To be published :

WORKSHOP IX

Cell Division and the Replicon
eds W. Fangman, T. Kishimoto, M. Kohiyama and C. Coath

WORKSHOP X

Axis Formation in the Vertebrate Embryo
eds S. Ang, R. Behringer, H. Sasaki, J. S. Altman and C. Coath

WORKSHOP XI

Neuroenergetics: Relevance for Functional Brain Imaging
eds P. J. Magistretti, R. G. Shulman, R. S. J. Frackowiak and J. S. Altman

ISBN : 92-9164-007-7 ISSN : 1026-9819

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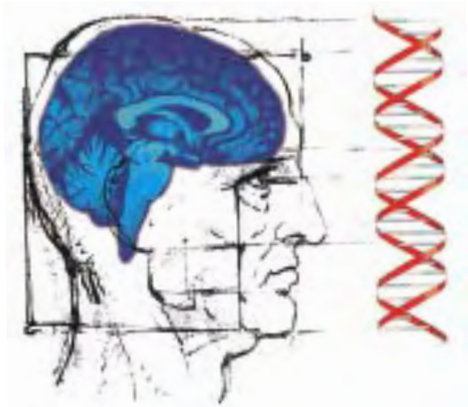
THE REGULATION OF SLEEP

VOL.8
APRIL
2000

HUMAN FRONTIER



WORKSHOP VIII

THE REGULATION OF SLEEP

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PART III

SLEEP AS A REGULATED AND REGULATORY SYSTEM

Although many clues to the functions of sleep can be gathered from examining the cellular mechanisms governing the sleep–wake cycle, the full answer will come only when these mechanisms are placed in the context of the whole system. The following articles take a variety of approaches to sleep as a system but they have in common questions about the significance of the structure of sleep.

Humans — and rats — spend the larger part of the sleep cycle in non-REM sleep, with REM sleep occupying a smaller proportion of total sleep time. However, in humans the time spent in non-REM sleep typically decreases and that in REM sleep increases as sleep progresses. Unlike the slow-wave dominated EEG of non-REM sleep, in REM sleep the EEG is similar to that in the awake state, with rapid non-rhythmic cortical activity. In animals other than humans, the theta (θ) rhythm, a 6–9-Hz oscillation originating in the hippocampus, is also seen. In contrast to this cortical activity, the tone in the body musculature is at its lowest in REM sleep, except for the rapid movements of the eyes. Michel Jouvet proposes that REM sleep may help to maintain the psychological variability that differentiates individuals of a species and he delineates the ponto-geniculo-occipital system as the probable regulator of REM sleep. The role of sleep in memory consolidation, a recurring theme in *Parts I* and *II*, is picked up by Bruce McNaughton, who shows that place-sensitive neurons in the hippocampus of the rat replay their daytime activity during non-REM sleep. However, a similar conclusion cannot yet be reached for REM sleep.

The homeostatic relationship between non-REM and REM sleep has been long debated. The established position is that the need for both accumulates during the awake state. Using data

from elegant experiments on rat pups, Craig Heller demonstrates that non-REM sleep develops before REM sleep, which supports his alternative view that only the need for non-REM sleep depends on the awake state; the need for REM sleep is determined by non-REM sleep. Hibernation, a state specially adapted to the rigours of winter, is usually considered to be a form of sleep but studies on the Djungarian hamster from Siberia lead Irene Tobler to argue that hibernating animals are sleep deprived and she provides further support for the restorative nature of non-REM sleep.

The circadian rhythm of the sleep–wake cycle is generated in a part of the hypothalamus known as the suprachiasmatic nucleus. Lesions in or damage to this area in humans (Cohen and Albers, 1991) and non-human primates (Edgar *et al.*, 1993) abolish the circadian sleep–wake rhythm, in particular the ability to stay asleep or awake. Each neuron in the suprachiasmatic nucleus contains a circadian oscillator and Martha Gillette examines how this intrinsic rhythm is reset by external signals to entrain it to environmental conditions. Lastly, Derk-Jan Dijk discusses the relationship between the circadian and homeostatic regulation of sleep in humans and the changes in this relationship as we age.

Cellular regulators of circadian timing

Martha U. Gillette

The regulation of sleep is intricately related to the circadian system, which determines the timing of the sleep and awake state, as well as the ability to maintain sleep. The continuous interaction between the circadian system and sleep homeostatis determines the daily changes in sleep propensity (see *Introduction*; Dijk, this volume). These rhythmic processes, which persist in the absence of time cues and have a period of about a day — hence the term circadian (for review, see Gillette, 1998) — are generated by an endogenous 'clock' with two unique properties, timekeeping and gatekeeping. External and internal signals, such as light or arousal state, can shift the phase of the rhythm, re-aligning the animal's physiology with the daily alternation of light and dark or body state (see *Fig. 86*).

In mammals, the circadian clock is based in the oscillatory properties of individual neurons in the suprachiasmatic nucleus (SCN) of the hypothalamus. This nucleus is a highly integrative region that receives inputs from many other parts of the brain, mediated by a wide range of neurotransmitters and modulators. SCN slices maintained in vitro conserve both timekeeping and gatekeeping properties: they can generate a stable circadian rhythm that is sensitive to phase-shifting signals and can coordinate appropriate phase adjustments. The molecular events underlying the rhythms generated by these pacemaker neurons in the SCN are the subject of intensive investigation (for review, see Dunlap, 1999).

We are using SCN slices from rats to investigate the gatekeeping function of the circadian clock, i.e., how it ensures that clock phase can be reset only by signals relevant to temporal desynchronization. I discuss the role of the glutamate- and acetylcholine-mediated inputs to the SCN in the control of this phase resetting and the identity of the intracellular cascades mediating these processes.

Properties of the clock

SCN neurons are autonomous clocks with intrinsic periods similar to the period of the circadian rhythm *in vivo*, one measurement of which is activity in a running wheel (*Fig. 86;* Liu *et al.*, 1997*a*; Herzog *et al.*, 1998). Lesions in the SCN disrupt the expression of circadian rhythms; subsequent transplantation of SCN tissue can restore rhythmicity but the period is that of the donor tissue, confirming that the endogenous rhythms are generated in the SCN (Ralph *et al.*, 1990). This timekeeping property of the clock enables it to generate endogenous rhythms, such as the sleep–wake cycle, fluctuations in body temperature and melatonin production (Gillette and Tischkau, 1999).

The rhythms can be reset, or entrained, by external and internal signals (*Fig. 86*), a process experienced at its most extreme by anyone who has suffered from jet-lag. Entrainment is fundamental for synchonizing physiological processes to the daily light–dark cycle. Light is the dominant external factor determining the phase of the clock and its effect provides a good example of the gatekeeping property of the clock, because the sensitivity of the clock to resetting by light pulses varies during the circadian cycle. Animals maintained in environments such as constant darkness, that lack periodic signals, continue to show these changes in sensitivity. Under constant conditions, nocturnal rodents are active in the part of the circadian cycle that corresponds to night in normal light–dark conditions. Light pulses given early in this 'subjective night' evoke phase delays, whereas later in the subjective night they result in phase advances. In the subjective day, coresponding to the light portion of the normal light–dark cycle, they are ineffective (see *Fig. 88b*). SCN slices maintained in vitro display similar changes in sensitivity to light pulses, according to the light–dark cycle in the animal colony from which the slice donor came.

Light information reaches the SCN through a direct pathway from the retina, mediated by the neurotransmitter glutamate.

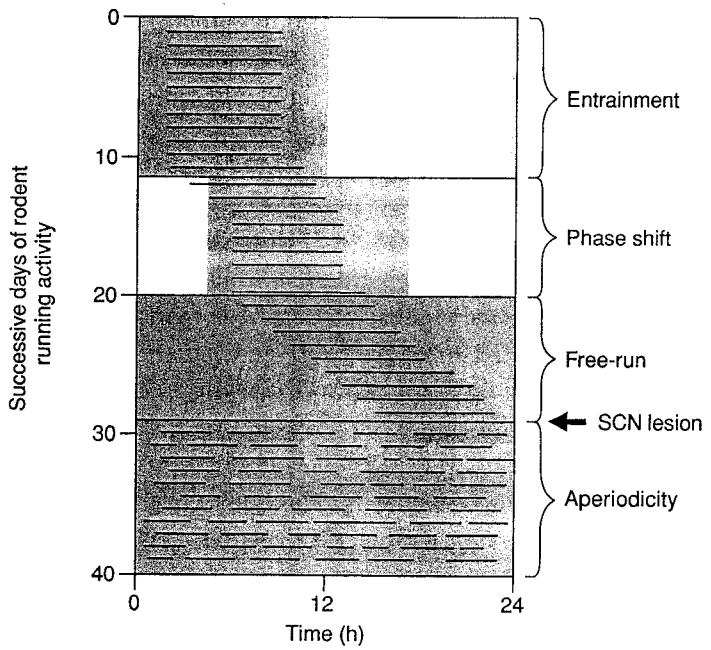

Figure 86. *Fundamental circadian principles illustrated schematically by records of the activity of a hamster when wheel-running. The horizontal bars within each day's record represent intense bouts of activity. Initiation of wheel-running activity, the phase-marker for this behavioural rhythm, occurs shortly after the onset of night (shaded area). On days 1–11, activity is entrained to the normal dark period. On d 12, onset of darkness is delayed, inducing a delay in the phase of the rhythm that brings the activity pattern back into the original phase relationship to the onset of darkness. In constant darkness (d 20–29), activity remains rhythmic but the daily cycle becomes slightly longer than 24 hours, so the pattern shifts to the right on successive days. After a lesion of the suprachiasmatic nucleus (SCN), the cycle of wheel running and sleep loses its periodicity.*

A second important input is from cholinergic neurons in the sleep–wake regulatory centres of the basal forebrain and mesopontine tegmental nuclei (see *Part II Introduction*; McCarley, this volume), which may be the neuroanatomical basis for the interactions between the sleep–wake and circadian cycles. Other strong regulatory inputs come from the intergeniculate leaflet in

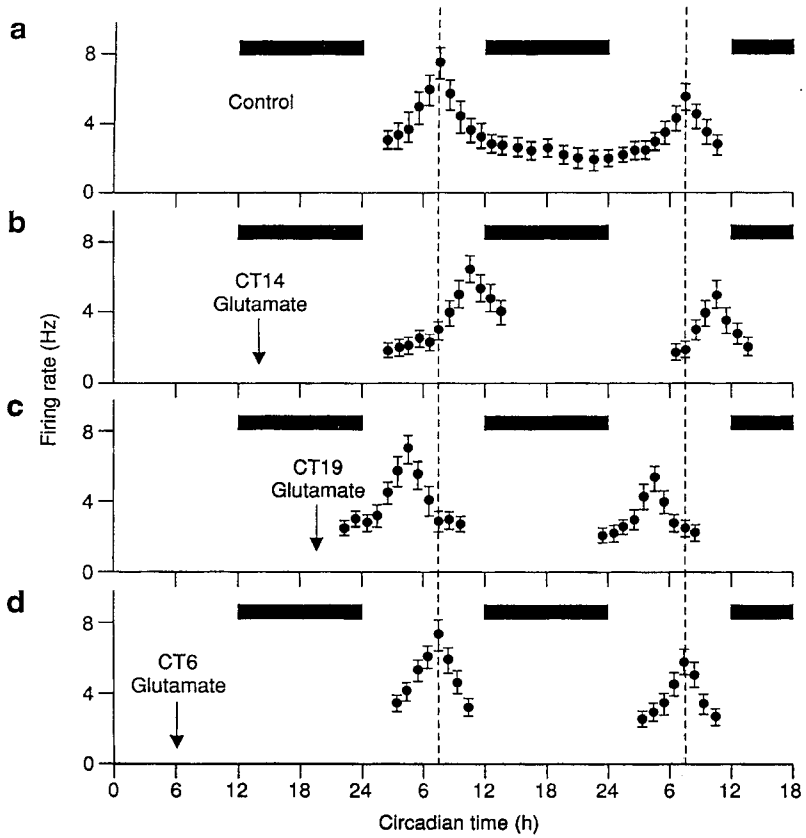

Figure 87. *Sensitivity of the endogenous circadian rhythm of neuronal discharge in the SCN to phase resetting depends on clock phase. Glutamate was applied to the surface of SCN slices from rats for 10 min and the effect on the phase of the rhythm over 38 h on the following 2 d recorded from an ensemble of neurons.* ***a****, circadian rhythm in controls (unperturbed or media-treated slices). Dashed vertical lines mark the normal peak of activity at circadian time (CT) 7, used as the phase marker in all experiments on SCN slices. Horizontal bars, subjective night, corresponding to the dark phase of the 12-h light:12-h dark cycle in the animal colony.* ***b****, glutamate applied at CT 14, during early subjective night induced a 3-h delay in the subsequent activity peaks.* ***c****, glutamate applied at CT 19, i.e., late subjective night, induced a 3-h advance in the activity peaks.* ***d****, glutamate had no effect when applied in the middle of the subjective day. Points represent 2-h running averages of the discharge rates recorded from 82–124 single neurons (± SEM). Modified from Ding* et al., *1994.*

the thalamus (mediated by neurotransmitters neuropeptide Y and GABA) and the 5-HT neurons of the dorsal raphe nucleus. The input from the pineal gland, mediated by the hormone melatonin, seems to be involved in regulating the phase of the clock at dawn and dusk (McArthur *et al.*, 1997).

Glutamate and acetylcholine as regulators of clock phase

Glutamate or acetylcholine applied in the subjective night mediates phase-shifting effects on circadian rhythms generated in the SCN slice. Glutamate on the surface of SCN slices for 10 min induced delays or advances in the clock phase over the next 2 d but the effect depended on the circadian time in the preparation when it was administered (*Fig. 87*). Early in the subjective night, it caused a phase delay, later a phase advance (cf. *Fig. 87b,c*; Ding *et al.*, 1994). However, it had no effect on circadian timing when administered in the subjective day (*Fig. 87d*). The phase–response plot for the phase-shifting effect of glutamate on the SCN slice confirmed that the clock phase is insensitive to glutamate during subjective day (*Fig. 88a*) and has a similar shape to the plot obtained from rats when the phase of the behavioural circadian rhythm was measured by running on a wheel in total darkness after a 1-h exposure to light at various times in the circadian cycle (*Fig. 88b*).

Acetylcholine or its analogue carbachol applied to the slice preparation similarly had no effect on phase in the subjective day but, in contrast to glutamate, in the subjective night it always induced phase advances. These were up to twice as large as those induced by glutamate (*Fig. 89*; Liu and Gillette, 1996). The natural stimulus that is mediated by acetylcholine is unknown but is likely to be involved in the arousal systems that regulate the sleep–wake cycle (see *Part II Introduction*; McCarley, this volume).

Intracellular mechanisms of clock gating

We are identifying components of the intracellular cascades in

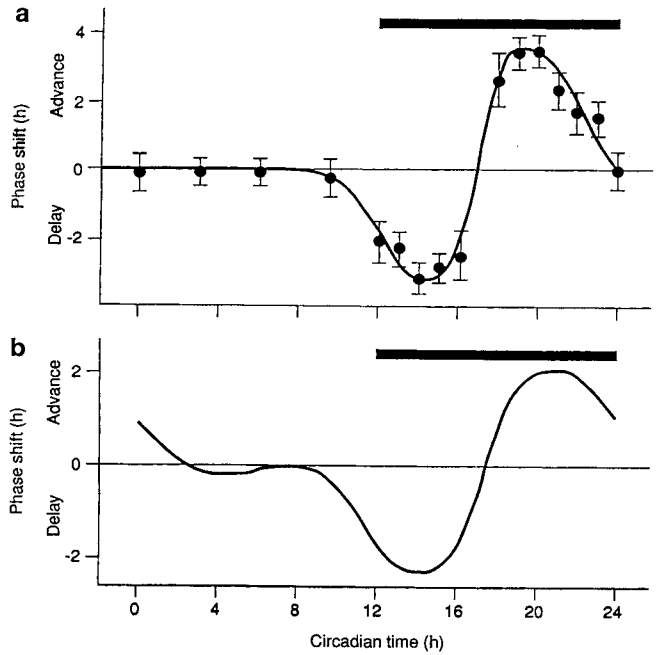

Figure 88. *The phase–response curve obtained by resetting the neuronal rhythm in the isolated SCN by applying glutamate compared with that obtained by resetting the rhythm of wheel-running activity in rats by light.* ***a****, the effect of glutamate applied directly to the SCN for 10 min at various times in the circadian cycle on the phase of the circadian rhythm generated by SCN neurons. Throughout the subjective day, the phase of the rhythm was unaffected but glutamate delayed the clock phase early in the subjective night and later advanced it.* ***b****, the circadian rhythm of locomotor activity of rats in constant darkness reset by a 1-h light pulse at different times in the 24-h period. Modified from* ***(a)*** *Ding* et al., *1994;* ***(b)*** *Summers* et al., *1984.*

the SCN neurons that ultimately lead to the observed phase shifts (Ding *et al.*, 1994, 1997, 1998; Liu *et al.*, 1997*b*). Glutamate induces membrane depolarization and the activation of NMDA receptors, which results in a rapid influx of extracellular Ca^{2+}. This in turn stimulates nitric oxide synthase and the production of nitric oxide (NO; *Fig. 90*). In the absence of glutamate, pharmacological treatments that increase intracellular

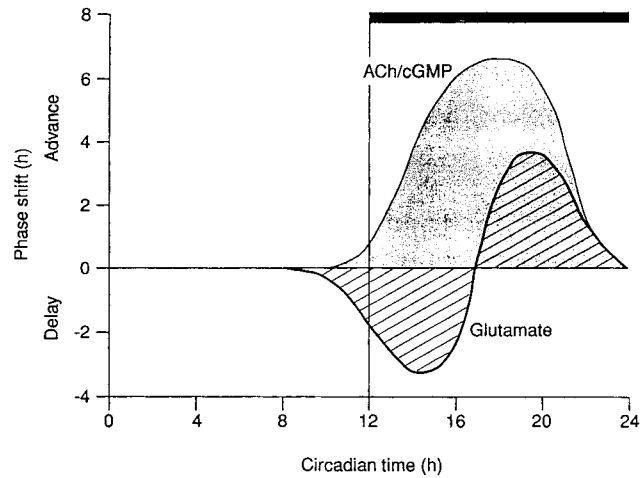

Figure 89. *Phase–response curves for glutamate and acetylcholine (ACh) reveal different patterns of response. Stimulating the acetylcholine pathway with the analogue carbachol or an analogue of the intracellular messenger cGMP induced phase advances with a maximum of 6.5 h when applied to the SCN at CT 16, during the subjective night. Glutamate induced a smaller phase advance with a maximum of 3.5 h between CT 19–20 and a phase delay of 3.5 h at CT 14. Dark bar, subjective night. Data from Prosser* et al., *1989; Ding* et al., *1994; Liu and Gillette, 1996.*

NO resulted in phase shifts similar to those produced by light in vivo and glutamate in vitro: a phase delay in the early subjective night, advance in the late subjective night and no phase shift in the subjective day. The induction of NO thus seems to have different consequences for intracellular signalling at different points in the circadian cycle.

Using compounds that selectively block or activate components of the intracellular signalling pathways, we have confirmed that the consequences of NO induction differ in the early and late parts of the subjective dark period (*Fig. 90*). In the late part it stimulates the production of cyclic GMP (cGMP) and activation of protein kinase G (PKG), whereas signalling in the early part of the subjective dark is through stimulation of ryanodine

receptors, releasing Ca^{2+} from intracellular stores (see Berridge, this volume). For instance, only during the delaying period early in the night was the phase shift induced by light or glutamate blocked by dantrolene, a selective inhibitor of ryanodine receptors; phase advances were not affected (Ding *et al.*, 1998). Pharmacological agents that activate ryanodine receptors all cause phase delays in early subjective night but have no effect on clock phasing in late night or day.

The cholinergic input to the SCN also activates the cGMP–PKG signalling pathway, so it partly overlaps with the glutamate pathway in the late night (*Fig. 90*). Acetylcholine activates metabotropic m1 muscarinic receptors, which stimulate guanylyl cyclase and raise the concentration of cGMP (Liu and Gillette, 1996; Liu *et al.*, 1997*b*). Analogues of cGMP had a similar effect on the circadian rhythm generated in the SCN slice preparation to that of the muscarinic agonist carbachol (*Fig. 89*; Prosser *et al.*, 1989; Liu and Gillette, 1996) and carbachol applied to the SCN in the subjective night increased cGMP production and PKG activity (Liu *et al.*, 1997*b*). Specific inhibitors of guanylyl cyclase and PKG blocked phase shifts induced by carbachol.

We have preliminary data showing that activation of $InsP_3$ receptors mediates the phase-shifting effects of stimulating m1 receptors in the SCN (Kuriashkina *et al.*, 1999). Carbachol applied to the SCN slice in the middle of the subjective night doubled $InsP_3$ synthesis and the carbachol-induced phase advance was blocked by xestospongin, an $InsP_3$-receptor antagonist. Like ryanodine receptors, $InsP_3$ receptors stimulate the release of intracellular Ca^{2+} (see Berridge, this volume). A picture is beginning to emerge from these as yet incomplete experiments: in the early subjective night an increase in intracellular Ca^{2+} produced by activation of ryanodine receptors induces phase delays, whereas that induced by $InsP_3$ results in phase advances. How such differential effects on phase in the early night are both influenced by intracellular Ca^{2+} remains to be determined.

Targets of the signalling pathways

Both of the pathways stimulated by light → glutamate → NO produce a rapid and transient response involving phosphorylation of the gene transcription factor CREB (Ginty *et al.*, 1993; Ding *et al.*, 1997). The concentration of phosphorylated CREB (P-CREB) reached a peak 10 min after the beginning of a light pulse (150 lux for 10 min). It remained significantly elevated for 60 min but returned to basal level by 120 min. As a light stimulus in vivo has the same effect as a pulse of glutamate in vitro, P-CREB is likely to be involved in light-induced phase resetting

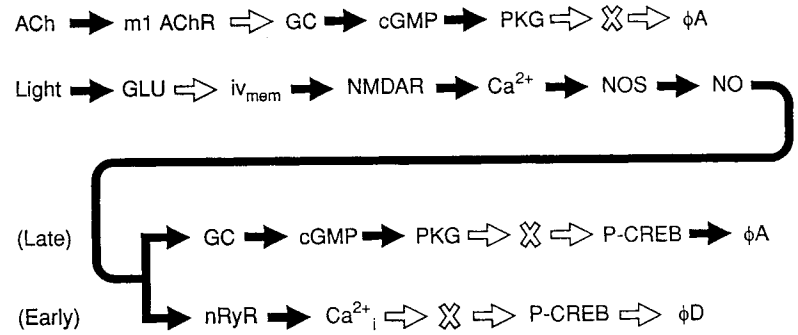

Figure 90. *Complex intracellular signalling pathways mediate the phase shifts of the SCN circadian clock at night. Stimuli mediated by acetylcholine (ACh), probably originating in the basal forebrain and brain stem tegmentum, advance clock phase (ϕA) through activation of the cGMP–protein kinase G (PKG) cascade by stimulation of m1 muscarinic receptors (m1 AChR). Light stimuli are transmitted by glutamate (GLU) released from the terminals of retinal ganglion cells on to the SCN neurons, activating NMDA-type glutamate receptors (NMDAR), Ca^{2+} influx and the generation of nitric oxide (NO). Early in the night this pathway leads to a phase delay (ϕD) through activation of neuronal ryanodine receptors (nRyR) releasing Ca^{2+} from intracellular stores (Ca^{2+}_i). Late in the night, it results in an advance of clock phase mediated by a cGMP–PKG pathway. Both early and late pathways must pass through a critical gating site (X), still to be identified, before stimulating the phosphorylation of CREB (P-CREB). Open arrows indicate unknown steps in the cascades. See text for further details. GC, guanylyl cyclase; iv_{mem}, membrane potential; NOS, nitric oxide synthase.*

in the subjective night by activating transcription of immediate-early genes, e.g., c-*fos*, and possibly genes implicated in the generation of mammalian circadian rhythms, such as *period* and/or *timeless* (Dunlap, 1999; Tischkau *et al.*, 1999).

These signalling pathways are all involved in phase resetting during the night period of the clock. Nevertheless, the critical elements, NMDA receptors, NO synthase and CREB, are all present throughout the circadian cycle. In addition, direct stimulation during subjective night of molecular steps in the pathway from receptor to NO causes a full-amplitude phase shift. As the concentration of CREB protein is constant at all points sampled during day and night, there must be a critical, clock-controlled molecular 'gate' between NO and CREB, which the clock uses to regulate its own temporal sensitivity (-X- in *Fig. 90*). The identity of this gate, i.e., one or several molecular reactions that are permissive to nocturnal shifts but non-permissive for shifts in daytime, is unknown. Determining this, as well as the extent to which glutamate and acetylcholine stimulate common permissive gating molecules in the late night but act through different pathways in the early night, will be important contributions to understanding the cellular regulators of the circadian clock and thus their contribution to the regulation of the sleep–wake cycle.

A circadian perspective on human sleep–wake regulation and ageing

Derk-Jan Dijk

in collaboration with

Jeanne F. Duffy

The distribution and quality of both the sleep and awake states are regulated by the interaction between circadian and homeostatic

direct evidence for processing of wake-related information during sleep (McNaughton). These events occur in non-REM sleep, leaving an open question over the role of REM sleep in information processing or retrieval. The possibility that the periodic PGO bursts during REM sleep activate a programme for integrating genetic and epigenetic information (Jouvet) remains to be explored. One argument against a central role for REM sleep is that its suppression by antidepressants has no clear consequences for memory (Gillin).

Looking to the future

Progress in sleep research, already rapid in the past few years, is now accelerating, thanks to the closer cooperation that is developing between the traditional areas of sleep science and the cellular and molecular neurosciences. Applying the concepts of Ca^{2+} regulation (Berridge), genetic analysis (Mignot) and differential gene expression (Tononi), together with the techniques used in these types of investigation, should facilitate rigorous testing of the current theories about the functions of sleep. Important among these is the question of sleep as a local brain process and whether its elementary manifestations and functions could reside at the cellular or subcellular level. From another direction, the increasing understanding of the cellular mechanisms underlying circadian rhythms (Gillette) may provide new leads into the mechanisms that regulate sleep.

Lastly, these advances at the cellular, molecular and genetic levels will have to be integrated into the recently developed computer models of sleep rhythms. The computer models will then become a powerful tool for identifying the essential processes that bridge the levels between the macroscopic EEG and the operations in neuronal networks, subcellular processes and genetic regulation. A multi-disciplinary approach will be the future benchmark for progress in understanding the functions and regulation of sleep.