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Conditions of Transfer of Morphine Tolerance by Brain Extracts* (33539)

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In a previous publication (1) we described experiments in which tolerance to morphine could be induced in mice by treating them with extracts of brain taken from animals rendered tolerant to the drug by repeated exposure. The present paper reports further examples of this transfer of tolerance and discusses the probable causes of failure in attempts made to replicate the experiments (2, 3).

Materials and Methods. The brain extracts used were prepared by the procedures previously described (1). Sprague-Dawley rats were made tolerant to morphine by a series of three daily subcutaneous injections of morphine sulfate at doses progressively increased from 10 to 100 mg/kg during a period varying from 14 to 20 days. On the last day, 4–10 hr after the last injection, the rats were decapitated and their brains were immediately frozen on dry ice. Extracts were made by dialyzing a homogenate of brain, concentrating the dialyzate and partitioning it with phenol. The active material was precipitated out from the phenol phase with 20 vol of cold acetone. The precipitate, dissolved in distilled water at the concentration of the equivalent of 2 g of wet weight of brain per ml, was the preparation used in most experiments. Procedures used for further purification of the active material will be mentioned below.

The extracts were injected intraperitoneally into male Swiss albino mice (20–25 g). Control animals were either left uninjected or were treated with similarly prepared extracts of brain taken from normal, nontolerant rats. It was established previously that the latter preparations did not induce tolerance to morphine.

Analgesic effect was tested by the method

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TABLE I. Analgesic Effect of Morphine Sulfate (6 mg/kg) at Different Intervals after Treatment of Mice with Low and High Potency Brain Extracts from Tolerant Rats.[a,b]

Interval (hr):	3			7			24			96		
Extract (g/mouse)	R	N	%	R	N	%	R	N	%	R	N	%
None	4	8	33	5	7	42	3	9	25	4	8	33
"Low"												
0.2	7	5	58	6	6	50	7	5	58	9	3	75[c]
0.4	4	8	33	7	5	58	8	4	67	9	3	75[c]
1.0	7	5	58	7	5	58	9	3	75[c]	8	4	67
"High"												
0.2	6	4	60	—	—	—	7	3	70[c]	7	3	70
0.4	7	3	70	—	—	—	7	3	70[c]	8	2	80[c]
0.6	8	2	80[c]	—	—	—	8	2	80[c]	7	3	70

[a] The analgesic effect was tested in random sequence by a "blind" procedure.

[b] Abbrev.: R = No. of mice responding to stimulus; N = No. of mice not responding to stimulus; and % = Percentage tolerant.

[c] Significantly different from controls by χ^2 test.

of Bianchi and Franceschini (4) based on the response of mice to a clip applied to the root of the tail. Before injection the mice were screened and those not responding to the stimulus were eliminated. The criterion of analgesia was a lack of response within 30 sec. Tests were done 30–60 min after subcutaneous injection of 6 mg/kg of morphine sulfate. In some experiments the reaction times of the mice were also recorded.

In one series of experiments the hypothermic effect of morphine was measured. Temperature was recorded in mice, immobilized in plastic holders, through intrarectal probes connected with a telethermometer (Yellow Springs Instrument Co.). Readings were made at 15-min intervals for 1 hr before and 2 hr after subcutaneous injection of 10 mg/kg of morphine sulfate.

Mortality curves were obtained after subcutaneous administration of the drug. Survival times were recorded for 2 hr after injection. Lethal doses, their confidence limits and ratios were calculated by the method of Litchfield and Wilcoxon (5).

Results. It was noted in the previous paper (1) that extracts taken from rats were more potent than those made of dog brain. Subsequently, we found that the potency of the extract varied considerably within the same species of donors, in spite of the apparently identical schedule of injections given to them and a similar preparation procedure. Table I shows the difference in potency between two extracts of rat brain taken from identically treated donors and prepared by the same procedure. The action of the more potent extract can be detected 3 hr after injection while the other becomes active only after 24 hr. It is also obvious that, in terms of equivalent amounts of brain, the latter requires a higher dose to produce the same degree of tolerance.

When analgesia is expressed by increase in the graded reaction time instead of the quantal terms of response or no-response, the difference in potency is still apparent (Table II), but the statistical significance between the experimental and control groups has disappeared so that one could conclude that no transfer had taken place.

Table III shows the results of experiments in which development of tolerance was tested by the hypothermic effect of morphine. The fall in temperature was consistently smaller in the recipients of brain extracts than in the controls but the difference fell short of the usually accepted criteria of significance.

The simplest way to demonstrate the existence of tolerance is by a shift in the lethal dose. Figure 1 shows the mortality caused by morphine in untreated mice, in mice injected

TABLE II. Same Experiment as in Table I but the Analgesic Effect of Morphine Sulfate Was Measured in Terms of the Reaction Time to Stimulus (applied for 30 sec).[a]

Extract (g/mouse)	Reaction time (sec) ± SD				
Interval (hr):	3	7	24	96	*N*
None	23 ± 10	26 ± 7	24 ± 10	23 ± 10	12
"Low"					
0.2	26.5 ± 4	23 ± 6	20 ± 12	16 ± 12	12
0.4	23 ± 9	20 ± 5	19 ± 8	16 ± 13	12
1.0	24 ± 7	21.5 ± 5	20 ± 8	15 ± 14	12
"High"					
0.6	15 ± 10	—	14 ± 12	12 ± 8	10

[a] Before morphine, the mean reaction time of all the mice was 7 ± 8 sec.

24 hr previously with extract of normal rat brain and two extracts of brain from tolerant rats. In the two control groups the LD_{50} is, respectively, 265 mg/kg (95% c.l. 217–323) and 300 mg/kg (c.l. 252-357). The ratio between the two doses is 1.13 and the difference is not significant. In the animals treated with the low potency extract the LD_{50} was 480 mg/kg (c.l. 375–613) and in those that received the highly potent extract it was 960 mg/kg (c.l. 785–1122). Both were significantly different from either of the controls. They were given at the comparatively high dose of 1 g of brain per mouse but the effect of smaller doses can be demonstrated by measuring the survival at shorter periods. Table IV shows that the brain extracts delayed

TABLE III. Hypothermia Produced by Morphine Sulfate (10 mg/kg) in Mice Injected with Extract of Brain from Morphine Tolerant and Normal Rats.[a]

After MS (min)	Fall in temperature (°) MTRB ± SD		NRB ± SD	
30	—1.95	1.4	—2.3	1.8
45	—2.0	1.2	—2.6	1.9
60	—1.65	0.9	—2.5	2.0
75	—1.3	0.8	—2.2	1.4

[a] Before injection of morphine the mean temperature of all the mice was 37.6 ± 0.4°. MTRB = mice injected with extract of brain from morphine tolerant rats; NRB, from normal rats. Dose equivalent to 1 g of fresh brain, injected 24 hr before morphine.

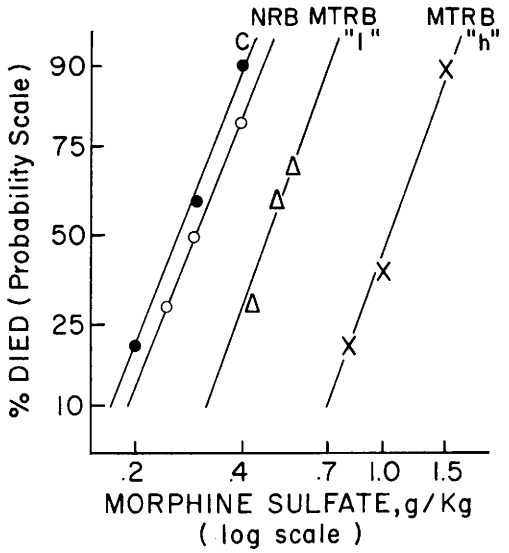

FIG. 1. Mortality curves by morphine in control mice (C), mice injected with extract of normal rat brain (NRB), and two extracts of brain taken from morphine-tolerant rats (MTRB): abscissa: dose of morphine sulfate, g/kg, subcutaneous (log scale); ordinate: percentage mortality (probability scale). All the brain extracts, NRB, MTRB "l" (low potency) and MTRB "h" (high potency) were injected intraperitoneally, at the dose equivalent to 1 g of brain, 24 hr before morphine.

death in the mice even when they did not allow indefinite survival.

Discussion. In their study of the "replicability" of our experiments. Smits and Takemori (3) did four series of experiments. In three of them, all the recipients were injected with homogenates equivalent to 200–240 mg

TABLE IV. Mortality by Morphine 1 and 2 hr after Injection in Mice Injected with Extract of Brain from Tolerant Rats.[a]

Morphine sulfate (mg/kg)	Mortality (%)					
	1 hr			2 hr		
Dose of brain (g/mouse):	0	0.5	1.0	0	0.5	1.0
300	30	0	0	50	20	0[b]
425	50	30	10[b]	80	70	30[b]
550	90	40[b]	30[b]	100	90	50[b]

[a] Groups of mice were given "low potency" extract 24 hr before morphine.

[b] Significantly different from controls by χ^2 test.

of brain from tolerant rats. All animals were tested once, 3 hr after injection. In all three experiments there was a slight difference suggesting greater tolerance in the recipients of tolerant brain. As in the 3-hr tests of our experiments (Table I), the difference was far from being significant, but had the tests been done at longer intervals or at higher doses, they would probably have approached the criteria of significance. The fourth experiment of Smits and Takemori was completely negative. It was done with a brain preparation (perchloric acid filtrate) which gave negative results in our hands too, probably because the active material is either precipitated by perchloric acid or is adsorbed on the precipitate.

In another attempt at "replication," Tirri (2) used brain homogenates from mice rendered tolerant by a schedule of injections similar to the one we used in rats. Each recipient received the equivalent of one donor brain (about 250 mg) and was tested 1–5 days after injection. The results were entirely negative and this agrees with our own experience with mice being used as donors. Extracts of brain taken from mice which had been made tolerant to morphine under the same conditions as rats and dogs did not produce tolerance in the recipient mice. Doses of 300–600 mg of brain were given per mouse and the animals were tested 24, 48, and 72 hr after injection of the extract. Mice tolerate about three times more morphine than rats, and it is possible that they could become satisfactory donors if they were made tolerant under different conditions. Tirri used both analgesia and hypothermia to test for tolerance. It was seen above (Table III) that hypothermia, like other graded responses, is not a satisfactory method for demonstrating transfer of tolerance to a significant degree.

The results reported in this paper indicate that preparations of brain taken from tolerant animals may differ quantitatively in their tolerance-inducing effect. It seems certain that the donors have to be more than minimally tolerant. The potency of the extracts depends on the doses of morphine given to the donors and the length of their exposure to the drug, as well as on other factors which as yet are unknown. Consequently, the preparations cannot be tested by an arbitrary procedure utilizing a fixed dose and assuming a fixed onset of action. These preparations are like many natural products, familiar to pharmacologists, whose potency has to be determined by bioassay. Their activity cannot be denied unless all reasonable doses and intervals give negative results.

In replication experiments, a great deal of time and effort could be saved by avoiding the pitfalls of either changing radically the conditions of the original experiment or arbitrarily narrowing down the experimental conditions. These precautions are especially applicable to areas of research which are just beginning to be explored and in which many variables still escape our control (6).

Attempts have been made to isolate the transfer material from the brain extract. Gel filtration and ion exchange chromatography of the dialyzate yielded a fraction that produced tolerance at the dose of 0.1–0.2 μg/mouse. The material is ninhydrin negative but its hydrolysis yields ninhydrin positive products. Although analysis shows that it has constituents other than amino acids, its activity depends on the presence of some peptide linkages, since it was shown to be destroyed by chymotrypsin (1).

Summary. The experiments reported give further support of the possibility of transferring morphine tolerance by administration of

material extracted from the brain of tolerant animals. However, for reasons still unknown, brain extracts identically prepared from donors subjected to the same treatment show widely varying potencies. To demonstrate the effect of low potency extracts, higher doses have to be given with a longer interval between administration of the extracts and testing of their effect. Not all tests are equally suitable for showing the transfer of tolerance: graded responses are less satisfactory than quantal effects. The shift in the mortality curve of morphine, although less sensitive than the analgesic effect, is particularly suitable for an easy demonstration of the transfer of tolerance. Purification of the transfer factor, although still incomplete, has reached the point where administration of 0.1 to 0.2 μg of material per mouse induces significant tolerance to the analgesic effect of morphine.

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Lack of Effect of Paralyzation with Gallamine and Decamethonium on Duration of Hippocampal and Amygdaloid After-Discharge (33540)

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Paralyzation with gallamine triethiodide (Flaxedil), but not with decamethonium, increases the duration of cortical after-discharge (1–3). We have previously interpreted this effect of gallamine as being due to an attenuation of the accompanying motor seizure (1). Further, we have suggested that the difference in effect between gallamine and decamethonium lies in their differential effect on muscle spindles (3).

The purpose of the present investigation was to examine the effect of gallamine and decamethonium on the duration of after-discharge elicited from the hippocampus and amygdala. These experiments were suggested by the fact that after-discharge from these brain areas is not accompanied by a motor seizure, at least during early trials (4). Thus it was reasoned that paralyzation with gallamine should not have an effect on their duration.

Methods. Twelve adult cats of both sexes were used in these experiments. The animals were prepared under pentobarbital sodium (25 mg/kg) anesthesia. Bipolar electrodes, consisting of parallel strands of 32-gauge stainless steel wire, bared for approximately 0.5 mm at the tip, were inserted into the dorsal hippocampus of 6 cats and into the lateral amygdala of 6 other animals. Stainless steel screws were threaded into the skull for recording the electroencephalogram (EEG). The electrode leads were externalized via a Winchester plug which was cemented to the skull with dental acrylic.

Preliminary trials to determine the after-discharge threshold were begun after a 2-week recovery period. Each cat was stimulated with a 5-sec train of 1 msec, monophasic square wave pulses at 25 pulses/sec (delivered from a Grass S-4 stimulator via a Grass SIU-4B stimulus isolation unit and Grass CCU-1A constant current unit) starting from 0.5 mA and with increments 0.5 mA. The current was increased until after-discharge was recorded from the site of stimulation. At