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MORPHINE-INDUCED CHANGES IN THE ACTIVITY OF ENZYMES INVOLVED IN NEUROTRANSMITTER METABOLISM IN SPECIFIC BRAIN REGIONS OF THE TOLERANT RAT

G. N. Catravas
C. G. McHale
S. L. Cohan

ARMED FORCES RADIOBIOLOGY RESEARCH INSTITUTE
Defense Nuclear Agency
Bethesda, Maryland

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G. N. CATRAVAS
C. G. McHALE
S. L. COHAN

D. O. Carpenter

D. O. CARPENTER
Chairman
Neurobiology Department

Myron I. Varon

MYRON I. VARON
Captain MC USN
Director

ARMED FORCES RADIOBIOLOGY RESEARCH INSTITUTE
Defense Nuclear Agency
Bethesda, Maryland

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FOREWORD
(Nontechnical summary)

The brain is composed of special cells known as neurons. Brain activity depends on and results from the interaction of neurons with each other. Nerve cells communicate with each other by releasing special chemical substances known as neurotransmitters into the synaptic cleft, i.e., the space between the axon terminal of one neuron and the cell body of its adjacent neuron. Neurotransmitter biosynthesis takes place inside the neurons, and a multitude of enzymes are involved in their production and also degradation.

Drugs, such as narcotics, act on the central nervous system by mechanisms not yet understood. They may affect the CNS by influencing biochemical mechanisms responsible for the normal function of neurons. It is therefore possible that they produce their effects by altering the levels, storage, uptake and release of neurotransmitters and by inducing changes in the activities of enzymes responsible for the production and breakdown of the various neurotransmitters.

The objective of this study was to determine the changes which are produced in the activities of brain enzymes involved in neurotransmitter metabolism by chronic administration of morphine. Adult male rats were made tolerant to morphine and sacrificed at predetermined times between 15 minutes and 24 hours after the last morphine injection. The morphine-induced changes in the activities of choline acetyl transferase and acetylcholinesterase, enzymes responsible for the synthesis and breakdown of acetylcholine, respectively, and of monoamine oxidase, the enzyme responsible for the breakdown of catecholamines and indoleamines, were investigated in the

hypothalamus, thalamus, hippocampus, cerebral cortex and basal ganglia. It was found that the activities of these enzymes are affected in animals made tolerant to morphine and that the direction and magnitude of these changes depend on the time of sacrifice of the animals after the last morphine injection.

ABSTRACT

The morphine-induced changes in the activity of enzymes involved in the metabolism of neurotransmitters in specific areas of the brain of rats made tolerant to morphine have been investigated. The activities of choline acetyl transferase (E.C.2.3.1.6.), acetylcholinesterase (E.C.3.1.1.7.) and monoamine oxidase (E.C.1.4.3.4.) were determined in the hippocampus, thalamus, hypothalamus, cerebral cortex and basal ganglia of groups of rats sacrificed at predetermined times following the last scheduled morphine injection. It was found that, in animals that showed a paradoxical activation reaction shortly after the last morphine injection ("jumping" rats), monoamine oxidase activity decreased in all brain areas studied with lowest values between approximately 30 and 60 minutes, and returned to nearly normal values by 6 hours postinjection. Acetylcholinesterase activity showed oscillations above control values. Oscillating changes were also observed in the activity of choline acetyl transferase depending on the time of sacrifice of the animal. No appreciable changes in monoamine oxidase activity were observed in "nonjumping" tolerant rats.

I. INTRODUCTION

Current literature on the effects of opiates on the metabolism of neurotransmitters is often contradictory and to a large extent a reflection of species differences, dosage and type of the opiate, and time of sacrifice of the animal following administration of the drug. Furthermore many of these studies have used whole brains of experimental animals and did not take into consideration the regional distribution of certain neurotransmitters and the role which the various areas of the brain may play in the manifestation of opiate tolerance and dependence.

Conflicting results have been reported on the effects of chronic administration of morphine on brain acetylcholine levels.[9,15] On the other hand administration of single doses of morphine was found to increase the acetylcholine levels in the brain of rats.[8,11] No significant changes in the levels of endogenous brain norepinephrine were observed by some investigators in morphine dependent rats.[17] In contrast, others reported that in rats which received repeated doses of morphine the levels of brain norepinephrine and also its biosynthesis increased.[1,3] Although no changes in the levels and synthesis of serotonin were observed in the brain of mice made tolerant to morphine,[2,14] Way et al.[18] indicate that the levels and turnover rate of this neurotransmitter increased in the brain of such animals. Furthermore it was found that inhibition of serotonin synthesis with p-chlorophenylalanine inhibited the development of tolerance.

The objective of the present study was to determine the changes which occur in the activity of enzymes involved in the metabolism of neurotransmitters in specific areas of the brain of rats made tolerant to morphine.

II. MATERIALS AND METHODS

Animals. A total of 228 Sprague-Dawley male rats 9-10 weeks old, weighing 240 to 260 grams, were used in these series of experiments. The animals were kept in a temperature-controlled room at 22°C individually housed in cages and had free access to water and food (Purina chow pellets). They were equally divided into 38 groups of 6 rats each. Twenty-six of these groups were used for the morphine experiments and the remaining 12 served as controls.

Morphine injection. The experimental animals were made tolerant to morphine by injecting the drug, 30 mg/kg body weight of morphine sulfate (Lilly) per injection intraperitoneally, twice daily for 8 days. Control animals were injected with sterile physiological saline in volumes corresponding to those of the morphine solution. After the 5th or 6th day of morphine administration, for the first 10 to 20 minutes after each injection of the drug the rats showed a paradoxical hyperactivity reaction characterized by increased irritability, spasmodic jumping and gnawing ("jumping" rats). However, a number of rats of which 72 were selected at random did not exhibit this hyperactivity reaction ("nonjumping" rats). These animals were kept and assayed separately.

Preparation of enzyme systems. At the end of the 8th day, the experimental as well as control groups were sacrificed by decapitation at 15, 30, 60, or 90 minutes, 6 or 24 hours after the last injection. Their heads were instantly frozen in liquid nitrogen in a Dewar flask. They were later removed from the liquid nitrogen and stored at -90°C until time of assay. Storage at this temperature for as long as 5 days was found to cause no detectable loss in enzymic activity. Enzymic activity determinations were performed within 24 hours after sacrifice of the animals. Rapid freezing of the

rat heads in liquid nitrogen usually resulted in bilateral splitting of the skull and brain, facilitating removal of the brain areas under investigation. The frozen heads were partially thawed in a cold room kept at 2-3°C and the hippocampus, thalamus, hypothalamus, cerebral cortex and basal ganglia were dissected out and homogenized in the appropriate media using glass homogenizers of the Potter-Elvehjem type with Teflon pestle kept in crushed ice. For composition of homogenization media, conditions of homogenization and centrifugation see appropriate references.[7,13,16,19]

Choline acetyl transferase. Choline acetyl transferase activity was determined according to the method of McCaman and Hunt[16] with some modifications. The assay mixture contained 3.5 μmoles phosphate buffer pH 7.4; 0.25 μmole choline hydrochloride; 0.01 μmole eserine; 1.0 μmole $MgSO_4$; 2.5 μg bovine serum albumin; 0.5 μmole 1-^{14}C acetyl CoA (approximately 200,000 counts/min); 0.1 mmole NaCl and 0.2 ml enzyme preparation to a total volume of 0.6 ml. The mixture was incubated for 30 minutes at 37°C and treated as previously described.[16]

Acetylcholinesterase. The activity of acetylcholinesterase was determined colorimetrically according to the method of Ellman et al.[7] as modified by Maletta et al.[13]

Monoamine oxidase. Monoamine oxidase activity was assayed by a modification of the method of Weissbach et al.[19] The assay mixture contained 75 μmoles Tris HCl buffer pH 7.4; 0.45 μmole kynuramine-di-HBr and 0.3 ml enzyme preparation to a total volume of 1.9 ml. Following incubation for 90 minutes at 37°C the mixture was made up to 3 ml with water. After the addition of 0.2 ml 0.5 N NaOH and 0.4 ml 10 percent $ZnSO_4$ it was shaken and centrifuged at 10,000 x g. The concentration of the reaction product 4-hydroxyquinoline was determined in the supernatant

spectrophotometrically by measuring the absorbance (appearance of peak) at 330 nm. A blank cuvette was prepared by replacing kynuramine with water. As Figure 1 shows, the height of the peak at 330 nm is directly proportional to the amount of 4-hydroxyquinoline present in the solution. Enzymic activities were expressed per milligram of protein. Protein determinations were performed according to the method of Lowry et al.[12]

The data obtained from the control animals were normalized at 100 for comparison with the other values.

Chemicals utilized in this study were purchased from Sigma Chemical Company, St. Louis, Missouri. Radioactive compounds were purchased from New England Nuclear Corporation, Boston, Massachusetts.

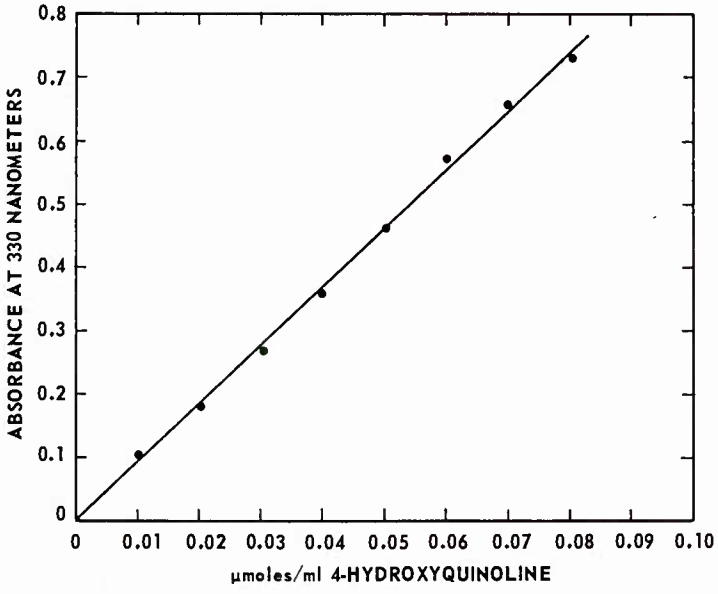

Figure 1. Absorbance at 330 nm (appearance of peak) of various concentrations of standard solutions of 4-hydroxyquinoline

Data presentation. Data are presented as the mean plus or minus standard error. Student's two-tail "t" test was used for statistical analysis.

III. RESULTS

The effects of morphine administration on the activity of the brain enzymes under investigation in the tolerant rats which showed the hyperactivity reaction (jumping rats) are shown in Figures 2-4. Figure 2 shows the morphine-induced changes in the activity of choline acetyl transferase. Depending on the time of sacrifice of the animal following the last injection of the drug, changes of an oscillating nature occurred in the activity of this enzyme either above or below control values.

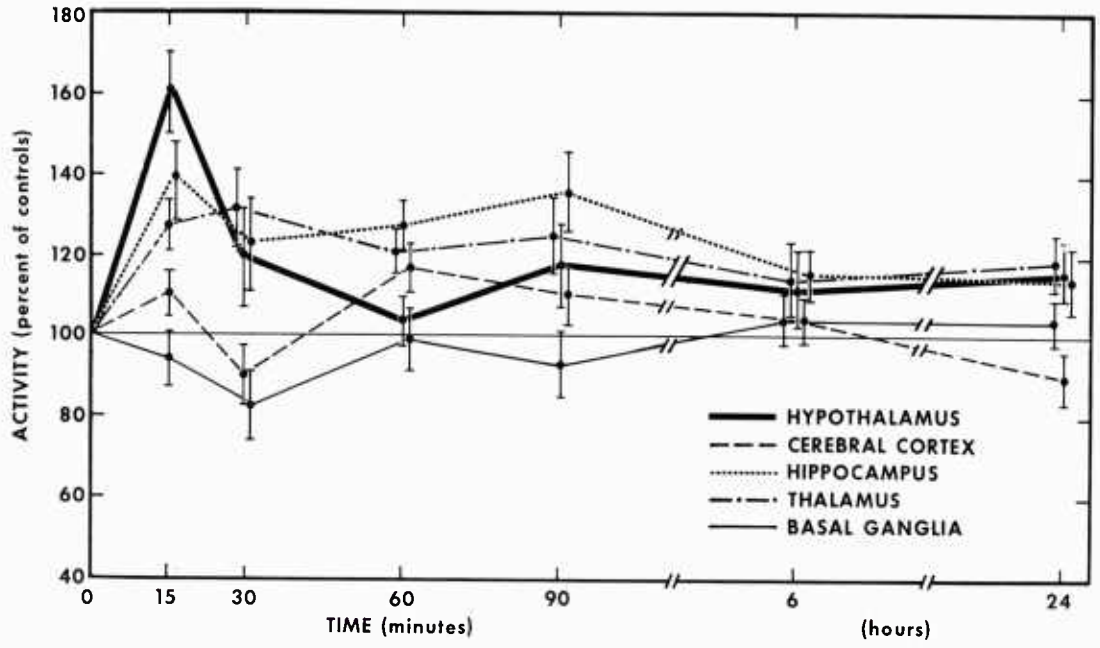

Figure 2. Effect of chronic administration of morphine on the activity of choline acetyl transferase in various brain areas of tolerant jumping rats. Each plotted point is the mean for 12 to 14 rats and the vertical bars denote ± S.E.M.

There is an activity peak (approximately 60 percent increase; $p < 0.025$) in the hypothalamus and possibly in the hippocampus at about 15 minutes after the last morphine injection.

The morphine-induced changes in the activity of acetylcholinesterase are shown in Figure 3. As in the case of choline acetyl transferase, it was found that, depending

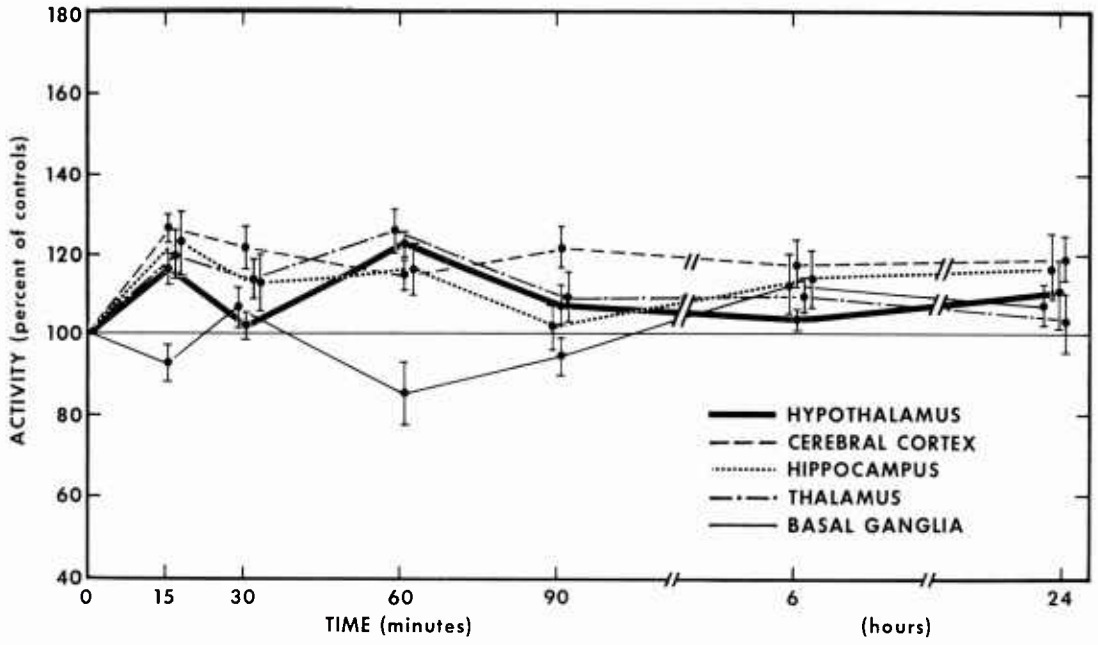

Figure 3. Effect of chronic administration of morphine on the activity of acetylcholinesterase in various brain areas of tolerant jumping rats. Each plotted point is the mean for 12 to 14 rats and the vertical bars denote ± S.E.M.

on the time of sacrifice of the animals following the last morphine injection, acetylcholinesterase activity oscillated also in all brain areas studied. However, the magnitude of oscillation was less pronounced than that of choline acetyl transferase. Furthermore, compared with choline acetyl transferase, no appreciable peak in the

activity of this enzyme was observed in the hypothalamus at 15 minutes after the last injection of the drug.

Figure 4 shows the morphine-induced changes in the activity of monoamine oxidase in animals which showed the hyperactivity reaction. Within minutes after the last injection of the drug, monoamine oxidase activity decreased in all brain areas

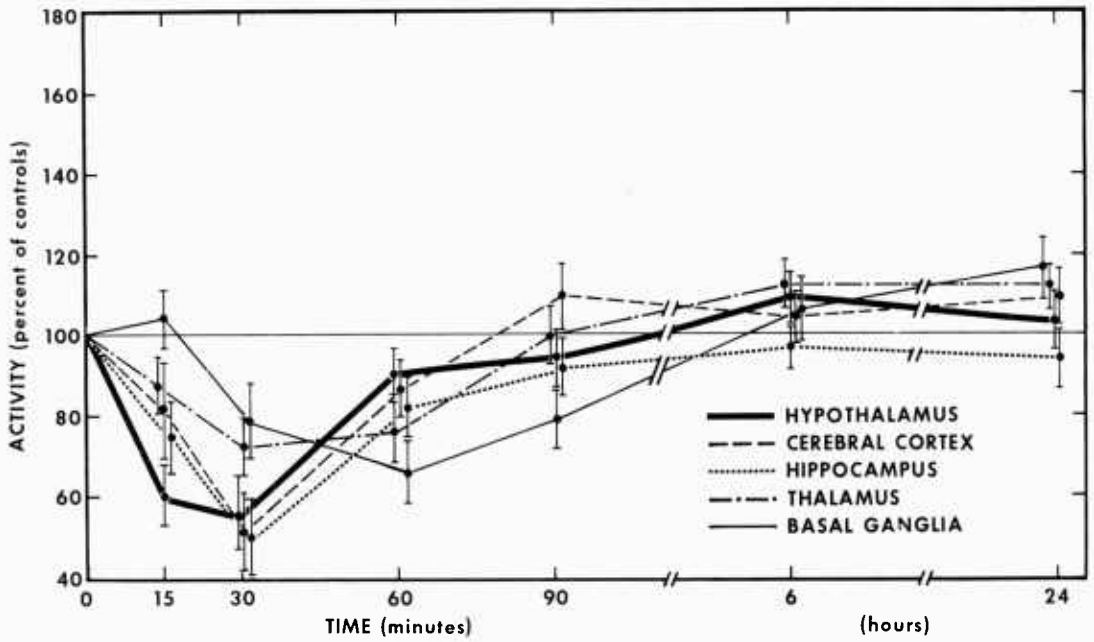

Figure 4. Effect of chronic administration of morphine on the activity of monoamine oxidase in various brain areas of tolerant jumping rats. Each plotted point is the mean for 12 to 14 rats and the vertical bars denote ± S.E.M.

investigated reaching lowest levels (up to 50 percent decrease in the hippocampus, hypothalamus and cerebral cortex) at approximately 30 minutes postinjection. In rats sacrificed at 90 minutes or longer the activity of this enzyme returned to nearly normal levels. No statistically significant differences in the activities of brain choline

acetyl transferase and acetylcholinesterase were observed between jumping and non-jumping tolerant rats. However, results with monoamine oxidase were different. In the jumping rats which were sacrificed during the first 60 minutes after the last morphine injection, monoamine oxidase activity decreased markedly in all brain areas investigated. In contrast, in the nonjumping animals sacrificed during the same post-injection time, no change or some increase in the activity of this enzyme was observed (Table I).

Table I. Changes of Monoamine Oxidase Activity in Morphine Tolerant Jumping and Nonjumping Rats

Time after last injection	Hypothalamus		Cerebral cortex		Hippocampus		Thalamus		Basal ganglia	
	Jumping	Non-Jumping	Jumping	Non-Jumping	Jumping	Non-Jumping	Jumping	Non-Jumping	Jumping	Non-Jumping
15 min	62 ± 8*	121 ± 11	80 ± 9	123 ± 8	75 ± 7	119 ± 12	88 ± 9	109 ± 8	105 ± 6	
30 min	57 ± 7	100 ± 9	49 ± 6	101 ± 12	53 ± 8	104 ± 7	74 ± 5	134 ± 10	79 ± 13	
60 min	91 ± 9	108 ± 6	85 ± 11	110 ± 9	80 ± 6	125 ± 9	77 ± 6	93 ± 7	69 ± 6	
90 min	94 ± 9	72 ± 7	108 ± 7	73 ± 5	89 ± 7	84 ± 7	101 ± 8	71 ± 6	81 ± 9	
6 h	108 ± 6	73 ± 9	103 ± 8	99 ± 6	96 ± 9	100 ± 9	111 ± 7	96 ± 8	105 ± 8	
24 h	103 ± 8		107 ± 7		97 ± 9		111 ± 6		118 ± 9	

Results are expressed as percent of controls
* Values are means ± S.E.

IV. DISCUSSION

Previous studies have shown that the levels of acetylcholine in the brain of rats increase after administration of morphine.[8,11] Similar changes have been also observed in the caudate nucleus of rats 1 hour after administration of single doses of morphine but not after repeated daily injections of the drug.[5] On the other hand, it

has been reported that no significant changes in the levels of acetylcholine occurred in the brain of rats which received relatively small doses of morphine and were sacrificed 1/2 hour later. However, remarkable acetylcholine depleting effects of the drug were observed.[6] Results on the effect of morphine on brain catecholamines and indoleamines have also been contradictory.[1-3, 14, 17, 18] The purpose of this research was to study the effects of chronic administration of morphine on the activities of enzymes involved in neurotransmitter metabolism in specific brain areas of the rat in terms of time after last injection of the drug. It is obvious from the results obtained that, under our experimental conditions, the activities of the enzymes studied are affected and that, depending on the time of sacrifice of the animals, oscillating changes in the activities of choline acetyl transferase and acetylcholinesterase occur during a period of several hours after the last morphine injection. It is of interest to note that oscillating changes in the activity of acetylcholinesterase have also been observed by Datta[4] after single doses of morphine, irrespective of dose. As mentioned earlier, in our experiments with tolerant rats acetylcholinesterase activity showed oscillating changes. However, except in basal ganglia, it remained above control values. There was relatively little change in the activity of this enzyme (approximately 18-20 percent increase) in the hypothalamus of rats sacrificed at 15 minutes after the last morphine injection. If one considers that at 15 minutes postinjection choline acetyl transferase activity in the hypothalamus increased by 60 percent, this would suggest that active acetylcholine synthesis took place during the time when the morphine tolerant animals show the paradoxical activation reaction. Furthermore, the oscillating nature of the activity changes of these two enzymes observed in this study could

probably explain the inconsistency of results obtained in other studies in which varying single times of sacrifice of the animals were chosen after administration of the drug.

Our results indicate that, in animals which showed the paradoxical activation reaction (jumping tolerant rats) shortly after the last morphine injection, monoamine oxidase activity decreased in all brain areas investigated reaching lowest values in animals sacrificed at 30 to 60 minutes after the last injection. It then returned to nearly normal levels. In contrast, in rats that did not show this hyperactivity and were sacrificed during the same period of time, monoamine oxidase activity remained around control values or increased slightly. The pronounced decrease in the activity of this enzyme observed in the jumping animals indicates that, during this time, a marked decrease in oxidative deamination of biogenic amines took place in the brain of these animals. It is of interest to note that two different populations of animals ("jumpers" and "nonjumpers") were also observed by other investigators[10] in experiments in which biogenic amine levels were determined after naloxone precipitated withdrawal. These authors reported that in mice and rats which jumped after administration of naloxone, brain dopamine levels increased above control values but did not increase in the nonjumping animals. The increase in the levels of this neurotransmitter observed by these investigators in naloxone treated jumping animals could be explained by an increase in dopamine synthesis and/or a decrease in its oxidative deamination, i.e., decreased monoamine oxidase activity. Similarly, a decrease in the activity of this enzyme was found to occur in our experiments with morphine tolerant jumping rats but not with nonjumping animals.

The morphine-induced changes in the activity of tyrosine hydroxylase and tryptophan hydroxylase, the rate-limiting enzymes in the synthesis of catecholamines and indoleamines, respectively, were not determined in this series of experiments. One cannot therefore draw conclusions as to what changes occurred in the rate of synthesis of catecholamines and indoleamines as a result of chronic administration of morphine.

Monoamine oxidase activity was assayed using kynuramine as substrate. During these determinations it was found that, if instead of measuring the decrease in absorbance at 360 nm,[19] one measures the increase in absorbance (appearance of peak) of the reaction product 4-hydroxyquinoline at 330 nm, at least a threefold to fourfold increase in the sensitivity of the reaction can be achieved. As shown in Figure 1 there is a linear relationship between the height of the peaks at 330 nm and the corresponding 4-hydroxyquinoline concentrations. It should be mentioned that, since it has been shown that monoamine oxidase in the brain of rats exists in multiple forms with different substrate specificities,[20] determination of its activity using kynuramine as substrate does not give a true picture of the morphine-induced changes in the activities of these forms. However, experiments are currently under way to separate the various monoamine oxidase forms and determine the morphine-induced changes in the activity of the individual isomers.

REFERENCES

1. Akera, T. and Brody, T. M. The addiction cycle to narcotics in the rat and its relation to catecholamines. Biochem. Pharmacol. 17:675-688, 1968.

2. Cheney, D. L. and Goldstein, A. Narcotic tolerance and dependence: lack of relationship with serotonin turnover in the brain. Science 171:1169-1170, 1971.

3. Clouet, D. H. and Ratner, M. Catecholamine biosynthesis in brains of rats treated with morphine. Science 168:854-855, 1970.

4. Datta, C. Influence of morphine on adenosine triphosphatase and acetylcholinesterase activity of mouse and rat cerebral tissues in vivo. Indian J. Exptl. Biol. 9:18-20, 1971.

5. Datta, K. and Wajda, I. J. Morphine-induced kinetic alterations of choline acetyltransferase of the rat caudate nucleus. Brit. J. Pharmacol. 44:732-741, 1972.

6. Domino, E. F. and Wilson, A. Effects of narcotic analgesic agonists and antagonists on rat brain acetylcholine. J. Pharmacol. Exptl. Ther. 184:18-32, 1973.

7. Ellman, G. L., Courtney, K. D., Andres, V., Jr. and Featherstone, R. M. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharmacol. 7:88-95, 1961.

8. Giarman, N. J. and Pepeu, G. Drug-induced changes in brain acetylcholine. Brit. J. Pharmacol. 19:226-234, 1962.

9. Hano, K., Kaneto, H., Kakunaga, T. and Moribayashi, N. Pharmacological studies of analgesics. VI. The administration of morphine and changes in acetylcholine metabolism in mouse brain. Biochem. Pharmacol. 10:441-447, 1964.

10. Iwamoto, E. T., Ho, I. K. and Way, E. L. Elevation of brain dopamine during naloxone precipitated withdrawal in morphine dependent mice and rats. Proc. West. Pharmacol. Soc. 16:14-18, 1973.

11. Large, W. A. and Milton, A. S. The effect of acute and chronic morphine administration on brain acetylcholine levels in the rat. Brit. J. Pharmacol. 38:451P-452P, 1970.

12. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265-275, 1951.

13. Maletta, G. J., Vernadakis, A. and Timiras, P. S. Acetylcholinesterase activity and protein content of brain and spinal cord in developing rats after prenatal X-irradiation. J. Neurochem. 14:647-652, 1967.

14. Marshall, I. and Grahame-Smith, D. G. Unchanged rate of brain serotonin synthesis during chronic morphine treatment and failure of parachlorophenylalanine to attenuate withdrawal syndrome in mice. Nature 228:1206-1208, 1970.

15. Maynert, E. W. Effects of morphine on acetylcholine and certain other neurotransmitters. Arch. Biol. Med. Exptl. 4:36-41, 1967.

16. McCaman, R. E. and Hunt, J. M. Microdetermination of choline acetylase in nervous tissue. J. Neurochem. 12:253-259, 1965.

17. Neal, M. J. Failure of morphine dependence in rats to influence brain noradrenaline turnover. J. Pharm. Pharmacol. 20:950-953, 1968.

18. Way, E. L., Loh, H. H. and Shen, F. Morphine tolerance, physical dependence and synthesis of brain 5-hydroxytryptamine. Science 162:1290-1292, 1968.

19. Weissbach, H., Smith, T. E., Daly, J. W., Witkop, B. and Udenfriend, S. A rapid spectrophotometric assay of monoamine oxidase based on the rate of disappearance of kynuramine. J. Biol. Chem. 235:1160-1163, 1960.

20. Youdim, M. B. H., Collins, G. G. S. and Sandler, M. Multiple forms of rat brain monoamine oxidase. Nature 223:626-628, 1969.

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13. ABSTRACT

The morphine-induced changes in the activity of enzymes involved in the metabolism of neurotransmitters in specific areas of the brain of rats made tolerant to morphine have been investigated. The activities of choline acetyl transferase (E.C.2.3.1.6.), acetylcholinesterase (E.C.3.1.1.7.) and monoamine oxidase (E.C.1.4.3.4.) were determined in the hippocampus, thalamus, hypothalamus, cerebral cortex and basal ganglia of groups of rats sacrificed at predetermined times following the last scheduled morphine injection. It was found that, in animals that showed a paradoxical activation reaction shortly after the last morphine injection ("jumping" rats), monoamine oxidase activity decreased in all brain areas studied with lowest values between approximately 30 and 60 minutes, and returned to nearly normal values by 6 hours postinjection. Acetylcholinesterase activity showed oscillations above control values. Oscillating changes were also observed in the activity of choline acetyl transferase depending on the time of sacrifice of the animal. No appreciable changes in monoamine oxidase activity were observed in "nonjumping" tolerant rats.

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