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Long-Term Memory: Disruption by Inhibitors of Protein Synthesis and Cytoplasmic Flow[1]

JAMES F. FLOOD[2] AND DENNIS W. LANDRY

Department of Psychiatry and Biobehavioral Sciences, The Neuropsychiatric Institute, School of Medicine and Department of Psychology, University of California, Los Angeles, CA 90024

EDWARD L. BENNETT

Chemical Biodynamics Division, Lawrence Berkeley Laboratory University of California, Berkeley, CA 94720

AND

MURRAY E. JARVIK

Department of Psychiatry and Biobehavioral Sciences, The Neuropsychiatric Institute Department of Pharmacology, School of Medicine, University of California, Los Angeles, CA 90024 and Veterans Administration Medical Center, Brentwood Los Angeles, CA 90073

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FLOOD, J. F., D. W. LANDRY, E. L. BENNETT AND M. E. JARVIK. *Long-term memory: Disruption by inhibitors of protein synthesis and cytoplasmic flow.* PHARMAC. BIOCHEM. BEHAV. 15(2) 289–296, 1981.—Colchicine (60 μg/kg), an inhibitor of axoplasmic transport, administered subcutaneously to mice had no detectable effect on retention when given shortly after active avoidance training, nor did a pretraining injection of anisomycin (ANI) have an amnesic effect. However, when ANI was administered shortly prior to training and colchicine was administered after training, retention performance was impaired. The amnesic effect was dependent on the time at which colchicine was administered. The amnesic effect was also obtained when ANI was combined with either vinblastine (6 μg/kg) or podophyllotoxin (3 μg/kg), drugs that inhibit axoplasmic transport. Intracerebral injections of colchicine (60 ng to 60 pg) caused amnesia in subjects pretreated with ANI, but not in subjects pretreated with saline. Lumicolchicine, an isomer of colchicine, which has similar central nervous system effects but has a low binding affinity for microtubule protein, did not impair retention in ANI pretreated mice. It is suggested that axonal transport of recently synthesized protein is required for long-term memory storage.

Memory Passive avoidance Active avoidance Protein synthesis inhibition
Inhibition of axonal transport Anisomycin Colchicine Vinblastine Podophyllotoxin

SUBSTANTIAL evidence has been obtained that protein synthesis is essential for long-term memory formation (for reviews see [9, 14, 16]). It has been suggested [5] that once the protein has been synthesized, microtubules may be involved in the transport from the cell body to axonal and dendritic endings of proteins and other neurochemicals needed for conversion of short-term memory to long-term memory.

Colchicine, a plant alkaloid, inhibits fast axoplasmic flow by binding to tubulin, the principal structural protein of microtubules [20,35]. Rose and Sinha [29] reported that colchicine administered intraperitoneally to rats at a dose of 40 μg/kg impaired the accumulation of protein with a radioactive label into synaptosomes. Crothers and McCluer [6] demonstrated that intracranially administered colchicine impaired the axoplasmic transport of protein in mice.

Colchicine (60 μg) administered intracranially to goldfish impaired long-term memory formation of shock avoidance training when administered prior to training but it did not cause amnesia when given after training [5]. Cherfas and

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[2]Present address: Psychobiology Research Laboratory, VA Hospital, Sepulveda, CA 91343.

Bateson [3] found that high doses (256 μg/kg) of colchicine impaired overall responding by chicks on a passive avoidance task but did not clearly impair either acquisition or short-term memory. Murakami [22] has reported that vinblastine administered either peripherally (50 μg) or intracerebrally (0.5 μg) one week prior to training impaired acquisition by mice. Retention test performance was impaired by similar doses administered immediately after training. However, these high doses of vinblastine appear to disrupt performance rather than processes specifically involved in learning and memory storage since mice that were trained previously and subsequently administered vinblastine also performed poorly on the retention test.

The doses used in the above studies appeared to be high, since 40 μg/kg of colchicine administered peripherally to rats has been reported to inhibit fast axoplasmic flow in the central nervous system [28,29]. In addition, high doses of colchicine are associated with a variety of side effects such as blocking the release of neurotransmitters [2, 30, 33] inhibiting uptake of dopamine and norepinephrine [33], and inhibiting the action of vasopressin [32]. The purpose of this study was to obtain additional evidence for a role of axoplasmic transport in the formation of long-term memory using three inhibitors of this process, colchicine, vinblastine and podophyllotoxin.

METHOD

Subjects

The subjects, Swiss Webster (CD-1) male mice, 60–80 days old at training, were obtained from Charles River Breeding Laboratories, Wilmington, MA at 6 weeks of age. They were housed singly 24 hr prior to training and until the retention test.

Drugs

Unless otherwise indicated, all drug and control injections were administered subcutaneously, ANI at a dose of 20 mg/kg and colchicine at 60 μg/kg. Both of these drugs were prepared in physiological saline at a pH of 5–6. ANI was obtained from Pfizer Diagnostics, Clifton, NJ, vinblastine sulfate from Sigma Chemical Co., St. Louis, MO, and podophyllotoxin from Polyscience, Inc., Warrington, PA. Colchicine was obtained from Eli Lilly, Indianapolis, IN in sealed ampoules containing 500 μg/ml. Lumicolchicine was prepared by the ultraviolet irradiation (Xenon lamp source, $CuSO_4$ filter) of colchicine in absolute ethanol [35]. Conversion to lumicolchicine was 96% based upon the final ultraviolet spectrum. The lumicolchicine solution was evaporated to dryness *in vacuo* and the product was dissolved in saline.

Training Procedures

Step-through passive avoidance. The procedure for training and testing mice for the one-trial, step-through passive avoidance task has been described previously [10,11]. In brief, the apparatus consisted of a black start compartment joined to a white shock compartment by a partition containing a mouse hole through which the subjects could enter the white compartment. In the white compartment, footshock was given until the mouse returned to the black compartment. Acquisition of this task is controlled by the latency of the subject to enter and to escape the shock compartment and the footshock intensity [10]. To reduce individual differences in acquisition, only subjects with latencies to enter and to escape the shock compartment of 2 sec (recorded in tenths of sec and rounded to the nearest sec) were used; other subjects were discarded. Less than 15% of the subjects were discarded. The footshock intensity was used to control overall training strength; it was 0.28 mA in Experiment 1 and 0.38 mA in Experiment 4. In order to test retention, the mice were again placed in the black compartment one week after training and the latency-to-enter the white compartment was taken as a measure of retention. Subjects not entering the white compartment within 180 sec were removed and the test was terminated. A latency-to-enter the white shock compartment on the test day of 20 sec or less was defined as amnesia, as this represents the longest latency of the naive subjects entering the white compartment during training. Training and testing were done between 0730 and 1400 hrs. In all experiments, solutions were coded, and "blind" procedures were used.

T-maze active avoidance. The T-maze has been previously described [12]. It consisted of a black plastic start alley with a start box at one end and two goal boxes at the other; a brass floor grid ran throughout the entire maze. Each goal box was fitted with a clear plastic liner, the bottom of which extended below the shock grid. The liner was used to remove the mice from the goal box. The start box was separated from the start alley by a black plastic guillotine door which prevented the mouse from moving down the alley until the training started. Mice were not permitted to explore the maze prior to training. The conditioned stimulus was a loud doorbell-type buzzer. The footshock level was set at 0.35 mA; the intertrial interval was about 45 sec. Subjects were aroused prior to training by rotating their cages every 5 min, starting 15 min prior to training. A training trial consisted of placing the mouse in the start box, then raising the guillotine door and simultaneously sounding the buzzer. Mice not moving to the correct goal box within 5 sec were shocked until they did so. On the first training trial each mouse had to go to the side opposite its first (preferred) response to escape shock. On subsequent trials, the correct goal box was the initially non-preferred side for each mouse. At the end of each trial the mouse was removed to its home cage by carefully removing the goal-box liner and placing it into the mouse cage; then the mouse was placed again into the start box. All subjects received 5 training trials. As training proceeded, a mouse could make one of two types of responses: (a) a response latency longer than 5 sec was classified as an escape since the mouse received shock and escaped from it to the goal box; (b) a response latency less than or equal to 5 sec was classified as an avoidance since the mouse did not receive shock. To reduce the learning variability among subjects, those not having an escape latency on trial 4 or 5 of between 1.5 and 3.4 sec were discarded. Mice not reversing side preference on the first trial within 20 sec were also discarded. The criteria, applied to all subjects, eliminated primarily slow learners and a very few subjects which learned quickly enough to actually make an avoidance. Less than 22% of the subjects were discarded. The test of memory was given one week after training and consisted of retraining to a criterion of one avoidance response. Retraining to the first avoidance response was used because previous work showed that in this training situation the majority of subjects continue to make avoidances after the first occurrence and the additional trials used by criterion measures can confuse the issue of retention and maintenance of the avoidance habit [12]. Mice requiring more than three trials to make the first avoidance response were classed as

amnesic. This criterion provides the optimal separation of well-trained and naive subjects [12].

EXPERIMENT 1

Effect of Colchicine on Retention for Passive Avoidance Training

The purpose of this experiment was to determine if colchicine (60 μg/kg) administered subcutaneously prior to training would impair retention. So that we might have an optimal situation for observing an amnesic effect of colchicine, the footshock level was set low enough so that even control subjects would show forgetting over a 5 week retention period and possibly colchicine would show an even greater rate of forgetting. Colchicine or saline were administered 1 hr prior to one-trial, step-through passive avoidance training. Retention was tested in three separate sets of saline- and colchicine-injected mice at either 1, 3, or 5 weeks after training.

RESULTS

Forgetting increased from 1 to 5 weeks in both saline and colchicine treated mice. The percentage forgetting and the numbers of subjects per group for tests given 1, 3, or 5 weeks after training were as follows: saline 20% (N=20); 40% (N=20); 69% (N=18) versus colchicine 29% (N=28); 50% (N=24); 75% (N=24). While colchicine-treated mice consistently showed poorer retention at each test period, the largest difference between the saline and colchicine groups was only 10%. Since the distribution of test latencies in passive avoidance was bimodally distributed for both colchicine- and saline-treated mice, a Chi-Square Test was used to test for any significant difference in the main effect of all saline versus all colchicine-treated subjects, and a comparison of saline versus colchicine at each retention period. No statistically significant differences were found. Since amnesic effects are more easily obtained with passive avoidance than with active avoidance, and pilot data indicated colchicine did not cause amnesia when given prior to active avoidance either, we discontinued testing for amnesic effects with pre-training colchicine administration.

EXPERIMENT 2

Effect of Both ANI and Colchicine Administration on Retention

In Experiment 1, colchicine might have failed to cause amnesia because in low doses it did not completely block cytoplasmic flow of fast axonal transport [25]. If a large amount of "memory-related" protein was synthesized during and immediately after training, then a partial blockade of axoplasmic or dendritic flow might not be sufficient to prevent long-term memory from forming. We reported previously that as protein synthesis occurs at later intervals after training, the less likely is the synthesis to establish long-term memory [13]. In this experiment, anisomycin (ANI) was used to delay protein synthesis for 2 hr until the capacity for synthesis to establish long-term memory was reduced. Colchicine was administered immediately after training to test if transport of protein into the neuronal processes was important for long-term memory formation. T-maze active avoidance was employed in this experiment because we knew that a single pre-training injection of ANI would not have a measurable effect on retention even with minimal conditions of training needed to insure good retention in the control groups.

TABLE 1

EFFECT OF ANI AND COLCHICINE ON RETENTION (N=20/GROUP)

Treatment Groups	% Forgetting	Trials to Criterion* Mean ± S.E.M.
ANI/Colchicine	70	4.10 ± 0.32
ANI/Saline	10	2.65 ± 0.24
Saline/Colchicine	15	2.35 ± 0.29
Saline/Saline	15	2.65 ± 0.30

*Mean number of trials to make first avoidance response.
ANI/Colchicine differed significantly from ANI/Saline, $\chi^2=15.0$, $p<0.001$, and Saline/Colchicine, $\chi^2=12.38$, $p<0.001$.

The subjects were trained on T-maze active avoidance as described above. ANI or saline was administered 15 min prior to training. The saline- or ANI-injected subjects also received a subcutaneous injection of either colchicine (60 μg/kg) or saline immediately after training. The N per group was 20. The retention test was given one week after training. Forgetting was defined as failing to make an avoidance response on or before the third retention trial.

RESULTS

Retention of the saline controls was good, with 15% of the subjects classed as forgetting and mean trials to make their first avoidance of 2.65 (Table 1). Neither ANI nor colchicine alone had an effect on retention. However, when ANI was administered prior to training and colchicine was administered after training, significantly more mice were classed as forgetting than controls (70% forgetting, mean trials to first avoidance equal to 4.1).

EXPERIMENT 3

Comparison of the Effect of Three Inhibitors of Axoplasmic Flow on Retention

This experiment compared the amnesic effects of three inhibitors of fast axoplasmic flow given after training (colchicine, vinblastine or podophyllotoxin) when ANI was given prior to training. It also tested the colchicine isomer lumicolchicine. These two isomers have similar effects on the central nervous system (e.g., inhibition of vasopressin action, inhibition of nucleoside uptake, [25]), but lumicolchicine has a low binding affinity for microtubule protein; therefore, it is considerably less effective as a blocker of axoplasmic flow [8, 19, 21]. ANI, in combination with lumicolchicine, was used to determine if ANI+Colchicine-induced amnesia was related to side effects *not* associated with blocking axoplasmic/dendritic flow.

The training and testing were the same as in Experiment 2. ANI was administered 15 min prior to training and each of the following was administered immediately after training: colchicine (60 μg/kg), vinblastine (6 μg/kg), podophyllotoxin (3 μg/kg), lumicolchicine (60 μg/kg), or saline. The doses of the three inhibitors were based in part on the work of Paul-

TABLE 2

AMNESIC EFFECTS OF ANI AND THREE INHIBITORS OF AXOPLASMIC FLOW

Treatment Groups	(N)	% Forgetting	Trials to Criterion* Mean ± S.E.
ANI/Colchicine	23	78	4.43 ± 0.24
ANI/Vinblastine	24	83	4.38 ± 0.27
ANI/Podophyllotoxin	25	88	4.16 ± 0.23
ANI/Saline	26	19	2.65 ± 0.21
ANI/Lumicolchicine	20	20	2.75 ± 0.29
Saline/Saline	23	22	2.69 ± 0.31

*Mean number of trials to make first avoidance response.

ANI/Saline differed significantly from ANI/Colchicine, $\chi^2=16.9$, ANI/Vinblastine, $\chi^2=22.32$, and ANI/Podophyllotoxin, $\chi^2=24.19$, and with p values greater than 0.001. ANI/Colchicine also differed significantly from ANI/Lumicolchicine, $\chi^2=17.08$, $p<0.001$. Preliminary tests of Saline/Colchicine, Saline/Vinblastine, Saline/Podophyllotoxin, and Saline/Saline yielded no amnesic effects (% forgetting and N respectively were: 19%, 16; 25%, 16; 17%, 18; and 20%, 15).

son and McClure [25] from which we estimated the ratio of doses needed to obtain similar degrees on inhibition of axonal transport relative to the dose of colchicine being used. The N's are given in Table 2. Retention was tested one week after training.

RESULTS

The retention of the saline control group was good (22% forgetting). Preliminary tests showed that mice given saline prior to training and colchicine, vinblastine, or podophyllotoxin after training did not develop amnesia (Table 2, footnote). However, when ANI was administered prior to training and any of the three inhibitors of axoplasmic flow were administered after training, significantly more subjects were classed as forgetting than controls given only ANI or saline (Table 2). Lumicolchicine given in combination with ANI had no impairing effect on retention.

EXPERIMENT 4A

Interaction Between Colchicine and Protein Synthesis

In a previous study, a "pulse" of protein synthesis could be created by altering the normal schedule under which ANI was administered. ANI administered every two hours maintained continuous inhibition of protein synthesis at 80% or greater, but additional delay beyond the 2 hr interinjection interval allowed recovery of protein synthesis proportional to the length of the delay period [13]. This pulse of protein synthesis against a background of extensive inhibition prevented amnesia when the delay in the injection schedule was sufficiently long. This suggested that protein needed for long-term memory was synthesized during the pulse period. If colchicine blocks the transport of protein(s) needed for long-term memory, then blocking axoplasmic/dendritic flow before, during, and after the pulse should yield a time-dependent amnesic effect with respect to the time of colchicine administration.

Step-through passive avoidance was used in this experiment so that we could test whether the amnesic effect obtained with the ANI-colchicine treatment is generalizable to passive avoidance and because the passive avoidance task requires a small total number of ANI injections. The training procedure was the same as in Experiment 1 except that the footshock intensity was changed from 0.28 mA to 0.38 mA. Mice received three successive injections of ANI or saline at 2 hr intervals beginning 15 min prior to training. Several additional groups received one injection of ANI 15 min prior to training: the second injection was delayed 90 min beyond the usual 2 hour interinjection interval and was given 3.5 hr after the first ANI injection, and the third injection was given 2 hr after the second ANI injection (ANI−90−ANI+ANI). Pilot data indicated that the additional 90 min delay between the first and second ANI injections prevented amnesia induced by ANI+ANI+ANI treatment. The ANI−90−ANI+ANI treated subjects were divided into groups that received either colchicine or saline. Colchicine was administered 1, 2, 3, or 4 hr after the first ANI injection (designated colchicine$_1$, colchicine$_2$, etc.). Saline was administered in place of colchicine either 1 or 4 hr after the first ANI injection. The number of subjects per group is indicated by the numbers in parentheses in Fig. 1. The retention test was administered 1 week after training and drug treatment. Statistical evaluation of the data was by the Chi-Square-Test since the test latencies are distributed bimodally so parametric statistics would not be appropriate.

RESULTS

The test latencies indicate that three successive injections of ANI at 2 hr intervals yielded a significantly higher percentage of mice classed as forgetting than occurred in saline controls (85% vs 10% forgetting, $p<0.001$). An additional 90 min delay in the injection schedule between the first and second ANI injection blocked amnesia (85% vs 12% forgetting for ANI+ANI+ANI vs ANI−90−ANI+ANI/Saline$_{1\ or\ 4}$; either $p<0.001$). Thus protein synthesis needed for long-term memory formation occurred during the 90 min delay period. This replicated previous findings [13]. When colchicine was administered either 1 or 2 hr after the first ANI injection (both injections occurring prior to the pulse of protein synthesis) as much forgetting occurred as with subjects receiving three successive injections of ANI at 2 hr intervals (Fig. 1). The ANI+ANI+ANI group did not differ significantly from ANI−90−ANI+ANI/Colchicine$_{1\ or\ 2}$ (85%, 73%, 65% forgetting, respectively). However, when colchicine was administered 3 or 4 hr after the first ANI injection (during and after the pulse of protein synthesis), this yielded fewer subjects classed as forgetting.

EXPERIMENT 4B

Colchicine might induce amnesia by reducing the availability of catecholamine transmitters at the synapses. A test of this alternative was also made in Experiment 4. AMPT, which blocks the synthesis of dopamine and norepinephrine, was injected with ANI before training or in place of colchicine after training to determine if the ANI-colchicine treatment could have induced amnesia by reducing catecholamine availability. AMPT was either administered simultaneously with ANI or administered 1 hr after the first ANI injection (ANI+AMPT−90−ANI+ANI/Saline$_1$, ANI−90−ANI+ANI/AMPT$_1$).

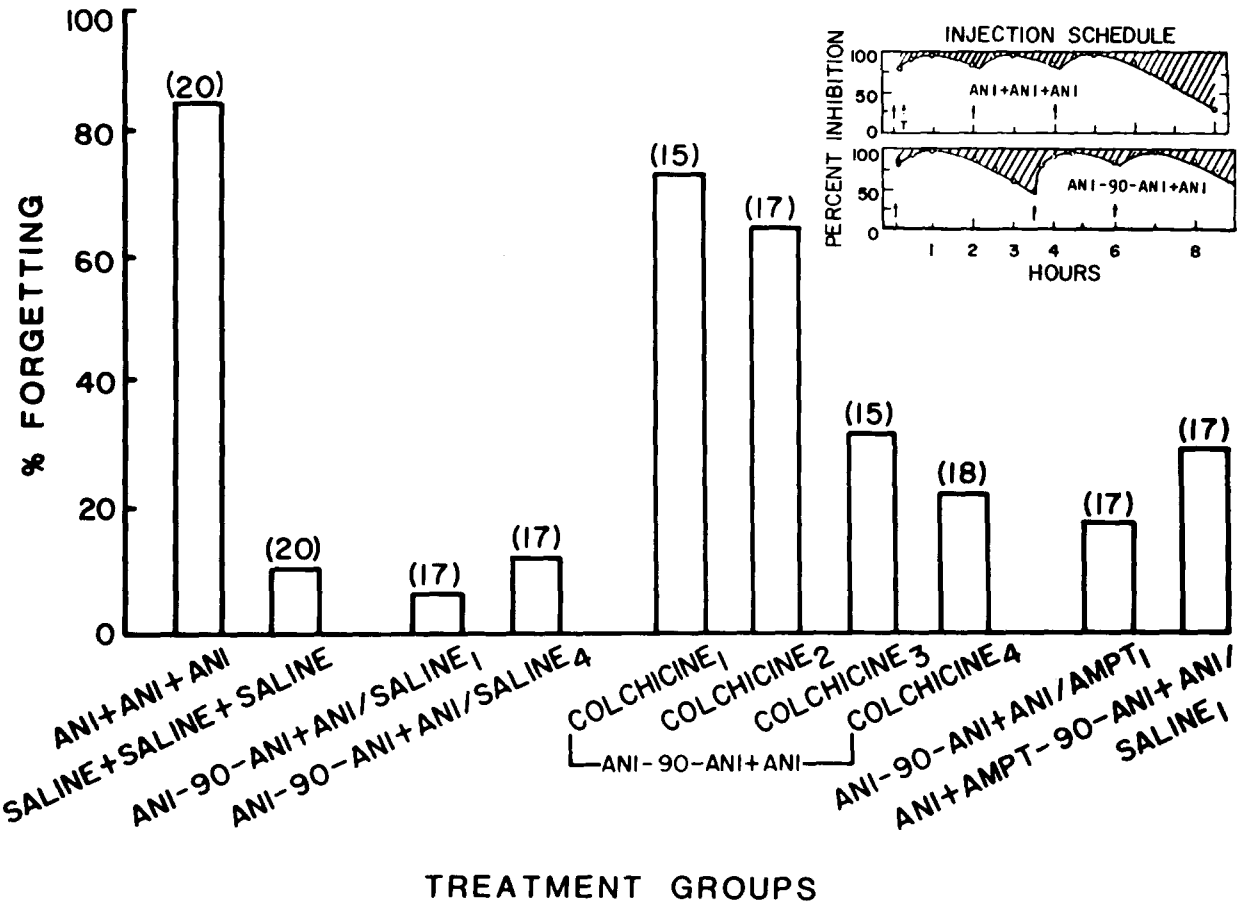

FIG. 1. The effect of colchicine and controlled protein synthesis on retention. The inset shows the effect of an additional 90 min delay in the ANI injection schedule on protein synthesis inhibition. The altered injection schedule results in some recovery of protein synthesis between 2 and 3.5 hrs. The main graph illustrates the results of Experiments 4A and 4B. The numbers above the bars indicate the N per group. Subscripts indicate the time in hours after the first ANI injection that colchicine or saline was injected. Since the test latencies of passive avoidance training are bimodally distributed, non-parametric statistics were used to test for the significance of differences in the number of subjects classed as forgetting and not forgetting (Fisher Exact Probability Test). Three successive injections of ANI significantly impaired retention (ANI+ANI+ANI vs SALINE+SALINE+SALINE, $p=1.6\times10^{-6}$). The 90 min delay in the injection schedule significantly reduced the number of subjects classed as forgetting (ANI−90−ANI+ANI/Saline$_1$ or Saline$_4$ vs ANI+ANI+ANI, $p=8.8\times10^{-6}$, $p=4.9\times10^{-5}$, respectively). The time at which colchicine was administered to ANI-treated subjects affected the number of subjects classed as forgetting (ANI−90−ANI+ANI/COLCHICINE$_{1\text{ vs }4}$, $p=0.004$). Neither group receiving AMPT differed significantly from ANI−90−ANI+ANI/Saline$_1$.

RESULTS

AMPT administered either simultaneously with the first ANI injection or 1 hr after the first ANI injection did not significantly alter the retention of groups having the pulse of protein synthesis (ANI+AMPT−90−ANI+ANI/Saline$_1$, 29%; ANI−90−ANI+ANI/AMPT$_1$, 18%; and ANI−90−ANI+ANI/Saline$_1$, 6% forgetting). The slightly higher percentage forgetting in the groups receiving AMPT is consistent with reports that pretraining AMPT administration can cause amnesia [27].

EXPERIMENT 4C

The purpose of this experiment was to test the generality of the results of Experiment 4A using the active avoidance paradigm. The subjects were as in the previous experiments and were trained on T-maze active avoidance as described in Experiment 2. Four successive injections of ANI (ANI^4) were given at 2 hr intervals starting 15 min prior to training. An additional delay of 90 min (210 min total interinjection interval) between the first and second injection of ANI (ANI−90−ANI^3) was used to block the amnesic effect of ANI^4. The ANI−90−ANI^3 treated subjects were divided into groups which received colchicine 1 or 3 hr after the first injection of ANI or saline 1 hr after the first ANI injection. As in Experiment 4A, subscripts on colchicine or saline indicate the time (in hrs) after the first ANI injection that colchicine or saline was injected. All groups and their N's are indicated on Table 3. Retention was tested one week after training.

RESULTS

Four successive injections of ANI at 2 hr intervals yielded a significantly greater mean number of trials to first

TABLE 3

EFFECT OF COLCHICINE ON THE ANTI-AMNESIC EFFECT OF CONTROLLED PROTEIN SYNTHESIS IN AN ACTIVE AVOIDANCE PARADIGM

Treatment Groups*	(N)	% Forgetting	Trials to Criterion Mean±S.E.M.
ANI^4	18	89	4.50±0.28
ANI^1	20	10	2.65±0.19
$Saline^4$	18	17	2.50±0.21
$ANI-90-ANI^3/Saline_1$	20	10	2.65±0.19
$ANI-90-ANI^3/Colchicine_1$	18	78	4.44±0.34
$ANI-90-ANI^3/Colchicine_3$	19	26	2.79±0.23
$Saline-90-Saline^3/Colchicine_1$	20	15	2.35±0.29
$Saline-90-Saline^3/Colchicine_3$	18	11	2.39±0.26

*Superscripts indicate the number of injections given 2 hrs apart; −90− indicates an additional delay of 90 min in addition to the 2 hr interjection interval; subscripts indicate the time in hours when either saline or colchicine was injected after the first ANI injection. Statistical evaluation of percent forgetting vs not forgetting was by the Chi-Square Test. ANI^4 differed significantly from $Saline^4$, $\chi^2=18.84$, $p<0.001$. $ANI-90-ANI^3/Saline_1$ did not differ significantly from $Saline^4$ indicating the 90 min additional delay in the injection schedule blocked the amnesic effect of four ANI injections. $ANI-90-ANI^3/Colchicine_1$, did not differ significantly from ANI^4, indicating the colchicine given 1 hr after the first ANI injection (prior to the pulse of protein synthesis) prevented the anti-amnesic effect of the 90 minute delay while $ANI-90-ANI^3/Colchicine_1$, differed significantly from $ANI-90-ANI^3/Colchicine_3$, $\chi^2=9.77$, $p<0.01$. Colchicine administered to saline-treated groups did not yield significant amnesic effects.

TABLE 4

EFFECT OF COLCHICINE INJECTION INTO THE CAUDATE ON RETENTION

Quantity Colchicine Injected†	(N)	% Forgetting	Trials to Criterion* Mean ± S.E.M.
	Anisomycin Pretreated Mice		
30 ng	29	69	4.03 ± 0.32
3 ng	28	67	4.28 ± 0.32
300 pg	25	68	4.36 ± 0.41
30 pg	26	53	3.50 ± 0.35
3 pg	23	21	2.43 ± 0.33
	Saline Pretreated Mice		
30 ng	29	10	2.48 ± 0.15
3 ng	27	11	2.26 ± 0.25
300 pg	23	13	2.21 ± 0.27
30 pg	26	15	2.42 ± 0.26
3 pg	25	8	2.44 ± 0.19
ANI with Saline intracerebrally	24	21	2.83 ± 0.34
Saline with Saline intracerebrally	22	18	2.55 ± 0.18

*Mean number of trials to first avoidance response.

Colchicine administration in ANI pretreated subjects yielded significant anmesia with a dose as low as 30 pg (ANI/Saline vs $ANI/Colchicine_{30\ pg}$, $\chi^2=5.71$, $p<0.02$. The ANI/Colchicine groups all differed at $p<0.001$ from their respective Saline/Colchicine controls except for the 3 pg dose.

†The colchicine was administered in 0.5 μl bilaterally at a concentration of 6×10^{-2} mg/ml for 30 ng which was reduced by a factor of 10 for each smaller dose.

avoidance than four successive injections of saline or a single injection of ANI (ANI^4 vs Saline, $t=6.85$, $df=34$; ANI^4 vs ANI, $t=4.00$, $df=36$, both giving $p<0.001$, one-tailed test). Groups with an additional 90 min delay between the first and second ANI injection and given an injection of saline 1 hr after training ($ANI-90-ANI^3/Saline_1$) did not differ significantly from $Saline^4$ or $Saline-90-Saline^3/Colchicine_1$ treated groups. Subjects pretreated with ANI and administered colchicine prior to the pulse of protein synthesis ($ANI-90-ANI^3/Colchicine_1$) required a significantly greater mean number of trials to make their first avoidance than groups given colchicine at the end of the pulse period ($ANI-90-ANI^3/Colchicine_3$) or $ANI-90-ANI^3/Saline$ treated subjects (ANI with colchicine at 1 hr vs ANI with colchicine at 3 hr, $t=3.84$, $df=35$; ANI with colchicine at 1 hr vs ANI with Saline at 1 hr, $t=3.73$, $df=36$, both having $p<0.001$, one-tailed test). When subjects were classed into groups designated as forgetting or not forgetting, similar statistical differences between groups were found (Table 3). This experiment replicated the principal findings of Experiment 4A, thus establishing generality of the results across both active and passive avoidance.

EXPERIMENT 5

Effect of Central Colchicine Administration on Retention

Colchicine does not readily cross the blood-brain barrier [1]. This raised the question whether the amnesic effect of colchicine in ANI-treated mice resulted from central or peripheral effects of the drug. We tested for central mediation of the amnesic effect by giving ANI subcutaneously prior to training and by giving colchicine intracerebrally after training. The mice were prepared for the intracerebral injections by surgery performed 24 to 48 hrs prior to training. This was done so that only the shortest possible time was needed to administer colchicine or saline under ether anesthesia in a stereotaxic instrument. Prior to beginning this study, an extensive effort was made to verify that the stereotaxic coordinates for bilateral injections into the anterior region of the caudate were correct. Thionin injected into the area showed that a volume of 0.5 μl was reliably found in the anterior, medial portion of the caudate and over a 30 min period did not diffuse into the surrounding structures.

The operation consisted of deflecting the scalp and drilling one hole over the caudate in each hemisphere (0.5 mm anterior to bregman, 2 mm left and right of the central suture). The holes were covered with a light application of bone wax and the subject was returned to its cage. Immediately after training, the subjects were anesthetized with ether and returned to the stereotaxic instrument. Colchicine or saline was injected intracerebrally within 3 min after training using a microsyringe fitted with a 31 gauge needle and anchored to the micromanipulator. A volume of 0.5 μl was

injected at a depth of 3.2 mm into each hemisphere of the caudate. After the injection, bone wax was reapplied, the wound closed and the subject returned to its cage. Operated, injected controls were included in every session.

Mice were trained on T-maze active avoidance 24 to 48 hr after preliminary surgery as described above. The results of Experiments 2 through 4 indicated that the amnesia was due to an interaction of ANI and colchicine. In a previous study, we found that injections of ANI into each hemisphere of the caudate caused amnesia [15]. Thus it seemed likely that the caudate could be one area that might mediate the interaction. To determine if a relationship between the dose of colchicine and amnesia existed, colchicine was administered in 0.5 μl into each caudate hemisphere in ANI pretreated mice at the following concentrations: $6\times10^{-2,\ -3,\ -4,\ -5,\ \text{or}\ -6}$ mg/ml. Other groups were given saline 15 min prior to training and bilateral injections of one of the 5 concentrations of colchicine into the caudate. In addition, two groups received either a pretraining injection of ANI or saline followed by a 0.5 μl injection of saline. The groups and their N's are shown in Table 4.

RESULTS

Subjects pretreated with saline and given any of the 5 concentrations of colchicine after training did not differ from subjects given saline before training and intracerebral injections of saline after training. However, subjects that received ANI subcutaneously prior to training and colchicine after training forgot significantly more than ANI-injected subjects given saline injections after training. Colchicine was an effective amnesic treatment in combination with ANI at quantities from 30 ng/hemisphere to 30 pg/hemisphere. Mice given injections of colchicine at 3 pg/hemisphere did not differ significantly from ANI subjects given intracerebral injections of saline (Table 4). The lowest quantity of colchicine injected into the brain that caused amnesia was similar to the amount of colchicine detected in the brain as a result of systemic administration [1].

DISCUSSION

Three types of hypotheses that might account for the amnesic effect of administering both an inhibitor of protein synthesis and an inhibitor of axoplasmic transport are these: (a) potentiation of one of the two effects of the drugs may occur without any interaction; (b) some side effect of colchicine, vinblastine or podophyllotoxin may interact with ANI to cause amnesia or (c) ANI reduces the synthesis of protein(s) needed for long-term retention and colchicine impairs the transport of the residual protein to sites where it is needed to consolidate memory.

Synergistic Mechanism of Action

Research results indicate that colchicine probably does not inhibit the uptake of labeled amino acid into protein as do the protein synthesis inhibitors cycloheximide or puromycin [5, 17, 18, 29]. In addition, Rose and Sinha [29] studied the effect of cycloheximide on axoplasmic flow and concluded that cycloheximide has no greater effect on transport of labeled amino acid than would be expected from its inhibitory effect on protein synthesis. While the evidence suggests that inhibitors of protein synthesis and axoplasmic transport do not mimic each other's action, it is still possible that the combination of inhibitors potentiates the effect of either. Thus the amnesia could have resulted either because an inhibitor of axoplasmic flow increased the duration of protein synthesis inhibition or because an inhibitor of protein synthesis increased the interference with axoplasmic flow. We have not done biochemical studies to test possible synergistic actions of these two classes of drugs.

Side Effects as a Mechanism of Action

Colchicine, vinblastine, and podophyllotoxin block fast and slow axoplasmic transport by binding to the protein in microtubules [6, 20, 25, 35]. A variety of side effects of colchicine or vinblastine administration have been reported which might in part account for the amnesic effect. These inhibitors are known to block the transport of NE [31] or its release [2,33]. The uptake of NE, DA, gamma-aminobutyric acid and glutamate in a rat brain synaptosomal fraction was inhibited by vinblastine (0.05 to 0.25 mM) and colchicine (0.1 to 1.0 mM); the lowest concentrations were clearly less effective at blocking uptake than the high concentrations [23]. Colchicine (1 mM) and vinblastine (0.1 mM) blocked acetylcholine transmission when perfused into the superior cervical ganglion in cat [34]. Colchicine (40 μg) injected intraocularly in pigeons depressed evoked potentials in the optic tectum [26]. RNA synthesis can be inhibited by vinblastine with the high dose of 2 mg/kg [4]. Certain changes in the ultrastructures of neurons in the supraoptic nucleus occurred after administration of colchicine (40 μg) into the subarchnoid space in rat: (a) the Golgi complex was enlarged; (b) the number of mitochondria, lysosomes and phagosomes increased; (c) the nuclear membrane was deeply folded; and (d) the granular endoplasmic reticulum was distended by reticular and filamentous material [24]. Colchicine administration intraocularly at 100 to 500 μg induced an enlargement of the synaptic vesicles, abnormal mitochondria and glycogen granules [7]. While we cannot rule such side effects as part of the cause of the amnesia obtained with ANI and any of the inhibitors of axoplasmic flow, the dosages or concentrations used to demonstrate these effects are considerably greater than used in our experiments.

Possible Role of Protein Synthesis and Cytoplasmic Flow in Memory Processing

A hypothesis based on the principal effect of ANI and colchicine suggested that amnesia resulted from a decrease in protein synthesis and from a reduction of transport of protein to important areas of the cell where it was needed for long-term memory storage. Experiments 4A and C show most clearly how this might work in that long-term retention depended on some recovery of protein synthesis over a 90 min period. When colchicine was administered prior to a resumption of protein synthesis, amnesia occurred; when administered after a resumption of protein synthesis, amnesia decreased as the duration of protein synthesis increased.

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