Biochemical Pharmacology, Vol. 24, pp. 1983–1987. Pergamon Press, 1975. Printed in Great Britain.

# EFFECT OF CHRONIC MORPHINE TREATMENT ON BRAIN CHROMATIN TEMPLATE ACTIVITIES IN MICE

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(*Received* 13 *September* 1974; *accepted* 14 *February* 1975)

**Abstract**—Chromatins have been isolated from both placebo- and chronic morphine-treated mice. The specific activity of chromatin-directed UTP incorporation was measured. Evidence is presented to show that the chromatin template activity isolated from tolerant animals is increased. The increase may be due to the alteration of non-histone protein in chromatin. This is a narcotic-specific phenomenon, since the morphine-induced increase can be blocked by naloxone.

The synthesis of RNA can be inhibited by levorphanol, a synthetic analogue of morphine [1–3]. The authors of these studies emphasized that the most intense and consistent inhibitory effect of levorphanol is upon ribosomal RNA synthesis, although non-ribosomal RNA synthesis is also reduced. Recent reports [4–6] have shown that the development of tolerance to the analgesic effect of morphine in rats or mice can be reduced or prevented by drugs which, in different ways, inhibit the synthesis of RNA and/or protein in the brain.

In studies of RNA metabolism in chronic morphine-treated rats, Datta and Antopol [7] reported that chronic administration of morphine produced dose-dependent decreases in uridine and thymidine incorporation in liver and brain homogenates. Clouet [8] reported the synthesis of a new RNA in the nuclei after the chronic administration of morphine. Castles *et al.* [9] further indicated in their study that newly formed RNA from brains of tolerant rats was not lost as rapidly as RNA from brains of non-tolerant rats.

In the present study, attempts have been made to determine if the changes in RNA synthesis during tolerance development are actually due to the alteration of chromatin template activity.

## METHODS

ICR male mice (20–25 g) from Simonsen Laboratories, Gilroy, Calif., were rendered tolerant to morphine by the implantation of a 75-mg morphine pellet for a period of 72 hr [10]. The control animals received placebo pellets. When naloxone pellets (10 mg) were used to antagonize morphine effects, four groups of mice were treated as follows. The mice were implanted with: (1) a placebo pellet which was then followed 2 hr later by a second placebo pellet; (2) a naloxone pellet followed 2 hr later by a placebo pellet; (3) a placebo pellet followed 2 hr later by a morphine pellet; and (4) a naloxone pellet followed 2 hr later by a morphine pellet. The animals were implanted with both pellets for a total of 3 days.

The degree of tolerance was measured by the decrease in "analgetic" response to morphine sulfate as estimated by the tail-flick method [10]. After the mean baseline response was established, the tail-flick reaction time was re-determined 30 min after s.c. morphine sulfate administration. A quantal response to at least three doses of morphine was used to determine the median analgetic dose ($AD_{50}$) and the 95 per cent confidence limits by the method of Litchfield and Wilcoxon [11]. The $AD_{50}$ was determined 6 hr after the removal of both pellets.

All animals were sacrificed by decapitation, brains were removed immediately and kept in cold TKM–0·25 M sucrose buffer (Tris-HCl, 50 mM, pH 7·5, 23°; $MgCl_2$, 10 mM; KCl, 25 mM). The brains were homogenized in 2 vol. TKM–0·25 M sucrose solution and mixed with equal volume of TKM–2·3 M sucrose. Nuclei were isolated according to the method of Blobel and van Potter [12] and chromatins were prepared according to the method of Spelsberg and Hnilica [13]. For each preparation of chromatin, brains from 50 mice were used. After centrifugation through TKM–2·3 M sucrose in a Beckman SW27 rotor at 27,000 rev/min for 90 min, the nuclei were washed three times with 3 ml of 0·08 M NaCl–0·02 EDTA (pH 6·0). The suspension was centrifuged at 5000 *g* for 10 min. The sediment which contained the chromatin was washed once as above with 1 ml of 0·15 M NaCl. The chromatin was then washed twice with 1 ml of 1·5 mM NaCl–0·15 mM sodium citrate (pH 7·0) and sedimented by centrifugation at 20,000 *g* for 10 min. For the excessively washed preparation of chromatin, 10 times the volumes given above were used in each one of the six washings. The chromatin was finally resuspended in 5 ml of 1·5 mM NaCl–0·15 mM sodium citrate (pH 7·0) and could be kept in −10° for at least 3 weeks without detectable loss of activity. Histone-free chromatin was prepared according to the method of Fambrough *et al.* [14]. Protein and DNA

* I. K. Ho is a recipient of a PMAF Faculty Development Award in Basic Pharmacology.

† H. H. Loh is a recipient of an NIMH Research Scientist Development Award, K2-DA-70554.

were determined according to the methods of Lowry *et al.* [15] and Ceriotti [16] respectively.

The chromatin-dependent UTP incorporation assay system is essentially the same as that reported by Spelsberg and Hnilica [13], except that $^3$H-UTP (50 Ci/m-mole, New England Nuclear) was used instead of $^{14}$C-UTP. Each 0·25-ml reaction mixture contained 20 μmoles Tris-HCl (pH 8·3, 23°); 25 μmoles NaCl; 0·1 μmole UTP (0·4 μCi $^3$H-UTP); 0·25 μmole each of GTP, ATP and CTP; 1·5 μmoles Dithiothreotol; 5 μg Bentonite; and *Escherichia coli* K-12 RNA polymerase (nucleoside triphosphate: RNA, nucleotidyltransferase; EC 2.7.7.6) with or without chromatin. The reaction mixture was incubated at 37° for 60 min or as otherwise indicated and the reaction was terminated by the addition of 1·5 ml of cold 5% trichloroacetic acid containing 1% Na pyrophosphate (TCA + PPi). The precipitate was collected on glass fiber filter (Whatman) and washed twice with 15 ml cold TCA–PPi and twice with 15 ml cold ethanol. The air-dried filter was then counted. Background incorporation without added chromatin was substracted from the total incorporation. Experiments were carried out in triplicate and the data presented are the mean of three assays. RNA synthesis is expressed as nmoles UTP incorporated into the TCA insoluble fractions.

## RESULTS

Results indicated that the rates of DNA-dependent RNA formation were linear up to 2 hr, when mouse brain chromatin was used as DNA template. Figure 1 indicates that the $^3$H-UTP incorporation into RNA was proportional to the amount of DNA added. Chromatin isolated from tolerant mice showed higher template activity than that isolated from non-tolerant animals. The rate from the former was linear up to at least 5·0 μg DNA, whereas the latter began to level off above 3·0 μg DNA. In the routine assay, a limited amount of DNA was always used. Table 1 shows that

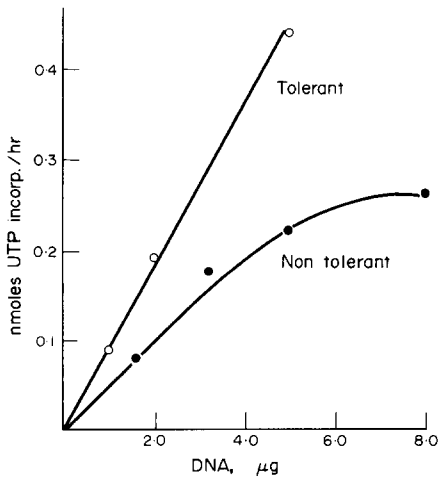


Fig. 1. Rate of UTP incorporation with various amounts of chromatin extracted from tolerant or non-tolerant mice. The reaction mixture is the same as described in the Methods. The rate of UTP incorporation is expressed as nmoles/hr.

Table 1. Effect of chronic morphine treatment on chromatin template activity isolated from mouse brain*

| Treatment | Specific activity ± S.E. $^3$H-UTP incorporation (nmoles/mg DNA) |
|---|---|
| Non-tolerant | 41·96 ± 3·38 (9)† |
| Tolerant | 65·55 ± 7·87 (9)† |

* Chromatins were prepared according to the regular washing procedure described in the Methods.
† Number of preparations performed. $P < 0·01$.

chronic morphine treatment resulted in increased specific activity of brain chromatin when it served as DNA template. When exogenous RNA polymerase was omitted from the reaction mixture, there was negligible incorporation; thus, the difference in chromatin activities cannot be accounted for by RNA polymerase contamination in isolated chromatin. Morphine, up to 0·1 mM added to the reaction medium produced no significant change in the rates of $^3$H-UTP incorporation.

When the chromatins were washed excessively, as described in Methods, chromatin template activity from non-tolerant animals increased nearly 2-fold, whereas the activity of chromatin isolated from tolerant animals remained essentially the same (Table 2). Therefore, the template activity of chromatin extracted from tolerant animals following excessive washing was approximately the same as or slightly lower than the activity of chromatin from control mice.

The differences in template activities of chromatin isolated either from control or tolerant mice cannot be due to ribonuclease (RNase) contamination. Table 3 shows that, when actinomycin D was added at the beginning of incubation, the incorporation of UTP was inhibited about 85 and 80 per cent for control and tolerant groups respectively. However, when actinomycin D was present after initiation of RNA synthesis, the incorporation rate was slowed down, but there was no indication of hydrolysis. Therefore, it is concluded that there is no RNase activity present in the chromatin preparations.

Table 4 shows the results when the histones were removed from chromatins with $H_2SO_4$ extraction. The histone-free chromatins exhibited approximately a 6- to 10-fold increase in template activity. However, excessively washed chromatin from placebo-treated animals still exhibited higher activity than the regu-

Table 2. Effect of excessive washing on chromatin activity in directing $^3$H-UTP incorporation into RNA

| Treatment | Specific activity ± S. E. $^3$H-UTP incorporation (nmoles/mg DNA) |
|---|---|
| Regular wash | |
| Non-tolerant | 47·75 ± 2·60(4)*,† |
| Tolerant | 80·75 ± 15·70(4)* |
| Excessive wash | |
| Non-tolerant | 84·50 ± 7·93(4)† |
| Tolerant | 72·12 ± 8·73(4) |

* $P < 0·05$. † $P < 0·01$.

Table 3. Effect of actinomycin D on chromatin template activity*

| Treatment | Specific activity $^3$H-UTP incorporation (nmoles/mg DNA) |
|---|---|
| Non-tolerant | |
| Total 60-min incubation time | |
| None | 69·1 |
| Actinomycin (25 μg/ml) | 10·3 |
| Total 75-min incubation time | |
| None | 81·6 |
| Actinomycin (25 μg/ml) added at 60-min incubation | 75·5 |
| Tolerant | |
| Total 60-min incubation time | |
| None | 84·1 |
| Actinomycin (25 μg/ml) | 17·5 |
| Total 75-min incubation time | |
| None | 100·8 |
| Actinomycin (25 μg/ml) added at 60-min incubation | 93·5 |

* Chromatins were prepared according to the regular washing procedure described in the Methods.

larly washed chromatin isolated from placebo controls. Apparently, the difference in their template activity cannot be due to the difference in the histones.

However, when the chromatin was extracted with 2 M NaCl in which 90 per cent of the protein was removed, the chromatin template activity was increased at least 30-fold. In this case, the activity of DNA from non-tolerant mice was the same as that of DNA from tolerant mice.

In order to ascertain whether the phenomenon was narcotic specific, a 10-mg pellet of naloxone base, a morphine antagonist, was implanted together with a morphine pellet. In mice receiving a morphine pellet implant for 72 hr, tolerance to morphine was highly developed. However, in mice receiving a naloxone pellet implantation 2 hr prior to a morphine pellet implantation, tolerance to morphine was prevented completely. As shown in Fig. 2, the $AD_{50}$ of morphine in non-tolerant mice receiving two placebo pellet implantations was 5·5 mg/kg. In mice receiving a placebo pellet implantation followed by a morphine pellet implant, the degree of tolerance to morphine was increased 8·7-fold, as evidenced by an increase in morphine $AD_{50}$ to 48 mg/kg. However, in mice implanted

Table 4. Comparison of chromatin template activities after the removal of histones

| Treatment | Specific activity $^3$H-UTP incorporation (nmoles/mg DNA) |
|---|---|
| Chromatin (histone-bound) | |
| Non-tolerant | |
| Regular wash | 47·6 |
| Excessive wash | 66·6 |
| Tolerant | |
| Regular wash | 73·3 |
| Excessive wash | 53·6 |
| Chromatin (histone-free) | |
| Non-tolerant | |
| Regular wash | 282 |
| Excessive wash | 458 |
| Tolerant | |
| Regular wash | 380 |
| Excessive wash | 360 |

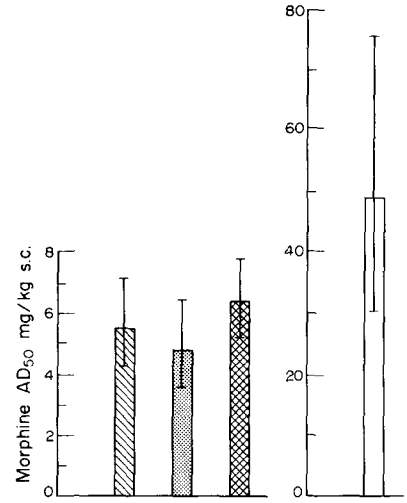


Fig. 2. Effect of naloxone on the development of morphine tolerance in the mouse. Mice were rendered tolerant by morphine pellet implantation. A naloxone or placebo pellet was implanted 2 hr before either placebo or morphine pellet. The hatched bar denotes the control group receiving the two placebo pellets. The dotted bar represents the group receiving a naloxone pellet 2 hr before morphine pellet implantation. The crossed bar represents the group receiving a naloxone pellet 2 hr before a placebo pellet. The white bar denotes the group receiving a placebo pellet, followed 2 hr later by a morphine pellet. The $AD_{50}$ of morphine and the 95 per cent confidence limits were determined 6 hr after the removal of both pellets.

with a naloxone pellet 2 hr prior to a morphine pellet implant, the morphine $AD_{50}$ was only 6·4 mg/kg, which was not significantly different from that of the non-tolerant animals. In animals receiving both a naloxone and a placebo pellet, the $AD_{50}$ of morphine was 4·8 mg/kg, which was also not significantly different from that of the control animals with two placebo pellets implanted. Thus, the simultaneous implantation of naloxone and morphine pellets blocked entirely the development of tolerance to morphine.

Chromatins were also prepared from the four groups of mice as described above. Chromatin template activities were measured. It can be seen from the data in Table 5 that the chromatin isolated from the mice treated with naloxone plus placebo showed activity in directing $^3$H-UTP incorporation similar to that of the placebo–placebo control; chromatin activity from mice given placebo plus morphine was 53 per cent higher than the chromatin activity isolated from placebo–placebo mice. However, when both

Table 5. Effect of naloxone on chronic morphine-induced chromatin activity*

| Treatment | Specific activity $^3$H-UTP incorporation (nmoles/mg DNA) |
|---|---|
| Placebo–placebo | 87·5 |
| Naloxone–placebo | 95 |
| Placebo–morphine | 135 |
| Naloxone–morphine | 95 |

* Chromatins were prepared according to the regular washing procedure described in the Methods.

naloxone and morphine are implanted in the mice, the increased chromatin activity induced by morphine is clearly abolished. We also observed that acute morphine administration (morphine sulfate, 40 mg/kg, s.c., 30 min) had no effect on chromatin template activity.

## DISCUSSION

The data presented here demonstrate that, when mice are rendered tolerant to morphine by pellet implantation, the rate of chromatin-dependent UTP incorporation is increased. The increase is not due to the presence of more DNA, since the specific activity is calculated per mg of DNA. Omission of chromatin or RNA polymerase resulted in negligible incorporation.

If the chromatin is prepared using the excessive wash procedure (i.e. 10 times the regular volume of solution used for each wash), there is a 2-fold increase in chromatin template activity from placebo-implanted mice. However, if the chromatin is isolated from morphine-tolerant mice, template activity is the same, whether the excessive or the regular wash preparation procedure is used. This suggests that some components which control the activity of DNA-dependent UTP incorporation have been removed or altered during the excessive wash. Chromatin activity from tolerant animals remains the same regardless of the washing procedure, indicating that the "regulator" is either absent or is very labile in tolerant animals.

Chromatin contains mainly histone and non-histone (acidic) proteins. The role of histone proteins has been under intensive investigation [17]. However, the non-histone proteins are much more complex and less understood. It should be mentioned that histones, in general, inhibit RNA synthesis *in vivo* [18,19]. Removal of the basic proteins is accompanied by dramatic increases in the RNA synthetic capacity of the nucleus. The non-histone proteins which may include a derepressor of a certain genome, a steroid-binding protein, or some enzymes involving gene expression, such as RNA polymerase, are believed to stimulate the rate of transcription from the appropriate DNA template *in vitro* [20, 21]. However, one should not over-simplify the system, since it is known that both histone and non-histone proteins are also subject to enzymatic modifications. Although the biological significance of the modification is not fully understood, it is generally believed that the phosphorylation of either histone or non-histone proteins would result in positive control of template activity [22–24]. Furthermore, nuclear acidic proteins may stimulate RNA synthesis in isolated chromatin fractions by augmenting the DNA–histone complexes [25].

Since some of the chromatin proteins may be loosely associated with the complex, it is possible that the complex may be mechanically damaged during the preparation which results in the removal of more protein from one group than from the other. However, it is not likely, because the data presented in Table 1 are the average values of nine preparations. The preliminary results obtained from acylamide gel electrophoretic pattern in our laboratory indicated that the general patterns of chromatins obtained from control animals or chronic morphine-treated animals are not different except for two regions. In those two regions, there are apparently more proteins in preparations from tolerant animals than in those from controls*. Purification and identification are being made for the chemical and enzymatic properties of these proteins.

Removal of histone from chromatin did not change the fact that the tolerant group still showed higher activity than the placebo group. However, "naked" DNAs (devoid of 90 per cent protein) from either placebo or tolerant animals have identical template activities. This indicates that the difference in template activity between placebo and tolerant animals is not due to the difference in DNA. Additions of actinomycin D (25 μg/ml), after the initiation of RNA synthesis, result in no hydrolysis of synthesized RNA, indicating that there is no RNase contamination in the chromatin preparation. However, other interference, such as the presence of RNA polymerase cofactor, protein kinase or phosphorylase, cannot be ignored. Further studies are necessary to identify the non-histone proteins and to study their role in the regulation of chromatin activity.

It should be noted here that the increase in template activity in chromatin was also observed in the rat. Our preliminary data indicate that chromatin activity isolated from morphine-tolerant rats was approximately 50 per cent higher than chromatin isolated from placebo controls. A recent article by Hodgson *et al.* [26] reported that brain chromatin isolated from morphine-tolerant rats exhibited a significantly lower capacity to promote RNA synthesis than did chromatin from control rats. However, the method used to isolate chromatin by these investigators was different from ours. Since it has been shown in our work that the washing procedure used to prepare chromatin is critical, the difference between these studies may be resolved.

The observations reported in this communication indicate that the chromatin activity has been altered due to chronic morphine treatment. The proteins of the chromatin may be responsible for the change. It is a narcotic-specific phenomenon, since the effect can be blocked by naloxone. Further studies are now in progress in this laboratory to characterize the chromatin protein and to study the consequences of the change in chromatin activity after chronic morphine treatment.

*Acknowledgements*—These studies were supported in part by NIMH grants DA-00563 and DA-00564 and U.S. Army Research Contract DADA-17-73-C-3006.

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