Proc. Nat. Acad. Sci. USA
Vol. 72, No. 11, pp. 4660–4663, November 1975
Psychology

Studies on memory: Inhibitors of protein synthesis also inhibit catecholamine synthesis

(acetoxycycloheximide/anisomycin/cycloheximide/puromycin)

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Contributed by Louis B. Flexner, August 29, 1975

ABSTRACT **The rates of accumulation of newly synthesized catecholamines and endogenous catecholamine levels in mice were determined after treatment with cycloheximide, acetoxycycloheximide, puromycin, and anisomycin. The rates of accumulation were found to be decreased by all antibiotics tested, weakening the assumption that their amnestic effects are due solely to inhibition of protein synthesis.**

The hypothesis that protein synthesis is essential for long-term memory has developed from the observation in several species that treatment with an inhibitor of protein synthesis before or shortly after training leads to amnesia of the training experience (1–6). There is evidence, however, that important side-effects on the central adrenergic system may be common to all inhibitors of protein synthesis and that these side-effects may contribute to the amnesia. For example, in mice and rats the amnesia caused by puromycin, acetoxycycloheximide (AXM), and cycloheximide (CXM) is attenuated by adrenergic stimulants or monoamine oxidase inhibitors (7–9). Also consistent with this possibility are the reports that AXM, CXM, and anisomycin (Ani) cause a reduction of tyrosine hydroxylase activity (10, 11). This further suggests that the amnestic effects of the antibiotics may depend in part on a reduction of the functional pools of newly synthesized catecholamines (CAs) (12, 13). We found support for involvement of these functional pools by observing that a large amnestic dose of AXM strikingly reduced the rate of accumulation of CAs from circulating tyrosine for at least 12 hr, greatly exceeding in degree and duration what might be anticipated from the loss of tyrosine hydroxylase activity *in vitro*. We also found that the antibiotic, in spite of its inhibitory effect on the rate of accumulation of newly synthesized CAs, increased the endogenous levels of CAs for 4 hr after treatment, indicating that it interferes with aspects of CA metabolism in addition to biosynthesis (14).

The present experiments extend this approach to CXM, Ani, puromycin, and to the smallest dose of intracerebrally injected AXM found to be amnestic in mice (15). We find all of these antibiotics to have substantial effects on central CAs. In some ways, these effects resemble those produced by inhibitors of tyrosine hydroxylase and dopamine β-hydroxylase, agents reported to have the same amnestic properties as those of CXM in active or passive avoidance (16, 17) and food-motivated discrimination tasks (18).

Male Swiss-Webster and ICR mice (28–32 g) were housed as previously described (14). CXM (120 mg/kg) and Ani (25–30 mg/kg; gift of Pfizer Inc.) were injected subcutaneously in 0.2 ml of water. AXM (10 μg/injection site; gift of the National Cancer Institute) or puromycin (90 μg/injection site; neutralized with NaOH) were injected bitemporally in 12 μl of water under light Evipal (150 mg/kg) anesthesia. Controls were injected in the same way with 12 μl of water per injection site. The inhibitor of tyrosine hydroxylase, α-methyl-*para*-tyrosine (methyl ester-HCl; 50 mg/kg) (AMPT) was injected intraperitoneally in 0.2 ml of water.

For the rate studies, at various times after treatment with the antibiotics or AMPT, we injected mice intraperitoneally with 20 μCi of L-[3,5-^{3}H]tyrosine and terminated the experiment 10 min later by cervical dislocation. Because of its wide use in behavioral experiments, we followed the effects of CXM for 24 hr using two mice at both the beginning and at the end of each of the periods given in Table 1. Observations with the other antibiotics were made to determine if they had the same qualitative effects as CXM; those with AMPT to gain an approximate idea of the duration of its action. Rates with puromycin and AXM were determined no sooner than 4 hr after the intracerebral injections to avoid the effects of the anesthetic (14). All mice were sacrificed from 1 to 3 p.m. The cerebral hemispheres were rapidly removed, frozen on dry ice, and stored overnight at −50°.

Levels of endogenous CAs were measured at 1 and 4 hr after treatment with CXM, puromycin, and AXM to test for an increase in CA concentration as was found with a large amnestic dose of AXM (14). In view of its shorter period of inhibition of protein synthesis (19), this period was reduced to 1–2 hr for Ani. Assays were extended to 17 hr with CXM and AXM to determine the effect of reduced rates of synthesis on levels of CAs. With AMPT, assays were made relatively early after treatment to determine the effect of its inhibition of synthesis on CA levels and relatively late to test for recovery of these levels.

We have slightly modified the analytical procedures previously described (14). Briefly stated, we followed the method of Anton and Sayre (20) for the extraction of CAs and their adsorption onto alumina. We used the method of Neff *et al.* (21) for separation of [^{3}H]norepinephrine from [^{3}H]dopamine on Dowex 50W×4 columns maintained at 21–22°. These fractions were freeze-dried and the residue taken up in 0.05 M perchloric acid. Their radioactivities were corrected for cross contamination (4% of norepinephrine appeared in the dopamine fraction; 2% of dopamine in the norepinephrine fraction), for recovery (50–60%), and for contamination from [^{3}H]tyrosine and its non-CA derivatives. Tyrosine and its non-CA derivatives were absent in the norepinephrine fractions but contributed about 10% of the dopamine fractions of control mice. Endogenous norepinephrine was determined according to Anton and Sayre (20), endogenous dopamine according to Adler (22). The specific radioactivity of the precursor tyrosine was determined after passing the tissue extract through a column of Dowex 50W×4(H^+-form). Half of the eluate was used for measure-

Abbreviations: AMPT, α-methyl-*para*-tyrosine; Ani, anisomycin, AXM, acetoxycycloheximide; CXM, cycloheximide; CAs, catecholamines.

Table 1. Effect of inhibitors of protein synthesis and of AMPT on rates of accumulation of cerebral CAs from circulating tyrosine and on concentration of cerebral tyrosine, all in terms of the control values

Hr after treatment	% Control rate of accumulation			% Control concentration
	Norepinephrine	Dopamine	Total CAs	Tyrosine
		CXM (120 mg/kg, sc)		
0.5—1 (4)	53**	36***	39***	217***
2—4 (4)	74	57**	59***	185***
8—12 (4)	48**	59**	55***	87
17—24 (4)	87	100	98	88
		Puromycin (180 μg, ic)		
4—6 (6)	50***	95	85*	154***
		Ani (25—30 mg/kg, sc)		
2 (7)	51***	44***	48***	190***
		AXM (20 μg, ic)		
4 (4)	65	36***	41***	243***
		AMPT (50 mg/kg, ip)		
1 (2)	35*	11*	14*	97†
7 (2)	85	107	103	128†
24 (4)	85	106	103	106†

The means ± SEM of the controls in Swiss-Webster (n = 10) and ICR (n = 12) for norepinephrine = 0.20 ± 0.03 and 0.21 ± 0.02 μg/g per hr, respectively; for dopamine = 0.80 ± 0.12 and 0.91 ± 0.08 μg/g per hr; for total CAs = 6.35 ± 0.34 and 7.08 ± 0.05 nmol/g per hr; and for tyrosine = 14.0 ± 0.71 and 14.3 ± 0.73 μg/g. Swiss-Webster mice were used with CXM and puromycin; ICR mice with the remainder. Number of mice is in parentheses. P values (Mann-Whitney U test, 2 tailed): * = 0.05; ** ≤ 0.008; *** = 0.002. sc = subcutaneous; ic = intracerebral; ip = intraperitoneal.

† These values for tyrosine may be somewhat high because its column separation from AMPT was incomplete. AMPT applied to the column gave a fluorometric reading 25% as great as an equimolar quantity of tyrosine.

ment of radioactivity; half for determination of tyrosine by the method of Waalkes and Udenfriend (23).

The quantities of norepinephrine and dopamine derived from tyrosine during 10 min were calculated by dividing their radioactivities by the mean specific radioactivity of tyrosine corrected for the loss of a tritium atom in the conversion of [3,5-^{3}H]tyrosine to norepinephrine and dopamine. Over this time period, Zigmond and Wurtman (24) found the rate of synthesis of CAs to approach linearity. After intraperitoneal injection, we found the average radioactivity of [^{3}H]tyrosine in the cerebral hemispheres of both control mice and mice injected intracerebrally with a high dose of AXM (120 μg) to be 54% of the 10-min value (14). We have used this value in the present experiments to derive the average specific radioactivity of tyrosine.

As shown in Table 1, CXM, Ani, and AXM reduced the rates of accumulation of both norepinephrine and dopamine, whereas puromycin reduced only that of norepinephrine. Substantial reductions in rate of accumulation of total CAs were found up to 12 hr after injection of CXM; recovery was evident at 17 and 24 hr. AMPT had severe effects relatively early after injection, with recovery occurring long before 24 hr.

As noted above, we found that a large amnestic dose of AXM increased the endogenous levels of CAs for 1–4 hr after treatment in spite of inhibition of synthesis of CAs. This effect was also present 1 hr after injection of CXM (Table 2). Additionally, no significant reduction in concentration of CAs was found up to 17 hr after CXM was administered although the synthetic rate was lowered for at least 12 hr. This conservation of CAs suggests that CXM, in addition to its inhibition of synthesis, may also decrease the loss of CAs by actions that interfere with release or enzymatic degradation or by increasing uptake of CAs. With the low dose of AXM, only dopamine was conserved throughout the period of observation. The early rise in concentration of CAs was observed only with CXM.

The mechanisms responsible for the effects of inhibitors of protein synthesis on CA synthesis are unknown. Two possibilities appear applicable to the present findings. The inhibition of tyrosine hydroxylase activity found *in vitro* for a short time and to a limited degree with AXM, CXM, and Ani may be importantly enhanced *in vivo*. Substrate inhibition must also be considered since all the antibiotics increased cerebral tyrosine levels (Table 1). Substrate inhibition was first noted by Shiman *et al.* (25), who found that purified bovine adrenal tyrosine hydroxylase, in the presence of the naturally occurring cofactor, was 30% inhibited at only twice the normal tissue concentration of tyrosine. Kaufman recently found that tyrosine hydroxylase isolated from the corpus striatum of rats is equally sensitive to substrate inhibition (personal communication). Although similar inhibition was reported in homogenates of rat caudate nucleus (26), the importance of this phenomenon *in vivo* is uncertain. High levels of tyrosine have been reported not to affect endogenous cerebral CA levels (27) nor rate of accumulation of dopa after decarboxylase inhibition (28). However, both these findings are less than conclusive. The first measurement may be insensitive to changes in CA synthetic rate, while in the second no assessment was made of the effects of

Table 2. Effect of inhibitors of protein synthesis and of AMPT on endogenous levels of cerebral CAs in terms of the control values

Hr after treatment	% Control concentration		
	Norepinephrine	Dopamine	Total CAs
	CXM (120 mg/kg, sc)		
1 (6)	113**	129**	127**
4 (3)	100	100	100
7 (3)	96	102	101
17 (3)	122	150	143
	Puromycin (180 μg, ic)		
1 (3)	104	104	104
4 (3)	92	90	89
	Ani (25–30 mg/kg, sc)		
1–2 (6)	104	104	104
	AXM (20 μg, ic)		
1 (3)	93	104	102
4 (6)	81**	110	106
7 (3)	79*	101	97
17 (3)	79*	91	90
	AMPT (50 mg/kg, ip)		
4 (3)	69*	64**	65**
16 (3)	96	91	92

The means ± SEM for the controls in Swiss-Webster (n = 12) and ICR (n = 12) mice for norepinephrine = 0.30 ± 0.01 and 0.33 ± 0.01 μg/g, respectively; for dopamine = 1.31 ± 0.11 and 0.89 ± 0.12 μg/g; for total CAs = 10.20 ± 0.29 and 7.80 ± 0.81 nmol/g, all corrected for 80% recovery. P values: * ≤ 0.05; ** ≤ 0.02. Other details are given in legend of Table 1.

drug treatment on tyrosine hydroxylase activity. Feedback inhibition by increased levels of endogenous CAs may occur but only with CXM and then only shortly after treatment.

If substrate inhibition is responsible for the reduced rate of accumulation of newly synthesized CAs, all inhibitors of protein synthesis might be anticipated to share this side-effect. However, the finding that CA synthetic rates are decreased at 8–12 hr after CXM treatment despite lack of elevation of cerebral tyrosine levels indicates that other factors must also contribute to the reductions. Also unexplained is the finding that puromycin only affects norepinephrine synthetic rates.

We thought it possible that inhibition of [^{3}H]tyrosine uptake into nerve terminals might contribute to the decreased rate of accumulation of [^{3}H]CAs. Such an inhibition might result from the loss of rapidly turning-over permease systems (29) or increases in intracellular amino acid pools (30). Consequently, we measured the accumulation of [^{14}C]tyrosine into synaptosome-rich preparations, using a slight modification of the method of Grahame-Smith and Parfitt (31). At 1 and 5 hr after CXM treatment, no reduction in [^{14}C]tyrosine uptake was detected at either 0.08 mM or 0.24 mM tyrosine, in agreement with the findings of Squire *et al.* (11). Apparently, changes in [^{3}H]tyrosine uptake do not contribute to the decrease in CA synthetic rates.

Does the inhibition of CA synthesis and probable interference with other aspects of CA metabolism that we have observed provide an adequate explanation for the amnesia caused by inhibitors of protein synthesis? A satisfactory explanation must account for the transient amnesias that have been found with AXM and CXM (4–6). CXM, for example, causes amnesia, not due to illness (18), 24 hr after administration, with recovery of memory 1 day later (5). In view of our failure to find any abnormality at 24 hr after injection of CXM, we would have expected return of memory at this time. With AMPT, transient amnesia has also been observed 24 hr after treatment (18), at a time when we found no evidence of its characteristic biochemical effect. Perhaps all of these agents have prolonged effects on certain critical areas of brain obscured by our use of the whole cerebral hemispheres. Perhaps they disrupt other aspects of CA function or affect other transmitter systems.

The fact that AXM, CXM, puromycin, and Ani have different mechanisms of action has been used to support the hypothesis that protein synthesis inhibition, rather than any side-effect, is responsible for their effects on memory. Our observation that all these agents cause marked changes in cerebral tyrosine levels and rate of accumulation of newly synthesized CAs indicates that these drugs do indeed share side-effects. Consequently, their behavioral manifestations may not be attributable solely, or at all, to inhibition of protein synthesis.

We thank Mrs. Sophie Conroy for her excellent technical assistance. This work was supported by National Science Foundation Research Grant GB42753 and National Institutes of Health Grant GM02046.

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