variants of MR and GR that rapidly change over the duration of a single ultradian pulse. This may differ depending on the area of the brain resulting in changing states of responsiveness to environmental input. The current model system in which we impose hourly ultradian pulses provides a unique position to address such mechanistic issues in future studies.

7.8 Perspectives

Clinical relevance

Until now, the clinical relevance of the pulsatile nature of glucocorticoids was poorly understood or sometimes even regarded as not important. As described in this thesis, disturbances in glucocorticoid pulsatile patterns and aberrant HPA axis functioning are well recognised signs in patients suffering from stress-related disorders. Deviations from the optimal pulsatile pattern are considered to precipitate disease as daily variations in glucocorticoid hormone concentrations are thought to be fundamental for the maintenance of physiological, metabolic, cognitive and behavioural well being (Dallman et al. 2003, de Kloet et al. 2005, Herbert et al. 2006). Newly acquired insights in glucocorticoid hormone pulsatility could therefore be of great importance for the elucidation of the aetiology and pathophysiology of stress-related disorders (Young et al. 2004, Lightman et al. 2008). In addition, this will also result in more insight into the consequences of long term glucocorticoid excess or deficiency as seen in many stress-related disorders.

Knowledge of pulsatility also has important implications for the therapeutic application of glucocorticoids. 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), as manipulation of the temporal aspect is already a successfully used approach in clinical therapy for instance for growth hormones (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 may therefore greatly contribute to the prognosis and treatment of disease.

Future directions

It is currently not known if disorganisation of rapid fluctuations in glucocorticoid levels are causally linked to the aetiology of stress-related disease or vice versa. Unravelling the mechanisms that underlie the secretory pattern of corticosterone release, but also under conditions of stress, is thus essential. The generation of MR and GR specific transgenic knockout models but also the development of infusion systems that allow to control ultradian pulses, now provides a unique position to address such mechanistic issues in future studies. Many scenarios come to mind and a few are outlined in some detail below.

As extensively discussed is the previous paragraphs, we suggest that the different components of ultradian pulses (i.e phase and amplitude) have different effects on stress-activated circuitry. It is thus very likely that distinctly different mechanisms are operating simply due to the difference in the duration of glucocorticoid exposure. In that respect, the balance between rapid non-genomic and slower genomic effects via membrane-bound and nuclear variants of MR and GR could rapidly change over the duration of a single ultradian pulse. Moreover, the recruitment of MR and/or GR during stress, superimposed on the ultradian and circadian pattern, would also depend on the phase of the pulse. One approach would be to use either pharmacological or genetic knock-out models to systematically study the role of MR and GR in basal and stress-induced HPA axis activity and behavioural activity as a function of these different pulse characteristics to unravel the underlying mechanism. A complicating factor is that genetic models tend to be based on mice, while the rapid sampling/infusion system that we used exists only for rats (and humans). Besides an endocrine and/or behavioural context, it would also be very interesting to study at a more fundamental level the involvement of (membrane) MR and GR in rapid modulation of synaptic transmission and neuron excitability by ultradian corticosterone rhythms.

Measurement of HPA axis secretagogues (i.e. AVP and CRH) may also be an appropriate approach to understand acute effects of ultradian glucocorticoid pulses. The primary action of AVP within the axis is the potentiation of CRH action on the pituitary (Gillies et al. 1982,

Rivier & Vale 1983). Thus increased release of AVP may sensitise corticotrophs to the pulsatile CRH signals reaching them. Besides potentiating the magnitude of each ACTH burst, pulsatile administration of AVP to corticotrophs also causes a more rapid rise in ACTH release during each CRH pulse (Evans et al. 1988). The measurement of these peptides may therefore provide more insight in supra-pituitary integration of glucocorticoid pulsatility. Furthermore, it has recently been demonstrated that the circadian rhythm in glucocorticoids is partly attributed to rhythmic steroidogenesis in the adrenal gland (Son et al. 2008), which could gate adrenal sensitivity to ACTH (Oster et al. 2006). It is however not known to what extent ultradian corticosterone secretion is explained by these observations and how ultradian ACTH release relates to changes in adrenal responsiveness.

The endocannabinoid system works as an important neuromodulatory system by negative modulation of basal and stress-induced HPA axis activity (Cota et al. 2007). Endocannabinoids are suggested to modulate CRH, ACTH and glucocorticoid levels and fast negative feedback via rapid non-genomic effects of glucocorticoids (Manzanares et al. 1999). Glucocorticoids induce retrograde signalling of endocannabinoids from CRH-containing neurons and thereby inhibit excitatory transmission via activation of presynaptic CB1 receptors in the PVN (Di et al. 2003). How endocannabinoids are exactly involved in ultradian glucocorticoid pulsatility and negative feedback is not known but it has been proposed that ultradian oscillations are generated by inhibitory ultra-short cycles in negative feedback inhibition within the hypothalamus (Windle et al. 1998b, Lightman et al. 2008), but also between the pituitary and adrenal gland (Walker et al. 2010). Endocannabinoids could underlie such a self-sustaining mechanism in ultradian fluctuations as the rapid rise in corticosterone levels during every secretory phase (but also during stress) could potentially generate a fast feedback signal. Consequently this may result in inhibition of corticosterone secretion and thus cyclic levels of glucocorticoids. In addition, endocannabinoids are known to be under negative glucocorticoid regulation in the hippocampus and striatum (Di et al. 2003, Hill et al. 2005b) but in fact increase activity in the limbic forebrain after glucocorticoid exposure which may contribute to the region-specific effects of glucocorticoids (Hill et al. 2005a).

Finally, ultradian glucocorticoid pulses induce consecutive waves in GR signalling and transcriptional initiation of target genes in cultured cells (Stavreva et al. 2009). However, just a few studies have addressed the consequences of ultradian corticosterone exposure for nuclear receptor signalling in the brain [(Conway-Campbell et al. 2007) and chapter 4]. In that respect large scale gene expression profiling studies of for instance the hippocampal transcriptome or analysis of single target genes, would be very valuable in understanding the significance of ultradian hormone fluctuations for MR and GR signalling in the brain. Also, the consequences for protein levels have not been thoroughly examined. Furthermore, as new technologies are rapidly developing, whole genome-wide receptor binding studies together with visualisation techniques could provide the means to unravel the continuous com-

petitive cycling between MR and GR together with the transcription and proteasome machinery on the chromatin of glucocorticoid response elements according to rapidly changing hormone levels which eventually determine transcriptional output.

7.9 General conclusions

The results presented in the current thesis have evolved in a new concept in glucocorticoid endocrinology. Even though the link between the different markers used here is uncertain, glucocorticoid signalling as well as behavioural and neuroendocrine responsiveness to stressors are all rapidly and transiently modulated by ultradian corticosterone pulses. We thus provide strong evidence for functional consequences of ultradian glucocorticoid pulsatility. Moreover, we have revealed that in particular the GR is affected when glucocorticoid pulsatility is disrupted and could thus provide an excellent target for therapy to normalise the downstream effects of disturbances in glucocorticoid rhythms in stress-related disease. It also provides a handle to tackle long term problems seen after withdrawal of high glucocorticoid exposure, for instance such as seen postoperatively in 'cured' Cushing's disease. The newly acquired insights are thus of great importance for the understanding of glucocorticoid biology but also for the elucidation of the pathophysiology and aetiology of stress-related disorders. As continuous administration of glucocorticoids attenuates many of the investigated parameters, we conclude that the significance of glucocorticoid pulsatility lies in 'priming' the HPA axis and different brain circuits through rapidly alternating feedforward and feedback modes providing full responsiveness and 'resilience' in the coordination of stressful events.

Chapter 8

Summary

The 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 cortisol and corticosterone. In mammals including humans, glucocorticoids are characteristically released in approximately hourly hormone bursts by the adrenal gland. These rapid ultradian hormone pulses increase in amplitude, and to a lesser extent in frequency, in anticipation of the active phase providing a circadian pattern of hormone release. Interestingly, pulse amplitude, frequency and phase all seem to be major determinants in the acute response of the HPA axis. It is however not clear how rapid ultradian oscillations contribute to physiology such as target tissue responsiveness of brain and the response to stress. The objective of this thesis is therefore to assess the role of glucocorticoid pulsatile patterns in brain glucocorticoid signalling and the neuroendocrine and behavioural response to stress.

Chapter 2 describes the screening of a panel of GR primary antibodies in order to find the most suitable antibody to study GR nuclear translocation as a potential marker for tissue responsiveness using immunohistochemistry and confocal imaging. Hereto, ADX rats and cultured AtT20 cells were stimulated with a high dose of glucocorticoids. Interestingly, while some antibodies (i.e. BuGR2, H300) were consistent between the two conditions, we found opposing results in GR nuclear translocation patterns for some GR primary antibodies (i.e. M20, P20) between cultured cells and rat CA1 cells. The latter findings were confirmed in another hippocampal cell population, granule dentate gyrus cells. We therefore concluded that findings from cell culture studies cannot always be extrapolated to in vivo situations and that the outcome of antibody-dependent studies can depend heavily on the choice of the antibody. The H300 GR primary antibody consistently demonstrated nuclear translocation patterns in both cultured cells and in brain sections and was therefore chosen to continue further studies.

In **chapter 3** we further characterised receptor nuclear translocation in the rat hippocampus as a potential marker for glucocorticoid signalling. Hereto, both intact and ADX rats were injected with a high dose of corticosterone mimicking the stress response. Subcellular distribution patterns of MR and GR at different time points after injection were measured in single cells in the different hippocampal subfields using the same methodology as chapter 2. We found significant subregion-specific differences in translocation patterns for both MR and GR, with respect to the extent and timing of nuclear translocation. Moreover, specific differences between ADX and intact animals that are most prominent in the dentate gyrus. We conclude that in response to a single stress-like increase in corticosterone distinct region-specific MR and GR-dependent translocation patterns exist in the rat hippocampus.

The effects of long-term disturbances in endogenous corticosterone pulsatile patterns on molecular markers for target tissue sensitivity to an additional glucocorticoid challenge mimicking the stress response are described in **chapter 4**. Diurnal and ultradian corticosterone fluctuations were clamped at constant levels in intact animals by exogenous administration of corticosterone by means of subcutaneous corticosterone pellet implantation. Additionally, recovery from constant exposure was studied in groups that had the pellet removed 24 hours prior to the challenge. Molecular markers for receptor responsiveness to this acute challenge were examined in the rat hippocampal area. The constant exposure to the steroid resulted in dose-dependent down-regulation of GR and attenuated MR and GR translocation to the acute challenge. Interestingly, while glucocorticoid responsive expression of Gilz to the challenge was already attenuated during stable daily average levels (40%), resistance of Sgk-1 gene expression to the challenge was only observed after constant high corticosterone exposure (100%). Washout of 100% cort recovered all molecular markers (partial) while removal of the 40% cort pellet still attenuated responsiveness to the challenge. This finding suggests differential responsiveness of the two genes due to treatment. Using these molecular markers we conclude that tissue responsiveness to glucocorticoids is maintained by pulsatile rather than constant exposure to the hormone.

In **chapter 5** flattening of ultradian and circadian glucocorticoid pulsatility via subcutaneous pellet implantation was verified and validated by high frequency automated blood sampling with 10 min intervals. Corticosterone rhythms were effectively flattened around daily average (40%) or at levels seen during stress-related disease (100% cort). Removal of the corticosterone pellet resulted in rapid recovery of ultradian pulsatility in the 40% group but not in the 100% group, most likely due to a local depot formation in the skin. However, we found a dissociation between neuroendocrine and behavioural responsiveness to noise stress. Animals previously exposed to 40% corticosterone showed less and shorter behavioural activity to noise stress compared to 100% corticosterone and vehicle treated animals while their endocrine response was similar to vehicle animals. This data suggest that even though HPA axis activity adjusts in a reversible fashion to alterations in the characteristic pulsatile pattern of corticosterone, this happens in spite of longer lasting changes in the brain affecting stress responsiveness.

Chapter 6 describes the neuroendocrine and behavioural response to stress in relation to ultradian corticosterone pulses. We have addressed this question by infusing identical amounts of corticosterone but in constant or hourly pulsatile patterns in ADX rats. We also varied the pulse amplitude without altering total administered dose by adjusting both the concentration and duration of infused corticosterone. At the end of infusion – and either during the rising or falling phase of our exogenous pulse – animals were exposed to 10 minutes of 99 dB noise stress. Constant administration of corticosterone resulted in a blunted ACTH response to stress, while pulsatile administration facilitated a brisker response

markedly greater in the rising than in the falling phase of a corticosterone pulse. This differential phase-dependent effect was also seen in the behavioural response to noise which was much greater in the rising phase. Interestingly, we found strong region-specific differences in c-fos neuronal expression patterns as a function of either pulse amplitude or phase, indicating that under stress different brain circuits are differentially affected by glucocorticoid pulsatility. We conclude that the current data provide strong evidence for pattern-dependent stress responsivity in which different brain circuits show differential sensitivity to the phase and amplitude of pulsatile corticosterone exposure.

As discussed in **chapter 7**, a new concept in the endocrinology of glucocorticoids has evolved from the data presented in this thesis showing that pulsatile release of glucocorticoids is a major determinant in 'resilience' of glucocorticoid signalling and stress responsiveness. Moreover, we have revealed that in particular the GR is affected when glucocorticoid pulsatility is disrupted and could thus provide a excellent target for therapy to normalise the downstream effects of disturbances in glucocorticoid rhythms in stress-related disease. Potential mechanisms and suggestions for future research are extensively discussed in the general discussion.

Samenvatting

Stress, zowel fysiek als psychisch, zorgt voor snelle fysiologische veranderingen in het lichaam. Activering van het sympathische zenuwstelsel resulteert via adrenaline en noradrenaline in o.a. een verhoogde hartfrequentie, bloeddruk en bloedtoevoer naar de spieren. Dit stelt een individu in staat om vrijwel direct en adequaat op de situatie te reageren. Daarnaast zorgt activering van de hypofyse-bijnier as [in het Engels: hypothalamic-pituitary-adrenal (HPA) axis] voor acute afgifte van de glucocorticoïd stresshormonen (cortisol bij de mens, corticosteron bij knaagdieren). Deze hormonen zijn essentieel in het vrijmaken van beschikbare energie voor de reactie op stress maar controleren ook een overvloed aan functies in de periferie en de hersenen zoals cognitie, emotie en perceptie. De acties van glucocorticoïden worden gemedieerd via twee receptoren, de mineralocorticoïd en de glucocorticoïd receptor (MR en GR), die samen HPA-as activiteit handhaven. MR wordt geacht tonische HPA-as activiteit en de drempel van het systeem te reguleren ('pro-actieve' modus), terwijl GR het herstel van stress te bevordert ('reactieve' modus).

Het is recentelijk beschreven dat vrijwel alle hormonen in het lichaam, inclusief de glucocorticoïden, worden afgegeven volgens een bepaald pulsatiel patroon. Dit is het ultradiane ritme en zorgt voor de uurlijkse afgifte van glucocorticoïden door de bijnier in alle zoogdieren, waaronder de mens. Het circadiane ritme dat onder invloed van de biologische klok in de hersenen staat superponeerd hier dag/nacht fluctuaties op. Het circadiane en ultradiane ritme werken nauwgezet samen om zo intrinsieke ritmiciteit van de HPA-as te controleren vanwege de grote invloed op onder andere gezondheid, vitaliteit en het immuunsysteem.

Het is op het moment bekend dat tijdens chronische stress of ziekte de vatbaarheid voor stress-gerelateerde en autoimmuunziekten toeneemt. Bovendien zijn stoornissen in het karakteristieke pulsatiele patroon van glucocorticoïd afgifte vaak beschreven in stress-gerelateerde pathologie. Het is echter niet duidelijk hoe snelle ultradiane oscillaties in glucocorticoid niveaus bijdragen aan fysiologie, stress responsiviteit en receptor signalering in het brein. Het doel van het beschreven onderzoek in dit proefschrift is daarom gericht op de rol van glucocorticoïd pulsatiele patronen op glucocorticoïd signalering in de hersenen, HPA-as activiteit en de neuroendocriene en gedragsrespons op stress.

Hoofdstuk 2 beschrijft een studie waarin wordt gezocht naar het meest geschikte antilichaam om het proces van GR nucleaire translocatie te visualiseren als mogelijke marker voor weefsel responsiviteit. Met behulp van immunohistochemische technieken en confocale microscopie werd een panel van GR primaire antilichamen getest in zowel een cellijn als hersenweefsel. Om nucleaire translocatie van GR te induceren zijn bijnierloze ratten en AtT20 cellen gestimuleerd met een hoge dosering glucocorticoïd. Het blijkt dat hoewel

sommige antilichamen (bijv BuGR2 en H300) overeenstemmende resultaten vertonen tussen de twee condities, we tegenstrijdige resultaten in translocatiepatronen tussen gekweekte cellen en rat hippocampale CA1 cellen met andere GR antilichamen vinden (bijv M20, P20). De laatste bevindingen werden ook bevestigd in granulaire dentate gyrus cellen, een andere hippocampale celpopulatie. Wij concluderen dat de bevindingen in cellijnen niet altijd kunnen worden geëxtrapoleerd naar de in vivo situatie en dat de uitkomst van antilichaamafhankelijke onderzoeken sterk kan afhangen van de keuze van het antilichaam. Aangezien de GR H300 antilichaam consistent nucleaire translocatie patronen in zowel gekweekte cellen als in hersenen secties heeft aangetoond werd deze gekozen om verdere studies voort te zetten.

In **hoofdstuk 3** hebben we receptor nucleaire translocatie in de hippocampus van ratten als een mogelijke marker voor glucocorticoïd signalering verder gekarakteriseerd. Zowel intacte als bijnierloze ratten zijn geïnjecteerd met een hoge dosis corticosteron om de stressrespons na te bootsen. Vervolgens is met dezelfde methode als beschreven in hoofdstuk 2 de subcellulaire localisatie van MR en GR op verschillende tijdstippen na injectie gemeten in verschillende subgebieden van de hippocampus. We hebben significante subregio-specifieke verschillen in de translocatiepatronen voor zowel MR en GR gevonden met betrekking tot de omvang en de timing van de nucleaire translocatie. Bovendien waren de specifieke verschillen tussen intacte en bijnierloze dieren het meest prominent in de gyrus dentatus. We concluderen dat in reactie op een met stress vergelijkbare stijging in corticosteron niveaus er regio-specifieke verschillen in MR en GR translocatie patronen bestaan in de hippocampus van ratten.

De effecten van langdurige verstoringen in glucocorticoïd pulsatiele patronen op moleculaire markers voor weefselgevoeligheid worden beschreven in **hoofdstuk 4**. Exogene toediening door middel van subcutane corticosteron pellet implantatie werd gebruikt om zowel circadiane als ultradiane corticosteron fluctuaties op een constant niveau in intacte dieren vast te 'klemmen'. Om stressachtige weefselresponsen te induceren werden alle dieren na een week geïnjecteerd met een extra dosering glucocorticoïd. Eventueel herstel van constante blootstelling werd onderzocht in groepen waarin 24 uur voorafgaand aan de extra injectie de corticosteron pellet was verwijderd. Vervolgens zijn verschillende moleculaire markers voor receptor functie onderzocht in de hippocampus van de rat. Constante blootstelling aan de steroïden resulteerde in een dosis-afhankelijke vermindering van GR niveaus en resistentie in MR en GR translocatie na de acute injectie. Interessant is dat constante blootstelling aan dagelijks gemiddelde niveaus (40%) de glucocorticoïd-afhankelijke inductie van target gen Gilz al verminderde, terwijl de expressie van Sgk-1 alleen werd beïnvloed na blootstelling aan constante hoge corticosteron niveaus (100%). Deze bevinding suggereert gen-specifieke verschillen in glucocorticoid responsiviteit, afhankelijk van het patroon van corticosteron toediening. Het uitwassen van hoge concentraties resulteerde verder in vrijwel

volledige normalisatie van alle moleculaire markers, terwijl 24 uur na het verwijderen van de 40% cort pellet weefsel responsiviteit nog steeds aangedaan was. Met behulp van deze moleculaire markers concluderen we dat de flexibiliteit in weefselreactiviteit op glucocorticoïden wordt onderhouden door pulsatiele in plaats van voortdurende blootstelling aan het hormoon.

In **hoofdstuk 5** is met behulp van een geautomatiseerd bloedafnamesysteem met hoog frequente intervallen (elke 10 minuten) het afplatten van ultradiane en circadiane glucocorticoïd pulsatiliteit via subcutane pellet implantatie gecontroleerd en gevalideerd. Uit de resultaten blijkt dat na beide manipulaties corticosteron ritmes inderdaad effectief zijn afgeplat rond het dagelijks gemiddelde (40% cort) of op een niveau vergelijkbaar met stress-gerelateerde ziekte (100% cort). Verwijdering van de corticosteron pellet resulteerde in een snel herstel van ultradiane pulsatiliteit in de 40% groep, maar niet in de 100% groep. Dit laatste is waarschijnlijk te wijten aan de formatie van een lokaal depot in de huid. Interessant genoeg vonden we een dissociatie tussen de neuroendocriene en gedragsreactie op geluidstress 24 uur na verwijdering van de pellet. Dieren blootgesteld aan 40% corticosteron vertonen verminderde en verkorte reactiviteit op geluidstress in vergelijking met 100% corticosteron en controle dieren, terwijl hun endocriene respons vergelijkbaar was met de controle dieren. Deze gegevens wijzen erop dat hoewel HPA- as activiteit flexibel omgaat met veranderingen in het karakteristieke pulsatiele patroon van corticosteron, dit gepaard gaat met langer durende veranderingen in de hersenen die de gedragsmatige stress responsiviteit bepalen.

Hoofdstuk 6 beschrijft de relatie tussen ultradiane corticosteron pulsen en de neuroendocriene en gedragsreactie op stress. Hiertoe zijn bijnierloze ratten geïnfuseerd met gelijke hoeveelheden corticosteron, maar in óf constant, óf uurlijkse pulsatiele patronen. Door aanpassing van zowel de concentratie en de duur van corticosteron-infusie hebben we ook gevarieerd in de amplitude van de puls, zonder de totale toegediende dosis te wijzigen. Aan het einde van de infusie - en zowel tijdens de stijgende of dalende fase van een uurlijkse puls – werden de dieren blootgesteld aan 10 minuten 99 dB geluidstress. Constante toediening van corticosteron resulteerde in een afgeplatte ACTH respons op stress, terwijl pulsatiele administratie resulteerde in een aanzienlijk grotere respons die meer prominent was tijdens de stijgende dan in de dalende fase van een corticosteron puls. Dit fase-afhankelijke effect hebben we ook waargenomen in de gedragsreactie op geluidsstress. Verder hebben we sterke regio-specifieke verschillen in c-fos neuronale expressie patronen als functie van pulsamplitude of fase gevonden, waaruit blijkt dat onder stress de hersenen verschillende circuits differentieel worden beïnvloed door glucocorticoïd pulsatiliteit. De huidige gegevens verstrekken sterk bewijs voor patroon-afhankelijke stress responsiviteit in de hersenen waarin verschillende circuits differentiële gevoeligheid tonen voor de fase en amplitude van pulsatiele corticosteron blootstelling.

Het functioneren van het stress systeem onder invloed van de pulsatiele afgifte van glucocorticoïd stress hormoon is nog niet eerder onderzocht. In de fysiologie staat hormoon pulsatiliteit centraal en kan een grote rol spelen bij ziekte. Kennis over het functioneren van dit systeem en begrip van de celprocessen in het brein kan dus van groot belang zijn voor het gericht ontwikkelen van nieuwe farmaca en therapieën voor het behandelen van stress-gerelateerde ziekten zoals bijvoorbeeld depressie. Zoals uitvoerig besproken in **hoofdstuk 7** dragen de resultaten van dit proefschrift bij aan een nieuw concept in de neuroendocrinologie van glucocorticoïd stresshormonen. Pulsatiele blootstelling aan glucocorticoïden blijkt een belangrijke factor in de 'veerkracht' van glucocorticoïd signalering en stress reactiviteit. Verder hebben we aangetoond dat met name de GR wordt beïnvloed wanneer glucocorticoïd pulsatiliteit is verstoord. Dit eiwit zou dus uitstekend als doelwit kunnen functioneren voor therapie en normalisatie van de downstream effecten van stoornissen in glucocorticoïd pulsatiele patronen in stress-gerelateerde ziekten. Mogelijke mechanismen en suggesties voor toekomstig onderzoek worden uitvoerig besproken in de algemene discussie van dit proefschrift.

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Curriculum vitae

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Sarabdjitsingh RA, Conway-Campbell BL, Leggett JD, Waite E, Meijer OC, de Kloet ER, and Lightman SL. Stress responsiveness varies over the ultradian glucocorticoid cycle in a brain region specific manner. Submitted for publication.

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Oral presentations

Leiden / Amsterdam Center for Drug Research (LACDR) Spring Symposium. April 13, 2010, Amsterdam. Title: Disrupted glucocorticoid pulsatility affects glucocorticoid signalling in the rat brain.

Dutch Endocrine Meeting, January 30, 2010, Noordwijk. Title: Disrupted glucocorticoid pulsatility affects glucocorticoid signalling in the rat brain.

Marius Tausk Masterclass with prof. dr. J. Cidlowski. November 5, 2009, Leiden. Title: Selective brain responses to amplitude and phase of glucocorticoid pulses

Leiden Center for Translational Neuroscience (LCTN) Symposium, October 15, 2009, Leiden. Title: Disrupted glucocorticoid pulsatility affects glucocorticoid signalling in the rat brain.

7th Dutch Endo-Neuro-Psycho meeting. June 4, 2008, Doorwerth. Title: Tissue responsiveness to chronically elevated levels of glucocorticoids: nuclear dynamics of MR and GR in the rat brain.

IRTG Summerschool "Psychobiology of stress: steroid hormone action". June 9, 2008, Leiden. Title: Tissue responsiveness to chronically elevated levels of glucocorticoids: nuclear dynamics of MR and GR in the rat brain.

6th Dutch Endo-Neuro-Psycho meeting. June 6, 2007, Doorwerth. Title: Dynamics of nuclear receptor activation: Mineralocorticoid & Glucocorticoid receptors

Annual meeting of the Physiological Society. December 6, 2006. Title: Different translocation patterns of MR and GR in rat hippocampal subfields

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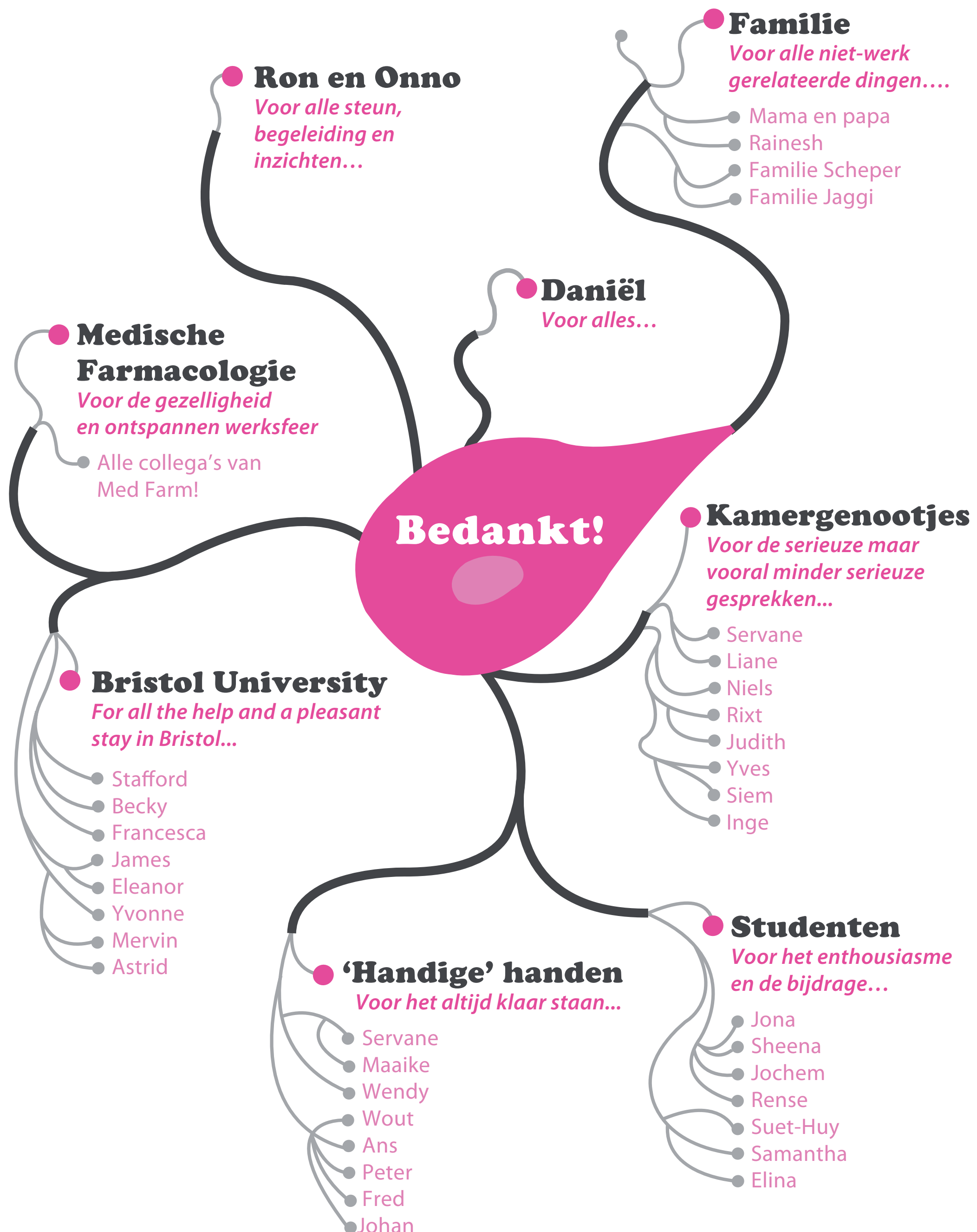

Soms heb je van die dromen die je wakker houden…
tot je ze uitvoert
Loesje