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Glucocorticoids and the circadian clock

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Abstract

Glucocorticoids, hormones produced by the adrenal gland cortex, exert numerous functions in body homeostasis and the response of the organism to external stressors. One striking feature of their regulation is a diurnal release pattern, with peak levels linked to the start of the activity phase. This release is under control of the circadian clock, an endogenous biological timekeeper which acts to prepare the organism for daily changes in its environment. Circadian control of glucocorticoid production and secretion involves a central pacemaker in the hypothalamus, the suprachiasmatic nucleus, as well as a circadian clock in the adrenal gland itself. Central circadian regulation is mediated via the hypothalamic-pituitary–adrenal axis and the autonomic nervous system, while the adrenal gland clock appears to control sensitivity of the gland to the adrenocorticotropic hormone ACTH. The rhythmically released glucocorticoids in turn might contribute to synchronisation of the cell autonomous clocks in the body and interact with them to time physiological dynamics in their target tissues around the day.

Introduction – the circadian clock

The physiology of most organisms changes significantly between day and night. This represents a key adaptation to cope with the different environmental challenges the organism faces at different times of day, such as changes in lighting conditions and temperature, food availability or the presence of predators. Many of the rhythmic physiological changes are not simply "driven" by environmental changes, because the rhythms persist even under constant conditions. Rather, these biological rhythms are generated by an endogenous biological "clock" or "pacemaker" which keeps time even in the absence of environmental cues (Dunlap et al. 2004). Characteristically, the period length of this clock is slightly shorter or longer than 24 hours when measured in constant conditions, hence it is termed "circadian" (= about one day) clock. In order to avoid drifting out of phase with respect to the environment, the circadian clock is reset, or "entrained", to the local conditions by environmental timing cues such as light or temperature. In the absence of such cues, the clock "free-runs" according to its endogenous period.

A central pacemaker in the brain – the suprachiasmatic nucleus of the hypothalamus

The simplest concept of a circadian clock is that of an endogenous oscillator, which receives timing information from the environment through an input pathway and drives physiological rhythms via an output pathway. In mammals, the search for the endogenous oscillator identified a small paired nucleus in the anterior hypothalamus, the **s**upra**c**hiasmatic **n**ucleus (SCN), as the seat of the pacemaker driving rhythms in behaviour and many aspects of physiology, including hormonal secretion (Klein et al. 1991). The SCN receives

input from the eyes through the retino-hypothalamic tract. The principal light detecting system conveying timing information to the SCN are not the rods and cones, the dedicated visual photoreceptors of the retina, but rather a subset of directly light sensitive retinal ganglion cells which express a special photopigment, melanopsin (Berson 2007; Hankins et al. 2008), and which project directly to the SCN. Here, light input triggers a signalling cascade which changes gene expression and the firing rate of SCN neurons (Antle and Silver 2005; Maywood et al. 2007). Output projections from the SCN target many different brain regions, amongst others the subparaventricular zone, the dorsomedial nucleus and the paraventricular nucleus of the hypothalamus (Fig. 2, (Buijs and Kalsbeek 2001; Kalsbeek and Buijs 2002; Saper et al. 2005)), and may underlie the circadian changes observed in processes governed by these structures.

The molecular clockwork – a transcriptional negative feedback loop

Interestingly, single SCN neurons in dispersed culture also show circadian rhythms of electrical activity (Welsh et al. 1995). This, together with the presence of circadian clocks in unicellular organisms (Roenneberg and Merrow 2002; Mackey and Golden 2007), suggested that the oscillator works in a cell-autonomous manner. Combined with genetic studies in a variety of organisms, notably in *Drosophila* and mouse, these findings have led to the elucidation of the basic molecular mechanisms of the circadian clock (Fig. 1; for review see e.g. (Rosato et al. 2006), (Ko and Takahashi 2006)). The core clock elements turned out to be transcription factors participating in a delayed feedback loop that leads to oscillating gene transcription. In vertebrates, a heterodimer of the bHLH-PAS family members CLOCK and BMAL1 activates transcription of the *period (per)*

and *cryptochrome (cry)* genes upon binding to so-called E-box regulatory sequences in their promoters. Protein accumulation of PERs and CRYs and their localisation to the nucleus takes several hours and peaks at the end of circadian daytime. The CRY and PER proteins then act to inhibit transcriptional activity of the CLOCK/BMAL1 heterodimer and thereby shut off their own transcription. This core negative feedback loop is modulated by a number of different mechanisms, which all seem to contribute to the stability and exact timing of the clock cycles. Thus, an accessory feedback loop drives rhythmic expression of the *bmal1* gene through RORE (**R**etinoic acid-related **O**rphan receptor **R**esponse **E**lement) regulatory sequences in its promoter. These sequences are sequentially bound by the nuclear receptor proteins RORα, which acts as an activator, and REV-ERBα, acting as a repressor (Duez and Staels 2008). Also posttranslational regulation influences the properties of the feedback loop (Gallego and Virshup 2007): Several kinases have been shown to phosphorylate clock genes and thereby alter their stability, nuclear localisation or transcriptional activity. Additionally, F-box protein containing ubiquitin ligase complexes have been implicated in feedback loop regulation (Busino et al. 2007; Godinho et al. 2007; Siepka et al. 2007). In the cyanobacterial clock system, which uses genes dissimilar to the vertebrate clock, three clock proteins alone are sufficient to create circadian cycles of phosphorylation in a test tube, suggesting that, at least in this system, a circadian rhythm can be generated without a transcription-translation feedback loop (Nakajima et al. 2005). However, recent results indicate that the phosphorylation rhythm is not strictly required for circadian rhythm generation in the bacterial cells and that the transcription-translation feedback loops appear to be necessary for clock function at low temperature (Kitayama et al. 2008) (for an alternative

interpretation of these results see (Brunner et al. 2008)). Also in *Drosophila* and in vertebrates, there is evidence that transcriptional regulation plays a crucial role in the clock mechanism and does not serve just as a means of linking clock oscillations to clock output (Sato et al. 2006; Kadener et al. 2008). Thus, despite the increasingly recognised importance of protein modifications in clock regulation, transcription-translation feedback loop mechanisms still appear as an important player in the clock of all systems studied so far (Brunner and Schafmeier 2006; Zheng and Sehgal 2008).

"Central" and "peripheral" pacemakers

It had long been thought that the circadian clock was the function of a limited number of specialized pacemaker structures such as the SCN or, in lower vertebrates, also the retina and pineal gland (Menaker et al. 1997). These so-called "central pacemakers" were considered to be responsible for generating all circadian rhythms within the organism. However, some early work also reported the existence of autonomous circadian rhythms in explant cultures of "peripheral" organs, notably in the adrenal gland (Andrews and Folk 1964; Andrews 1971) and the heart (Tharp and Folk 1965). These findings were confirmed after the cloning of clock genes: Basic clock components are not only expressed in the central pacemaker structures, but throughout the animal, where they also show oscillating expression (Balsalobre 2002). These rhythms persist in organ cultures (Whitmore et al. 1998), as also revealed by real-time imaging of such cultures from bioluminescent transgenic animals carrying clock gene reporter constructs (Yamazaki et al. 2000; Yoo et al. 2004; Kaneko et al. 2006). Even many non-neural cultured cell lines exhibit circadian expression of clock genes (Balsalobre et

al. 1998; Whitmore et al. 2000; Nagoshi et al. 2004; Welsh et al. 2004; Carr and Whitmore 2005). Thus, autonomous peripheral clocks exist in most cells and tissues. These findings have changed the view of the central pacemakers, which are now no longer considered to be the direct drivers of most peripheral rhythms, but instead appear to interact with and coordinate the many autonomous peripheral oscillators.

Circadian regulation of the transcriptome

Which biological processes are under control of this "web of pacemakers" (Schibler and Sassone-Corsi 2002)? Before the advance of microarray gene expression monitoring, few genes had been identified that were regulatory targets of clock output pathways (e.g. *dbp* (Wuarin et al. 1992), *hlf* (Falvey et al. 1995), *tef* (Fonjallaz et al. 1996), *pdf* (Renn et al. 1999)). However, studies in cyanobacteria (Liu et al. 1995; Van Gelder et al. 1995) indicated that a large proportion of the genome might be expressed in a circadian fashion. These estimations have been validated by microarray studies ((Duffield 2003; De Haro and Panda 2006), see also (Bozek et al. 2007; Keegan et al. 2007) and references therein), showing oscillating transcripts e.g. for 8-10 % of genes in different mouse tissues (Panda et al. 2002; Storch et al. 2002). The number of cycling proteins might be still higher, as a recent liver proteomics study reports circadian changes in up to 20% of soluble liver proteins, even if their corresponding mRNAs do not cycle (Reddy et al. 2006). Cycling genes fulfil a broad range of functions in all the tissues studied, including peptide synthesis, processing and secretion; vesicle trafficking; detoxification and metabolism (Claridge-Chang et al. 2001; McDonald and Rosbash 2001; Panda et al. 2002; Storch et al. 2002). The circadian regulation of

metabolic pathways observed in these studies often occurs at the level of rate limiting enzymes (Claridge-Chang et al. 2001; McDonald and Rosbash 2001; Panda et al. 2002; Reddy et al. 2006).

Interestingly, there is only little overlap in cycling transcripts between different tissues: even though about 60 % of the genes cycling in one of the tissues are also expressed in the other, they do not cycle there (Panda et al. 2002; Storch et al. 2002). While this certainly reflects tissue specific circadian regulation, some caution has to be exerted when interpreting the microarray data, since recent meta-analyses show that the overlap of genes identified as cycling between different studies using similar samples is quite limited (Bozek et al. 2007; Keegan et al. 2007). This is most likely due to differences in the mathematical methods for analysis of rhythmicity or to lab-dependent differences in microarray protocols and platforms. Nevertheless, the data suggest a model of transcriptional hierarchies with some conserved elements such as E-boxes or ROREs at the top that drive gene expression in certain phases of the circadian cycle (Ueda et al. 2005). These elements could then interact with tissue specific transcription factor networks to create tissue specific cycling gene profiles, which all the same influence a largely overlapping set of cellular processes. The exact structure of these transcriptional hierarchies and the links between direct clock targets and downstream processes in different tissues are still largely unknown.

Integration of central and peripheral pacemakers

How do the central and the peripheral clocks interact in the circadian control of physiology within the animal? Tissue-specific disruption of the liver clock abolishes circadian transcription of most genes in the liver, illustrating the

importance of the peripheral pacemaker for circadian regulation of transcription (Kornmann et al. 2007). On the other hand, following ablation of the SCN no circadian rhythm can be detected in the liver transcriptome (Akhtar et al. 2002). Interestingly, organ cultures derived from SCN-ablated animals still show rhythmic clock gene expression, but with different phases for each cultured organ sample, indicating a loss of synchronisation of peripheral pacemakers within the animal (Yoo et al. 2004). When different samples are pooled for microarray analysis, these desynchronised phases will cancel each other out, and no circadian rhythm is detected. Strikingly, the liver specific clock arrest also revealed some genes whose circadian expression was independent of the peripheral pacemaker and appeared to be directly driven by centrally derived cues (Kornmann et al. 2007).

The search for mechanisms integrating central and peripheral clock regulation of physiology has brought endocrine rhythms back into focus. Many hormones show circadian patterns of release and are therefore well placed as candidate signals that may coordinate central and peripheral rhythms (Aschoff 1979; Hastings et al. 2007; Haus 2007). Notably glucocorticoids have been implicated in the regulation of peripheral circadian rhythms and are themselves subject to circadian regulation at many levels of organisation.

Here, we review both the circadian regulation of synthesis and release of glucocorticoids as well as their interaction with clock controlled processes. We will first examine SCN control of the **h**ypothalamic-**p**ituitary-**a**drenal (HPA) axis and then turn towards an alternative route from the SCN to the adrenal gland, namely the autonomic nervous system. We will study the role that the peripheral

pacemaker of the adrenal gland itself plays in circadian glucocorticoid secretion and discuss how it might interact with the central control mechanisms. After touching upon potential roles for other peripheral clocks in the nervous system and briefly reviewing circadian regulation of glucocorticoid feedback and stress reactions, we will consider functions of circadian glucocorticoid changes in their target tissues. A role for glucocorticoids in coordinating peripheral pacemakers throughout the body and in the brain will be explored, and we will examine how glucocorticoids interact with peripheral clocks in the control of target tissue dynamics. Finally, we will discuss potential mechanisms by which the cell-autonomous circadian clock might modulate the glucocorticoid signalling pathway, and we will end with an outlook upon some areas for future research.

Circadian patterns of glucocorticoid release

Glucocorticoid hormones fulfil many different functions in body homeostasis and stress responses (Norris 2007). Their secretion shows pronounced temporal regulation, with both pulsatile ("ultradian" = shorter than a day) and circadian rhythmicity (Chrousos 1998; Haus 2007; Lightman et al. 2008). The circadian peak in corticosterone release is locked to the activity phase of the animal: it occurs in the early morning in diurnal and in the early night in nocturnal animals (Cheifetz 1971; Perlow et al. 1981; Lincoln et al. 1982; Klemcke et al. 1989; Van Cauter 1990; Lefcourt et al. 1993; Irvine and Alexander 1994). Globally, the ultradian pulses occur with a frequency of about one to two per hour (Weitzman et al. 1971; Holaday et al. 1977; Tapp et al. 1984; Jasper and Engeland 1991; Loudon et al. 1994; Sarnyai et al. 1995; Windle et al. 1998a; Windle et al.

1998b), and their amplitude increases towards the end of the day period in nocturnal animals (Chrousos 1998; Windle et al. 1998a; Windle et al. 1998b; Lightman et al. 2008). These rhythms are paralleled by similar rhythms in the **A**drenoc**c**orticot**t**ropic **H**ormone (ACTH), albeit less robust and of lower amplitude (Carnes et al. 1988; Carnes et al. 1989; Veldhuis et al. 1990; Sarnyai et al. 1995; Gudmundsson and Carnes 1997; Chrousos 1998). ACTH is released by the corticotrope cells of the pituitary ("C" in Fig. 2), binds to melanocortin type II receptors in the adrenal cortex (mainly zona glomerulosa and fasciculata (Abdel-Malek 2001)) and acts to stimulate corticosteroid synthesis (Fig. 3A, (Norris 2007)). ACTH release in turn can be stimulated by the secretagogues **C**orticotropin **R**eleasing **H**ormone (CRH), **A**rginine **V**aso**p**ressin (AVP) and oxytocin, which are released into the portal blood stream of the median eminence from nerve terminals projecting from the **p**ara**v**entricular **n**ucleus (PVN) of the hypothalamus (Fig. 2, (Whitnall et al. 1985; Engler et al. 1989; Chrousos 1998; Jacobson 2005)). Also CRH synthesis seems to be under circadian control, as its pre-mRNA shows diurnal variations (Watts et al. 2004). However, ACTH release precedes the onset of *crh* gene transcription considerably, implicating that coupling of the two processes might be only loose.

Experimental difficulties brought about by the pulsatile nature of release patterns may in part explain the abundance of conflicting reports in the literature regarding the exact parameters of the circadian and ultradian rhythms in the hypothalamic-pituitary-adrenal (HPA) axis. There is also evidence that the mechanistic link between ACTH and glucocorticoid secretion might indeed be less tight than previously thought: under various physiological and pathophysiological conditions, the pulses of ACTH and glucocorticoids do not correlate, indicating

dissociation between the two factors (reviewed in (Bornstein et al. 2008), see also below). Thus, uncoupling among elements of the HPA axis can be seen at multiple levels.

SCN control of HPA axis activity

Neuroanatomy of SCN – HPA axis connections

The circadian aspects of the rhythmic release of glucocorticoids and their tropic hormone ACTH are under control of the SCN, since SCN lesions abolish the rhythms (Moore and Eichler 1972; Abe et al. 1979; Szafarczyk et al. 1979; Cascio et al. 1987). Interestingly, the SCN also exhibits ultradian rhythms of activity, but at present it is unknown if and how these rhythms might contribute to the ultradian rhythms of the HPA axis and of glucocorticoid release (Yamazaki et al. 1998; Lowry 2002). Since SCN grafts into SCN lesioned hamsters are able to restore circadian locomotor rhythms, but fail to restore the circadian endocrine rhythms including those of glucocorticoids, axonal connections seem to be required for their circadian control (Meyer-Bernstein et al. 1999). The SCN shows few direct projections to the **m**edio**p**arvocellular division of the PVN (mpPVN) (Vrang et al. 1995; Engeland and Arnhold 2005), where the CRH and AVP expressing neurons projecting to the median eminence are located (Fig. 2, broken black arrow, (Antoni et al. 1983; Swanson et al. 1983; Sawchenko et al. 1984a; Sawchenko et al. 1984b)). However, many neighbouring areas in the PVN are also innervated by the SCN ("I" in Fig. 2) and they may contact the hypophysiotropic neurons as well ((Berk and Finkelstein 1981; Stephan et al. 1981; Watts et al. 1987; Buijs et al. 1993b; Vrang et al. 1995) and references therein). Furthermore, the SCN contacts

the **subp**ara**v**entricular **z**one (subPVZ) and the **d**orso**m**edial nucleus of the **h**ypothalamus (DMH), which in turn send projections to the mpPVN and to many other brain areas (Fig. 2, (ter Horst and Luiten 1986; Buijs et al. 2003; Engeland and Arnhold 2005)). It has been proposed that this pattern of SCN projections to central relay stations allows for an efficient contact with many different brain regions, enabling simultaneous circadian changes in various brain outputs (Watts et al. 1987; Buijs et al. 1993b) and integration with other environmental cues (Saper et al. 2005). Other brain regions targeted by the SCN that may influence PVN output include the arcuate nucleus, the preoptic and supraoptic nuclei of the hypothalamus and the paraventricular nucleus of the thalamus (Saeb-Parsy et al. 2000; Buijs et al. 2003; Pecoraro et al. 2006).

Function of the SCN in HPA axis control

Morning (trough) corticosterone levels have been reported to increase after ablation of the SCN in rats, indicating that at least part of the circadian control might operate in an inhibitory fashion (Buijs et al. 1993a). One of the main neurotransmitters in the SCN projections towards the PVN/DMH areas is AVP (Buijs et al. 1993b; Buijs et al. 1999), and it shows a diurnal release rhythm in the cerebrospinal fluid and the SCN (Reppert et al. 1981; Kalsbeek et al. 1995). Infusion of AVP into the paraventricular/dorsomedial hypothalamus area inhibits corticosterone release in SCN ablated rats (Kalsbeek et al. 1992), indicating 1) that this peptide alone can mimic the inhibitory effect of the SCN and 2) that the paraventricular/dorsomedial hypothalamic region mediates this SCN effect. In support of this idea, corticosterone release is stimulated when AVP is blocked by antagonist infusion into these brain regions during the circadian peak times of AVP

release (Kalsbeek et al. 1992; Kalsbeek et al. 1996a; Kalsbeek et al. 1996b). Thus, AVP acts twofold in the hypothalamic control of the HPA: a) its release by the mpPVN neurons into the median eminence stimulates ACTH secretion, and b) it acts as an inhibitory neurotransmitter in the SCN control of the mpPNV activity, most likely via the indirect neural pathways detailed above. Interestingly, the inhibitory role of AVP in the nocturnal rat is reversed in the diurnal rodent *Arvicanthis ansorgei*: Hypothalamic AVP infusion leads to stimulation of corticosterone release, whereas infusion of an antagonist blocks the endogenous corticosterone peaks (Kalsbeek et al. 2008). Thus, whereas the timing of clock gene expression, neural activity and transmitter release by the SCN are similar in both species, the effect on the brain targets is different. The authors propose that the SCN projections might contact different interneurons in the subPVZ and/or DMH areas, inhibitory GABA-ergic neurons in the case of the rat and excitatory glutamatergic neurons in *A. ansorgei*, which would then exert their effects on PVN neurons. More detailed anatomical and neurochemical studies will be needed to validate this attractive hypothesis.

In addition to the inhibitory SCN control of corticosterone secretion, there is also evidence for a stimulatory role of the SCN in corticosterone release in the rat. Thus, in adrenalectomised animals supplemented with corticosterone pellets (avoiding confounding corticosterone feedback effects and increasing daily ACTH rhythms), the evening *rise* in ACTH is abolished by SCN ablation (Cascio et al. 1987). Also, the increases in ACTH and corticosterone secretion upon AVP antagonist infusion are most pronounced during the second half of the light period, consistent with the stimulatory activity appearing during this period (Kalsbeek et al. 1996b). The nature of this stimulatory agent is still elusive, although

Neuromedin-U has recently been proposed as a candidate (Graham et al. 2005; Kalsbeek et al. 2006).

An alternative pathway of circadian control: the autonomic nervous system

Changes in glucocorticoid release are not always dependent on ACTH changes

In the above discussion, we have focused our attention on the HPA axis and potential SCN actions on circadian glucocorticoid release via this pathway. However, there is evidence that the SCN also regulates circadian changes in adrenal activity through alternative routes. In hypophysectomised animals, e.g. gulf killifish (Srivastava and Meier 1972) or rats (Meier 1976), rhythms of corticosteroid secretion are still present in the absence of cycling ACTH levels. Interestingly, the corticosterone rhythms are lost in the hypophysectomised rats once their adrenal glands are denervated, arguing for the involvement of a neural control mechanism (Ottenweller and Meier 1982).

A neural SCN-adrenal gland connection

Significant evidence now points to a neural SCN-adrenal gland connection via the autonomic nervous system (Fig. 2, blue arrows; (Buijs et al. 1999; Ueyama et al. 1999); reviewed in (Engeland and Arnhold 2005)). Transneuronal retrograde virus tracing revealed a multisynaptic pathway from the SCN to the adrenal gland, which passes via pre-autonomic PVN neurons as a first relay station to the **i**nter**m**edio**l**ateral column (IML) of the spinal cord, the second relay station. The

pre-autonomic PVN neurons contact sympathetic preganglionic neurons in the IML, which then innervate the adrenal gland through the splanchnic nerve (Buijs et al. 1999). Interestingly, this pathway apparently does not overlap with the SCN connections to the PVN via the subPVN and DMH described above, indicating a separation of the autonomic from the HPA axis targeted pathways of SCN control at the level of the PVN.

The autonomic neural control of circadian glucocorticoid secretion has been demonstrated through two lines of evidence: 1) the neural pathway transmits light information to the adrenal gland that then leads to corticosterone release changes without accompanying changes in the HPA axis, and 2) the pathway modulates the adrenal sensitivity to ACTH.

Light influences the adrenal gland via the autonomic SCN pathway

In rats, light induces a fast (within 5 min) repression of corticosterone release when given at the beginning of the night, but not later in the night or at the beginning of the day (Buijs et al. 1999). In a similar study, Ishida et al. did not report such a decrease in mice, but rather saw an increase after 1h during the subjective night only, not during the day (Ishida et al. 2005); this slower response was not examined by Buijs et al. Importantly, these changes were not accompanied by changes in ACTH, demonstrating that they must be mediated outside the HPA axis. The light input depends on the SCN, since SCN ablation abolished the light dependent glucocorticoid release changes (Buijs et al. 1999; Ishida et al. 2005). The light signal is most likely transmitted through the autonomic SCN-adrenal pathway described above, as light exposure increased adrenal nerve activity, but only if the SCN was intact, and adrenal denervation blocked the light induced

corticosterone release changes (Niijima et al. 1992; Niijima et al. 1993; Ishida et al. 2005). The transmission of the signal to the adrenal cortex might involve adrenaline release by the adrenal medulla after the light pulse (Ishida et al. 2005), and such paracrine medulla-cortex interactions have also been described previously upon splanchnic nerve stimulation (Bornstein et al. 1990; Ehrhart-Bornstein et al. 1998). It will be interesting to examine if these light pulse evoked mechanisms are also involved in the generation of the normal circadian glucocorticoid surge observed at the end of the light phase in nocturnal rodents, a time point not tested in the above studies, and whether similar mechanisms are involved in the glucocorticoid rise at the dark-light transition in diurnal species.

Neural control of adrenal gland sensitivity to ACTH

The second evidence for autonomic control over circadian glucocorticoid secretion involves regulation of the adrenal sensitivity to ACTH (reviewed in (Engeland and Arnhold 2005; Ulrich-Lai et al. 2006)). This sensitivity is tested by measuring the corticosterone release response to exogenous ACTH when the endogenous ACTH rhythm is blocked by dexamethasone treatment. Dexamethasone is a glucocorticoid receptor agonist which causes downregulation of ACTH secretion via central glucocorticoid feedback mechanisms (see also below). Under these conditions, ACTH sensitivity shows a diurnal rhythm in rats, with higher sensitivity leading to higher corticosterone release in the evening (Dallman et al. 1978). Ablation of the SCN abolishes these rhythms (Sage et al. 2002) and denervation of the adrenal glands leads to a decrease of corticosterone secretion and of ACTH sensitivity in the evening (Dijkstra et al. 1996; Ulrich-Lai et al. 2006). Together, these findings suggest that neural control mechanisms are

involved in ACTH sensitivity regulation and that circadian control might be mediated via the SCN-autonomic-adrenal pathway. Since the ACTH sensitivity studies generally are carried out in the presence of dexamethasone, it will be important to examine potential contributions of direct effects of this drug on sympathetic innervation (Brown and Fisher 1986) or the adrenal gland itself (Gummow et al. 2006). Indeed, there is some evidence that corticosterone levels can modulate the SCN driven ACTH sensitivity changes (Sage et al. 2002).

One possible mechanism at work in autonomic control of glucocorticoid release from the adrenal gland might be illustrated by recent studies on the role of the autonomic innervation of the liver in daily glucose rhythms (Cailotto et al. 2008). This work revealed that a balance of sympathetic and parasympathetic inputs is required for the glucose rhythm to occur: The rhythm is abolished by either sympathectomy or parasympathectomy, but present when both inputs are removed simultaneously. Future studies will reveal whether similar balance mechanisms could be at work in the regulation of adrenal glucocorticoid secretion.

The adrenal gland peripheral pacemaker

The peripheral clock of the adrenal gland might gate ACTH sensitivity

In addition to rhythmic HPA axis activity and neural connections a third player has emerged in the circadian control of glucocorticoid secretion: the peripheral clock of the adrenal gland itself. The existence of such an organ-autonomous clock was suggested by pioneering work of Andrews and colleagues, who showed that adrenal glands exhibit circadian rhythms of metabolic activity and glucocorticoid release even in culture (Andrews and Folk 1964; Andrews

1971). Later, clock genes were found to be expressed and to cycle also in the adrenal gland (Bittman et al. 2003; Ishida et al. 2005; Lemos et al. 2006; Oster et al. 2006a; Oster et al. 2006b; Torres-Farfan et al. 2006; Fahrenkrug et al. 2008). Experiments by Oster et al. (Oster et al. 2006b) suggest that this autonomous adrenal clock contributes to the ACTH sensitivity rhythm: adrenal slice cultures from wildtype mice showed a stronger response to ACTH stimulation in the evening, when the natural peak in glucocorticoid secretion would occur, than around the morning nadir timepoint. This difference was absent in adrenal cultures derived from *per2/cry1* double mutant mice, which lack a functional molecular clock. Subsequent *in vivo* experiments were consistent with these findings: Adrenal gland transplants from wild type mice into *per2/cry1* double mutant hosts still secreted corticosterone in a diurnal fashion, even though no ACTH rhythm was present in their clockless hosts. This rhythmic secretion ceased after transfer to constant darkness, which caused a loss of rhythmic clock gene expression in the transplanted adrenals. Thus, a circadian rhythm in adrenal ACTH sensitivity in the transplants would translate the constant ACTH levels in the mutant hosts into different amounts of corticosterone secretion in the morning and the evening. To support this hypothesis, it would be interesting to test if the differences in response to exogenous ACTH application observed in the slice cultures are also present in these transplanted adrenals *in vivo*. One caveat for these experiments stems from the fact the adrenal glands might become reinnervated upon transplantation in rats (Ulrich-Lai and Engeland 2000). It would therefore be important to examine if such ectopic innervation occurs under the surgery protocol used in the mice and if it might give time cues to the adrenal glands independent from the tissue autonomous clock.

Clock regulation of the adrenal transcriptome

These caveats aside, it appears that the adrenal gland clock gates ACTH input into glucocorticoid synthesis to certain times of day (Fig. 3, C). Indeed, among the many genes showing circadian mRNA expression in the adrenal gland, there is the ACTH receptor itself as well as components of its downstream signalling pathway, such as *Adcy5*, several G proteins, protein kinase A, and protein phosphatase 1 subunits (Oster et al. 2006a; Oster et al. 2006b). It will be instructive to see whether these rhythms are directly driven by the adrenal clock machinery or if systemic cues also contribute to their rhythmic expression. This would require comparison with the trancriptome of a clockless adrenal gland *in vivo*, similar to the liver studies by Kornmann et al. (Kornmann et al. 2007). Another interesting question is whether the proteins of the ACTH pathway genes, or their posttranslational modifications, equally exhibit circadian rhythms that could then be directly linked to the rhythms in ACTH sensitivity.

Other genes implicated in the biosynthesis of glucocorticoids, such as regulators of cholesterol transport, also show circadian rhythms of expression; interestingly however, the genes previously demonstrated as rate-limiting for the synthesis, like steroidogenic acute regulatory protein (*Star*) or cytochrome P450 side-chain cleavage enzyme (*Cyp11a1*) (Payne and Hales 2004; Stocco et al. 2005), are not amongst them (Oster et al. 2006b). This is in contrast to findings in the liver, which showed rate-limiting steps of different physiological pathways as clock targets ((Panda et al. 2002) and see above). One could speculate that the rapid regulatory requirements of acute stress response and feedback regulation

preclude a tight circadian control of these genes, pushing clock control to other parts of the synthesis pathway.

Circadian expression analysis of the core clock gene mRNAs themselves in the adrenal gland shows differences in expression levels and in cycling between the medulla and different parts of the cortex, reflected also by the somewhat conflicting reports in the literature (Bittman et al. 2003; Ishida et al. 2005; Lemos et al. 2006; Oster et al. 2006a; Oster et al. 2006b; Torres-Farfan et al. 2006; Fahrenkrug et al. 2008). Such expression differences of core clock genes might lead to tissue-specific changes in clock controlled gene expression. Indeed, differential requirements of certain clock genes for clock function in different tissues and for different target genes are currently being discussed (Oishi et al. 2000; Reick et al. 2001; Debruyne et al. 2006; Kennaway et al. 2006; DeBruyne et al. 2007a; DeBruyne et al. 2007b; Bertolucci et al. 2008).

Does the autonomic nervous system regulate the adrenal clock?

The adrenal clock might be the link which integrates the findings on neural control of corticosterone secretion into a common framework. Nocturnal light exposure signalled via the splanchnic nerve elevated *per1* gene expression in the adrenal (Ishida et al. 2005), indicating that environmental light information might act to reset the peripheral clock via this pathway. Changes in clock gene expression might then be passed on to gene expression in the ACTH pathway and thereby change adrenal gland receptiveness for the hormone (Fig. 3, B and C). At present, it is not known if and how the light induced *per1* expression leads to sustained changes in clock rhythms. A role for the autonomic nervous system in peripheral clock regulation is supported by findings that chemical denervation of sympathetic

liver input led to flattened rhythms of clock gene expression ((Terazono et al. 2003), but see also (Cailotto et al. 2005) for different results), and sympathetic innervation has also been shown to modulate clock gene expression in submaxillary salivary glands in rats (Vujovic et al. 2008). Thus, denervation of the adrenal gland might lead to desynchronisation of clock gene expression, and thereby influence ACTH sensitivity rhythms. An examination of clock gene expression in denervated adrenals will be needed to explore this possible mechanism.

Alternatively, signals from the autonomic nervous system might have more direct (and maybe faster) effects on corticosterone synthesis or ACTH sensitivity in the adrenal gland. To functionally dissect adrenal clock dependent and independent mechanisms in circadian glucocorticoid secretion, tissue specific arrest of the peripheral clock in the adrenal gland will be necessary, e.g. using the methods recently applied to the liver clock (inducible overexpression of *rev-erbα* to block the feedback loop (Kornmann et al. 2007)) or the retina clock (tissue-specific deletion of the *bmal1* gene (Storch et al. 2007)). Gene expression patterns from such clockless adrenals could then be compared with those of wildtype glands, throughout the circadian cycle or after a nocturnal light pulse. This will reveal adrenal clock independent mechanisms of circadian glucocorticoid control (Fig. 3A and C). Such systemic mechanisms doubtlessly exist, as adrenal glands from the clockless *cry1/per2* mutants transplanted into wild type hosts still secrete corticosterone rhythmically, albeit only with 40-50% of the amplitude seen in wildtype control grafts (Oster et al. 2006b). The full extent of these control mechanisms *in vivo* will only be revealed in normally innervated adrenals lacking a circadian clock.

Since adrenal medulla-cortex interactions have been proposed to play a role in glucocorticoid rhythms (see above), one could try to generate medulla and cortex specific clock arrest animals and examine tissue specific clock contributions to the rhythms. In this context, it would also be interesting to look at a potential role for the clock in the intra-adrenal CRH/ACTH feedback system (Ehrhart-Bornstein et al. 1998).

Do nervous system clocks outside the SCN contribute to glucocorticoid rhythms?

Peripheral clocks in the nervous system

The proposed interaction of the adrenal peripheral clock with systemic cues raises the interesting possibility that clocks in the pathway downstream of or parallel to the SCN might also contribute to circadian glucocorticoid secretion. Indeed, circadian rhythms of clock gene expression have been described in explants of the PVN (Abe et al. 2002) and of the pituitary (Yoo et al. 2004), but very little is known about the functions of these autonomous circadian rhythms. As in the case of the adrenal gland, tissue specific arrest of the clock will clarify if and how glucocorticoid rhythms depend on these clocks.

The "food-entrainable oscillator" clock

One such potential additional clock that might influence the circadian rhythm of glucocorticoid secretion is the postulated "food-entrainable oscillator" (FEO). The daily rest/activity cycle of the animal also generates a feeding/fasting cycle, with most of the food ingested during the night in rodents. When food

becomes restricted to certain times of the day, the animals display an increase in activity prior to the feeding time, which continues also in the absence of food on consecutive days and is therefore generated endogenously. The presence of an independent food-entrainable oscillator was suggested based on the observation that on such a time restricted feeding scheme, the rhythms of food anticipatory behaviour and physiology, including corticosterone secretion (Krieger et al. 1977), occur even in SCN lesioned animals (reviewed in (Hiroshige et al. 1991; Mistlberger 1994; Stephan 2002; Pecoraro et al. 2006; Mendoza 2007)). Some studies claim that the DMH, which we have already encountered as a major relay station for SCN input into the PVN (Fig. 2), may be the site of the FEO (Gooley et al. 2006; Mieda et al. 2006; Fuller et al. 2008), but this is questioned by others ((Landry et al. 2006; Landry et al. 2007), see also (Davidson 2006; Herzog and Muglia 2006; Gooley and Saper 2007; Landry and Mistlberger 2007)).

While food anticipatory activity rhythms remain in some clock mutants (Dudley et al. 2003; Pitts et al. 2003; Iijima et al. 2005), the clock genes *bmal1* and *per2* have been proposed to be crucial for the food-entrained oscillator (Feillet et al. 2006; Fuller et al. 2008). Fuller et al. injected adeno-associated viral vectors carrying the *bmal1* gene under control of its own promoter into the DMH of arrhythmic *bmal1* knock-out mice (Fuller et al. 2008). In this way, they were able to restore food-entrained rhythms in the mutant mice, indicating that clock function in this structure alone is sufficient for food entrainment. It will be interesting to see whether also corticosterone secretion patterns were restored by this procedure, or whether they would additionally require a rescue of the adrenal clock.

Circadian rhythms of feedback sensitivity and stress responsiveness

Glucocorticoids themselves contribute to shaping the circadian rhythm via negative feedback regulation of ACTH release (Jacobson 2005). This feedback is mediated by mineralocorticoid receptors (MR) and glucocorticoid receptors (GR) in brain and pituitary. Despite its name, the MR binds glucocorticoids with higher affinity than the GR. The GR appears to exert predominant effects in the pituitary, while MR mediates the brain effects. Since MR expression in the brain is highest in the hippocampus and septum (Arriza et al. 1988), the brain mediated feedback seems to stem mainly from extra-hypothalamic sites. The sensitivity of ACTH release to glucocorticoid feedback is highest during the trough point of the circadian glucocorticoid rhythm and appears to depend only on MR at this time. Feedback sensitivity is lowest at the peak point and involves binding of glucocorticoids to both MR and GR (Dallman et al. 1993; Jacobson 2005). Recently, it has been proposed that also a membrane bound mineralocorticoid receptor might play a role in feedback regulation by mediating more rapid responses than the classical "genomic" (= nuclear receptor-like) action of MRs (Joels et al. 2008).

Also the responsiveness of the HPA axis to stress has been shown to vary diurnally (e.g. (Sage et al. 2001; Kalsbeek et al. 2003; Atkinson et al. 2006) and references therein). The precise nature of the stress seems to be important in these responses: It has been suggested that, at least in rats, psychological stresses (such as a novel environment or restraint) elicit the largest response early during the day, in the rest phase. In contrast, physical stresses (such as hypoglycaemic shock) do so later in the day, at the onset of activity. Physical stress information appears to be relayed to the PVN mainly via the brainstem, whereas the psychological stresses

require interpretation from higher brain centres and involve the limbic system (Jacobson 2005). The SCN might differentially interfere with signals from these different brain structures to the PVN, e.g. inhibit input from physical stressors later in the day while enhancing input from psychological stressors at the same time point. More detailed neuroanatomical and functional studies are required to test this hypothesis. Stress also appears to feed back on the clock control of the glucocorticoid rhythms: Chronic stress can flatten the circadian rhythm of glucocorticoid production and increase the frequency of the ultradian pulses (Windle et al. 2001; Lightman 2008), thereby suggesting an involvement of the clock in stress related diseases. Insight into which of the various modes of circadian control over corticosteroid secretion discussed above (Fig. 3) are involved in the response to different stressors might pinpoint suitable entry points for therapeutic intervention in stress connected disorders.

Are different systemic pathways used for circadian vs. stress control of glucocorticoid secretion?

In summary, it appears that clock control of glucocorticoid secretion does not primarily operate through the HPA axis, but more via the adrenal clock itself and sympathetic input to the adrenal. In the circadian system, cycling of ACTH levels might just form an additional layer of security contributing to the circadian gating of corticosterone secretion by the adrenal gland. In contrast, ACTH-dependent glucocorticoid regulation might play a more prominent role in acute stress responses (Kalsbeek et al. 1996b; Kalsbeek et al. 2003). As mentioned earlier, dissociation of ACTH and glucocorticoid responses has been observed in

many other physiological and especially clinical situations and receives increasing attention (Bornstein et al. 2008). Hence, although ACTH is required for baseline glucocorticoid production, its role in glucocorticoid regulation seems to be more complex than previously thought.

Glucocorticoids and the regulation of peripheral clocks

Glucocorticoids are not just a circadian output of the adrenal gland, downstream of central and peripheral clocks, they can also influence the circadian clock itself and interact with other clock outputs in the circadian regulation of physiology (Fig. 4).

Thus, glucocorticoids have been implicated in the synchronisation of circadian gene expression by serum shock in cultured fibroblasts (Balsalobre et al. 1998): Transient treatment of rat-1 fibroblasts with the glucocorticoid receptor agonist dexamethasone alone leads to circadian rhythms of clock gene expression similar to those seen after a serum shock (Balsalobre et al. 2000). Dexamethasone injections were also able to phase shift clock gene expression in the liver *in vivo*. These phase shifts were not present in the livers of mice carrying a liver specific GR gene disruption, indicating that this receptor mediates the phase shifts. Interestingly however, clock gene expression in the GR-less livers was identical to wildtype livers, arguing that glucocorticoids are not strictly required for the synchronisation of this peripheral oscillator. In another experimental setup, restraint stress was used as a more physiological means to raise glucocorticoid levels. Under these conditions, *per1* was reported to be acutely induced in mouse peripheral tissues, most likely through a functional glucocorticoid response

element (GRE) in its promoter (Fig. 1). However, it is the only core clock gene for which a response was observed, therefore overall clock function seems not to be perturbed via this induction (Yamamoto et al. 2005). Finally, transcription of the clock accessory loop element REV-ERBα has been shown to be repressed by glucocorticoids (Fig. 1, (Torra et al. 2000)), but the precise mechanism of this control and its function in clock regulation remain unexplored. It is possible that glucocorticoid repression of *rev-erbα* might be more associated with the additional roles of this gene in metabolism regulation (Duez and Staels 2008).

Glucocorticoids modulate food entrainment of peripheral clocks

Taken together, the effect of glucocorticoid administration on peripheral clock entrainment is not well understood. Nevertheless, a contribution of glucocorticoids to liver oscillator entrainment is revealed under conditions of daytime restricted feeding. Limiting food availability to the day, the period of rest in nocturnal rodents, leads to a decoupling of peripheral clocks from the central pacemaker. Clock gene expression in the SCN remains linked with the light phase, while the phase of peripheral pacemakers adapts to the feeding regime, leading to phase shifts of up to 12 hours (Damiola et al. 2000; Hara et al. 2001; Stokkan et al. 2001). As mentioned above, corticosterone secretion also adapts to the new feeding schedule by showing a food anticipatory peak. Interestingly, injections of corticosterone into rats at the time when this food anticipatory peak would occur did not induce the phase shifts caused by restricted feeding (Stokkan et al. 2001). Therefore, the changes in endogenous glucocorticoid secretion observed under restricted feeding appear not to be the reason for the food induced phase shifts. However, glucocorticoids may play a modulatory role in food entrainment: In

adrenalectomised mice and mice with a liver specific GR mutation, the phase shifts towards daytime feeding occur more rapidly, whereas phase shifts back to nocturnal feeding are unaffected (Le Minh et al. 2001). Thus, it appears that glucocorticoids can inhibit an as yet unknown signalling pathway that promotes adaptation to non-natural feeding times. This modulation might be particularly strong when the potent GR agonist dexamethasone is injected, leading to phase shifts even when the other hypothetical pathway shows unaltered activity.

Glucocorticoids are required for cycling expression of clock genes in some brain regions

Some peripheral clock genes in brain regions outside the SCN might require glucocorticoids for their circadian expression. *per2* expression in the oval nucleus of the bed nucleus of the stria terminalis (BNSTov) and in the central nucleus of the amygdala is severely blunted in adrenalectomised animals (Amir et al. 2004; Lamont et al. 2005). Interestingly, these rhythms can be restored through corticosterone replacement via the drinking water, which leads to a diurnal rhythm of plasma corticosterone in the animals, but not via implantation of constant-release pellets that result in constant peak levels of corticosterone (Segall et al. 2006). Thus, although *per2* expression at least in the BNSTov is also affected via neural projections from the SCN (Amir et al. 2004), circadian corticosterone changes seem to directly contribute to the rhythms, with constant levels not sufficient for rescuing circadian expression. In one model, the changing glucocorticoid levels might directly drive *per2* expression changes (Fig. 4A). Glucocorticoids might also interact with other hormones shown to regulate circadian *per2* expression in the brain (Amir and Robinson 2006; Perrin et al.

2006), or affect its expression via targeting the peripheral clock machinery itself (Fig. 4B). To decide between these possibilities, it will be important to examine whether glucocorticoids also affect expression of other clock genes and core clock function in these brain structures, and whether a functional peripheral clock is required for the glucocorticoid regulation of *per2* expression. Interestingly, purely systemic regulation of circadian *per2* expression was observed in animals with clockless livers (Kornmann et al. 2007)(see also (Reddy and Maywood 2007)), indicating that a drive mechanism may contribute to clock gene regulation. Redundancy of systemic and peripheral control of clock gene expression might enhance the robustness of the circadian system.

Glucocorticoid-dependent circadian gene expression in certain brain regions might also be involved in feedback of glucocorticoids to the SCN (Buijs and Escobar 2007): The circadian transcription of *tryptophan hydroxylase-2*, the rate-limiting enzyme of serotonin synthesis, in the raphe nucleus is abolished in adrenalectomised rats supplemented with basal (=diurnal) corticosterone levels, but can be reinstated by corticosterone replacement in the drinking water (Malek et al. 2007). Serotonergic projections from the raphe nucleus to the SCN, which have been suggested to affect light entrainment of the clock (Meyer-Bernstein and Morin 1999; Morin 1999; Sage et al. 2004), might thus be a site of glucocorticoid feedback to the central clock. In addition, many other brain regions involved in the control of adrenal glucocorticoid release receive serotonergic input from the raphe nucleus (reviewed in (Lowry 2002)) and might be influenced by glucocorticoid dependent circadian serotonin synthesis.

Glucocorticoid interactions with transcriptional clock outputs in peripheral tissues

Part of the circadian liver transcriptome is dependent on the adrenal gland

Besides their actions on peripheral clock gene expression, other mechanisms might link glucocorticoids to circadian gene expression changes in peripheral tissues. Oishi et al. examined circadian gene expression changes in the liver of adrenalectomised mice compared to sham-operated controls (Oishi et al. 2005). For 100 of the 169 cycling genes identified in this study circadian expression was dependent on the adrenal gland. Expression of several clock genes was not affected by adrenalectomy, in line with the findings in liver-specific GR knock-out mice (Balsalobre et al. 2000). To more clearly define the glucocorticoid contribution to liver transcriptome regulation, as opposed to that of other adrenal gland derived cues, it would be informative to compare the transcriptomes from adrenalectomised animals with those from liver GR-deficient animals. Some of the adrenal gland dependent genes (e.g. the biosynthetic pathway genes *HMG CoA reductase*, *ornithine decarboxylase* and *lipin 1*) were shown to be induced transiently upon bolus dexamethasone injections, but this did not reinstate circadian transcription with the appropriate phase within the 24 h examined. Therefore, the authors suggest that circadian glucocorticoid rhythms might drive circadian expression of these genes without involvement of the peripheral clock (Fig. 4A), but a more thorough test of this hypothesis would require analysis for longer than 24 h and in a clockless liver (Kornmann et al. 2007).

Also indirect effects on other hormonal pathways might play a role: Insulin levels are low in adrenalectomised mice (Dallman et al. 1993), suggesting that lack

of this hormone might cause some of the effects of adrenalectomy. This is supported by the finding that circadian *glucokinase* expression in the liver, which is dependent on the adrenal gland, is also abolished when insulin levels are decreased by streptozotocin-mediated pancreatic β-cell destruction (Oishi et al. 2005). In contrast, *HMG-CoA reductase* was not affected by insulin reduction and appears therefore more directly dependent on the glucocorticoids. The importance of such indirect hormonal crosstalk mechanisms in liver transcriptome regulation under more physiological conditions remains to be established.

Glucocorticoids might help to synchronise circadian liver gene transcription via HNF4α

As mentioned above, the circadian transcription in the liver is desynchronised after SCN lesion. Reddy et al. recently reported that a single dexamethasone treatment of SCN-lesioned mice is sufficient to synchronise about 60% of the circadian liver transcriptome (as followed through three timepoints over 36 hours (Reddy et al. 2007)). Also in muscle tissue, roughly 50% of the circadian regulated genes have been reported as glucocorticoid responsive genes (Almon et al. 2008). A caveat for these studies is that the glucocorticoid regulation seen with these treatments may just reflect a glucocorticoid response to homeostasis threats, as in stress, which acts independently of or even overrides the circadian regulation. To clarify this issue, a comparison with stress induced transcriptome changes in these organs would be insightful (similar to e.g. (Reyes et al. 2003; Kassahn et al. 2007)).

Analysis of a subset of the synchronised genes from the liver study showed that two thirds of the examined promoters contain GRE elements, often combined

with E-boxes. Another enriched element were binding sites for HNF4α, which itself is a glucocorticoid-sensitive circadian gene. Overall, these findings suggest that glucocorticoids may influence circadian transcription in the liver at multiple levels: through direct effects via GREs, via indirect effects through other target genes such as HNF4α, via effects on clock gene expression itself and by combinations of any of these mechanisms on different promoters. As for the adrenalectomy studies, further dissection of the relative contributions of these mechanisms will require more detailed analysis of the transcriptomes of clock-less and GR-less livers.

Glucocorticoids and circadian cell cycle rhythms

One important process under circadian regulation is the cell cycle. Rhythms of cell proliferation are evident from cyanobacteria to humans (Bjarnason and Jordan 2000; Mori and Johnson 2000), and several cell cycle genes have been shown to be under transcriptional control by circadian clock factors (Reddy et al. 2005; Hunt and Sassone-Corsi 2007; Vallone et al. 2007). Our recent study in zebrafish larvae has indicated a role for glucocorticoids in the regulation of these rhythms (Dickmeis et al. 2007). Circadian cell cycle rhythms are present both in the larvae and in zebrafish-derived cell lines, implying a cell-autonomous clock regulation also in this species (Dekens et al. 2003). However, examination of pituitary mutant larvae revealed that, *in vivo*, the rhythms depend on systemic input from this gland (Dickmeis et al. 2007). Analysis of mutants with overlapping pituitary cell type deficiencies pinpointed the corticotrope lineage as the source for the required signal. Mutants possessing only reduced numbers of the corticotrope

cells contained lower cortisol levels than wildtype larvae. Importantly, cell cycle rhythms in these mutants could be rescued by tonic treatment with dexamethasone. Thus, glucocorticoids might act as permissive signals which enable peripheral clock control of the cell cycle (Fig. 4D); alternatively, the peripheral clocks might gate glucocorticoid input into the cell cycle machinery (Fig. 4C). The circadian changes of glucocorticoid levels themselves are not required for their action on the circadian cell cycle rhythms, but might constitute an additional level of security to ensure proper signalling input to the cell cycle machinery (and their cycling levels might also be of essential importance for other processes, see above). It will be interesting to see whether similar mechanisms also operate in the mammalian system, e.g. in the circadian rhythms observed in liver regeneration after partial hepatectomy (Matsuo et al. 2003), where liver specific disruption of the GR would allow direct testing of the glucocorticoid contribution.

Circadian modulation of GR signalling in the target tissues?

The glucocorticoid receptor pathway is the main signalling pathway activated by glucocorticoids, and it is modulated by and interacts with a wealth of other signalling pathways (Grad and Picard 2007; Kassel and Herrlich 2007; Stahn et al. 2007). Modulation can occur at various levels (Fig.5): pre-receptor control of glucocorticoid availability by glucocorticoid metabolising enzymes, association with heat shock proteins, phosphorylation of the receptor and interaction with transcriptional cofactors and other transcription factors in the nucleus. In several of these processes, circadian rhythms are observed, and they might cooperate with the circadian glucocorticoid signal in the generation of circadian outputs.

Local glucocorticoid metabolism

Local metabolism regulates access of the circulating glucocorticoids to their receptors (Chapman and Seckl 2008). 11β-hydroxysteroid dehydrogenase (11β-HSD) enzymes catalyse conversion between active 11-hydroxy-glucocorticoids and their inactive 11-keto forms. The type 2 enzyme shows high expression in mineralocorticoid target tissues, where it inactivates glucocorticoids and thereby allows mineralocorticoids to bind the MR despite this receptor's high affinity for glucocorticoids. The type 1 enzyme exhibits a more widespread expression and primarily catalyses the opposite reaction, thereby regenerating and "amplifying" glucocorticoid concentrations in the cell (Fig. 5, I). Rat *11β-hsd1* mRNA expression shows a diurnal rhythm in the hippocampus, but not in peripheral tissues (Buren et al. 2007). Circadian rhythms of glucocorticoids are flattened in *11β-hsd1* knock-out mice, mainly owing to an increase in diurnal levels. This has been suggested to reflect attenuated glucocorticoid feedback, as also stress induced increase in corticosterone is not properly inhibited by exogenous glucocorticoids (Harris et al. 2001). Surprisingly however, liver-restricted rescue of 11β-HSD1 expression in *11β-hsd1* knock-out mice is sufficient to rescue HPA axis dysfunction, including the circadian aspects of it (Paterson et al. 2007). Thus, hepatic metabolism of glucocorticoids seems to be of crucial importance for the overall glucocorticoid regulation. Therefore, the *in vivo* relevance of circadian 11β-HSD1 expression in the brain remains to be determined.

Circadian regulation of the glucocorticoid receptor

Before binding of its glucocorticoid ligand and subsequent nuclear translocation, the GR resides in the cytoplasm as a dynamic complex with several heat shock proteins (HSPs) and HSP associated proteins (Fig. 5, II, (Grad and Picard 2007)). Many HSPs show circadian regulation, and they have been suggested to convey systemic circadian temperature changes to peripheral tissues and thereby contribute to clock entrainment in the periphery (Brown et al. 2002; Kornmann et al. 2007; Reinke et al. 2008). Hence, circadian regulation of HSP/GR complexes, as recently reported for the hippocampus (Furay et al. 2006), might contribute to GR dependent circadian clock outputs. Also expression of the GR mRNA itself can be under circadian regulation (Fig. 5, III): A global survey of circadian nuclear receptor mRNA expression in mouse metabolic tissues found circadian GR expression in white and brown adipose tissue (interestingly, with different peak phases), but not in muscle or liver (Yang et al. 2006). Also in the brain, circadian rhythms of GR mRNA have been reported (Herman et al. 1993). Additionally, transcriptional cofactors of nuclear receptors might modulate GR signalling (Fig. 5, IV). Thus, the cofactor PGC1α has recently been implicated in circadian clock function (Liu et al. 2007) and can also interact with the GR (Knutti et al. 2000; Yoon et al. 2001). Finally, as mentioned above for HNF4α, GRs might interact with clock regulated transcription factors at the promoter level, including the core clock factors themselves (Fig. 5, V). These mechanisms of differential circadian regulation at the glucocorticoid target cell level could cause differential circadian receptiveness of different tissues and thereby gate glucocorticoid responses to certain times of day (Fig. 4C).

The emerging picture is that a dynamic glucocorticoid signalling machinery receives an equally dynamic glucocorticoid signal which then interacts with various dynamically regulated cofactors and signalling pathways to shape target tissue specific responses. A precise understanding of these processes will require combinations of *in vivo* imaging of the network components with systems biology approaches in order to generate predictive models of temporal regulation of tissue physiology.

Some areas for future research

As evident from the data presented above, much is still to be learned about the precise molecular mechanism by which glucocorticoids and the circadian clock interact to time target tissue dynamics. This includes the functions of the ultradian rhythms in glucocorticoid release and their relation to the circadian clock. There is evidence that the clock also contributes to ultradian pulsatility (Loudon et al. 1994), but the mechanisms through which this is achieved are unknown. Furthermore, more information is needed on how circadian dynamics in the target tissue integrate with these pulsatile dynamics. Interestingly, a proteasome dependent mechanism has been described recently that contributes to shutting off GR signalling at the trough of the glucocorticoid pulses (Conway-Campbell et al. 2007; Lightman et al. 2008), and one could speculate that circadian and ultradian rhythms might interact via such posttranslational mechanisms.

A second topic with need for further research is the establishment of the various dynamic regulations of glucocorticoid release during development. This is of importance, as prenatal stress or metabolic state of the mother can influence physiology and disease risk of the child, perhaps even into the next generation (Remacle et al. 2007; Darnaudery and Maccari 2008; Seckl 2008). It will be informative to determine if and to what extent such developmental programming acts via changes in the circadian rhythmicity of the HPA axis and glucocorticoid release.

This leads us to a third topic: How precisely do changes in the circadian regulation of corticosteroids contribute to pathology? In many diseases, including Cushing's syndrome, depression, Alzheimer's disease and the metabolic syndrome circadian glucocorticoid regulation is altered (Ferrari et al. 2001; Pasquali et al. 2006; Bao et al. 2008; Carroll et al. 2008). It will be important to know in which cases these changes are a mere symptom of the disease and in which cases they are a causal factor for the development of the disease. This information might then be translated into more physiological and more efficient therapies.

Figure legends:

Fig. 1.: The molecular clock mechanism. *"Core loop":* The bHLH transcription factors CLOCK and BMAL1 heterodimerise and bind to E-box enhancer elements. This activates transcription of *period* and *cryptochrome* genes, the products of which also heterodimerise and, after translocation to the nucleus, inhibit the activity of the CLOCK/BMAL1 dimer. This shuts down transcription of their own genes. Posttranscriptional and posttranslational modifications (not

shown) delay nuclear accumulation of PER and CRY, thereby leading to the approximate 24 hour rhythm of activation and repression on the *per* and *cry* promoters. *"Accessory loop":* The CLOCK/BMAL1 dimer also binds E-boxes in the promoter of the *rev-erbα* and *rorα* genes. RORα in turn binds a RORE element in the promoter of *bmal1* and activates its transcription. REV-ERBα competes with RORα for the RORE binding site and inhibits transcription of *bmal1*. The resulting cyclical transcription of *bmal1* is thought to enhance the core feedback loop. Glucocorticoids (GC) might modulate transcription of certain clock genes by binding to glucocorticoid response elements (GRE) in their promoters. Thus, a positive GRE element appears to mediate *per1* induction by glucocortioids (Yamamoto et al. 2005), whereas the promoter of *rev-erbα* has been proposed to contain a negative GRE (nGRE) which mediates glucocorticoid induced repression (Torra et al. 2000).

Fig. 2.: Neural pathways in circadian control of glucocorticoid release. See text for details. **Arrows***: Black arrows*: Neural projections targeting the HPA axis. *Blue arrows*: Neural projections via the autonomic nervous system. *Purple arrow:* projection from the DMH to the "autonomic PVN" distinct from the SCN-adrenal gland pathway. Also the subPVZ projects into this region. *Open arrows*: Humoral transport through the vasculature. *Clocks* symbolise autonomous pacemakers. **Abbreviations**: *Regions of the nervous system*: SCN – suprachiasmatic nucleus; subPVZ – subparaventricular zone; DMH – dorsomedial hypothalamus; PVN – paraventricular nucleus; mpPVN – medioparvocellular division of the PVN; I – interneuron of the PVN; C – corticotrope cell of the pituitary; IML – intermediolateral column of the spinal cord. *Hormones and*

neurotransmitters: AVP – arginine vasopressine; CRH – corticotropin releasing hormone; ACTH – adrenocorticotropic hormone.

Fig. 3: **Working models for circadian regulation of glucocorticoid secretion**. A) Rhythms in the HPA axis drive circadian ACTH release, which then stimulates glucocorticoid secretion. B) The autonomic nervous system, perhaps together with unknown other cues, entrains the circadian clock of the adrenal gland. The clock in turn regulates circadian glucocorticoid production and release. C) The adrenal gland clock, perhaps together with the autonomic nervous system, gates sensitivity of the gland to ACTH in a circadian manner. *Abbreviations*: ANS - Autonomic Nervous System, GC – glucocorticoids, SCN – suprachiasmatic nucleus.

Fig. 4: Working models for circadian regulation by glucocorticoids. A) Rhythms in glucocorticoid concentrations drive circadian output rhythms in the target tissue. B) Glucocorticoid changes entrain peripheral circadian clocks, which in turn control circadian rhythms in the tissue. C) Peripheral clocks gate the glucocorticoid input to physiological processes in a circadian manner. D) Glucocorticoids are required as permissive agents for circadian clock control to operate in rhythmic tissue processes. GC – glucocorticoids.

Fig. 5. Hypothetical intracellular mechanisms of circadian modulation of glucocorticoid signalling. I) 11β-HSD1 (11β, green) catalyses conversion of inactive keto-glucocorticoids (O=) to their active hydroxyforms (OH-). Its mRNA is under circadian regulation in certain tissues. **II)** Heat Shock Proteins (HSPs, H,

blue) form complexes with the glucocorticoid receptor (GR, red) in the cytoplasm, before GR binding of glucocorticoids and subsequent nuclear translocation. Expression of many HSPs shows circadian dynamics. **III)** The mRNA of the GR itself shows circadian cycling in some tissues. **IV)** Transcriptional cofactors (C, pink) for nuclear receptors, such as PGC1α, can be regulated in a circadian fashion. **V)** The glucocorticoid receptor can interact with various signalling pathways at the level of the promoter of its target genes. This may include: **Va)** other transcription factors which are subject to circadian regulation at the level of their mRNAs (X, turquoise, e.g. HNF4α), **Vb)** targets of other signalling pathways subject to circadian changes in their activity (Y, brown) and **Vc)** the core clock components themselves (yellow and orange, see Fig. 1).

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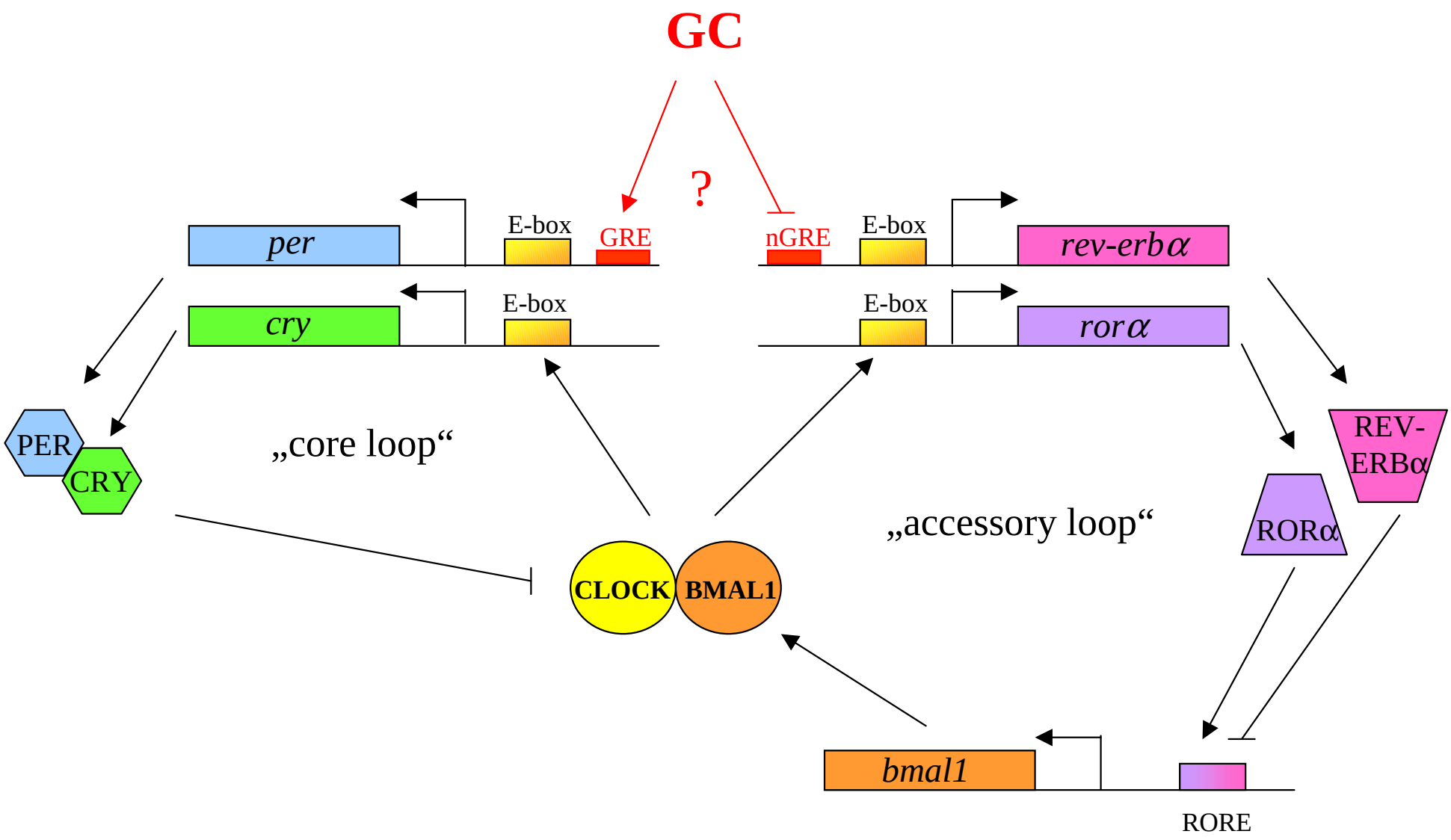
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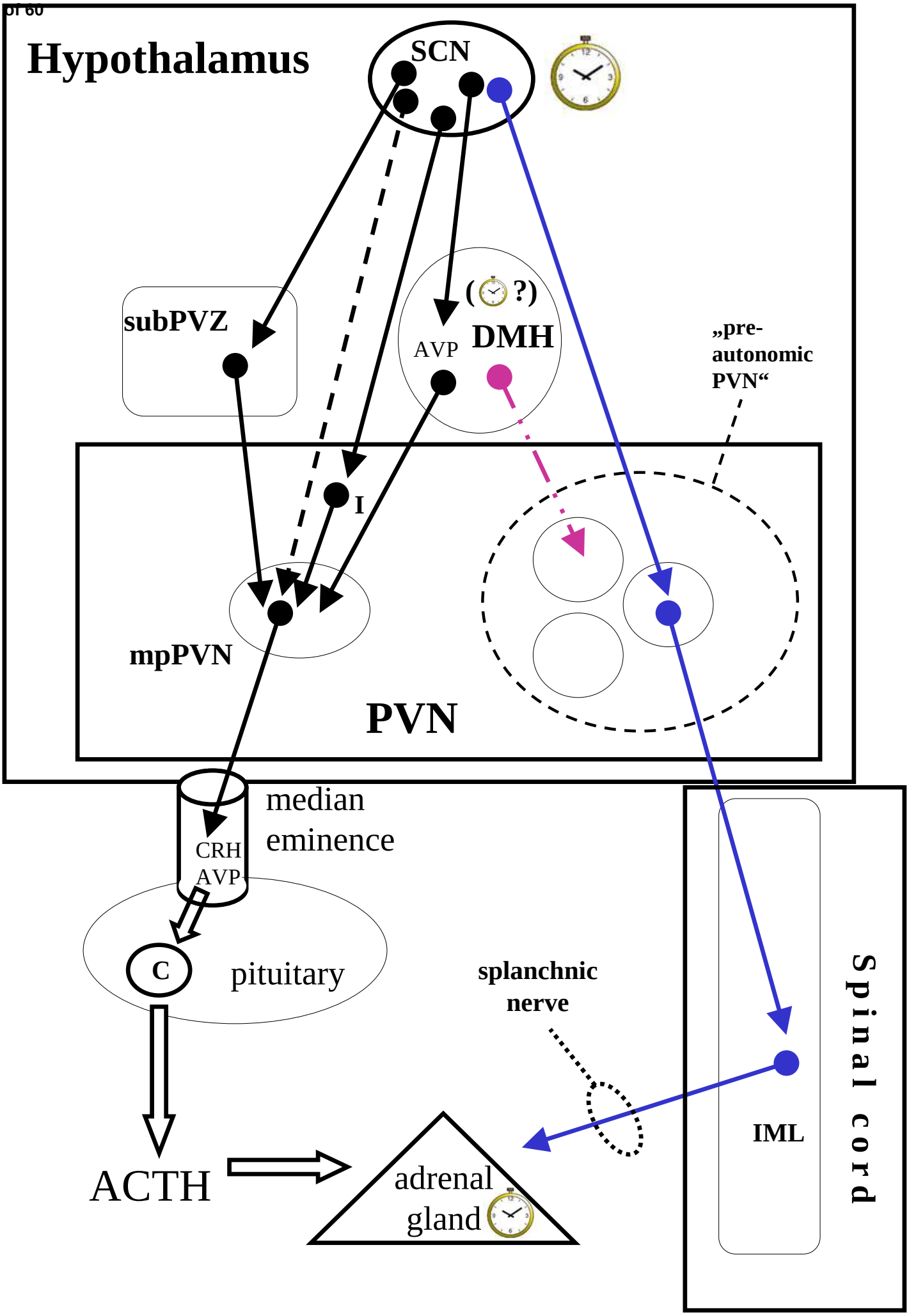
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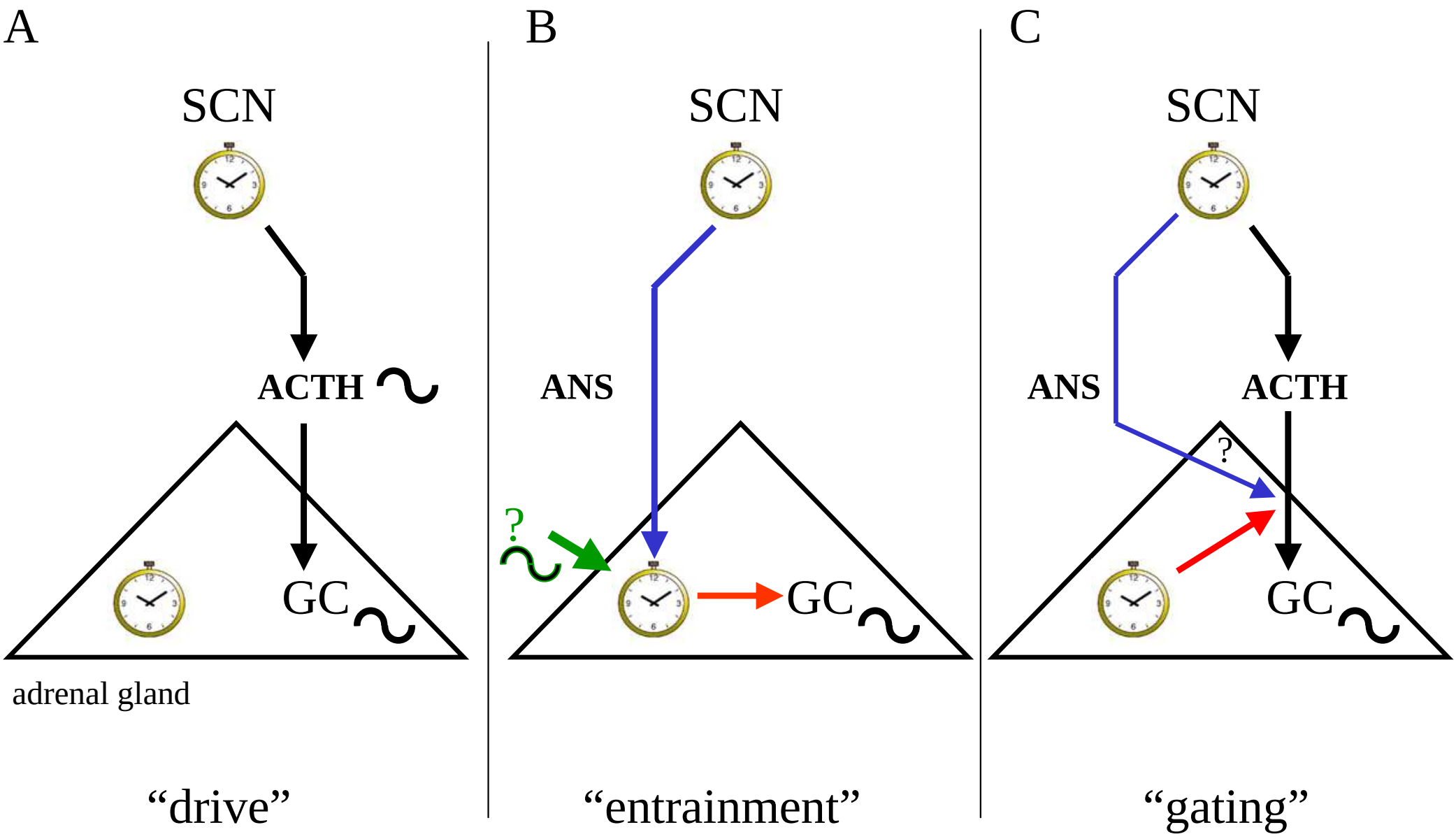
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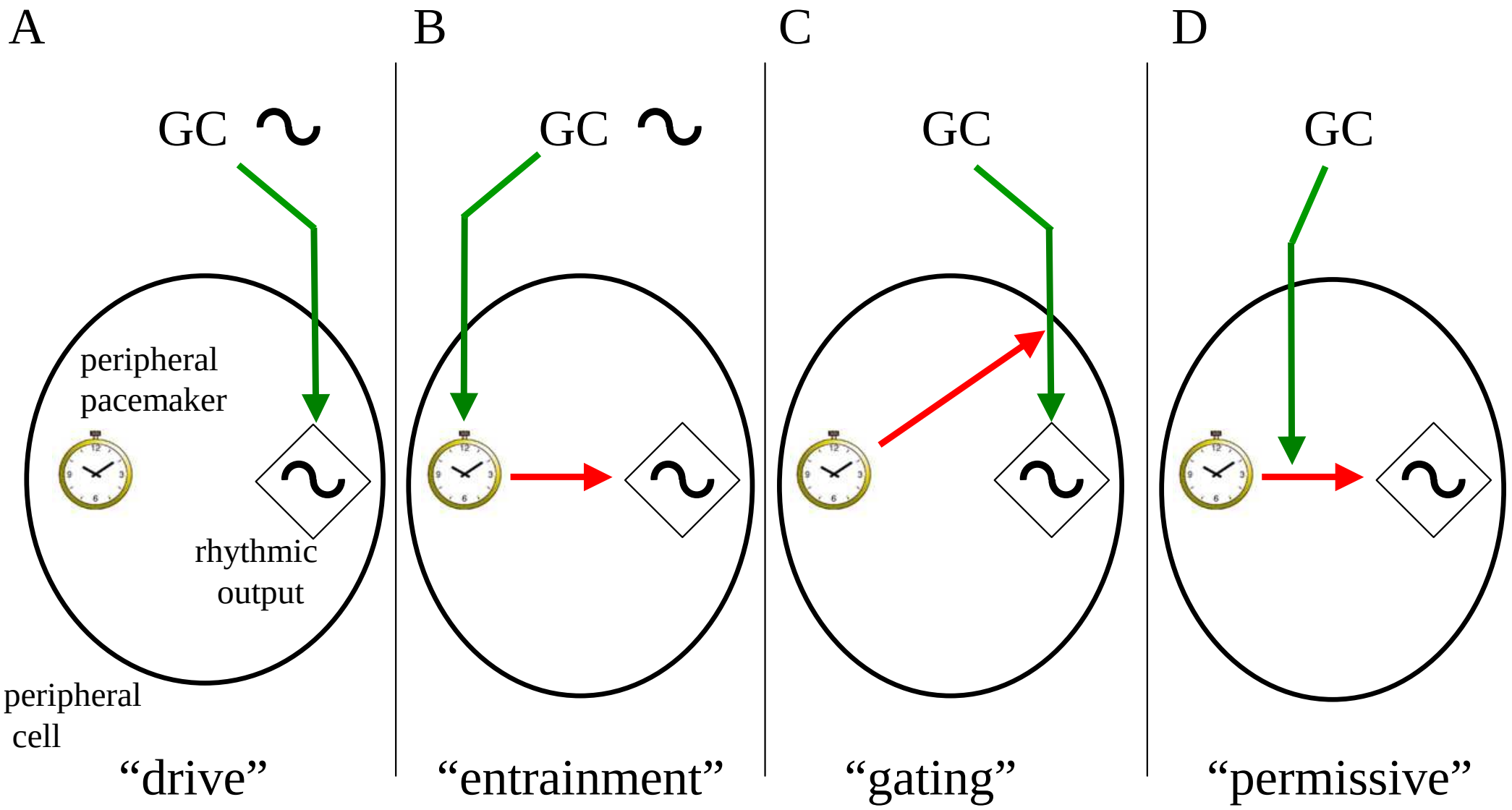
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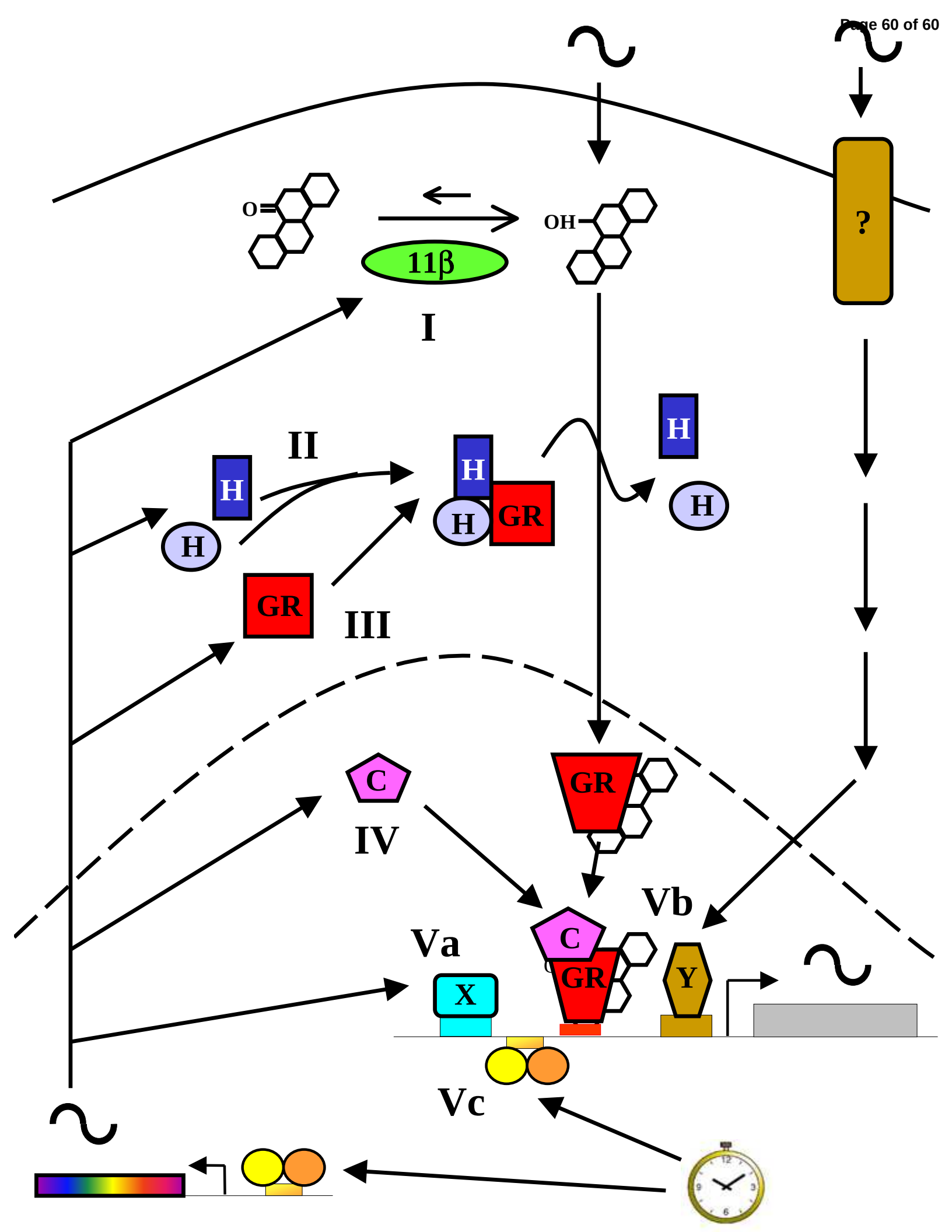
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