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CONTRIBUTION OF THE "KNOCK-OUT" TECHNOLOGY TO UNDERSTANDING THE ROLE OF SEROTONIN IN SLEEP REGULATIONS

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INTRODUCTION

The seminal work of Jouvet (12) has shown that serotonin (5-HT) plays a key role in the regulation of wakefulness and sleep. The mechanisms involved in such a control have been analyzed by using various tools as they progressively became available. They were firstly electrolytic lesions of serotonergic nuclei (12) and inhibitors of 5-HT synthesis (18), and secondly specific neurotoxins of the serotonergic system (1) as well as inhibitors of the 5-HT reuptake (17) or catabolism (ref in 22). More recently, the characterization of various kinds of 5-HT receptor types and subtypes (figure 1), and the use of their selective ligands, have allowed a better analysis of these phenomena (ref in 22). Schematically, it can be said that excessive levels of 5-HT in the extracellular space at terminal level (likely in the brainstem, 9) induce a decrease in the amounts of Rapid Eye Movement (REM) sleep.

At the present time, mutant mice that do not express one of the proteins involved in serotonergic neurotransmission open new opportunities to investigate these regulations. Compared to the pharmacological approach, the tools of the molecular biology field offer total selectivity. For example, deletion of the gene coding for a given type of receptor represents a much more specific impairment of 5-HT neurotransmission than using antagonist at this receptor, even if the latter is considered as a selective blocker.

Thus, we have analyzed the regulations of sleep and wakefulness in knock-out mice that do not express various types of serotonergic receptors, or the serotonergic transporter, or monoamine oxydase A (MAOA, a catabolitic enzyme of 5-HT) (Fig. 1). These studies have led to evidence a tonic inhibitory influence of 5-HT on the expression of REM sleep, and for the first time an involvement of 5-HT on some characteristics of sleep such as homeostasis and stress-induced response.

Previous pharmacological studies had established that the influence of 5-HT on sleep is mediated by 5-HT_{1A}, 5-HT_{1B}, and 5-HT_2 receptors (ref in 22). Indeed, activation of these receptors by selective agonists injected systemically induces an inhibition of REM sleep, sometimes associated with an enhancement of the time spent in wakefulness (2, 6, 21). In the same manner, treatment with a selective serotonin reuptake inhibitor (SSRI, ref in 15) or with an inhibitor of monoamine oxydase

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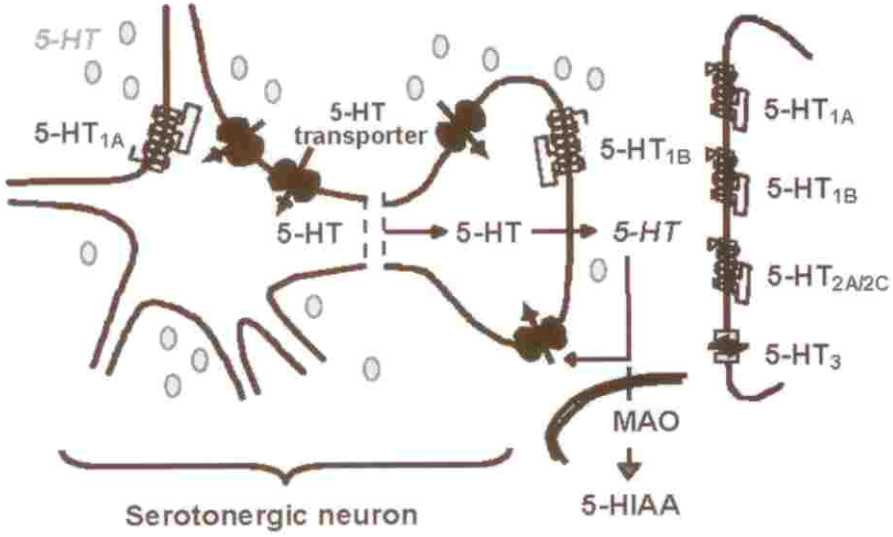

Fig. 1. - *Schematic representation of serotonergic neurotransmissio.*

Note that 5-HT_{1A} and 5-HT_{1B} receptors are located on both the serotonergic neuron (autoreceptors) and other neurons (in post-synaptic position relatively to the serotonergic neuron).
5-HT: serotonin; 5-HIAA: 5-hydroxy-indolacetic acid; MAO: monoamine oxydase.

(IMAO, ref in 3) induces a dramatic decrease of REM sleep amounts, probably due to enhanced activation of serotonergic receptors by extracellular 5-HT that accumulates as a result of blockade of its reuptake or degradation.

However, whether the effects of activating serotonergic receptors on vigilance are relatively well-known, notably regarding the 5-HT_{1A} and 5-HT_{1B} types, the consequences of their blockade are poorly understood. In such a context, mutant mice that do not express these receptor types reveal particularly relevant to complete the scarce and sometimes contradictory data obtained from the pharmacological approach. With regard to 5-HT_2 receptors, eventhough it is clear that antagonists induce an enhancement of deep slow wave sleep (SWS, 11), the respective involvement of the 5-HT_{2A} or 5-HT_{2C} sub-type in this effect remains an open question. Investigation of the vigilance states in knock-out mice that do not express these receptors should allow to provide some answers to these points.

RESULTS

1. - *Sleep-wakefulness regulations under baseline conditions*

Mutant mice that do not express 5-HT_{1A} or 5-HT_{1B} receptors

In both knock-out lines, animals exhibit the same circadian variations of sleep and wakefulness as their wild-type counterparts, with notably more sleep during the light

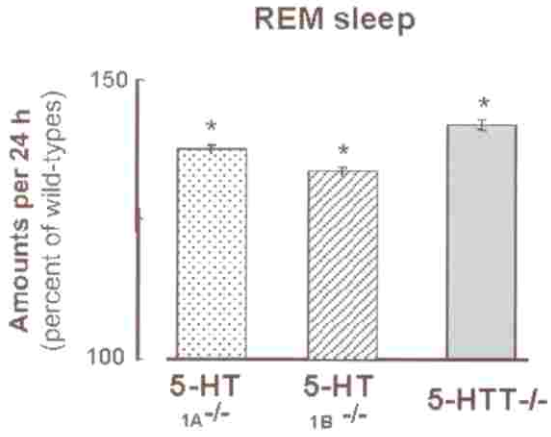

Fig. 2. - *Effect of various mutations on the amounts of REM sleep.*

Data (mean ± s.e.m.) are calculated during 24 hours, and expressed as percent of wild-type counterparts. * $p < 0.05$ significantly different from respective wild-type mice, Student's t test.

than during the dark period (4, 5). However, both 5-HT_{1A}-/- and 5-HT_{1B}-/- mutants express higher levels of REM sleep than wild-types (Fig. 2). This enhancement is more pronounced during the light period for both lines. Furthermore, in wild-type mice, pharmacological blockade of 5-HT_{1A} receptors on the one hand, and of 5-HT_{1B} types on the other hand, also induces an increase in REM sleep amounts, that mimics what is observed in 5-HT_{1A}-/- and 5-HT_{1B}-/- mutants, respectively (Fig. 3). Conversely, activation of these receptors by selective agonists induces an inhibition of REM sleep (4).

The fact that genetic or pharmacological inactivation of 5-HT_{1A} or 5-HT_{1B} receptors results in an enhancement of REM sleep suggests that 5-HT exerts a tonic inhibitory control, mediated by these two types of receptors, over this stage of sleep.

Mutant mice that do not express 5-HT_{2A} or 5-HT_{2C} receptors

Antagonists at 5-HT_2 receptors induce in humans (11) and in rats (6) an increase in SWS as well as in EEG delta power. However, the 5-HT_2 receptor sub-type(s) involved in this effect is unknown. Thus, we have examined sleep in 5-HT_{2A} receptor kock-out mice, and found that they spent more time in wakefulness and less time in SWS than their wild-type counterparts (19). The same profile has been described in 5-HT_{2C} receptor knock-outs (8). In contrast, treatment of wild-type mice with antagonists at 5-HT_{2A} and 5-HT_{2C} receptors is followed by an enhancement of SWS, concomitant with a decrease of REM sleep, similarly to what was observed in the rat (6).

Altogether, these data indicate that deletion of genes coding for 5-HT_2 receptors induces opposite effects on sleep that those of blockade of the same receptors. This apparent discrepancy could be due to development, in constitutive mutants, of adap-

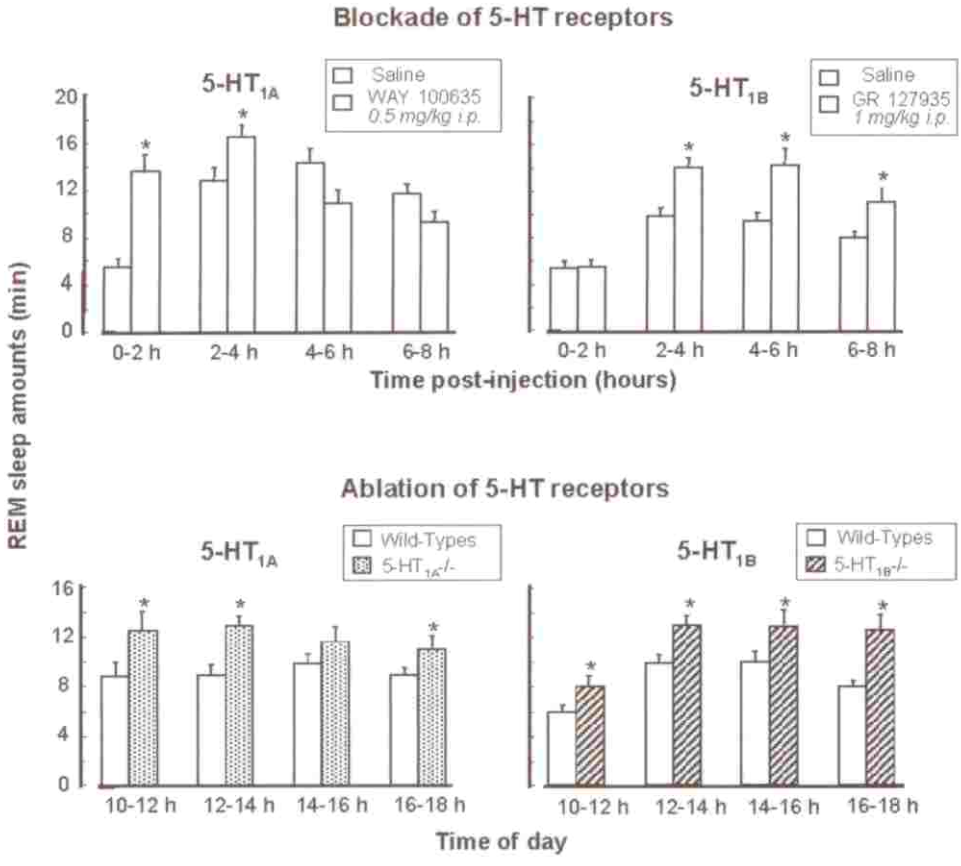

Fig. 3. - *Effect on REM sleep of the pharmacological blockade (top) or the genetic ablation (bottom) of 5-HT$_{1A}$ (left) and 5-HT$_{1B}$ (right) receptors.*

REM sleep amounts are expressed in minutes per 2 hours: top: after injection of saline (empty bars) or the ligand (filled bars), and bottom: in wild-type (empty bars) and knock-out (filled bars) mice.
The compounds (the 5-HT$_{1A}$ antagonist WAY 100635 and the 5-HT$_{1B}$ antagonist GR 126935) were injected at 10:00, and sleep-wakefulness were monitored from 10:00 until 18:00.
* $p < 0.05$: Top significantly different from injection of saline, paired Student's t test. Bottom: significantly different from respective wild-type mice, Student's t test.

tive modifications of serotonergic neurotransmission resulting from the gene invalidation.

Mutant mice that do not express 5-HT transporter

Knock-out mice in which the gene coding for the 5-HT transporter has been deleted (5-HTT-/-) exhibit locomotor and sleep-wakefulness cycles synchronized on the light dark phases, but the amplitude of this rhythm is reduced compared to that in their wild-type counterparts. In addition, 5-HTT-/- mice express more REM sleep, while the other states of vigilance are not modified.

In contrast to this REM sleep enhancement in 5-HTT-/- mutants, pharmacological blockade of the transporter (by injection of the SSRI citalopram) in wild-type controls induces an inhibition of this state of sleep. This effect is largely mediated by 5-HT_{1A} receptors since it is abolished in 5-HT_{1A}-/-, but not 5-HT_{1B}-/- mutants (15).

Thus, spontaneous sleep in 5-HTT-/- knock-outs does not mimick the effects of treatment with SSRIs. This might be explained, at least in part, by desensitization of 5-HT_{1A} and 5-HT_{1B} receptors in 5-HTT-/- mutants (4, 5). Such desensitization would result in a reduction of the serotonergic tone on REM sleep structures, and in turn in an increase of REM sleep.

Mutant mice that do not express monoamine oxydase A

MAOA-/- mice exhibit alterations of serotonergic neurotransmission, with notably a increase in extracellular concentration of 5-HT (7). These impairments are associated with modifications in the states of vigilance, without any change in circadian rhythms. In particular during the dark period, larger amounts of wakefulness and smaller quantities of REM sleep are observed in mutants compared to wild-types. The same modifications are induced in wild-type mice by treatment with MAO inhibitors.

Thus, the effect of deletion of the MAOA encoding gene on sleep organisation mimiks those of pharmacological inhibition of the enzyme, i.e., an inhibition of REM sleep (ref in 22).

2. - *Homeostasy, stress and sleep*

REM sleep processes undergo homeostatic regulations, illustrated notably by the REM rebound observed after selective deprivation of this sleep state (14, 20). Mutant mice in which molecular component of the serotonergic system have been ablated do not exhibit such a rebound (4, 5). Thus, REM sleep homeostatic processes in these mutants are substantially impaired.

Furthermore, it is known that acute stress triggers a REM sleep rebound in rats (10). In mice as well, an immobilization challenge of 90 minutes duration is followed by a REM sleep rebound during the 16 h-recovery period. However, this phenomenon is abolished in 5-HT_{1A}-/- (5), 5-HT_{1B}-/-, 5-HTT-/-, or MAOA-/- (3) mutants.

Thus, it seems that the homeostatic and stress-adaptive properties of REM sleep depend on the integrity of serotonergic neurotransmission.

DISCUSSION

1. - *The limits of the knock-out technology*

Genetic manipulations leading to the production of knock-outs is mostly performed on blastocytes of the 129Sv line. Then the genetic background of the mutant

line is progressively purified by means of successive backcrosses with another strain. Since significant differences in behaviour and sleep-wakefulness characteristics are found between various lines of mice (13), it is crucial to verify that the results obtained in mutants are specific of the mutated protein and not simply due to a difference in genetic background between knock-outs and wild-types. Such verification might be performed by investigating whether the same behavioural and sleep impairments are found in mutants backcrossed with other strains. Along this line, we have shown that 5-HTT-/- mice obtained on respectively the CD1 and the C57/Bl6 genetic backgrounds exhibit similar enhancement of REM sleep amounts compared to their respective wild-type counterparts.

It should also be mentioned that various compensatory mechanisms develop in constitutive knock-outs. However, since administration of 5-HT_{1A} and 5-HT_{1B} antagonists in wild-types mimics the respective knock-out phenotypes (Fig. 3), basal sleep expression in these mutants does not reflect long-term developmental changes. Meanwhile, other mutants such as the 5-HTT-/- ones, exhibit functional desensitization of 5-HT_{1A} and 5-HT_{1B} receptors. In the same manner in 5-HT_{1A}-/- mutants, the susceptibility of sleep to activation of 5-HT_{1B} receptors in enhanced (5), and conversely in 5-HT_{1B}-/- mutants regarding 5-HT_{1A} receptors (4). These adaptive phenomena at serotonergic neurotransmission (and most probably at other neurotransmitter systems as well) render the interpretation of the results difficult, since the impairments observed might be accounted for by other phenomena than directly the gene deletion.

In order to overcome this difficulty, further investigation of these adaptive processes can already be initiated by using pharmacological tools in mutants (15). In addition, it will be possible in the near future to compare the present data to those obtained in inducible knock-outs, i.e., in which the protein expression is eliminated acutely at adult age. In these models, it will in turn be particularly interesting to analyze the kinetics of possible compensatory mechanisms.

Finally, issues regarding pre- versus post-synaptic involvement of 5-HT_{1A} and 5-HT_{1B} receptors (Fig. 1) in sleep regulations (21) will be clarified by tissue-specific knock-out in which receptor gene is inactivated solely in serotonergic neurons.

CONCLUSION

In many ways, the data obtained in mutant mice concerning the serotonergic regulation of sleep and wakefulness have confirmed the hypotheses generated by the pharmacological approach. However, under several aspects, the genetic tool has allowed to evidence novel involvement of serotonergic neurotransmission in these functions. It is notably the case of the tonic inhibitory role of serotonin on REM sleep expression, which has been revealed by the sleep organisation in 5-HT_{1A}-/-, 5-HT_{1B}-/- and 5-HTT-/- mutants. It is also and even more the case for the facilitatory role of 5-HT on the REM sleep rebound that occurs after intrumental deprivation of this sleep state, or after acute stress. These functions would not have been evidenced by using solely the pharmacological approach.

Finally, it seems that heterozygous mutants should be more systematically studied (16). Indeed, the latter animals exhibit neurobiological, and possibly behavioural, impairments that are closer to pathological conditions than homozygous mutants. In this respect, they might represent relevant models of vulnerability to various sleep-related pathologies, in particular in the psychiatric and cardio-vascular domains (19).

In conclusion, we believe that association of genetic manipulations with relevant pharmacological ones should allow further progress in the understanding of sleep mechanisms.

SUMMARY

Genetic manipulation of the 5-HT system leads to alterations of 5-HT neurotransmission and provides new opportunities to investigate the role of 5-HT in sleep regulations. Indeed, it represents an alternative to the use of pharmacological tools and, to some extent, of localized lesions of the 5-HT system, which have been, from the 1960s until recently, the main approaches to investigate this question. The homologous recombinaison for the knock-out of genes coding for various proteins involved in 5-HT neurotransmission in the mouse, has allowed to explore further the role of the serotonin transporter (5-HTT), the momoamine oxydase A (MAO-A), or the 5-HT_{1A}, 5-HT_{1B} and 5-HT_2 receptors in the regulation of sleep.

In 5-HT_{1A}-/- and 5-HT_{1B}-/- knock-outs, REM sleep (REMs) was enhanced. Pharmacological blockade of these receptors had the same effects in wild-types. Thus, both receptor types exert a tonic inhibitory influence on REMs. In addition, 5-HT_{1A}-/- and 5-HT_{1B}-/- mutants exhibited hypersensitivity of other serotoninergic receptor types (notably the 5-HT_{1A} in 5-HT_{1B}-/- mice and vice versa), which suggests that adaptive changes at 5-HT neurotransmission develop in knock-outs.

In the same manner, 5-HTT-/- knock-outs exhibited increased REMs. This may be accounted for by a decrease of 5-HT_{1A} and 5-HT_{1B} receptor-mediated sleep regulations.

In contrast, MAO-A-/- knock-outs exhibited decreased REMs, associated with an enhanced response to 5-HTT blockade.

Finally, in 5-HT_{2A}-/- mice, we observed more wakefulness and less SWS than in wild-types. These effects could not be reproduced by 5-HT_{2A} blockade in wild-types.

To conclude, constitutive knock-outs undergo adaptive processes involving other proteins than those coded by the invalidated gene, which render the interpretation of the corresponding sleep phenotype difficult. Inducible knock-outs will probably help to overcome this difficulty. Finally, we believe that association of genetic manipulations with relevant pharmacological ones should allow further progress in the understanding of sleep mechanisms.

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