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Effect of α-Amanitin on Brain RNA and Protein Synthesis and on Retention of Avoidance Conditioning

PAOLA STROCCHI, NICOLA MONTANARO, ROSSELLA DALL'OLIO

Istituto di Farmacologia dell'Università di Bologna

FRANCESCO NOVELLO AND FIORENZO STIRPE

Istituto di Patologia generale dell'Università di Bologna, 40126 Bologna, Italy

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The effect of a sublethal dose of α-amanitin given intraventricularly to rats on retention of passive and active avoidance conditioning has been studied, together with the effect on brain RNA and protein synthesis in vivo. The toxin brings about a significant impairment of retention of both passive and active conditioning in rats poisoned 6 hr or 24 hr before training. Brain RNA synthesis is decreased at 6 hr after poisoning, whilst protein synthesis decreases at a later stage (not before 12 hr after poisoning). Thus in rats poisoned with α-amanitin memory consolidation is impaired when RNA synthesis is decreased, and before protein synthesis is affected.

α-Amanitin memory consolidation Brain RNA synthesis Retention of avoidance conditioning
Aggressiveness and hyperactivity

IN a previous paper [9] it has been reported that α-amanitin, a specific inhibitor of RNA polymerase II, was highly toxic to rats when injected intracerebrally and, at sublethal doses, brought about a temporary impairment of brain RNA polymerase II and of retention of passive avoidance conditioning. Furthermore, some of the poisoned animals showed an anomalous behavior, with signs of aggressiveness and hyperreactivity, while very few of them refused food [9]. The effects of α-amanitin on brain RNA polymerase II and on memory, tested with active and passive avoidance tests, were essentially confirmed in mice [11,12], although the dose used was highly toxic, as remarked by Dunn and Bondy [3].

The experiments reported in the present paper were undertaken to elucidate further the biochemical effects of α-amanitin on brain, and to correlate them with the effects on memory consolidation, as estimated from retention of both active and passive avoidance conditioning. The results indicate that α-amanitin depresses RNA synthesis and subsequently protein synthesis in the brain, and that it impairs long-term retention of memory as estimated with either method, without affecting appreciably the learning capacity. The effects on memory are already appreciable when RNA synthesis alone is impaired, and before protein synthesis is depressed.

METHOD

Chemicals

L-[U-^{14}C] leucine (specific activity 10 mCi/mmol), ^{14}C-labelled protein hydrolysate (specific activity 52 mCi/mmol) and 6-[^{14}C] orotic acid (specific activity 60.8 mCi/mmol) were purchased from the Radiochemical Centre, Amersham, Bucks., U.K. α-Amanitin was a generous gift from Professor Th. Wieland, Heidelberg, Germany. All other chemicals were of analytical grade.

Animals

Male rats of the Sprague-Dawley strain weighing 180–220 g were used. They were housed in an air-conditioned room artificially illuminated from 7 a.m. to 7 p.m. A commercial diet and tap water were given ad lib; food consumption and body weight, as well as changes in gross behavior, were recorded daily for 7 days after poisoning.

α-Amanitin (0.25 μg/rat, dissolved in 20 μl of sterile 0.9% NaCl) was injected into the right ventriculum under light ether anaesthesia with a stereotaxic apparatus, using the ventricular coordinates of a stereotaxic atlas [6].

Passive Avoidance Conditioning

An apparatus consisting of a two-compartment box divided by a guillotine door was used. The animal was put into the smaller lighted compartment (16 × 13 × 23 cm) starting an electronic timer measuring the time the animal remained in the compartment. As soon as the rat was released the guillotine door was opened and remained open throughout the training session. On entering the dark compartment (30 × 20 × 23 cm) the animal received a

scrambled d.c. footshock (0.8 mA, constant current) from which it could escape by coming back into the illuminated compartment. Subsequent entries the dark compartment resulted in other footshock administrations. The training session ended when the animal remained in the lighted compartment 2 min after the footshock. Most animals required 1–2 trials to show such short-term avoidance and none required more than 4 trials. There were no differences between control and poisoned rats ($\chi^2 = 5.25$; $df = 9$; $0.90 > p > 0.80$).

Immediately after the completion of the training session the animal was transferred back to its home cage. A memory test was performed 48 hr later by introducing the animal into the apparatus and recording the time spent in the lighted compartment as in the first trial of the training session. Our experimental data are given by latency times (in sec) of the first entry of the dark compartment in both sessions.

Active Avoidance

The apparatus consisted of a shuttle cage located into a soundproof box; the two compartments, each measuring 22 × 28 × 23 cm, were joined by an 8 × 10 cm opening. Timing of stimulus events were programmed electronically. The warning signal consisted of a buzzer which started 10 sec before delivering a scrambled d.c. footshock (0.8 mA constant current) which lasted for a further 10 sec, during which the buzzer continued. Whenever the animal crossed from one side of the cage to the other the stimulation was interrupted and crossing responses to buzzer alone were considered as conditioned responses. The intertrial interval was 30 sec.

Both training and test sessions (48 hr later) consisted of 25 trials, after a 10 min period of adaptation to the cage, without stimuli. In both sessions conditioning was expressed as percent of conditioned responses of each animal

$$\left(\frac{\text{number of conditioned responses}}{\text{number of trials}} \times 100\right).$$

Statistical Evaluation

Data of passive and active avoidance (log-reciprocal- and angular-transformed, respectively) were evaluated by means of hierarchal-factorial analysis of variance [14]. Results of the analyses of variance of transformed data were interpreted in terms of differences among the medians of the values in the original scale [7].

RNA Synthesis In Vivo

Rats were injected intraventricularly with [6-^{14}C] orotic acid (3 μCi per rat, dissolved in 20 μl of 0.9% NaCl) and were killed 20 min later. Brains were quickly removed and were homogenized with 5 ml of water. After addition of perchloric acid (0.2 M, final concentration) homogenates were centrifuged at 2,500 *g* for 15 min. Samples (0.4 ml) of the supernatant were put in counting vials with 6 ml of 2-methoxyethanol and 10 ml of scintillation fluid, and the acid-soluble radioactivity was determined in a Nuclear-Chicago Mark I scintillation spectrometer with an external standard. Nucleic acids were extracted from the sediment [10] and samples of the extract were taken for the determination of RNA [10] and of radioactivity. Radioactivity incorporated (cpm/mg of RNA) was corrected by dividing for acid-soluble radioactivity (cpm/mg of tissue). DNA was estimated by the method of Burton [2].

Protein Synthesis In Vivo

Rats were killed 20 min after an intraventricular injection of [U-^{14}C] leucine (1 μCi per rat, dissolved in 20 μl of 0.9% NaCl) and the brains were homogenized in 10 ml of 20% (w/v) trichloroacetic acid. The homogenate was centrifuged and 0.4 ml samples of the supernatant were taken to measure the acid-soluble radioactivity as described above. Protein was purified from the sediment as described by Verbin *et al.* [13], weighed samples (approximately 4 mg) were dissolved in Hyamine, and the radioactivity was measured after addition of 10 ml of scintillation fluid. Radioactivity incorporated (cpm/mg of protein) was corrected by dividing for acid-soluble radioactivity (cpm/mg of tissue).

Analysis of Polysomes

Polyribosomes were analysed and the incorporation of radioactivity was measured as described by Zomzely *et al.* [15]. Rats were injected with 10 μCi of ^{14}C-protein hydrolysate, given intraventricularly 10 min before killing. Post-mitochondrial supernatant from brain [15] was

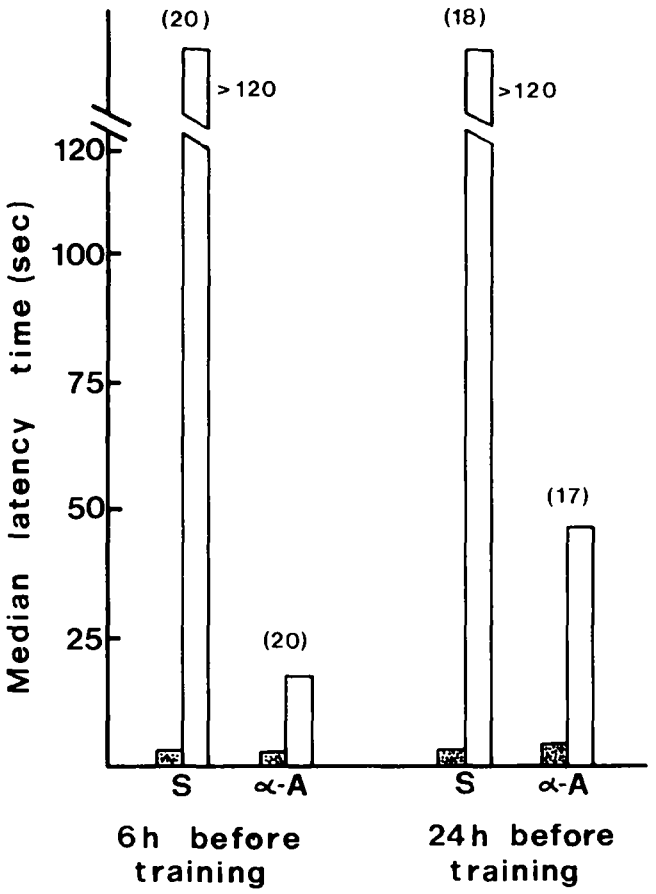

FIG. 1. Effect of poisoning with α-amanitin on passive avoidance conditioning, expresses as median latency times of the first entering the dark compartment in training session (hatched bars) and test session (empty bars). The numbers of animals are given in parentheses. S: rats injected with saline; α-A: rats injected with α-amanitin.

layered over a linear sucrose density gradient (15 -35%), containing 12 mM $MgCl_2$, 100 mM KCl, and 50 mM Tris-HCl buffer, pH 7.6. Gradients were centrifuged at 25,000 rpm for 4 hr at 0°C in the Spinco SW 25 swinging bucket rotor. Gradients were displaced from the bottom, the E_{254} was monitored, and 1 ml fractions were collected. The 80 S monomer peak was identified in separate experiments in which the postmitochondrial supernatant was treated with RNase. Trichloroacetic acid-insoluble material of each fraction was collected, washed [15] and its radioactivity was determined.

RESULTS

Gross Behavior

Food intake of rats injected intracranially with α-amanitin was recorded daily for 7 days and compared with that of rats injected with saline. α-Amanitin had an all or nothing anorexic effect, in that approximately 15% of the animals refused food until their death, beginning on the 2nd, 3rd or 6th day after poisoning. The food intake of the other poisoned rats was not significantly different from that of controls.

The body weight of the animals reflected their food consumption, decreasing only in the case of rats refusing food.

It was observed previously [9] that some rats poisoned with α-amanitin showed an anomalous behavior (defined as aggressive). These animals took a fixed posture when their cage was opened, rose on their hind limbs against the back wall of the cage when the experimenter's hand was introduced into the cage, and showed fear and aggressivity during handling. This behavior was observed again in 20% of poisoned rats, appearing 3 or more days after administration of α-amanitin. Most of these rats also showed the above mentioned changes in their feeding behavior. The results obtained from animals showing one or both anomalies were excluded from the evaluation of memory.

Passive and Active Conditioning

Figure 1 shows the results of the passive avoidance task expressed in terms of median latency times before the first entering the dark compartment in either session. As confirmed by the analysis of variance, rats poisoned with α-amanitin did not differ from controls in training session at both times of administration, $F(1,71)<1$, N.S., but showed significantly lower values of retention in the test session performed 48 hr after training, $F(1,71) = 5.18$, $p<0.05$. The amnestic effect appeared slightly more pronounced in rats poisoned 6 hr before training as compared with those poisoned 24 hr before training, although the difference between these two groups was not statistically significant, $F(1,71)<1$, N.S.

The results of active avoidance conditioning, reported in Fig. 2 in terms of median number of avoidance responses, showed a significantly lower number of avoidances by the α-amanitin-poisoned rats in the test session, $F(1,36) = 11.56$, $p<0.01$, with no difference between rats poisoned at 6 or at 24 hr before training, $F(1,36)<1$, N.S. The level of conditioning reached during the training session was the same in poisoned and control animals, $F(1,36)<1$, N.S.

RNA and Protein Synthesis

The time-course of RNA synthesis in brain after poisoning with α-amanitin is reported in Fig. 3. The synthesis of RNA decreased after poisoning to reach a minimum (- 30%) at 6 hr, when there was the maximum decrease (50%) of RNA polymerase II activity [9]. Subsequently RNA synthesis increased progressively, to reach an almost normal level at 48 hr.

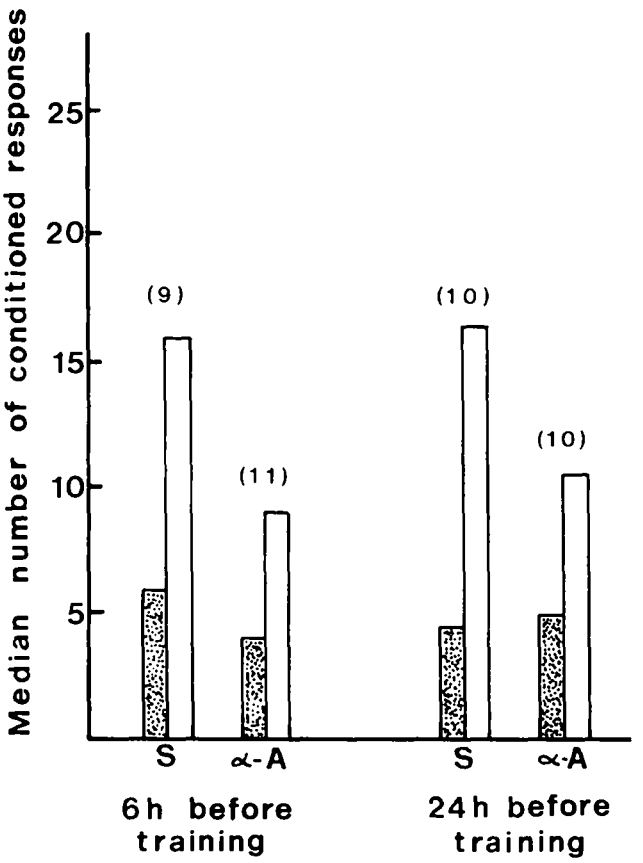

FIG. 2. Effect of poisoning with α-amanitin on active avoidance conditioning, expressed as median number of conditioned responses in training session (hatched bars) and in test session (empty bars). The numbers of animals are given in parentheses. S: rats injected with saline; α-A: rats injected with α-amanitin.

α-Amanitin affects also protein synthesis [8], although at a later time than RNA synthesis. Protein synthesis in the brain of poisoned rats begins to decrease at 12 hr, reaches a minimum (- 37%) at 24 hr and is still significantly lower than normal (-27%) at 48 hr after poisoning (Fig. 3).

At the dose used in these experiments α-amanitin did not alter the polyribosome distribution and brought about a reduction of amino acid incorporation into them at 24 hr but not at 6 hr (Fig. 4). When a higher dose is given, a reduction of polysomes and an increase of smaller particles (especially of dimers) is observed, together with a marked inhibition of amino acid incorporation (Fig. 4).

DISCUSSION

α-Amanitin given intraventricularly at the dose used in our experiments does not induce gross modifications of the general status of the animals, except in some cases in which a refusal of food and an anomalous behavior is observed. The cause of the latter may be a lesion of the hippocampal area, since unpublished experiments show this behavior could be reproduced more consistently by injecting α-

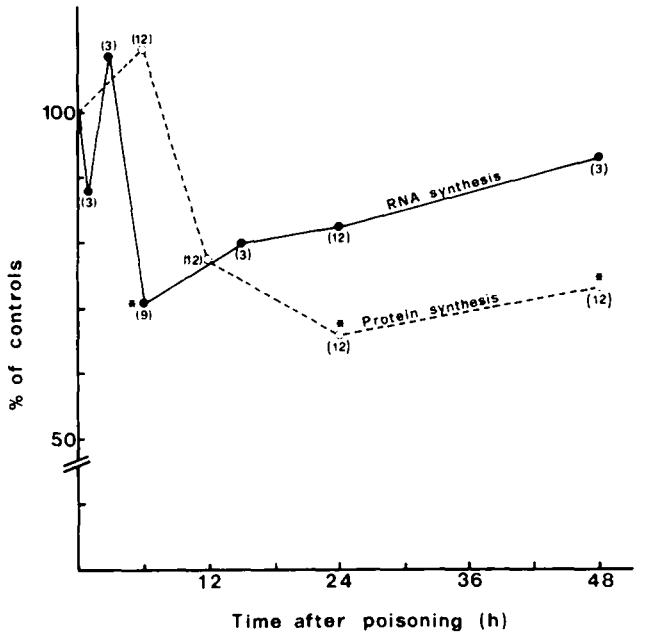

FIG. 3. Effect of poisoning with α-amanitin on RNA (●— —●) and protein (○ - - - ○) synthesis in rat brain *in vivo*. Results are given as percent of control values which are, for RNA synthesis 32 ± 1.6 (S.E.M.) cpm incorporated/mg of brain DNA, corrected for acid-soluble radioactivity (mean of 13 experiments) and for protein synthesis 10.4 ± 0.8 cpm incorporated/mg of protein, corrected for acid-soluble radioactivity (mean of 14 experiments). The numbers of animals are given in parentheses. Data marked * are significantly different from controls ($p<0.01$).

amanitin into the hippocampus. It is known that animals with sufficiently large hippocampal lesions are peculiarly hyperactive and hyperresponsive or reckless [1]. Thus, apart from these cases which are easily identified, α-amanitin does not have any apparent effect that could interfere with the testing of memory.

α-Amanitin administered 6 or 24 hr before training does not affect the learning capacity of rats either in an active avoidance task (as evaluated by the number of conditioned trials in the training session) or in a passive avoidance task, as indicated by the number of trails required to avoid the dark compartment in the training session. Further, in the latter task poisoned animals exhibit a median latency time for the first entering into the dark compartment which is equal to the median latency time of controls.

Administration of α-amanitin brought about a decreased retention of the learned task in both tasks, even though the comparison of the performance in the learning and test sessions indicates the presence of a mnemonic trace. The presence of amnesia for both passive and active avoidance suggests that the effect of α-amanitin is not caused by gross alterations of locomotor performance.

The reduced level of conditioning exhibited by α-amanitin-poisoned rats in the test session could be due to some sort of state-dependent learning. However, it should be considered that saline- and α-amanitin-injected rats do not differ in their exploratory activity in the shuttle-cage, as evaluated by the number of spontaneous crossings between the two compartments during the 10 min blank period before training and test session.

Our results are consistent with previous studies by us

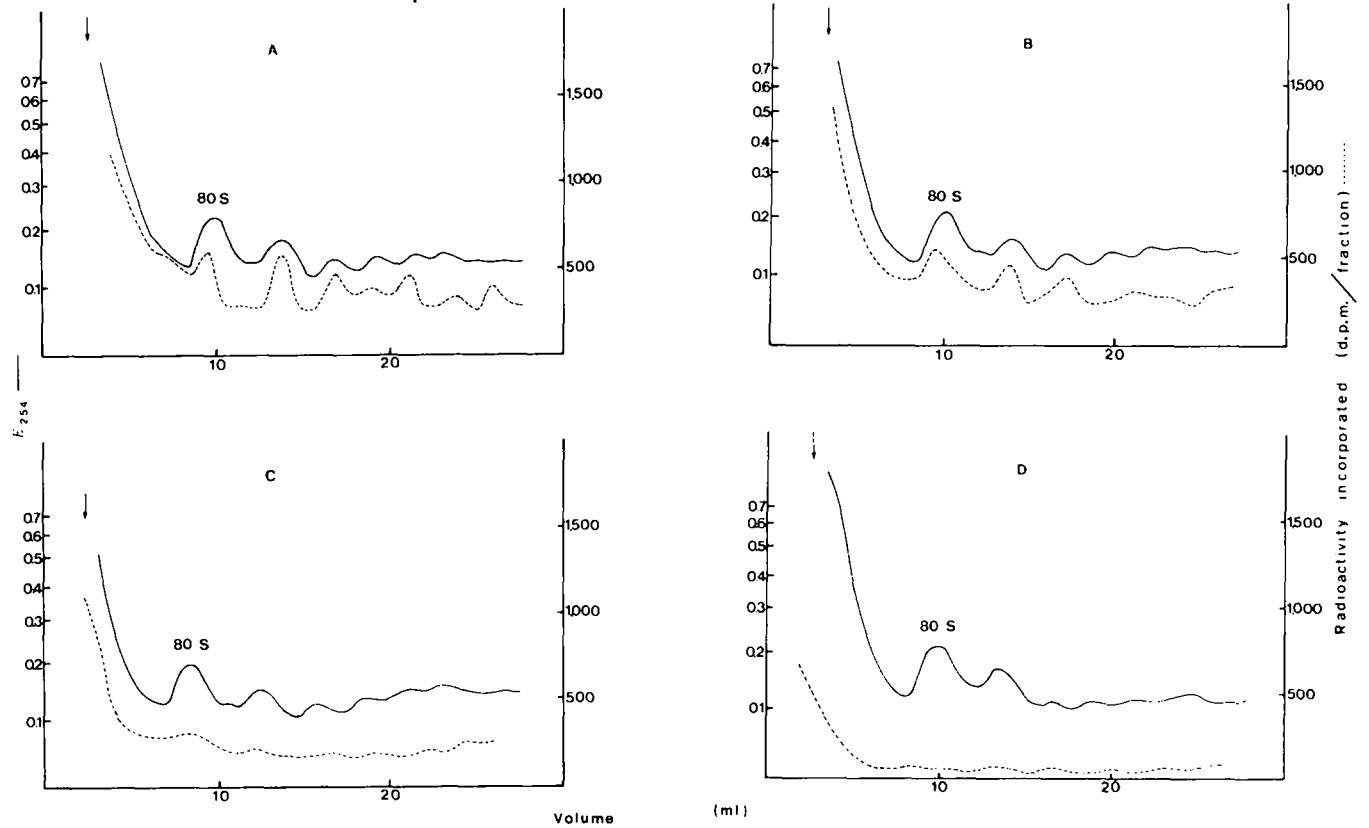

FIG. 4. Effect of poisoning with α-amanitin on polyribosomal profile and on incorporation of amino acids into polyribosomes *in vivo*. A: controls; B: 0.25 μg of α-amanitin 6 hr before sacrifice; C: 0.25 μg of α-amanitin 24 hr before sacrifice; D: 10 μg of α-amanitin 24 hr before sacrifice. The top of the gradients is indicated by the arrow.

[9] and by other investigators [11,12] although the latter obtained inhibition of both passive and active avoidance conditioning in mice treated with lethal doses of the toxin.

Intracerebral injection of α-amanitin induces a transient decrease of the RNA polymerase II activity of brain nuclei [9], the time-course of which is similar to that caused by α-amanitin in the liver [8]. As in the liver, this effect is approximately paralleled by a reduced synthesis of RNA, and later on it is followed by a decreased synthesis of protein. At the dose used for the experiments on memory, α-amanitin does not affect the distribution of polysomes, whereas a higher, lethal dose brings about alterations similar to those observed in the liver [8].

When the changes of RNA and of protein synthesis are compared with the effects on memory, it appears that consolidation of memory is affected already at a time (6 hr) when protein synthesis is normal. Thus it seems that a relatively modest reduction of RNA synthesis is sufficient per se to cause a significant amnestic effect. Thus the amnestic effects of inhibitors of protein synthesis should be evaluated considering that these substances affect also the synthesis of RNA [4].

The effect of α-amanitin, as well as of any other inhibitor, should be interpreted with great caution, since (a) it is not known whether other biochemical systems are affected by the toxin, and (b) the results obtained give an average of the rate of synthesis of all RNA and protein species in the whole brain. The possibility should be considered that the toxin could have different effects in some areas of the brain, or on the synthesis of some species of RNA and/or of proteins. It is known that α-amanitin inhibits specifically RNA polymerase II, and thus a reduced synthesis of heterogeneous and messenger RNA should be expected, whereas ribosomal RNA can still be produced by the unaffected RNA polymerase I. These considerations may explain the consequences on memory of a relatively modest inhibition of total RNA synthesis such as that observed in our experiments. Further, an increased synthesis of hippocampal RNA has been observed during learning in rats [5], and α-amanitin could affect memory simply by preventing this increase.

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