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Is the role of melatonin in induction of ovulation in the light-induced constant estrous anovulatory state mediated through the brain serotonergic system ?

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Summary. In animals in constant estrous-anovulatory (CEA) state, induced by continuous exposure to light (LL), the midbrain content of serotonin (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) decreased significantly, but no significant variations were detected in the basal hypothalamus. Late afternoon chronic melatonin treatment provoked resumption of ovulation and increased the midbrain 5-HT content. Similar results were evident after replacing LL-CEA rats in normal light-dark cycles. Melatonin appeared to mimic the effect of the daily darkness phase by eliciting ovulation ; this effect might be mediated via the midbrain serotonergic system.

Introduction.

In previous papers (Mess *et al.*, 1978 ; Trentini *et al.*, 1978), we demonstrated that the chronic injection of melatonin (MEL) in the late afternoon was able to overcome the constant estrous-anovulatory (CEA) state induced in the rat by exposure to continuous light (LL) for a period of 4 months. The luteinizing effect of MEL was significantly inhibited either by feeding the animals a tryptophan-poor diet or by injecting Methiothepin, a blocker of the central serotonergic and dopaminergic receptors. Moreover, it was demonstrated that both these treatments were able to significantly counteract the resumption of ovulation and of vaginal cyclicity that followed the replacement of LL-CEA rats in a normal light-dark (LD) environment (Mess *et al.*, 1979).

Several data in the literature (Bliss *et al.*, 1972 ; Héry *et al.*, 1975, 1976 ; Kordon and Ramirez, 1975 ; Wilson *et al.*, 1974, 1977) have suggested the relevant role played by the serotonergic system in the regulation of ovulation and the possible relationship linking the pineal gland to the central serotonergic system (for survey see Mess *et al.*, 1978 ; Trentini *et al.*, 1979).

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The present study provides more direct experimental evidence in favour of the hypothesis that MEL influences the brain serotonergic system which at least partly mediates the influence of the pineal gland on reproductive function.

Materials and methods.

Adult virgin female albino rats of our inbred Wistar strain were exposed to LL of uniform intensity for a period of 3 months. Vaginal smears were checked daily at 09.00 h after this date. Only rats which showed persistent vaginal cornification through the next 4 weeks and polyfollicular ovaries at laparotomy were considered to be CEA animals and were used for further experiments ; those showing any corpora lutea were discarded.

The LL-CEA rats were then divided into 3 experimental groups :
Group 1, treated daily with 0.1 ml of 1 p. 100 alcoholic saline ;
Group 2, treated daily with 100 μg of MEL in 0.1 ml of 1 p. 100 alcoholic saline ;
Group 3, replaced in a light-dark environment (14:10 hrs light-dark ; lights on 06.00-20.00 h, LD) and treated daily with 0.1 ml of 1 p. 100 alcoholic saline.

All injections were done subcutaneously at about 20.00 h and continued for a period of time varying from 5 to 15 days. Another group of animals (group 4) of the same age, used as a control, was maintained during the time of the experiment under the same light-dark conditions and treated as previously reported with alcoholic saline. Only rats showing regular 4-day vaginal cycles were used as controls. The animals of groups 2 and 3 were killed by decapitation on the estrous day of the first estrous cycle following the diestrous phase induced either by MEL treatment or by replacement in LD conditions. Immediately after sacrifice the midbrain, medial basal hypothalamus and a piece of the cerebral cortex were rapidly dissected and deeply frozen. Ovulation was tested by searching for tubal ova. Only specimens of animals positive for tubal ova were retained for biochemical determinations. Rats of groups 1 and 4 were killed in parallel with animals of groups 2 and 3. All the animals were killed between 11.00 and 12.00 h on the day of estrus.

The levels of 5-HT and of 5-hydroxyindoleacetic acid (5-HIAA) in the different areas of the brain were assayed spectrofluorimetrically using a modification of the method of Curzon and Green (1970). Individual specimens were homogenized in 7 ml of acid butanol and centrifuged at 6 500 × g at 2 °C for 10 min. The supernatant was pipetted into a 25-ml glass stoppered tube and shaken mechanically for 5 min with 1 ml of L-cysteine (0.1 N HCl 0.1 p. 100 solut.) and 25 ml of n-heptane. Two phases, A and B, were separated by 1 000 × g centrifugation for 3 min. In order to assay 5-HIAA, 10 ml of organic phase A were collected in a glass tube with 1 ml of 0.5 M phosphate buffer, pH 7, shaken mechanically for 5 min, and centrifuged at 1 000 × g for 3 min. 0.5 ml of the phosphate aqueous phase were added to 1 ml of concentrated HCl, 0.1 ml N HCl containing 0.1 p. 100 L-cysteine, and 0.2 ml of a 0.1 p. 100 O-phthalaldehyde (OPT) methanol solution. In order to determine 5-HT, 0.5 ml of the aqueous phase B were pipetted into a test tube with 0.1 ml of a 0.1 p. 100 OPT methanol solution and 1 ml of concentrated HCl. After stirring and heating in boiling water for 10 min, the tubes were cooled, and 5-HT and 5-HIAA fluorescence

was measured in an Aminco Bowman spectrophotofluorometer at wavelengths of 360/480 nm.

5-HT and 5-HIAA standard stock solutions were prepared as 100 μg/ml in 0.1 N HCl ; 0.5 μg was added to homogenized tissue blank and reagent blank. The tissue blank standards were processed as the. samples, including separation between butanol and aqueous phases. Reagent blank standards were added immediately before L-cysteine and OPT. The recovery of 5-HT and 5-HIAA was found to be 90 and 75 p. 100 respectively.

The significance of the differences between groups was determined using one-way ANOVA and the F-test. A value for P of < 0.05 was regarded as significant.

Results.

The exposure to LL provoked the CEA syndrome with polyfollicular ovaries in 92 p. 100 of the rats (table 1). It also significantly decreased the 5-HT content of

TABLE 1

Occurrence of ovulation in continuous light-constant estrous anovulatory (LL-CEA) *rats after melatonin treatment* (MEL) *or replacement in normal light-dark* (LD) *cycles*

Treatment	Occurrence of ovulation		Statistical significance
	No. ovulating / No. treated	Percent	
1. LL	2/25	8	
2. LL-MEL	17/25	68	$P < 0{,}01$ *vs* 1
3. LL-LD	15/20	75	$P < 0{,}01$ *vs* 1

the midbrain ($P < 0.01$), as compared with the levels of the control rats exposed to normal LD cycles, and significantly reduced the mesencephalic content of 5-HIAA (table 2). However, the hypothalamic levels of neither compound presented significant variations. In experimental groups 2 and 3, either MEL treatment or replacement in an LD environment provoked the resumption of ovulation in a large majority of the LL-CEA rats (table 1). Both treatments also induced significant changes in the 5-HT and 5-HIAA values. In particular, MEL treatment overcame the CEA syndrome and induced a significant rise in the 5-HT content of the midbrain that reached values similar to those of the LD controls. The 5-HIAA levels also presented an apparent increase which, however, did not reach the limit of statistical significance. On the other hand, the increase in the midbrain content of 5-HIAA, highly significant in animals replaced in LD cycles, was accompanied by a parallel increase of the 5-HT level. No significant variations were detected in the basal hypothalamus after either treatment, with the exception of a decrease of 5-HT content in MEL-treated animals, which was only significant when compared with that of the LD controls. Finally, no appreciable changes were detected in the 5-HT or the 5-HIAA content of the cerebral cortex under the different experimental conditions.

TABLE 2

Changes in hypothalamic and mesencephalic levels of serotonin (5-HT) *and of 5-hydroxyindoleacetic acid* (5-HIAA) *in LL-CEA rats ovulating after chronic melatonin* (MEL) *treatment or replacement in normal light-dark* (LD) *cycles*

Treatment	No.	Basal hypothalamus		Mesencephalon	
		5-HT (μg/g) [1]	5-HIAA (μg/g)	5-HT (μg/g)	5-HIAA (μg/g)
1. LL	23	5.052 ± 0.901	1.456 ± 0.317	1.850 ± 0.144	0.969 ± 0.148
2. LL-MEL...	10	2.668 ± 0.451	1.243 ± 0.421	2 544 ± 0.202 [b]	1.276 ± 0.129
3. LL-LD ...	11	4.475 ± 0.947	2.292 ± 0.845	3.187 ± 0.691 [b]	1.902 ± 0.326 [c]
4. LD	10	5.509 ± 0.521 [a]	1.976 ± 0.461	2.877 ± 0.291 [c]	1.624 ± 0.175 [b]

[1] Mean ± SEM ; [a] $P < 0.01$ *vs* group 2 ; [b] $P < 0.05$ *vs* group 1 ; [c] $P < 0.01$ *vs* group 1.

Discussion.

The present study demonstrates that exposure to LL induces a marked decrease in the 5-HT content of the midbrain and blocks ovulation and vaginal cyclicity at 12.00 h under our experimental conditions. LL does not appear to significantly modify the 5-HT metabolism of the basal hypothalamus. The results show, moreover, that the re-establishment of ovulation, induced by daily MEL treatment in the late afternoon (onset of darkness for control rats), was accompanied by a significant increase in the midbrain content of 5-HT and a lesser increase in 5-HIAA, which nearly reached the values presented by the control rats exposed to normal LD cycles. Similar changes in the midbrain serotonergic system accompanied the resumption of ovulation in LL rats replaced in normal LD cycles. On the contrary, neither MEL treatment nor replacement in LD cycles appeared to modify the 5-HT content of the medial basal hypothalamus, as compared with that of LL animals. However, MEL resulted in a significant decrease in the hypothalamic content of 5-HT, as compared with that of LD rats, but without significantly changing the 5-HIAA level. Further experiments are in progress to verify the true value of these data.

These results, demonstrating that the process of overcoming the CEA syndrome keeps pace with the apparent normalization of the midbrain 5-HT content; lend support to the previous hypothesis (Trentini *et al.*, 1978 ; Mess *et al.*, 1979) that MEL acts via the central serotonergic system in eliciting ovulation. On the other hand, it seems unlikely that these two effects take place independently, because similar effects were also observed in LL rats replaced in normal light-dark cycles. Thus, MEL administration in the late afternoon appears to mimic the action of light extinction on the midbrain serotonergic system in LL-CEA rats. Accordingly, the physiological role of the pineal gland is that of a biological clock informing the organism of the nyctohemeral rhythm, mainly through the secretion of MEL stimulated by darkness (Wurtman and Axelrod, 1965).

The present results also support the assumption (Trentini *et al.*, 1974, 1978) that a possible critical balance between the brain-stem 5-HT levels and the LHRH mobilizing mechanisms might be required to elicit ovulation.

Recent data favour the idea that 5-HT does not exert a simple inhibitory role in cyclic LH release, and a separate positive action has been repeatedly reported (Héry *et al.*, 1975, 1976 ; Kordon and Ramirez, 1975 ; Marko and Flückiger, 1976 ; Wilson *et al.*, 1974, 1977). Using intracerebral injections of 5,7-dihydroxytryptamine into the dorsal or median raphe of the rat, Meyer (1978) demonstrated a marked inhibition of ovulation and a significant decrease in 5-HT uptake in the suprachiasmatic nuclear region, indicative of the destruction of serotonergic inputs to that region. He concluded that this input to the suprachiasmatic nuclear region from at least the dorsal, and possibly the median, raphe nuclei seems to play a facilitating role in the control of rat ovulatory mechanisms.

The changes in the midbrain 5-HT concentration, demonstrated in the present experiments following MEL treatment, or return to normal LD cycles are associated with the resumption of ovulation provoked by MEL. Therefore, the anti-antigonadotropic effect exerted by MEL in the CEA syndrome, induced by exposure to LL, could be exerted via the midbrain serotonergic system.

In the light of these results, it might be correct to infer that either the antigonadotropic or the anti-antigonadotropic activity (for survey see : Mess *et al.*, 1978) exhibited by MEL in the regulation of ovulation, could represent only indirect effects, related to the functional state of the brain-stem serotonergic system. Accordingly, it could be hypothesized that the role played by MEL in the regulation of ovulation would express the general action that MEL exerts on the central nervous system through variations in the brain 5-HT levels (Antón-Tay *et al.*, 1971 ; Koella, 1969 ; Krapp, 1977 ; Sjoerdsma *et al.*, 1970).

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Résumé. Chez des rats en œstrus anovulatoire permanent (OAP) induit par une exposition en lumière continue (LL), une diminution significative du taux de 5-hydroxytryptamine (5-HT) et d'acide-5-hydroxyindole acétique (5-HIAA) mésencéphalique apparaît. Cependant, aucune variation significative de ces indoles n'est décelable dans l'hypothalamus basal. Un traitement chronique par la mélatonine, injectée tout à fait en fin d'après-midi, induit la reprise de l'ovulation et accroît le taux de 5-HT mésencéphalique. Des résultats semblables sont obtenus en replaçant les animaux OAP-LL dans un environnement à cycle jour-nuit normal. Il apparaît que la mélatonine mime l'effet de la phase obscure journalière en induisant l'ovulation et que cet effet pourrait s'exercer par l'intermédiaire du système sérotonergique mésencéphalique.

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