Archives Italiennes de Biologie, 142: 533-539, 2004.

GENES FOR PROSTAGLANDIN D SYNTHASE AND RECEPTOR AS WELL AS ADENOSINE A_{2A} RECEPTOR ARE INVOLVED IN THE HOMEOSTATIC REGULATION OF NREM SLEEP

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INTRODUCTION

I would first like to join the other speakers in congratulating Professor Jouvet for the 50th anniversary of his discovery of paradoxical sleep, and also to thank Dr. Luppi and other organizers for the kind invitation and warm hospitality. By way of introduction, I would like to briefly summarize our current view of how prostaglandin D_2 exerts its somnogenic activity and then in the main part of my talk, I shall discuss more recent experimental results with gene manipulated mice.

DISCOVERY OF SLEEP SUBSTANCES

The humoral theory of sleep regulation, namely sleep is promoted by a hormone-like chemical substance(s) rather than by a neural network was initially proposed by French neuroscientists Legendre and Piéron of Paris (6) and, independently and concurrently, by Professor Kuniomi Ishimori of Nagoya, Japan (3) almost one hundred years ago. They took samples of the cerebrospinal fluid 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 substances. However, the chemical nature of their sleep substances was not identified. During the next 90 years or so, more than 40 so-called endogenous sleep substances were reported by numerous investigators to be present in the brain and other organs and tissues of mammals. However, their physiological relevance remained uncertain in most instances. During the early 1980s, Professor Jouvet and his colleagues in Lyon found in the cerebrospinal fluid of cats a sleep-inducing factor, that was produced in the periventricular structures, including the choroid plexus (1, 5). By curious coincidence, our initial findings on prostaglandin D_2 and its somnogenic activity were published at about the same time from Kyoto, Japan.

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PROSTAGLANDINS AND SLEEP

Prostaglandin (PG)s are so-called tissue or local hormones. More than 30 different PGs are widely distributed in virtually all mammalian tissues and organs and exhibit numerous and diverse biological activities. In the late 1970's, we showed PGD_2 to be the most abundant prostanoid in the brains of rats and other mammals including humans (9), and that it is produced in the brain from the substrate PGH_2 by the action of an enzyme, PGD synthase (15). Since PGD_2 had long been considered as a minor and biologically inactive prostanoid, our findings suggested that it might be a unique constituent of the brain and might have some specific and important function in this organ. Sure enough, we soon found out that PGD_2 induced sleep when microinjected into the brains of rats (17). The results were soon confirmed and extended by more careful studies using a continuous infusion sleep bioassay system (16). When PGD_2 was infused continuously into the third ventricle of a freely moving rat during the night when rats were awake most of the time, both slow-wave sleep (SWS) and paradoxical sleep (PS) increased significantly during the time of infusion. The effect was specific for PGD_2 and dose-dependent. As little as several picomoles of PGD_2 per minute were effective in inducing excess sleep. Most importantly, however, sleep induced by PGD_2 was indistinguishable from physiological sleep as judged by several behavioral and electrophysiological criteria, including power spectral analyses.

PGD synthase (PGDS) is a monomeric glycoprotein with a molecular weight of approximately 25,000. The enzyme was purified from the brains of rats and humans and finally crystallized, and its tertiary structure has now been delineated. The free SH group of cysteine residue 65 is in the hydrophobic pocket and is in the active site of the enzyme. After an extensive search for an enzyme inhibitor, we found inorganic tetravalent (4+) selenium compounds to be potent, specific and reversible inhibitors of the brain PGD synthase (4). These compounds seem to interact with the free sulfhydryl group in the active center, because this inhibition is reversible and can be reversed by the addition of excess amounts of SH compounds such as glutathione (GSH) or dithiothreitol (DTT). We then administered selenium chloride into the third ventricle of a sleeping rat to see if it would have any inhibitory effect on sleep.

When selenium chloride was infused into the third ventricle of a rat during the day, SWS and PS were inhibited time- and dose-dependently (7). After about 2 hours from the start of the infusion, both SWS and PS were almost completely inhibited. The effect was reversible. When the infusion was interrupted, sleep was restored. Furthermore, the inhibition was reversed by the simultaneous infusion of SH compounds such as DTT and reduced GSH, as in the case of the *in vitro* enzyme activity. These results indicate that PGD_2 is essential for maintaining the sleep state.

In 1994, Hirata and coworkers cloned a cDNA for the mouse PGD receptor (DPR) and the structure was determined (2). It was a G protein coupled rhodopsin type receptor with putative seven transmembrane domains. More recently, we infused a PGD receptor (DPR) antagonist into the third ventricle of a sleeping rat. Both SWS

and PS were inhibited dose - and time - dependently and reversibly. These results are consistent with the conclusion that PGD synthase and the PGD receptor play a crucial role in the maintenance of natural sleep and therefore, PGD_2 is involved in sleep under physiological conditions.

We then determined the location of PGDS and DPR in the rat and mouse brain by *in situ* hybridization, immunohistochemical staining, and direct enzyme activity determination. The results obtained by three independent approaches were essentially in good agreement and clearly showed that PGDS was present mainly in the entire leptomeninges surrounding the brain as well as choroid plexus but DP receptor was localized exclusively in the ventro-rostral area of the basal forebrain. Very little, if any, of either protein, was detected in the brain parenchyma. Upon higher magnification, both the synthase and the receptor were found to be associated with the trabecular cells of the arachnoid membrane (8).

These results together with other experimental results which I do not have time to mention here today led us to our current tentative conclusion which is schematically shown in Figure 1.

PGD synthase is present mainly, if not exclusively, in the membrane system surrounding the brain, namely, the arachnoid membrane shown in red, and choroid

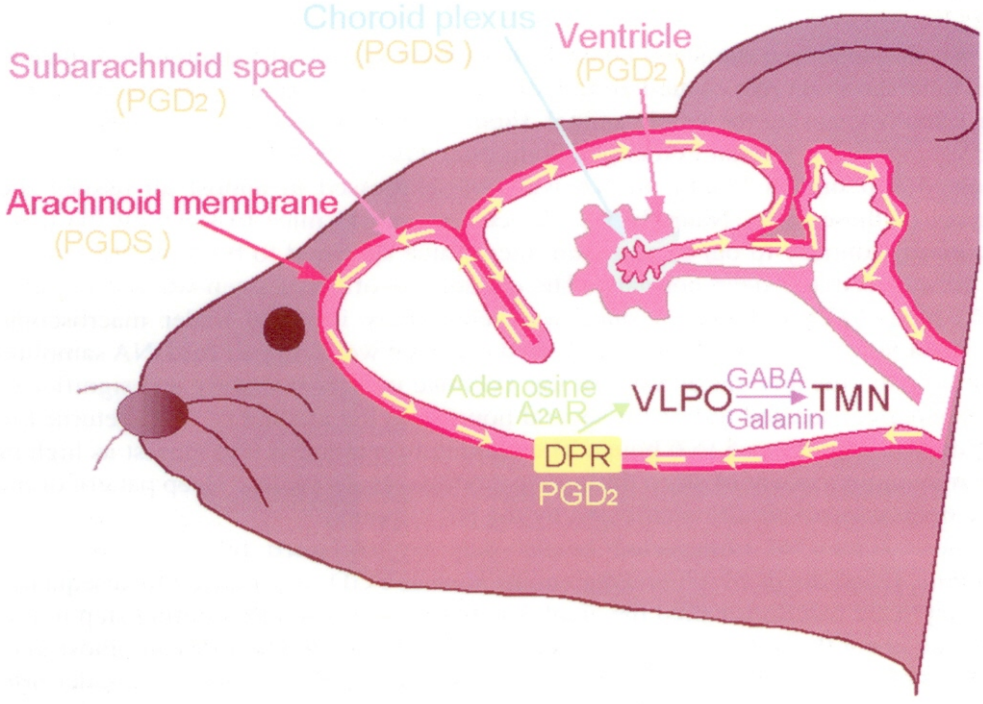

Fig. 1. *Molecular mechanisms of sleep-wake regulation by PGD_2 and adenosine.*

plexus, shown in blue, rather than in the brain parenchyma represented by the white area, although some enzyme activities are also present in oligodendrocytes in the brain parenchyma. PGD_2 is then secreted into the cerebrospinal fluid (CSF) and circulates in the ventricular and subarachnoidal space indicated by the pink color. PGD receptors are exclusively localized in a small area on the ventro rostral surface of the basal forebrain (8). PGD_2 in the CSF is then bound to the receptor, where a signal for sleep is generated.

C-Fos experiments were then carried out in collaboration with Scammell and Saper of Harvard Medical School (14). The signal initiated by the binding of PGD_2 to its specific receptor is transmitted into the brain parenchyma to the VLPO or ventrolateral preoptic area, a sleep center, where sleep-related neurons are activated. This process appears to be mediated via adenosine by way of adenosine A_{2A} receptor (11, 12, 13). The VLPO area projects heavily to the TMN or tuberomammilary nucleus, a wake center in the posterior hypothalamus, through GABAergic and galaninergic neurons, and sends inhibitory signals to the TMN to downregulate the neuronal activity involved in the maintenance of wakefulness. Thus sleep is promoted by upregulating the sleep neurons and at the same time downregulating the wake neurons by a flip-flop mechanism as mentioned by Dr. Saper in the preceding paper.

EXPERIMENTS WITH GENE MANIPULATED MICE

We then explored the sleep behavior of several genetically manipulated mice to elucidate the exact role of the PGD_2 system *in vivo* and to examine our current working hypothesis using the whole animal. First, we generated transgenic (TG) mice by incorporating the human PGDS gene into mice (10). Northern blot analyses clearly showed that human PGDS mRNA was over-expressed in almost all tissues and organs of these mice. Naurally we expected these TG mice to sleep all the time. However, contrary to our expectation, these mice appeared to be quite healthy and to grow, breed and sleep normally. The circadian profiles of sleep-wake patterns of wild type (WT) and mutant mice were essentially identical under macroscopic examination. However, when the tails of these mice were clipped for DNA sampling at 8:00 p.m., the amount of SWS of the TG mice increased sharply and significantly. This effect seemed to last for several hours, and the amount of SWS returned to the control level after 5 to 6 hours. The maximum increment was almost as high as the maximum amount of sleep during the daytime. However, the sleep pattern of the WT mice was essentially unaffected by the tail clipping.

These somewhat unexpected results may be explained by the sequence of enzymic reactions in which prostaglandins such as PGD_2 is produced by a sequence of enzymatic reactions called the arachdonate cascade. The rate limiting step in this cascade is not the synthase step but is generally the step in the upstream, most likely the cyclooxygenase step, especially the inducible COX II. Therefore, under normal conditions, the amounts of PGD_2 produced in the brain and elsewhere depends on the activity of COX but not on the synthase. This explains the fact that the sleep

patterns of WT and TG mice were essentially identical. It is possible, therefore, that the pain stimulus induced the cyclooxygenase, which then produced an excessive amount of PGH_2, the substrate for PGDS. The PGDS step then became the rate-limiting step under these conditions, thus leading to the production of a larger quantity of PGD_2 in the TG mice than in the WT mice and ultimately to an increased amount of SWS in the TG mice.

In order to prove this interpretation, we then measured the amount of PGD_2 produced in the brains of WT and TG mice before and after the tail clipping. The amount of PGD_2 in the brains of the TG mice increased sharply and significantly for about three hours after the tail clipping, and then started to decrease thereafter. These changes almost exactly paralleled the time course of changes in SWS. In contrast, the PGD_2 content in the brains of wild-type controls remained essentially the same. Thus, it seems reasonable to conclude that the increase in SWS in the transgenic mice after tail clipping was probably due to the induction of COX, or possibly another rate limiting enzyme in the upstream of the cascade, by the pain stimulus, resulting in an increased level of PGH_2 in the brain, which prostanoid was then converted to PGD_2 by the excessive amount of PGDS in these TG mice.

We then generated knock-out mice for PGDS and also for DPR. Circadian profiles of NREMS and REMS of the PGDS KO mice were essentially identical to those of WT mice. The lack of effect on phenotypes may be explained by the

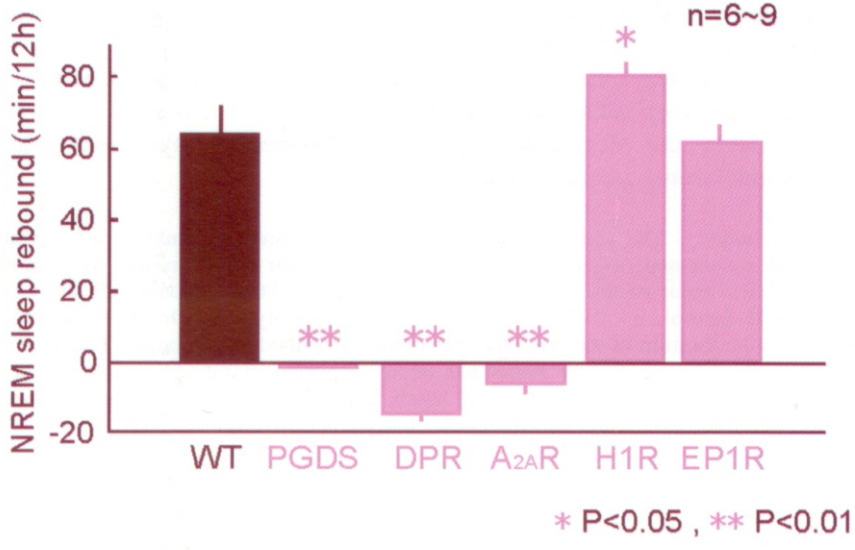

Fig. 2. *Amounts of NREM sleep rebound in WT and KO mice for PGDS, DPR, $A_{2A}R$, H1R and EP1R, after sleep deprivation.*

Mice were subjected to sleep deprivation for 6 hrs prior to the onset of the wake period. The amounts of NREM sleep rebound were determined during the following 12 hrs.

assumption that, because sleep is essential for life, the sleep-regulatory system is composed of a complicated network in which the deficiency of one system may be effectively compensated by other systems during development. When the WT mice were subjected to sleep deprivation, a pronounced rebound was observed, especially in NREMS, whereas little, if any, rebound occurred in NREMS in the mutant mice. The amount of PGD_2 increased significantly in the brains of WT mice while no increase was observed in the mutant mice.

Likewise, the circadian sleep profiles of WT and DPR, adenosine A_{2A} and other KO mice appeared to be essentially identical under macroscopic examination. However, when these mice were subjected to sleep deprivation, again a pronounced NREMS rebound was observed in the wild type mice, while no rebound was seen in the DPR and A_{2A} KO mice as in the case of PGDS KO mice. The total amount of NREM sleep rebound exceeded more than 60 min in the WT mice as well as in the histamine H1 receptor KO mice and prostaglandin EP1 receptor KO mice, which were used as controls (Fig. 2).

These results clearly show that the PGD system including the adenosine $A_{2A}R$ plays a crucial role in the homeostatic regulation of NREM sleep.

SUMMARY

(1) Prostaglandin D_2 is essential for the maintenance of the sleep state. (2) The adenosine and A_{2A} receptor system is a link between the humoral and neural mechanisms of sleep-wake regulation. (3) Prostaglandin D_2 plays a crucial role in the homeostatic regulation of NREM sleep.

Finally, it may not be too far-fetched to say that prostaglandin D_2 was most likely the endogenous sleep substance described by Piéron and Ishimori about 100 years ago, and possibly the sleep-inducing factor reported by Professor Jouvet and coworkers some twenty years ago.

Acknowledgements. - The authors would like to express sincere thanks to a large number of coworkers both inside and outside of my laboratory for their collaboration. We are especially indebted to Dr. E. Pinzar of the Osaka Bioscience Institute, Drs. T. Scammell and C. Saper of Harvard Medical School, Dr. J.-F. Chen of Boston University, and Dr. A. Mizoguchi of Mie University for collaboration in the crucial experiments described in this paper. The work from this laboratory described in the present paper has been supported mainly by grants-in-aid from the Ministry of Health, Labour and Welfare of Japan, the Ministry of Education, Culture, Sports, Science and Technology of Japan and the Osaka Bioscience Institute.

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