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Is Actinomycin D Suitable for the Investigation of Memory Processes?[1]

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WETZEL, W., T. OTT AND H. MATTHIES. *Is Actinomycin D suitable for the investigation of memory processes?* PHARMAC. BIOCHEM. BEHAV. 4(5) 515–519, 1976. – The influence of Actinomycin D (AMD) applied intrahippocampally at doses of 1–6 μg/animal, on the acquisition and retention of a shock-motivated brightness discrimination was studied on rats in a semiautomatic Y-maze. The injection of AMD 4 hr prior to training did not influence the acquisition, but causes, dose-dependent, a retention loss in relearning 24 hr after training. Twenty-eight hr after AMD application, naive rats exhibited a deterioration of acquisition performance increasing equally with the dose. At the same time, both circumscribed necroses in the hippocampus and signs of a general intoxication were observed. Considering the described pro- and retroactive effects, it is concluded that the use of the inhibitor AMD in learning experiments is not suitable to provide reliable evidence of the specific importance of the cerebral RNA synthesis for memory consolidation.

Actinomycin D Memory consolidation Hippocampus Toxicity Behavior

NUMEROUS experimental findings have suggested a specific importance of the cerebral RNA metabolism for the storage of acquired information.

The effect of an RNA synthesis inhibition by Actinomycin D (AMD) on the retention of learned reactions was investigated in a number of studies. However, these experiments often yielded contradictory results leading to contradictory conclusions. Thus, for example, after AMD application in learning experiments an inhibition of memory formation [1, 6, 8, 12, 25], no effect [4,7], non-consistent effects [2,11] or even an improvement of learning behavior [5] were found. In correspondence to some of the authors mentioned above, we believe that the degree of RNA synthesis inhibition cannot be held responsible for the contradictory behavioral findings.

Systemic application of AMD rapidly induces a general intoxication so that the amnesia observed under AMD may be the result of proactive effects [14]. Therefore, we chose a topical intracerebral application in which ADM was injected into a region necessary for memory consolidation, the hippocampus [20], at different doses, in volumes as small as possible.

METHOD

Studies were performed on 108 male Wistar rats of our own breeding stock, aged 12 weeks and weighing 160–230 g. For intrahippocampal injection, the animals were anesthetized with urethane and hexobarbital and a microcannula implanted in the dorsal hippocampus on either side 1 week prior to the learning experiment. The cannulae were inserted according to the stereotactical coordinates AP 3.2, lateral 2.5, and 3.2 mm deep [24] and fixed on the skull by means of Duracryl cement. To avoid bacterial contamination and to prevent the back-flow of the injected solution, the cannula tops were internally sealed by a rubber plug pierced through on injection [19].

Actinomycin D (AMD) was a gift from the Institut für Mikrobiologie und experimentelle Therapie der Akademie der Wissenschaften der DDR, Jena, G. D. R., through the generosity of Prof. Dr. H. Knöll. The substance was administered at different concentrations (0.5–3 μg/μl) in 4% mannitol solution. In all experiments, a volume of 1 μl per hippocampus was injected through the microcannula on the unanesthetized animal in not less than 10 sec [19]. This corresponds to a dosage ranging between 1 μg and 6 μg AMD per rat. Controls received 1 μl 4% mannitol solution per hippocampus.

To test the general toxicity of the substance after intrahippocampal injection of different doses, the animals' behavior was observed, weight and food intake noted, and measurements of the rectal temperature performed. Animals were killed at different times after AMD treatment and the brains fixed in formaldehyde were examined microscopically in the region of injection for morphological changes. In each case, the correct position of the microcannulas was histologically verified.

The training experiment consisted of learning a light–dark discrimination in a semiautomatic Y-maze [17]. The animals were trained to leave the start box after a 1 mA foot shock and run into the illuminated alley of the Y chamber.

Entering the dark alley was punished with foot shock.

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The position of light and dark alleys was altered after every third run. The average was 1 run per min. The number of incorrect runs (number of errors) was used for evaluation. Further details as to the methods were described in previous publications [16,18]. Training and relearning always consisted of 40 successive runs, with no definite criterion to be achieved. Two experimental series were performed (see Table 1): (A) Training 4 hr after application of AMD or mannitol and relearning 24 hr later. (B) Training 28 hr after application, no relearning. We calculated as a dimension for the retention the quantity % savings $[E_T - E_R/E_T] \cdot 100$ (E_T = number of errors in training; E_R = number of errors in relearning) [23]. Statistical evaluation of the results was done according to the t test.

TABLE 1

DESIGNATION OF EXPERIMENTAL GROUPS

Training 4 hr after injection, and relearning 24 hr after training		Training 28 hr after injection	
Group	Treatment*	Group	Treatment*
A 0	2 μl 4% mannitol	B 0	2 μl 4% mannitol
A 1	1 μg AMD	B 1	1 μg AMD
A 2	2 μg AMD	B 2	2 μg AMD
A 3	3 μg AMD	B 3	3 μg AMD
A 6	6 μg AMD	B 6	6 μg AMD

*Data as μl or μg per rat.

RESULTS

Learning Experiments

In training 4 hr after application, no significant difference was found between the rats which had received AMD (A1–A6) and the controls (A0); however, in the retention test 24 hr later a significant impairment was observed after 6 μg AMD (Fig. 1). The % savings for controls and animals treated with small AMD doses (A0–A3) ranged between 56% and 34%, but 0% after 6 μg AMD (A6). The results implies that the animals' relearning behavior after 6 μg AMD was identical to that of naive animals, i.e. they did not show any retention.

In order to examine whether this influence on relearning can be attributed to a specific effect of AMD on the processes of memory consolidation, or whether it is a proactive effect of the substance, the following experiments were carried out: Training 28 hr after injection of AMD or mannitol, i.e. at the same time, relative to application, when in (A) the animals were retrained. The results which are compared with relearning data in Fig. 1, reveal a dose-dependent impairment of performance in training 28 hr after injection which exhibits the same dose response relationship as the influence on relearning (A). The slope of the straight lines in parallel corresponds to the calculated coefficients of regression.

Toxicity

For assessment of the influence described above, on training and relearning 28 hr after application we studied the pattern of the general toxic effects after intra-

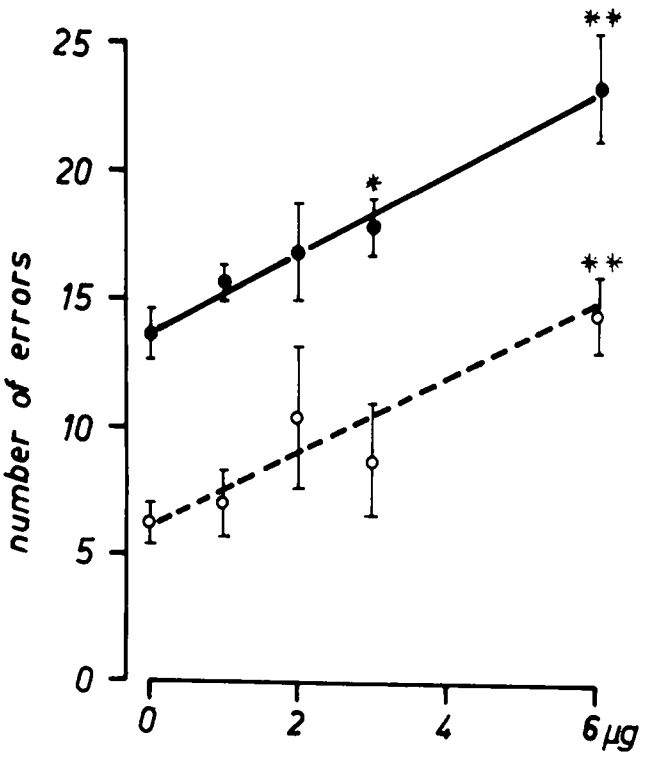

FIG. 1. Training performance (———) of different groups of rats in a brightness discrimination task 28 hr after intrahippocampally applied AMD compared with relearning performance (– – –) 24 hr after Training, i.e., 28 hr after injection. Each point represents the mean values ± SEM obtained from 5–15 animals. Abscissa: Dose of AMD in μg/rat. The slope function of the straight line corresponds to the coefficient of regression. * = Statistically significant difference ($p<0.05$) with reference to the corresponding control group (mannitol injection .)** = $p<0.001$.

hippocampal injection of AMD doses used in the learning experiments. For this, behavioral observations, weight and temperature measurements and microscopic studies for morphological changes in the region of injection were conducted.

Four hr after intrahippocampal application, the animals generally did not show remarkable behavioral changes even at high AMD doses, except for some cases of more pronounced excitability. After 24 hr, however, more or less striking deviations from normal behavior were observed, varying with the dose administered: overexcitability and even aggressiveness, reduced muscle tonus, reduced spontaneous activity, tendency to catalepsy, piloerection. After 12 μg AMD most of the animals were weakened so that they partially lay on their sides; consequently, this dose was not considered for retention tests after 24 hr. On the days following the injection, the above mentioned symptoms were intensified, with jumping seizures, nasal and rectal hemorrhage, dyspnea and cyanosis occurring and, finally, a state of passive lateral position onsetting, which, depending on the dosage, commenced sooner or later and was often of relatively long duration. After 6 μg or 12 μg the animals usually died as early as 1–3 days after AMD was administered. Whereas after smaller doses, they frequently lived upto 7–10 days. Body weights of AMD-treated animals at the time of retention test, i.e. 28 hr after

injection, revealed a loss of weight by some 10 g when comapred with the initial weight, while no significant weight decrease was observed in the controls (Table 2). On subsequent days, AMD-treated animals showed a dose-dependent and continuously progressing weight loss down to approximately 75% of the original weight. At the same time, food consumption diminished at an increasing rate and within a short period of time ceased completely. The rectal temperature of the rats was regularly elevated and some increase was observed on the day following AMD injection. This was also found in animals which, at that time, did not exhibit behavioral changes. A further temperature rise was observed on the second and third days, often exceeding 39°C (Table 3).

TABLE 2

WEIGHT LOSS IN g (MEAN VALUES; NUMBER OF ANIMALS IN PARENTHESES) 1 DAY AFTER INTRAHIPPOCAMPAL APPLICATION OF AMD

Control	1 μg	2 μg	3 μg	AMD/rat
1.8	6.0	11.7*	7.8	
(14)	(5)	(12)	(9)	

*Statistical significant difference ($p<0.01$) when compared with the control (mannitol injection).

Brain slices from animals which had lived 1 week after AMD treatment, were fixed in formaldehyde and studied microscopically. Intense and extensive destruction of the hippocampal region and adjacent structures became evident (Fig. 2b). Necroses of tissue at the site of injection were also observed 7 days after intracortical injection [9]. But in animals sacrificed 28 hr after intrahippocampal injection of AMD, we also found distinct morphological destructions of the hippocampus in the region of the locus of injection (Fig. 2a).

DISCUSSION

The present results show that the 24 hr retention of a brightness discrimination can be completely suppressed by an AMD dose applied intrahippocampally 4 hr before training, with the training behavior not altered. Identical pretreatment with AMD 28 hr before training revealed a dose-dependent impairment of the training performance, which was very similar to the dose-response pattern for the influence on retention (Fig. 1). These findings are consistent with the results reported by other workers, who found a retention impairment after AMD application in learning experiments [1, 6, 8, 12, 25], but also confirm the results reporting a proactive effect of AMD [14]. When training occurs 4 hr after AMD administration, no significant differences in learning were observed as compared with controls, but a distinct dose-dependent impairment occurred when training followed 28 hr after injection; it is reasonable to attribute this AMD impairment to effects other than intrahippocampally inhibited RNA synthesis.

Closer observation of the general toxic effects occurring relatively soon after AMD treatment showed that with regionally restricted application of AMD into the hippocampus a general intoxication occurs which is similar to that found after systemic application [3,21]. This general

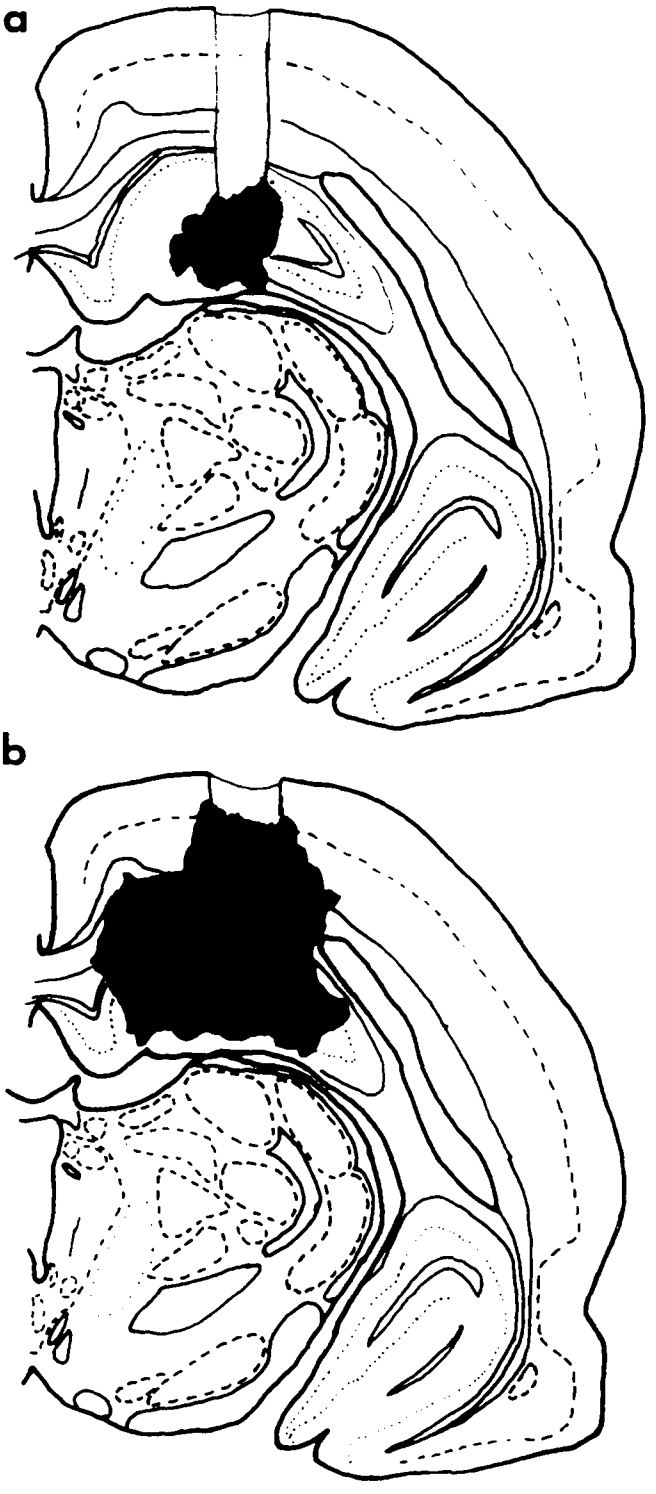

FIG. 2. Representative examples of the local effects of AMD. Black areas denote the extent of histologically verified destruction of hippocampal tissue. (a) 28 hr after 1.5 μg AMD per hippocampus (b) 1 week after 1.0 μg AMD per hippocampus.

TABLE 3

RECTAL TEMPERATURE 1-3 DAYS AFTER INTRAHIPPOCAMPAL APPLICATION OF AMD (MEAN VALUES; NUMBER OF ANIMALS IN PARENTHESES)

Day	Control	1 μg	2 μg	3 μg	4 μg	6 μg	8 μg	12 μg
0	37.4	37.4	36.8	37.5		37.1		
	(16)	(2)	(8)	(5)		(9)		
1	37.6	37.6	38.2	38.2		39.1†		
	(14)	(5)	(3)	(6)		(9)		
2-3	36.8	38.1*	38.5*	38.7†	38.7†		38.7†	39.3†
	(8)	(3)	(5)	(3)	(3)		(3)	(2)

*Statistical significant difference ($p<0.01$) when compared with the control (mannitol injection).
†$p<0.001$.

intoxication can be considered as a cause underlying the changes in the learning experiment. In particular, the finding that in animals not showing essential behavioral changes 28 hr after injection, weight loss and increase in body temperature were observed; the latter is possibly due to the release of endotoxins [21]. Alterations in electrical activity in the brain after local injection of AMD are described in the literature [9,13].

In addition to the above mentioned systemic effects due to the intrahippocampally applied AMD, side effects of AMD released by the local, irreversible inhibition of RNA synthesis must be taken into consideration. As demonstrated by the histological observations, at the time of retention test, the hippocampus already revealed partial destructions, i.e. the retention performance was tested under the conditions of a chemically induced hippocampectomy. Accordingly, the AMD-caused decrease in savings may be a consequence of various events. It is plausible, therefore, that the toxic effects of AMD persisting at the time of retention testing would be responsible for the observed disturbance in performance such that no effective examination of an optimally stored behavior could be possible. Thus, it can be assumed that AMD has no real influence on consolidation. This expectation is supported by the fact that both the acquisition and retention could be influenced in a dose-dependent manner. However, the possibility cannot be excluded that the AMD-induced retention deficit is caused by a direct disturbance of consolidation. The better relearning performance of animals treated 28 hr before relearning compared with the training performance of rats treated 28 hr before training is not necessarily in contradiction with this possibility. Conceivably, the behavior of naive rats may be more strongerly influenced than that of trained animals by AMD in terms of the proactive effects of this substance such as systemic toxicity and chemical hippocampectomy. For instance, it is a well known fact that depending on the learning task hippocamectomy can exert different effects on acquisition and/or retention performance [10]. From this point of view, the findings obtained for rats treated 28 hr before training present without doubt strong evidence for the proactive effects of AMD, but, on the other hand, a retroactive influence of AMD on the consolidation process cannot be excluded.

Since as reported in the literature, a maximum synthesis inhibition is known to occur after 4 hr [2], we chose this time interval between AMD administration and training.

Since our experiment resulted in only localized inhibition of cerebral RNA synthesis, we cannot necessarily exclude the possibility that the remaining RNA synthesis could be sufficient for memory storage. Summarizing, the conclusion to be drawn from our findings is that even under optimal experimental conditions the specific effect of AMD (inhibition of consolidation) cannot be differentiated from the unspecific action elicited by this substance (proactive deterioration of performance). For this reason it cannot be expected that experiments with AMD would help evaluate the hypothetical role of RNA for the storage of conditioned reactions to be elucidated. On the other hand, recently reported findings [15,22] suggested that Camptothecin, a reversible inhibitor of RNA synthesis, could be more suitable than AMD for further investigations in this field.

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