Brain Chromatin Activity of Morphine-Treated Rats[1] (36872)

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Recent reports suggest that morphine may alter intracellular gene regulation. First, multiple doses of morphine were reported to decrease rat-brain ribonuclease activity (1). Second, morphine appeared to cause a transient inhibition of brain RNA synthesis and a shift toward a more stable, heavier RNA (2, 3). Third, Friedler and Cochin (4) reported that morphine produces a residual effect in female rats which was passed on to her offspring in the form of a reduced growth rate. From these observations, it appears that morphine alters a genetic template necessary for regulating cell function. To investigate this possibility, we chose to study brain chromatin (*i.e.*, DNA-histone protein-nonhistone protein) which has been reported to preserve its *in vivo* template activity during isolation (5, 6) and to exhibit a high degree of tissue specificity within rodents (7).

Materials and Methods. Male rats (Charles River Strain) weighing 80–100 g were divided into two groups: (a) saline control, and (b) morphine-treated. Both groups were injected subcutaneously twice daily with either saline or morphine sulfate. The initial dose of morphine sulfate was 10 mg/kg/day. The dose was increased progressively to 100 mg/kg/day on the 13th day (3). All experiments were done on the morning of the 14th day, 12 hr after the last morphine dose.

Experimental animals were killed by decapitation and the brains rapidly homogenized in 12 volumes of cold 0.32 *M* sucrose. All operations were carried out at 4°. The homogenates of six brains were pooled, filtered through 10 layers of gauze, and the nuclei collected by centrifugation for 10 min at 750*g*. Nuclei were purified by resuspending the crude nuclear pellet in 0.1% Tween 80 and vigorously mixing on a Vortex mixer for 5 min (8). The nuclei were collected by centrifugation and washed three times in buffer A (0.01 *M* tris-HCl, pH 7.4, 0.001 *M* $MgCl_2$, 0.10 *M* NaCl).

DNA-dependent RNA polymerase was prepared from *E. coli* strain B (midlog phase, Miles Laboratories, Kankakee, Illinois) according to the methods of Chamberlin and Berg (9). The extraction was carried out to their Fraction 3 (F_3).

Chromatin was isolated from nuclei according to the methods of Paul and Gilmour (10) and assayed for its capacity to promote RNA synthesis according to the procedure of Chamberlin and Berg (9). Each 0.25 ml of reaction mixture contained 10 μmoles of tris-HCl (pH 7.9), 1.0 μmoles $MgCl_2$, 0.25 μmoles $MnCl_2$, 3.0 μmoles β-mercaptoethanol, RNA polymerase, chromatin or DNA, and 0.1 μmole of UTP, CTP, GTP and ATP (0.1 μc ^{14}C-8-ATP, New England Nuclear). Incubation was for 10 min at 37°. The reaction was stopped by the addition of an equal volume of cold 10% trichloroacetic acid (TCA). The precipitate was collected on Millipore filters (Millipore Filter Corporation, Bedford, Massachusetts), washed three times with 15 ml of cold 5% TCA, air dried, and counted in a toluene counting fluid containing 5.0 g of PPO and 0.3 g of POPOP per liter. Chromatin RNA synthesis is expressed as $\mu\mu$moles of ATP incorporated into the TCA insoluble precipitate.

DNA was determined by the diphenylamine method of Burton (11) with salmon sperm DNA (Calbiochem, Los Angeles, California) as a standard. RNA was determined by the orcinol reaction as described by Cerotti (12) using yeast RNA (Sigma Chemical Company, St. Louis, Missouri) as a standard. Histone and nonhistone proteins were extracted from chromatin according to the

[1] This work was supported by U.S. Public Health Service, NIMH Grant MH19625.

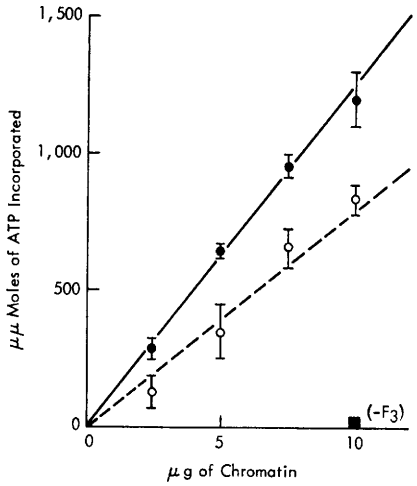

FIG. 1. Template activity of rat brain chromatin isolated from morphine treated O---O or control ●--● animals. The reaction mixture (0.5 ml) contained increasing amounts of chromatin and 134 μg of RNA polymerase. ATP incorporation by enzyme alone has been subtracted. Each point is an average ± standard deviation of three independent experiments. ■ samples incubated without added RNA polymerase.

procedure of Marushige and Bonner (13). Protein concentration was measured by the methods of Lowry *et al.* (14). Calf thymus histone (Calbiochem, Los Angeles, California) and bovine serum albumin (Sigma Chemical Company, St. Louis, Missouri) were used as standards for histone and nonhistone protein, respectively.

Results. The incorporation of C^{14}-ATP into RNA by chromatin from morphine-treated and control rats is shown in Fig. 1. Approximately 30% less C^{14}-ATP was incorporated into RNA by chromatin from morphine-treated rats. Similar decreases of 24.1%, 33.1% and 25.0% were seen when C^{14} UTP, C^{14}-CTP, and C^{14}-GTP were respectively substituted for C^{14}-ATP in the *in vitro* assay system. The omission of exogeneous RNA polymerase from the chromatin reaction mixture showed negligible incorporation ($-F_3$); thus the difference cannot be attributed to endogeneous RNA polymerase isolated with the chromatin. In addition, a comparison of the chemical composition of chromatin from both groups (Table I) showed no significant differences in DNA, RNA, histone protein, and nonhistone protein.

TABLE I. Chemical Composition of Rat-Brain Chromatin.[a]

Component	Mass ratios	
	Untreated chromatin	Morphine-treated chromatin
DNA	1.00	1.00
RNA	0.34	0.34
Histone protein	0.71	0.72
Nonhistone protein	1.10	1.08

[a] The values are the results of three independent preparations.

Three possible explanations for the morphine-induced decrease of chromatin RNA synthesis were explored: (1) increased ribonuclease activity; (2) RNA polymerase inhibition, and (3) a direct effect of morphine on the chromatin template. To check ribonuclease activity, chromatin from the brains of control and morphine-treated rats were incubated separately with C^{14}-ribosomal RNA (10564 dpm) for 10 min at 37°. The average amount of TCA-precipitated C^{14}-RNA was 10590 dpm and 10578 dpm for control ($n = 3$), and morphine treatment ($n = 3$), respectively. Thus, there appeared to be no ribonuclease extracted with the chromatin.

The possibility that an inhibitor of RNA polymerase was extracted with chromatin from morphine-treated animals was tested by incubating their chromatin with sperm DNA. If a substance (protein or morphine) extracted with chromatin, inhibited RNA polymerase, then sperm DNA activity should decrease also. The average sperm DNA activity alone ($n = 2$) and combined with chromatin ($n = 2$) was 4,399 μμmoles and 4,319 μμmoles, respectively. Thus, RNA polymerase was not significantly inhibited by a substance extracted with chromatin.

Evidence pointed toward a morphine-induced alteration in chromatin, which could result from an effect on DNA or on chromatin protein which is implicated in the regulation of the chromatin template.

A possible effect of morphine on DNA was tested by removing chromatin protein from chromatin isolated from both treatment

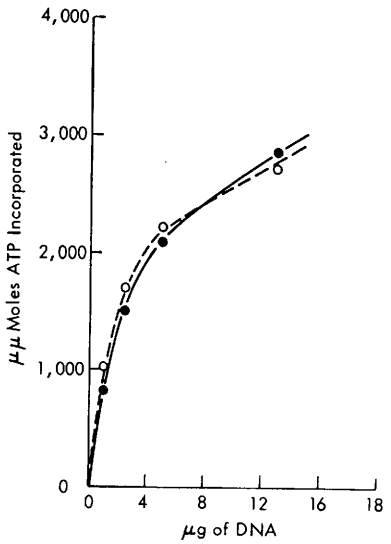

FIG. 2. Template activity of rat brain DNA extracted from morphine treated O---O or control ●—● animals. The reaction mixture (0.25 ml) contained increasing amounts of deproteinized DNA and 67 μg of RNA polymerase. ATP incorporation from enzyme alone has been subtracted. Each point is an average of three independent experiments.

groups so that only DNA remained. The DNA from each group was then tested for its capacity to synthesize RNA. Figure 2 shows that there are no apparent differences in the amount of C^{14}-ATP incorporated into RNA using equivalent amounts of control or morphine-treated deproteinized DNA. Thus, both DNA's show identical capacities for synthesizing RNA, indicating that the chromatin protein was necessary for the morphine effect.

TABLE II. Effect of Morphine on Template Activity.[a]

Morphine concentration	μμmoles of ATP incorporated
0	1031
5×10^{-12} *M*	1033
5×10^{-10} *M*	992
5×10^{-8} *M*	1075
5×10^{-7} *M*	1033
5×10^{-6} *M*	1044

[a] The incubation mixture (0.25 ml) contained 12.5 μg of DNA in the form of brain chromatin from control rats and 67 μg of RNA polymerase. Morphine sulfate was added in different concentrations and preincubated at 4° for 15 min before the addition of RNA polymerase. Incubation was for 10 min at 37°. ATP incorporation from enzyme alone has been subtracted. The results are the average values of two independent experiments.

In vitro experiments were performed to see if pharmacological concentrations of morphine sulfate would directly affect template activity of normal brain chromatin. The range of morphine sulfate concentrations was based on the report that 10 mg/kg of morphine sulfate given intraperitoneally to a tolerant rat produced a maximal brain concentration of 183 ng/g brain (*i.e.*, approximately 10^{-7} *M*) (15). Table II shows that *in vitro* morphine sulfate did not alter directly chromatin template activity, suggesting that the *in vivo* effect occurs at another step in the mechanism of gene regulation.

In light of the apparent indirect effect of morphine on chromatin, we considered two possible causes: fasting and sedation. In our experiments, chronically morphinized rats weighed approximately 30 g or 15% less than control rats at the end of the 13 day regimen. Because the dose was increased daily, a transient period of sedation (3–4 hr) occurred after each dose. To examine the role of these effects in the decrease of chromatin activity, we performed 2 experiments. In one experiment, we pair-fed the control rats to equalize their food intake to that of the morphine-treated rats. At the end of the 13 day treatment period, 10 μg of chromatin exhibited an activity of 1092 ± 26 (mean ± standard error, $n = 8$) and 924 ± 30 (mean ± standard error, $n = 8$), respectively, for the control and treated rats. Although this effect was not as great as observed in our initial experiment, it was significant at the $p < .001$ level. It indicates that fasting could be only partially responsible for the decreased chromatin template activity.

To test the role of sedation on the chromatin effect we utilized the rapid development of tolerance to sedation upon repeated injections of a constant dose of morphine sulfate (16). In these experiments, 10 mg/kg/day of morphine sulfate were injected subcutaneously for 13 days. By the end of 13 days we

observed no sedation after injection and thus assumed the rats to be tolerant to the sedative effects of morphine sulfate. Body weights of the treated and control groups were comparable. Chromatin activity in the control and treated groups was 1182 ± 15 ($n = 4$) and 1060 ± 36 ($n = 4$), respectively. This difference was significant at the $p < .025$ level, so it appears that sedation was not associated with the chromatin effect.

Discussion. These data show that morphine treatment will alter the template activity of brain chromatin. It appears that the effect is on the chromatin protein which is believed to perform an important role in gene regulation (*i.e.,* RNA and protein synthesis). There are several possible ways chromatin protein might be involved. One possibility is that brain chromatin from morphine-treated rats had more protein, thus fewer template sites. Perhaps the reason we found no quantitative differences in chromatin protein was a lack of sensitivity by our methods. Another possibility is that there was a rearrangement of chromatin protein to shift RNA synthesis toward a more slowly synthesized heavy RNA molecule or RNA molecules which contained less C^{14}-ATP. A third possibility is suggested by Misra *et al.* (17) who have reported a conjugate of morphine that remains in the brain for several days. It is possible such a conjugate might block template sites to reduce RNA synthesis.

The morphine regimen used in these experiments reduced food intake and caused sedation. When the food intake by the control and treated rats was equalized by pair-feeding, depressed chromatin template activity was still significant in the treated animals. However, the magnitude of the effect was less, which suggests that the reduced food intake may have contributed partially to the effect observed in our initial experiment.

Rats made tolerant to the sedative effects of morphine by injecting 10 mg/kg of morphine sulfate daily for 13 days, still exhibited a significant depression of brain chromatin template activity. Thus, sedation does not appear to be involved in the template effect.

Several pharmacological possibilities are suggested by morphine's action on brain chromatin. Since both analgesic tolerance and physical dependence to morphine have been related to nucleic acid and protein synthesis, our findings may reflect one or more of the mechanisms involved. If the observed effects are existent in other tissues, then these results provide a possible explanation for the growth retardation reported by Friedler and Cochin (4). Certainly, the relationship of the observed results and the pharmacology of morphine should be thoroughly investigated.

Summary. Brain chromatin isolated from morphine-treated rats exhibited a significantly lower capacity to promote RNA synthesis than chromatin from control rats. The effect was not caused by either ribonuclease or an RNA polymerase inhibitor extracted during the isolation of chromatin. Removal of protein from chromatin DNA abolished the effect. The *in vitro* addition of morphine to brain chromatin from untreated animals produced no effect.

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Received May 19, 1972. P.S.E.B.M., 1972, Vol. 141.