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α-MSH and MIF-I Effects on Serotonin Levels and Accumulation in Various Rat Brain Areas[1]

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SPIRTES, M. A., R. M. KOSTRZEWA AND A. J. KASTIN. *α-MSH and MIF-I effects on serotonin levels and accumulation in various rat brain areas.* PHARMAC. BIOCHEM. BEHAV. 3(6) 1011–1015, 1975. – Levels as well as accumulation of serotonin (5–HT) were measured in various brain regions of the rat after administration of α-melanocyte-stimulating hormone (MSH) and Pro-Leu-Gly-NH_2 (MIF-I). The method used in determining the serotonin measured both 5-OH-tryptamine (5-HT) and 5-methoxytryptamine (5-MT). No statistically significant changes in levels or accumulation of serotonin after pargyline injection were found when unoperated control rats were treated with either MSH or MIF-I. Similar treatment of hypophysectomized rats indicated that both peptides significantly ($p<0.05$) lowered serotonin accumulation only in the area of the frontal cortex; a similar but smaller, not statistically significant, decrease was seen in the hypothalamus and hippocampus of the hypophysectomized rat. Since only hypophysectomized rats were affected, no correlation between the behavioral effects of these peptides (which has been found to occur in both unoperated and hypophysectomized rats) and the biochemical changes could be made.

Melanocyte-stimulating hormone Melanocyte-stimulating hormone release inhibiting factor Peptides Brain Serotonin

MELANOCYTE-stimulating hormone (MSH) and an MSH-release inhibiting factor (MIF-I, prolyl-leucyl-glycinamide) recently have been shown to exert effects on the behavior of both normal and hypophysectomized rats [5, 7, 8]. Little is known, however, about changes in levels or turnover rates of monoaminergic neurotransmitters which possibly could accompany the behavioral changes observed after administration of either of these peptides. The present experiments were performed to investigate the possible occurrence of such relationships between reported behavioral changes after treatment of intact and hypophysectomized rats with α-MSH or MIF-I and serotonin levels or accumulation after injection of the monoamine oxidase inhibitor (MAOI) pargyline. To establish a correlation between behavioral and biogenic amine alterations, neurotransmitter changes should have been found for both the unoperated and hypophysectomized animal groups since behavioral changes due to both the above mentioned peptides have been established for them [5, 7, 8]. Such is not the case since significant inhibition of serotonin accumulation was found only in the frontal cortex of hypophysectomized but not of unoperated control rats.

METHOD

Chemicals

Synthetic α-MSH was measured by bioassay [6] and found to have maximal activity ($1-2 \times 10^7$ U/mg). Synthetic MIF-I was obtained from N. Plotnikoff of Abbott Laboratories, North Chicago, Illinois and showed a single spot after electrophoresis. Solutions of the peptides were made in 0.9 percent NaCl – 0.1 percent ascorbic acid and were kept at 4°C throughout each day's experiment. Five-hydroxytryptamine-creatinine sulfate and pargyline HCl were purchased from Regis Chemical Co., Chicago, Illinois. Five-methoxytryptamine was purchased from Sigma Chemical Co., St. Louis, Missouri and used without further purification. The ortho-phthaldialdehyde was obtained from Calbiochem Co., San Diego, California. All other organic and inorganic chemicals were of A. C. S. grade.

Procedure

Effect of α-MSH or MIF-I on endogenous levels of serotonin in various regions of the rat brain. Male, albino

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rats from Simonsen Laboratories, Inc., Gilroy, California weighing 150–170 g received an injection of either synthetic α-MSH (500 μg/Kg, IP) or MIF-I (100 mg/Kg, IP) at 24 hr intervals for 3 days and were sacrificed 24 hr after the final injection. The brains were rapidly removed and dissected into various regions according to the method of Glowinski and Iversen [3]. Tissues from each animal were frozen on dry ice and stored at −50°C until assayed. Regions of the frontal cortex, hypothalamus and pons-medulla were investigated in these assays.

Effect of α-MSH and MIF-I on serotonin accumulation in the regions of frontal cortex, hypothalamus, hippocampus and pons-medulla of the rat brain. In this study, groups of rats received either α-MSH (100 μg/Kg, IP) or MIF-I (20 mg/Kg, IP) at 24 hr intervals for a total of 3 treatments. Controls received the 0.9 percent NaCl – 0.1 percent ascorbic acid diluent. Levels of serotonin in the group receiving MIF-I were the same whether taken at 15 min or 60 min after the last injection. The MAOI pargyline HCl (75 mg/Kg, IP, free base) was injected 1 hr after the last injection of MIF-I or saline and 15 min after the last injection of α-MSH and the rats were sacrificed 5 or 20 min later. The appropriate zero-time group was sacrificed at the time when pargyline otherwise would have been given. Since brain dissection according to Glowinski and Iversen [3] can be accomplished with some practice in less than 10 min, the individual rats in each group were sacrificed at 10 min intervals. The same individual brain areas used for measurement of serotonin levels as well as the hippocampal area were frozen separately on dry ice immediately after being removed and then weighed. Serotonin assays were performed on each area from each rat after homogenization in acidified butanol by the method of Maickel as modified by Thompson *et al.* [15]. The same experiment as described above was repeated using rats hypophysectomized transaurally by Charles River Laboratories, Inc. 3 weeks before their sacrifice. Such animals were not included in our dissections if they gained substantial weight during the 3 week period in our laboratories or if the sella turcica was found to contain pituitary remnants.

All animals were kept on a 12 hr light and 12 hr darkness cycle and randomly sacrificed in the late morning and early afternoon hours (10 a.m. – 3 p.m.). Five or 6 animals were prepared for each point on the graphs.

Statistical analyses. Analyses for significance of differences in serotonin levels shown in Tables 1 and 2 between control and hormonally-treated groups of rats were performed using Dunnett's *t* test. The unoperated and hypophysectomized animals were treated separately even though they were both Sprague-Dawley descendant animals, since they differed not only in the presence of the pituitary but also in the dealers. For rates of serotonin accumulation, these two groups of animals were likewise treated separately; analyses of variance were performed and probabilities (p) calculated based on the F and df values. In the cases where the F values for controls and hormonally treated animals were significantly different, linear regression analyses were performed to obtain rate constants ± error term. Because, by the least squares regression method utilized, a single straight line for the 5 and 20 min points did not always give the smallest error terms, it was often necessary to draw one line from 0 to 5 min and another from 5 to 20 min. These lines of best fit then kept the error terms at a minimum. Percent difference between the control and peptide-treated animals was calculated from the rate constants obtained.

RESULTS

The values for endogenous serotonin levels found in intact rats 24 hr after the last hormonal or diluent injection are shown in Table 1. No statistically significant changes in serotonin levels were seen when these unoperated control and hormonally treated animals were compared.

Table 2 lists the levels of serotonin found in the hypothalamus, frontal cortex and hippocampus in hypophysectomized as well as unoperated rats sacrificed within an hour after receiving MSH or MIF-I. For the pons-medulla, only unoperated animals have been investigated. Serotonin levels remained the same whether the brains were removed at 15 min or 1 hr after the last peptide injection. Hypophysectomy did not cause a statistically significant change in serotonin levels in any part of the brain thus far examined with one exception: the level in the hippocampal area of hypophysectomized rats treated with MIF-I was statistically higher (15 percent, $p<0.05$) than that found in unoperated animals. It was also noted that there was a tendency in the hypothalamic area for the serotonin level to be higher for animals treated with MIF-I and lower for rats treated with α-MSH when compared with control rats treated with saline. These changes were not statistically significant. Endogenous serotonin levels at 0 min were not

TABLE 1

SEROTONIN CONTENT OF VARIOUS REGIONS OF INTACT RAT BRAIN 24 HR AFTER MIF-I AND α-MSH

Treatment	Frontal Cortex	Hypothalamus	Pons-Medulla
Saline	0.60 ± 0.02 (6)*	1.40 ± 0.04 (6)	0.79 ± 0.04 (5)
MIF-I	0.58 ± 0.02 (6)	1.35 ± 0.05 (7)	0.80 ± 0.02 (7)
α-MSH	0.59 ± 0.03 (6)	1.37 ± 0.05 (5)	0.79 ± 0.03 (6)

*Values expressed as mean μg/g wet weight ± SEM. Number of animals at each point in parentheses.

TABLE 2

SEROTONIN CONTENT OF VARIOUS REGIONS OF INTACT AND HYPOPHYSECTOMIZED RAT BRAIN WITHIN 1 HR AFTER MIF-I OR α-MSH

Type of Animal	Brain Area	Saline	MIF-I	α-MSH
Normal	Hypothalamus	1.36 ± 0.06 (6)*	1.47 ± 0.04 (6)	1.28 ± 0.03 (6)
Hypophysectomized		1.35 ± 0.04 (6)	1.45 ± 0.04 (6)	1.29 ± 0.04 (6)
Normal	Frontal Cortex	0.55 ± 0.01 (6)	0.58 ± 0.01 (6)	0.55 ± 0.01 (6)
Hypophysectomized		0.56 ± 0.02 (6)	0.57 ± 0.01 (6)	0.56 ± 0.01 (4)
Normal	Hippocampus	0.73 ± 0.06 (5)	0.68 ± 0.01 (6)	0.66 ± 0.01 (6)
Hypophysectomized		0.69 ± 0.05 (6)	0.78 ± 0.03 (5)	0.62 ± 0.02 (6)
Normal	Pons-Medulla	0.86 ± 0.01 (6)	0.87 ± 0.02 (6)	0.86 ± 0.02 (6)

*Values expressed as mean μg/g wet weight ± SEM. Number of animals at each point in parentheses.

altered in the other brain areas tested after either hypophysectomy or peptide treatment. Serotonin content decreased in the order: hypothalamus>pons-medulla>hippocampus>frontal cortex.

Figures 1 and 2 depicit the endogenous levels of serotonin at 0 min as well as the rates of accumulation of serotonin in the area of the frontal cortex from unoperated and hypophysectomized rats at 5 and 20 min after pargyline injection. Aside from the frontal cortex, the hypothalamus and hippocampus were investigated in a similar manner in both unoperated and hypophysectomized rats, while for the pons-medulla only unoperated rats were used. Analysis of variance for all values (5 and 20 min) of serotonin from the frontal cortex gave an $F(4,42) = 0.98$ and a $p>0.4$ for the unoperated animals; for the hypophysectomized animals a significant F value of 3.08 for the interaction of the saline-treated, MIF-I treated and α-MSH treated groups of rats was found with $df = 4,43$ and $p<0.05$.

A further analysis of the serotonin accumulation curve in the frontal cortex of hypophysectomized rats by calculation of linear regressions indicated that for the first five minutes there were no significant alterations in serotonin accumulation between the untreated controls and the rats injected with α-MSH and MIF-I. During the last 15 min, however, there was a decrease of 95 percent or more in serotonin accumulation rate for both α-MSH and MIF-I. As expected, similar calculations for the corresponding frontal areas of the brain from unoperated animals showed no significant differences in serotonin accumulation between the non-hormonally and hormonally treated animals.

Among the other brain areas of hypophysectomized rats investigated for serotonin accumulations, analysis of variance of the hypothalamus showed an F of 2.16, $p = 0.089$, slightly greater than the 0.05 level of significance.

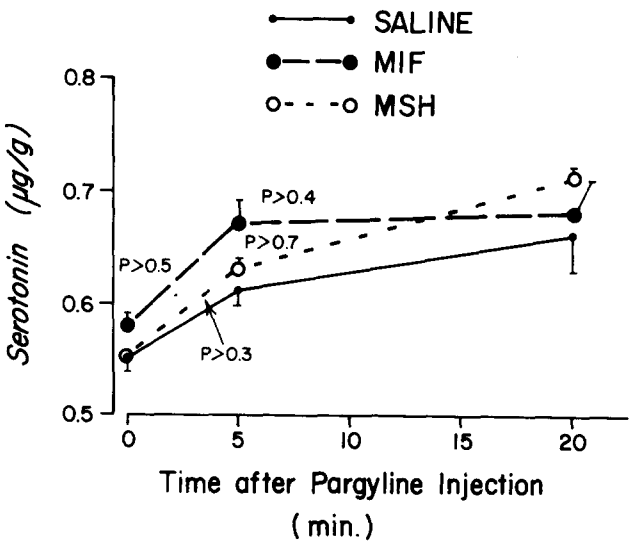

FIG. 1. Effects of pargyline (75 mg/Kg, IP) on endogenous accumulation of brain serotonin in the frontal cortex of control, MIF-I and MSH-treated intact rats. Each point represents the mean serotonin value from 5–6 animals ± the standard error of the mean. For statistical treatment, analysis of variance followed by linear regression analysis of the slopes of the 0–5 and 5–20 min periods was performed in order to compare each hormonal group with the control.

Both MIF-I and MSH caused some inhibition of serotonin accumulation in the hypothalamus. For the hippocampus, analysis of variance showed an $F = 1.86$, $p = 0.14$. In this case, only MSH caused some inhibition of serotonin accumulation.

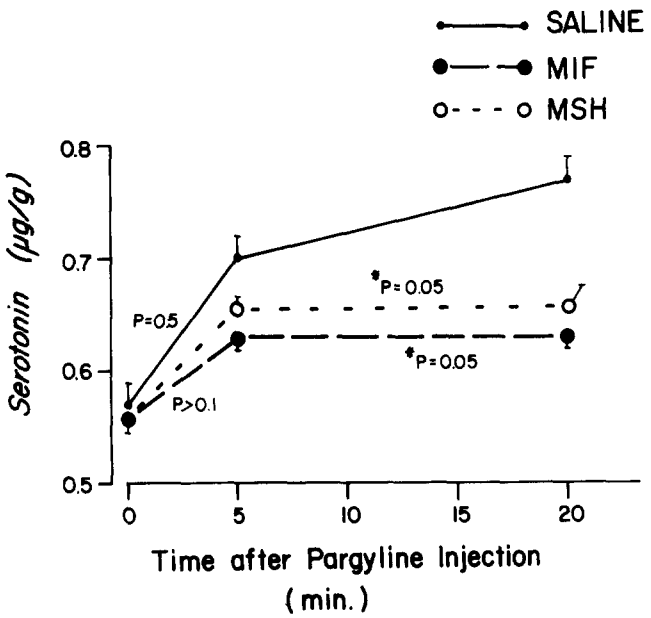

FIG. 2. Effects of pargyline (75 mg/Kg, IP) on endogenous accumulation of brain serotonin in the frontal cortex of control, MIF-I and MSH-treated hypophysectomized rats. Each point represents the mean serotonin value from 5–6 animals ± the standard error of the mean. For statistical treatment, analysis of variance followed by linear regression analysis of the slopes of the 0–5 and 5–20 min periods was performed in order to compare each hormonal group with the control.

DISCUSSION

The method used for the determination of serotonin in our experiments is relatively nonspecific in that the fluorescence developed has the same characteristics for 5-OH-tryptophan, 5-HIAA, 5-methoxytryptamine (5-MT), melatonin, N-diacetylserotonin and 5-HT [11,12]. However, in the presence of a sufficient concentration of pargyline (such as that used here), 5-HIAA should not be formed in significant amounts. In addition, 5-OH-tryptophan [2] and N-diacetylserotonin [9] are not known to occur in brain tissue in amounts likely to interfere with the fluorescence developed. Melatonin is not extracted from brain by the method used by us (acidified butanol) [11,12]. This leaves only 5-HT and 5-MT to contribute significantly to fluorescence in our extracts. 5-MT is thought by Bradley to act as a neurotransmitter much like serotonin [1]. By the methods of gas chromatography-mass spectroscopy, 5-MT has been shown to be present in the hypothalamus and other brain areas [9]. Essentially, therefore, what is called serotonin in our experiments is actually a mixture of 5-HT and 5-MT. A decrease in fluorescence has been interpreted by us as a decrease in 5-HT or 5-MT or both. An increase in fluorescence levels would then have to be interpreted as an increase in 5-MT or 5-HT or both.

In the past, the evidence indicating that α-MSH and MIF-I exerted direct effects on the brain was based mainly upon indirect animal behavioral and EEG studies as well as the use of analogues of ACTH which were known not to influence the secretion and release of peripheral endocrine hormones [5, 7, 8]. These compounds include peptides ($ACTH_{1-10}$, $ACTH_{4-10}$ and $ACTH_{4-10}$-D-phenylalanine) structurally related to α-MSH. They were found to influence rat behavior in both unoperated controls and hypophysectomized animals.

Leonard [10], for instance, studied $ACTH_{4-10}$ and $ACTH_{4-10}$(7-D-phenylalanine) for their behavioral and biochemical effects. The former was found to delay the extinction of a conditioned avoidance response and the latter to facilitate it. Their biochemical effects however were similar. Both peptides also produced a lowering of gamma-aminobutyric acid (GABA) and serotonin in the mid-brain [10]. It was also claimed that the same peptides increased the turnover of norepinephrine (NE) and reduced that of serotonin. Unfortunately no indication of the variability of the control, zero-minute levels of these transmitters was given so that it is difficult to interpret these statements concerning turnover rates. We also cannot explain why Leonard found a decrease in overall brain serotonin levels after treatment of rats with his two MSH-like peptides whereas we found that α-MSH treatment did not alter serotonin levels in a number of brain areas.

Versteeg *et al.* [16] stated that hypophysectomy itself, without any treatment with ACTH-analogues, caused a decrease in turnover rates of 5-HT, NE and dopamine (DA) in whole brain. However, a close perusal of these data raised the question as to whether the rate of disappearance of 5-hydroxy-indoleacetic acid (5-HIAA), a deaminated metabolite of 5-HT, in the presence of an MAOI like pargyline was directly related to the 5-HT formed in this type of experiment. No data on the direct determination of 5-HT were provided. The study of Neff and Tozer [13], upon which the technique used by Versteeg *et al.* is based, assumes that 5-HIAA is the major metabolite of 5-HT. The possibility must be considered however, that when an MAOI is used and the production of 5-HIAA is blocked, other pathways of 5-HT metabolism such as 0-methylation or N-acylation may substitute. It has already been mentioned that the presence of 5-MT in the hypothalamus has been established. Were the metabolic pathway to change, Neff and Tozer state that their model would have to be altered and 5-HIAA disappearance would not necessarily be equal to 5-HT synthesis rates. Versteeg *et al.* also employed tranylcypromine as the MAOI to inhibit serotonin breakdown. Tranylcypromine is known not only to block MAO but also to compete with serotonin, probably at its reuptake sites [4]. This effect could increase extracellular neurotransmitter levels and thus increase serotonin-0-methylation. Such a change in metabolism could also bring about a lower 5-HT level without affecting 5-HIAA disappearance. In contrast to the decreased turnover rates seen after hypophysectomy for 5-HT, NE and DA, Versteeg [16] found no changes in steady state levels or in "turnover rates" of these neurotransmitters after treatment of the operated animals with $ACTH_{4-10}$.

Plotnikoff *et al.* [14] injected normal mice daily for 5 days with as much as 20 mg/Kg of MIF-I. The animals were then sacrificed one hour after the last injection. No changes in the steady state levels of 5-HT, NE or DA in the whole brain of these mice were noted. Using normal unoperated rats, we were able to confirm Plotnikoff's results for serotonin, after a dose of 20 mg/Kg of MIF-I daily for 3 days. Likewise, our experiments with α-MSH (100 μg/Kg, daily for 3 days) injected into hypophysectomized rats tend to confirm the results of Versteeg *et al.* [16] that no changes in steady state levels of 5-HT and 5-MT occurred

under these conditions. What could not be supported were the experiments of Versteeg and colleagues indicating that serotonin synthesis was not affected after treatment of hypophysectomized rats with the MSH-like peptides, $ACTH_{4-10}$ and $ACTH_{1-10}$. Using α-MSH itself, we found it did cause a decrease in serotonin accumulation after pargyline injection in the area of the frontal cortex of the brain of hypophysectomized rats. MIF-I was found to accomplish the same change.

It is concluded from our results and those of others that no correlation can be made as yet between the behavioral changes appearing after α-MSH or MIF-I treatment of rats and changes in levels or accumulation of serotonin after pargyline injection. Preliminary results from experiments measuring norepinephrine breakdown after α-methyl DOPA injection do indicate a correlation in the midbrain area between behavioral changes and neurotransmitter alterations.

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