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Osamu Hayaishi

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THE ROYAL SOCIETY

Molecular mechanisms of sleep–wake regulation: a role of prostaglandin D_2

Osamu Hayaishi

Osaka Bioscience Institute, 6-2-4 Furuedai, Suita, Osaka 565-0874, Japan (hayaishi@obi.or.jp)

Prostaglandin (PG) D_2 is a major prostanoid in the brains of rats and other mammals, including humans. When PGD synthase (PGDS), the enzyme that produces PGD_2 in the brain, was inhibited by the intracerebroventricular infusion of its selective inhibitors, i.e. tetravalent selenium compounds, the amount of sleep decreased both time and dose dependently. The amount of sleep of transgenic mice, in which the human PGDS gene had been incorporated, increased several fold under appropriate conditions. These data indicate that PGDS is a key enzyme in sleep regulation. *In situ* hybridization, immunoperoxidase staining and direct enzyme activity determination of tissue samples revealed that PGDS is hardly detectable in the brain parenchyma but is localized in the membrane systems surrounding the brain, namely, the arachnoid membrane and choroid plexus, from which it is secreted into the cerebrospinal fluid (CSF) to become β-trace, a major protein component of the CSF. PGD_2 exerts its somnogenic activity by binding to PGD_2 receptors exclusively localized at the ventrorostral surface of the basal forebrain. When PGD_2 was infused into the subarachnoid space below the rostral basal forebrain, striking expression of proto-oncogene Fos immunoreactivity (FosIR) was observed in the ventrolateral preoptic area (VLPO), a putative sleep centre, concurrent with sleep induction. Fos expression in the VLPO was positively correlated with the preceding amount of sleep and negatively correlated with Fos expression in the tuberomammillary nucleus (TMN), a putative wake centre. These observations suggest that PGD_2 may induce sleep via leptomeningeal PGD_2 receptors with subsequent activation of the VLPO neurons and downregulation of the wake neurons in the TMN area. Adenosine may be involved in the signal transduction associated with PGD_2.

Keywords: prostaglandin D_2; sleep; PGD synthase; adenosine; Fos

1. INTRODUCTION

From the moment babies are born, they start to repeat sleep–wake cycles every day and night, and eventually spend almost one-third of their lifetime sleeping, in bed or elsewhere. And yet, the molecular mechanisms of sleep–wake regulation have so far been hardly understood. Furthermore, we are still not able to answer simple questions, such as 'What is sleep?', 'Why do we need to sleep?', etc. Thus, numerous basic as well as clinical problems surrounding sleep and wakefulness remain as enigmas despite the enormous progress in modern medical science.

According to recent reliable epidemiological surveys, more than 20% of the general population in the USA suffer from sleep problems (Wake up America 1993). Each year, these sleep disorders cost the US government at least $16 billion. The additional cost to society for such consequences as traffic and industrial accidents and low productivity and inefficiency cannot be accurately estimated. Thus sleep-related problems are now being envisaged not only as a medical issue but also as social and economic ones.

For the solution of these problems and an understanding of sleep and sleep disorders, multidisciplinary approaches are required. In this review, emphasis is placed on the biochemical and molecular biological approaches to challenge this formidable problem. The results are integrated and discussed in conjunction with the more conventional physiological studies.

2. PROSTAGLANDIN D_2 AND SLEEP

In the late 1970s, prostaglandin (PG) D_2 was shown to be the major prostanoid of the mammalian brain. Subsequent studies indicated that infusion of femtomolar quantities of PGD_2 into the third ventricle of rats induced both slow-wave sleep (SWS) and rapid eye movement (REM) sleep, and that the sleep induced by the infusion of PGD_2 was indistinguishable from physiological sleep as judged by inspection of electroencephalogram, electromyogram and other behaviours of the rat. The PGD_2 concentration in the rat cerebrospinal fluid (CSF) exhibited a circadian fluctuation in parallel with the sleep–wake cycle; it was higher during the day when rats are mostly asleep and lower during the night when they are mainly awake (Pandey *et al.* 1995). The PGD_2 concentration in the CSF of rats elevated time dependently with an increase in sleep propensity during sleep deprivation (Ram *et al.* 1997). These results indicate that PGD_2 is an endogenous sleep-promoting substance in the rat brain. Most of the early work up until April 1991 has been reviewed previously (Hayaishi 1988, 1991).

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3. PGD SYNTHASE: STRUCTURE AND FUNCTION

PGD synthase (PGDS, EC5, 3, 99, 2) catalyses the isomerization of a 9, 11-endoperoxide of PGH_2 to produce PGD_2. It has been characterized as a member of the lipocalin superfamily, and is sometimes referred to as brain-type PGD synthase or glutathione-independent PGD synthase. The other type of PGD synthase, identified as sigma-type glutathione S transferase, is sometimes referred to as haematopoietic PGD synthase based on its localization in the haematopoietic organs (Urade & Hayaishi 1999). The lipocalin-type PGD synthase, hereafter referred to as PGDS, is a monometric glycoprotein with a molecular weight of *ca.* 27 000 Da. We purified the enzyme from human, rat and frog brains, isolated the cDNAs encoding PGDS, and determined their nucleotide and amino-acid sequences (Urade *et al.* 1989). The primary structure of PGDS indicated that it is a member of the lipocalin superfamily and a small secretory protein present in the membrane system of the cell; namely, it is an ectoprotein.

The enzyme was finally crystallized and the tertiary structure is now being delineated. The free sulphydryl (SH) group of cysteine residue 65 is found only in PGDS and not in the other lipocalins. It resides in the hydrophobic pocket and is considered to be a part of the active site of the enzyme (figure 1). These findings will contribute to fabrication of a lead molecule for designing new functional proteins as well as new types of hypnotics and sleep-preventing drugs (Toh *et al.* 1996). We reasoned that if PGDS is indeed involved in the regulation of physiological sleep, then if the synthase activity during the day in rats could be inhibited by a specific enzyme inhibitor, sleep should also be inhibited and the amount of diurnal sleep should decrease. To find a selective inhibitor of PGDS, we then made an extensive search for a specific inhibitor and finally discovered that inorganic tetravalent (4+) selenium compounds are potent, specific, non-competitive and reversible inhibitors of brain PGDS (Islam *et al.* 1991). This is probably due to their interaction with this free SH group in the active centre, because this inhibition is reversible and can be reversed by the addition of excess amounts of sulphydryl compounds such as glutathione or diethyldithiothreitol (DTT). When selenium chloride was infused into the third ventricle of a rat during the day, sleep was inhibited both time and dose dependently. After about 2 h from the start of the infusion, both SWS and REM sleep were almost completely inhibited. The effect was reversible. When the infusion was interrupted, sleep was restored. Furthermore, the effect was reversed by the simultaneous infusion of SH compounds such as DTT and reduced glutathione, as in the case of the *in vitro* enzyme activity (Matsumura *et al.* 1991). These results clearly show that PGD synthase plays a crucial role in sleep regulation.

Roberts and co-workers (Roberts *et al.* 1980) in the USA reported that deep sleep exhibited by systemic mastocytosis patients is primarily due to the episodic release of PGD_2 from mast cells. Up to a 150-fold increase in endogenous production of PGD_2 has been observed in these patients. In 1990, Pentreath and co-workers (Pentreath *et al.* 1990) in the UK reported that the PGD_2 concentration was markedly (10^2–10^3-fold), progressively and selectively elevated in the CSF of patients with advanced African sleeping sickness induced by *Trypanosoma*. These clinical observations are consistent with the notion that excessive endogenous production of PGD_2 in man induces sleep under certain pathological conditions. However, animal experiments to verify this conclusion have so far been unsuccessful.

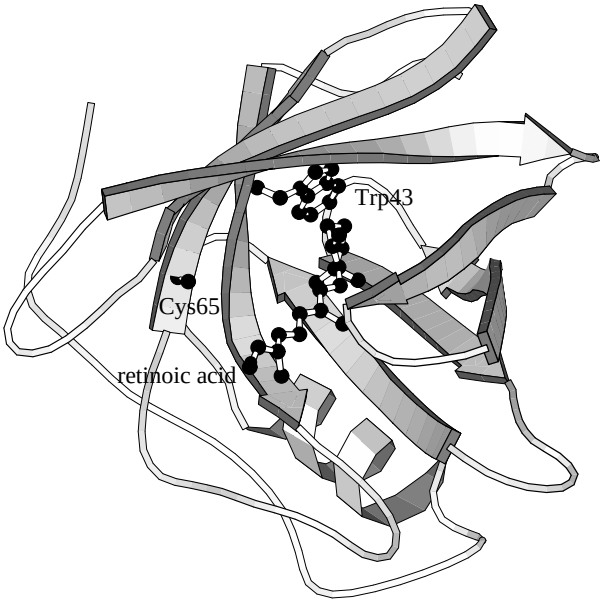

Figure 1. Ribbon model of the crystal structure of mouse lipocalin-type PGD synthase. The figure was produced by the use of the software MOLSCRIPT, originally developed by Kraulis (1991). Retinoic acid is a non-substrate ligand and a non-competitive inhibitor of this enzyme.

More recently, we generated transgenic mice in which the human PGDS gene had been incorporated and was overexpressed in all tissues and cells, as well as knockout mice devoid of the PGDS gene. Detailed analyses of the sleep behaviour of these genetically engineered mice are currently under way, but preliminary evidence indicates that, although these transgenic mice appeared to be quite healthy and to sleep normally, when the tails of these mice were clipped for DNA sampling, the amount of sleep almost doubled during the following several hours. On the other hand, the amount of sleep of the wild-type mice was essentially unchanged by the tail clipping. One possible explanation for this phenomenon is that by pain stimulation, cyclo-oxygenase II was induced to produce large amounts of PGH_2, the substrate for PGD synthase, and that the PGD synthase step became the rate-limiting step under these conditions. These experimental results and clinical observations from my own and other laboratories clearly show that PGD_2 is the endogenous sleep-promoting substance in the rat brain and that PGD synthase is the key enzyme in sleep regulation. Questions then arose such as 'In what region is the PGD synthase located in the brain?' and 'In what type of cells?'

4. LOCALIZATION OF PGDS IN THE BRAIN

To determine the localization of PGDS in the rat brain, we employed three independent approaches, namely, *in situ* hybridization to detect messenger RNA (mRNA), immunohistochemical staining to show the localization of

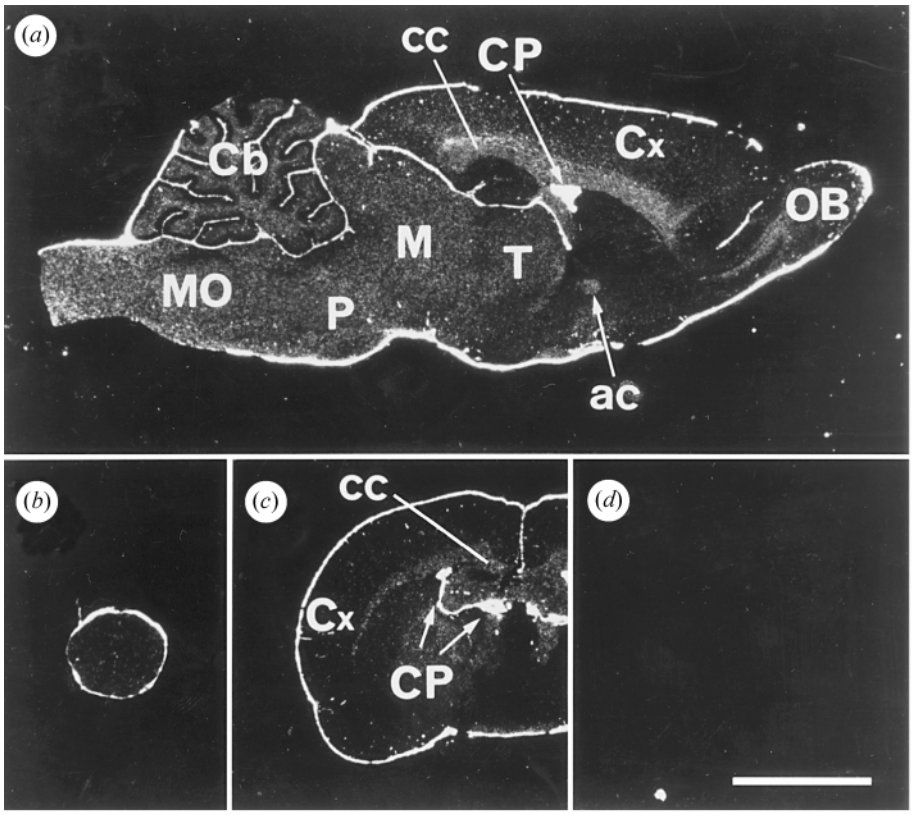

Figure 2. Distribution of mRNA for PGD synthase in the adult rat brain and spinal cord. Negative film images of *in situ* hybridization of (*a*) a parasagittal section, (*b*) coronal sections of the spinal cord, and (*c*, *d*) the brain are shown. Sections were hybridized with an antisense RNA probe for the enzyme in (*d*) the presence, or (*a–c*) the absence of a 100-fold excess of unlabelled probe. ac, anterior commissure; Cb, cerebellum; cc, corpus callosum; CP, choroid plexus; Cx, cerebral cortex; M, midbrain; MO, medulla oblongata; OB, olfactory bulb; P, pons; T, thalamus. Scale bar, 500 mm.

the enzyme protein and the direct enzyme assay to assess enzyme activity. The results provided several lines of important and unexpected information, which findings gave us a new insight into the mechanism of the somnogenic activity of PGD_2.

Typical results of the *in situ* hybridization studies for the detection of PGDS mRNA in the rat brain and spinal cord are shown in figure 2. The mRNA, shown by white spots and areas, was intensely expressed in the leptomeninges, namely, the arachnoid membrane and in the choroid plexus (CP), in the ventricles. But in the brain parenchyma it was expressed only faintly and diffusely, mainly in the white matter, especially in the corpus callosum (Urade *et al*. 1993).

The cellular localization of PGDS mRNA and enzyme protein was further investigated. The presence of mRNA as revealed by *in situ* hybridization as well as that of PGDS protein as revealed by the immunohistochemical technique clearly showed that the leptomeninges and choroid plexus contained an abundance of PGDS mRNA and enzyme protein. The oligodendrocytes were also positive for both, but little of either was seen in other cells, including neurons. In the human brain, the most intense staining for PGDS mRNA was observed in the epithelial cell layer of the CP (Blödorn *et al*. 1996)

We then determined the specific activity of PGDS in these tissues and also in the CSF of rats and humans. The PGDS activity in the CP and arachnoid membrane was several-fold higher than that in the whole brain. Furthermore, both rat and human CSF contained a remarkably large amount of PGDS activity. β-trace, which is the major protein in CSF, showed a very high specific activity, almost comparable to that of the most purified enzyme preparation from the rat brain. β-trace is the second most abundant protein in human CSF after albumin. It was discovered by Clausen in 1961 as a protein specific to the CSF (Clausen 1961), but its function has not yet been elucidated. Recently, two groups of investigators in Germany initially found the N-terminal 28-amino-acid sequence (Zahn *et al*. 1993) and subsequently the total sequence (Hoffmann *et al*. 1993), of human β-trace to be highly homologous to that of the human brain PGDS that we had reported previously. Watanabe *et al*. (1994) then found that β-trace and PGDS were structurally, enzymatically and immmunologically identical. As mentioned above, PGDS is a member of the lipocalin superfamily, all members of which are lipid transporters and secretory proteins. It is therefore reasonable to assume that PGDS is mainly, if not exclusively, produced in the membrane system surrounding the brain, namely, the arachnoid membrane and CP, and is then secreted into the CSF to become β-trace. β-trace and PGD_2 thus produced circulate in the CSF in the ventricular and subarachnoid spaces.

5. SITE OF ACTION OF PGD_2

To find the site of action of PGD_2, we employed a microdialysis probe to apply a picomolar amount of

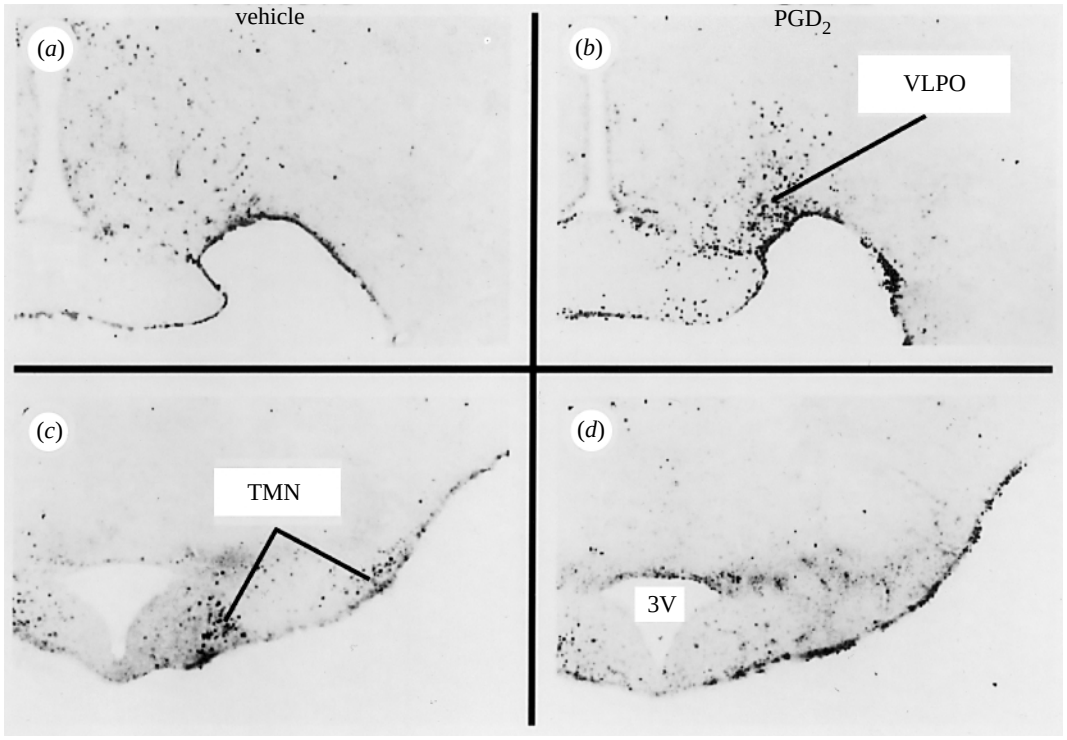

Figure 3. Effects of PGD_2 on Fos-IR in key sleep–wake regulatory regions. Bright-field photomicrographs of the preoptic area demonstrate little Fos-IR following (*a*) infusion of vehicle, but (*b*) showing numerous Fos-immunoreactive neurons in the VLPO following PGD_2 infusion. In the posterior hypothalamus, (*c*) the TMN contains many Fos-immunoreactive neurons following infusion of vehicle, but (*d*) not following that of PGD_2. Other abbreviations: oc, optic chiasm; 3V, third ventricle.

PGD_2 to more than 200 different areas in the rat brain. In the brain parenchyma, PGD_2 failed to induce sleep in all parts of the brain except in the preoptic area (POA), where a weak but consistent somnogenic activity was observed. The most pronounced sleep-inducing effect was observed when PGD_2 was applied to the subarachnoid space above the surface of the medial ventral region of the rostral basal forebrain (Matsumura *et al.* 1994). SWS was increased more than 120% up to the level of the peak of daytime sleep, indicating the presence of PGD_2 receptors on the surface of cells of the leptomeninges somewhat rostral to the POA and below the diagonal band of Broca.

The mouse prostaglandin D_2 receptor was cloned by Narumiya and co-workers (Narumiya *et al.* 1982), and its structure was delineated (Hirata *et al.* 1994). They also demonstrated (Oida *et al.* 1997) and we confirmed (Geraschenko *et al.* 1998) that the mRNA of this receptor was present exclusively in the arachnoid membrane in the rat brain, which is consistent with our finding that the site of action of PGD_2 is in the meninges surrounding the brain.

6. C-FOS EXPERIMENTS

The immediate early gene product cellular feline osteosarcome (c-Fos) is a useful marker of neuronal activation, and it has facilitated the identification of sleep- and wake-active neurons. To determine which neural regions are involved in the response to PGD_2, we used Fos immunohistochemistry to identify neurons activated or deactivated by infusion of PGD_2 into the PGD_2-sensitive zone in the subarachnoid space of the rostral basal forebrain. Infusion of PGD_2 into the subarachnoid space just anterior to the POA induced Fos-IR in the VLPO in association with an increase in SWS (figure 3). This neuronal activation was accompanied by increased Fos-IR in the basal meninges and in some other areas related to sleep-associated autonomic functions, along with a decrease in Fos in the putative wake-active neurons of the tuberomammillary nucleus (TMN). These observations suggest that PGD_2 may induce sleep via meningeal PGD_2 receptors with subsequent activation of VLPO neurons (Scammell *et al.* 1998). Our observation that PGD_2 induces Fos-IR in the leptomeninges suggests that PGD_2 activates the VLPO via leptomeningeal PGD_2 receptors. This route appears most likely because PGD_2 receptors are found only in the leptomeninges and are undetectable in the brain parenchyma (Oida *et al.* 1997). In addition, PGD_2 is most effective when infused into the subarachnoid space just anterior to the VLPO, but is much less effective in the parenchyma. Satoh *et al.* (1996) recently demonstrated that PGD_2-induced sleep is attenuated by an adenosine A_{2a} antagonist, KF 17837. Thus, PGD_2 may promote sleep by inducing meningeal cells to release paracrine-signalling molecules such as adenosine, which subsequently excite nearby sleep-active VLPO neurons.

The VLPO may induce sleep through inhibition of wake-promoting TMN neurons. The VLPO projects heavily to the proximal dendrites and soma of TMN neurons, and most of these axons contain GABA and galanin. Neurons of the TMN are tonically active during waking, less active during SWS and cease firing during REM sleep. The TMN is also near the site of action of PGE_2, a wakefulness-promoting substance (Hayaishi 1991). These results are summarized schematically in figure 4.

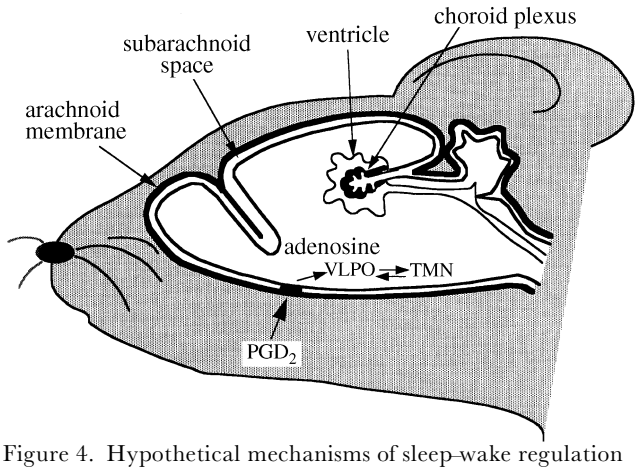

Figure 4. Hypothetical mechanisms of sleep-wake regulation by PGD_2.

7. POSSIBLE INVOLVEMENT OF ADENOSINE IN THE SOMNOGENIC ACTIVITY OF THE PGD_2 SYSTEM

In an attempt to define the mechanism of transduction across the meninges, we tested the effect of various neurotransmitters and neuroactive substances and found that adenosine A_{2a} agonists were the only compounds that could mimic the somnogenic activity of PGD_2, when applied to the PGD_2-sensitive zone.

When 2-[*p*-(2-carboxyethyl)phenylethylamino]-5′-*N*-ethylcarboxamidoadenosine (CGS21680), a selective A_{2a}-adenosine agonist, was infused continuously for 6 h during the night into the subarachnoid space underlying the ventral surface region of the rostral basal forebrain, both SWS and REM sleep increased in a dose-dependent manner up to 183% and 202% of their respective baseline levels. The increments produced by the infusion of CGS21680 were totally diminished when the rats had been pretreated with an intraperitoneal injection of (*E*)-1, 3-dipropyl-7-methyl-8-(3,4-dimethoxystyryl) xanthine (KF17837), a selective A_2 adenosine antagonist. Further, intraperitoneal injection of the latter compound dose-dependently attenuated the SWS increase produced by the infusion of PGD_2, indicating the possibility that the adenosine A_{2a} receptors are involved in the sleep-promoting process mediated by PGD_2.

8. PERSPECTIVES

The concept of humoral rather than neural regulation of sleep was initially proposed by a French neuroscientist, Henri Pieron of Paris, and independently and concurrently by Kuniomi Ishimori of Nagoya, Japan, in the first decade of the 20th century. They took samples of the CSF of sleep-deprived dogs and infused them into the brains of normal dogs. The recipient dogs soon fell asleep. Thus these researchers became the first to demonstrate endogenous sleep-promoting substance(s). However, the chemical nature of their sleep substances was not identified. During the next 90 years or so, more than 30 so-called endogenous sleep substances were reported by numerous investigators to be present in the brain, CSF, and other organs and tissues of mammals. However, their physiological relevance has remained uncertain in most instances (see Inoué (1989), for a general review). Recently, a more unified view of humoral regulation of sleep was proposed, one in which PGD_2 occupies an ultimate role in the cascade of sleep-regulatory events (Krueger *et al*. 1999). The data presented in this review also provide molecular interpretation of the somnogenic activity of PGD_2 and the unique signal transduction system in the mammalian brain, one that appears to be mediated by adenosine through the adenosine A_{2a} receptor. These results clearly show the link between the humoral and neural regulation of sleep. Further work is obviously necessary to delineate the details of this novel signal transduction mechanism and the interaction of humoral and neural mechanisms in sleep–wake regulation. To this end, further studies with multidisciplinary approaches, in particular molecular genetic ones, will be useful.

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