Effect of α -Amanitin on Ribonucleic Acid Polymerase II of Rat Brain Nuclei and on Retention of Avoidance Conditioning

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1. α -Amanitin inhibits *in vitro* the activity of RNA polymerase II of rat brain nuclei. 2. The toxin does not pass the blood–brain barrier, but when injected intracerebrally is highly toxic for rats, and causes inhibition of RNA polymerase II of isolated brain nuclei. 3. Intracerebral injection of α -amanitin 6 h before training to a passive avoidance task is followed by impaired performance of rats on retesting after 7 days, without affecting performance on retesting immediately after training.

Studies on the possible role of brain macromolecules in memory acquisition and consolidation have produced evidence suggesting (i) that an increased synthesis of RNA and of protein occurs in some parts of the brain during training, and (ii) that the establishment of 'long-term' memory is impaired by inhibitors of synthesis of RNA and protein (for review see Glassman, 1969).

The experiments reported below indicate that the establishment of long-term memory in rats is impaired by intracerebrally injected α -amanitin, a specific inhibitor of nuclear RNA polymerase II (Kedinger, Gniazdowski, Mandel, Gissinger & Chambon, 1970; Jacob, Sajdel & Munro, 1970; Lindell, Weinberg, Morris, Roeder & Rutter, 1970). It will be shown also that this toxin inhibits *in vitro* and *in vivo* the activity of RNA polymerase II of rat brain nuclei, although it does not pass the blood–brain barrier.

EXPERIMENTAL

Chemicals. α -Amanitin was a generous gift from Professor T. Wieland, Heidelberg, Germany; acetoxy-cycloheximide was purchased from Sigma Chemical Co., St Louis, Mo., U.S.A.; the chemicals for RNA polymerase assays were from the same sources as described by Novello & Stirpe (1970).

Animals. Male rats of the Wistar strain weighing 180–210 g were used. Intraventricular injections were given as described by Noble, Wurtman & Axelrod (1967).

Preparation of nuclei and determination of RNA polymerase activity. Nuclei were prepared from liver as described by Chauveau, Moulé & Rouiller (1956) in 2.2 M-sucrose containing 10 mM-magnesium acetate instead of CaCl_2 . Brain nuclei were isolated as described by Koenig, Lu, Jacobson, Sanghavi & Nayyar (1970). Purified

nuclei were resuspended in 0.25 M-sucrose containing 1 mM- MgCl_2 .

The activities of RNA polymerase I and II were determined with and without α -amanitin as described by Novello & Stirpe (1970). DNA was determined by the method of Burton (1956).

Testing of memory. This was performed with the apparatus described by Jarvik & Kopp (1967). Rats were put in the smaller, lighted compartment of a two-compartment box, communicating with the larger, darkened part. The animals stepped spontaneously from the lighted compartment into the darkened one, where they received a 350 μA electric foot shock, from which they escaped by returning to the lighted compartment. Jarvik & Kopp (1967) expressed the degree of training as the length of time the rats remained outside the dark compartment on retest. We observed that untrained animals entered the dark compartment very rapidly, and the variability between individual latencies was very small, whereas trained rats waited for a definitely longer time. Therefore, although the latency times on retest were recorded, in expressing results the animals were considered as 'negative' if they entered the dark compartment within 3 min, and 'positive' if they waited for a longer time.

RESULTS

Effect of α -amanitin, *in vitro* and *in vivo*, on the RNA polymerase activity of rat brain nuclei. Nuclei isolated from whole rat brain showed a low activity of RNA polymerase I and a relatively high activity of RNA polymerase II (Table 1). Addition of α -amanitin to the reaction mixtures inhibited polymerase II at concentrations (Fig. 1) similar to those effective on rat liver nuclei (Stirpe & Fiume, 1967), and without affecting significantly the activity of polymerase I. The intraperitoneal injection of α -amanitin at the dose of 5 $\mu\text{g}/10\text{g}$ body wt. is not

Table 1. *Activity of RNA polymerases I and II in isolated rat brain nuclei*

Reaction mixture 'Mg²⁺, low ionic strength' contained, in a final volume of 0.5 ml: 0.1 M-tris-HCl buffer, pH 8.0, 4 mM-MgCl₂, 14 mM-2-mercaptoethanol, 6 mM-NaF, 0.6 mM each of GTP, UTP and CTP, 0.03 mM-ATP, 0.01 mM-[8-¹⁴C]ATP. Reaction mixture 'Mn²⁺, high ionic strength' contained, in a final volume of 0.5 ml: 0.1 M-tris-HCl buffer, pH 8.0, 4 mM-MnCl₂, 0.28 M-(NH₄)₂SO₄, 1.8 mM each of GTP, UTP and CTP, 0.09 mM-ATP, 0.03 mM-[8-¹⁴C]ATP. α -Amanitin, when present, was at 1 μ g. The reaction was started in all cases with 0.1 ml of nuclear suspension. Incubation was at 37°C for 10 min. Mean results $\pm$ S.E.M. are given with the numbers of experiments in parentheses.

		RNA polymerase activity (pmol of AMP incorporated/10 min per mg of DNA)			
System	...	Mg ²⁺ , low ionic strength		Mn ²⁺ , high ionic strength	
α -Amanitin	...	Absent	Present (RNA polymerase I)	Absent	Present Difference (RNA polymerase II)
		130 $\pm$ 4 (3)	88 $\pm$ 9* (15)	1073 $\pm$ 76 (15)	159 $\pm$ 17 (15) 914 $\pm$ 66

* Mean of results obtained with the three preparations in the first column: 108 $\pm$ 13.

Fig. 1. Inhibition *in vitro* by α -amanitin of RNA polymerase of isolated rat brain nuclei. Experimental conditions were as described in Table 1 for the 'Mn²⁺, high ionic strength' system.

lethal for rats (Wieland, 1957) but causes a strong, temporary inhibition of RNA polymerase II of liver nuclei (L. Fiume, F. Novello & F. Stirpe, unpublished work). α -Amanitin given in this way did not affect the activity of RNA polymerase II of nuclei isolated from rat brain (Table 2). This could be due to lack of penetration of the toxin into the brain, which was circumvented by injecting α -amanitin intraventricularly. When administered in this way α -amanitin was much more toxic for rats than when given intraperitoneally, with an LD₅₀ of 0.5–0.8 μ g/rat (calculated by correcting for mortality due to the operation; Finney, 1964). The intraventricular injection of α -amanitin did not affect the activity of RNA polymerase I of

nuclei isolated from the brain, but was followed by a marked inhibition of RNA polymerase II. This occurred relatively slowly, in contrast with what was observed in the liver after intraperitoneal injection of the toxin (Stirpe & Fiume, 1967). The intraventricular injection of α -amanitin at an approximately LD₁₀ dose (2.5 μ g/rat) was followed by a 40% inhibition of RNA polymerase II of brain nuclei isolated 6 h after poisoning; the enzyme activity returned to normal values by 12 h after the injection (Table 2). The intracerebral administration of α -amanitin, even at the highest dosage given in these experiments, did not affect RNA polymerase II of liver nuclei, thus confirming that the toxin does not pass the blood-brain barrier.

Effect of α -amanitin on retention of avoidance conditioning. Retention of the avoidance conditioning was used to assess the training capacity of rats. The validity of the test used was ascertained in a preliminary experiment, in which it was confirmed that long-term retention was significantly impaired in rats injected with acetoxycycloheximide immediately after training. The acetoxycycloheximide was given at a much lower dosage than that used for similar experiments in mice (see e.g. Geller, Robustelli, Barondes, Cohen & Jarvik, 1969). This was because we observed that this inhibitor is much more toxic for rats (LD₅₀ 2.7–3.0 mg/kg body wt.) than for mice.

Rats trained 6 h after intracerebral injection of α -amanitin, i.e. when the activity of RNA polymerase II was depressed, showed a significant impairment of the response when retested 7 days later (Table 3), although they performed normally if retested within 15 min from training. α -Amanitin had no significant effect on the task performance if it was injected 48 h before or 24 h after training (Table 3).

Table 2. *Effect in vivo of α -amanitin on the activity of RNA polymerases I and II of rat brain nuclei*

α -Amanitin was injected intraventricularly in all experiments except the first one, and was dissolved in 20 μ l of 0.9% NaCl soln.; controls received the same amount of saline given by the same route. RNA polymerase activity was determined as described in Table 1. Results are means $\pm$ s.e.m. of the numbers of experiments given in parentheses.

Expt. no.	Treatment	RNA polymerase activity (pmol of AMP incorporated/10 min per mg of DNA)	
		I	II
1	Saline (2)	94	909
	α -Amanitin (5 μ g/10 g body wt.) intra-peritoneally 6 h before death (2)	59	955
2	Saline (1)	146	414
	α -Amanitin (100 μ g) 1 h before death (1)	123	374
3	Saline (1)	119	523
	α -Amanitin (100 μ g) 6 h before death (1)	217	26
4	Saline (1)	83	994
	α -Amanitin (10 μ g) 6 h before death (1)	47	251
5	Saline (4)	59 $\pm$ 13	1019 $\pm$ 83
	α -Amanitin (0.5 μ g) 6 h before death (7)	54 $\pm$ 6	407 $\pm$ 119*
	α -Amanitin (0.25 μ g) 6 h before death (6)	79 $\pm$ 17	627 $\pm$ 118†
6	Saline (2)	75	949
	α -Amanitin (0.25 μ g) 12 h before death (2)	71	955
7	Saline (2)	103	1103
	α -Amanitin (0.25 μ g) 24 h before death (2)	94	884
8	Saline (2)	106	1037
	α -Amanitin (0.25 μ g) 48 h before death (2)	94	899

* Significantly different from saline-injected controls ($P < 0.01$).

† Significantly different from saline-injected controls ($P < 0.05$).

Table 3. *Effect of α -amanitin on retention of avoidance conditioning*

α -Amanitin was injected intraventricularly at the dose of 0.25 μ g/rat, dissolved in 20 μ l of 0.9% NaCl soln. Controls received the same amount of saline. Retention was defined as negative if the animals remained outside the dark box for less than 3 min and positive if they waited a longer time. Statistical evaluation of results of the retest trial at 7 days (α -amanitin-injected rats versus saline-injected ones): Expt. 1, $\chi^2 = 13.01$, degree of freedom 1, $P < 0.01$; Expt. 2, $\chi^2 = 1.20$, degree of freedom 1, $0.30 > P > 0.20$; Expt. 3, $\chi^2 = 1.77$, degree of freedom 1, $0.20 > P > 0.10$.

Expt. no.	Treatment	Retention			
		15 min after training		7 days after training	
		Negative	Positive	Negative	Positive
1	Saline	0	26	5	21
	α -Amanitin 6 h before training	0	21	16	5
2	Saline	0	20	3	17
	α -Amanitin 48 h before training	0	20	7	13
3	Saline	0	17	0	17
	α -Amanitin 24 h after training	0	15	3	12

The intraventricular injection of α -amanitin (0.25 μ g/rat) did not cause apparent modifications in the behaviour of the rats, except that 15% of the animals surviving the injection showed aggressive behaviour, which lasted for at least 10 days, when the animals were killed for other purposes. Two of these animals refused food after the injection until their death.

DISCUSSION

Kato & Kurokawa (1970) reported that the RNA polymerase activity of guinea-pig brain nuclei is stimulated by high ionic strength, and to a greater extent than that of rat liver nuclei. Similar results were obtained in our experiments with rat brain nuclei, and the fact that the activity stimulated by

high ionic strength is inhibited by α -amanitin demonstrates the presence in brain of a high activity of RNA polymerase II (according to the nomenclature of Roeder & Rutter, 1969).

α -Amanitin seems not to pass the blood-brain barrier, since the brain polymerase is not affected when the toxin is given intraperitoneally, whereas liver polymerase is not inhibited after the intracerebral injections. When given in the latter way, α -amanitin is much more toxic for rats than when injected intraperitoneally. It is possible that this higher toxicity is due to the impermeability of the blood-brain barrier, which presumably does not allow a rapid elimination of the toxin from the brain.

The injection of sublethal doses of α -amanitin impairs the establishment of long-term memory in rats. It is tempting to correlate this effect with the inhibition of RNA polymerase II, as is also suggested by the fact that α -amanitin has no effect on memory if the training is given 48h after the poisoning, i.e. after the activity of RNA polymerase is restored. It is not certain, however, that α -amanitin affects only RNA polymerase II. Even assuming that this enzyme is the only direct target of α -amanitin, the consequences of its inhibition on other reactions are not yet known, and therefore it remains to be ascertained whether the effect of α -amanitin on memory is due to inhibition of RNA polymerase II itself, or to the possible consequent impairment of other reactions, and especially of protein synthesis. Whatever the exact mechanism, α -amanitin seems to affect the consolidation of memory from short- to long-term, since the toxin has no effect on the acquisition of short-term memory, nor does it suppress the long-term memory once this is established. These results exclude also an effect on retrieval rather than on fixation of memory, and on the ability to perform the assigned

task. Thus it seems unlikely that the observed effects on long-term memory are a non-specific consequence of the administration of a toxic substance, and it seems possible that α -amanitin affects memory in a manner similar to other inhibitors of RNA and of protein synthesis.

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