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Memory enhancement: Supra-additive effect of subcutaneous cholinergic drug combinations in mice

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Abstract. The amnesias characteristic of Alzheimer's disease and other age-related dementias are refractory to conventional pharmacotherapy. A recent treatment strategy is to combine present drugs to improve their memory enhancing effect. We used mice weakly trained on active avoidance in a T-maze to compare the effect of cholinergic drugs, given alone and in two-drug combinations, on retention test performance. All drugs were injected SC immediately after training. Memory retention was tested 1 week later. A dose-response curve was determined for each of four drugs (arecoline, edrophonium, oxotremorine, tacrine) and for each of the six possible two-drug combinations. Each drug and each combination improved retention test performance up to an optimal dose; the improvement decreased with further increases in dose. A striking reduction (66.2%–95.7%) in the optimal dose for enhanced retention was observed with these two-drug combinations.

Key words: Alzheimer's disease – Amnesias – Arecoline – Cholinergic – Edrophonium – Memory – Mice – Oxotremorine – Retention – Tacrine – Tensilon

Research on the mechanisms of memory processing indicates that acetylcholine, catecholamines, and some amino acid neurotransmitters can improve retention (Bartus et al. 1980, 1982; Drachman and Sahakian 1980; Hunter et al. 1977; Il'yuchenok 1976; Myers 1974; Sitaram et al. 1978; Sullivan et al. 1982; Yonkov et al. 1981). The clinical usefulness of drugs modulating these transmitter systems as a means of improving learning and memory may be limited by the high doses that are usually required and by the associated side effects. Recent clinical research has focused attention on cholinergic drug pharmacotherapy (Bartus et al. 1982; Drachman and Sahakian 1980; Ferris et al. 1979; Perry 1980; Peters and Levin 1979) of geriatric amnesias, such as those which are the hallmark of senile dementia.

What is required to improve memory is a drug that enhances memory at low doses without causing unpleasant side effects. Such a goal might be achieved by finding a combination of drugs that potentiate retention but not side effects (Cherkin and Riege 1983; Drachman and Sahakian 1980; Hollister 1981). Recently, we reported that intracerebrally injected cholinergic drugs given in combination improved retention in mice, with marked supra-additive effects on memory processing (Flood et al. 1983). A large decrease in drug dosage was thus made possible, compared to the dose required for equal improvement when the same drugs were administered singly (Flood et al. 1981, 1983). Clinical application of such interactions requires, however, that supra-additive drug effects be demonstrated to occur with peripheral or oral administration.

The present study reports the effect of SC administration of four cholinergic drugs, arecoline and oxotremorine (ACh agonists) and edrophonium and tacrine (anticholinesterases), alone and in combination, on retention test performance of mice 1 week after training and acute drug administration. The effects with SC drug administration confirmed our previous findings with intracerebroventricular injection (Flood et al. 1983). The dose-response curves, as a function of the route of administration and the drugs being combined, differed in detail but not in general shape, which was an inverted U.

Materials and methods

CD-1 male mice at 6 weeks of age were obtained from Charles River Breeding Laboratories, Wilmington, MA. After 1 week in the laboratory, each mouse was individually caged 24–48 h prior to training and until retention testing was completed 1 week later. Each experimental group had n–20 mice; each saline control group had $n=40$ mice. The median body weight was 35 g, with a range of 33–38 g.

Apparatus. The T-maze has been described previously (Flood et al. 1975; Flood et al. 1981). It consisted of a black plastic start alley with a start box at one end and two goal boxes at the other; a brass rod floor ran throughout the entire maze. Each goal box was fitted with a slotted plastic liner (the bottom of which went below the shock grid), which was used to remove the mice from the goal box without hand contact. The start box was separated from the start alley by a plastic guillotine door, which prevented the mouse from moving down the alley until the training started. The conditioned stimulus was a doorbell-type buzzer. The footshock was set at 0.35 mA. All mice were trained on a T-maze active avoidance task between 08:00 and 14:00 h.

Common training and testing procedures for experiments 1 and 2. A training trial started with a mouse placed into

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Table 1. Effects of single drugs on retention test performance (Dunnett's t test was used to determine if the difference between the mean trials to first avoidance for the optimal drug dose and control was significant.)

Dose (μg/mouse)	Arecoline hydrobromide								
	0	14	17.5	26	35	44	52	70	88
Mean trials to first avoidance	4.55	4.30	4.50	3.70	3.25	2.75[a]	3.00	4.15	4.15
SEM	0.21	0.35	0.31	0.33	0.34	0.26	0.30	0.36	0.30
Recall score (%)	25	35	30	45	60	75	70	45	30

Dose (μg/mouse)	Edrophonium chloride									
	0	35	70	105	140	210	245	280	350	420
Mean trials to first avoidance	4.35	4.40	3.80	3.50	3.15	3.10	2.85[a]	2.95	3.80	4.70
SEM	0.21	0.34	0.31	0.31	0.25	0.28	0.27	0.26	0.32	0.33
Recall score (%)	30	30	40	50	65	65	70	65	35	25

Dose (μg/mouse)	Oxotremorine sesquifumarate								
	0	3.5	8.8	17.5	43.8	87.5	131	154	175
Mean trials to first avoidance	4.33	4.00	3.40	3.10	2.85	2.65[a]	2.75	3.95	4.10
SEM	0.21	0.29	0.26	0.28	0.34	0.20	0.29	0.31	0.27
Recall score (%)	25	35	45	60	80	85	70	40	25

Dose (μg/mouse)	Tacrine hydrochloride sesquihydrate							
	0	17.5	35	52.5	70	87.5	105	175
Mean trials to first avoidance	4.15	4.15	3.10	2.45[a]	3.05	2.85	3.91	4.25
SEM	0.21	0.29	0.26	0.28	0.34	0.20	0.29	0.27
Recall score (%)	25	35	55	80	65	65	40	25

[a] Significant at $P<0.01$

the start box. The guillotine door was raised and the buzzer was sounded simultaneously, then 5 s later footshock was applied. The goal box that the mouse first entered on this trial was designated as "incorrect" and the footshock was continued until the mouse entered the other goal box, which on all subsequent trials was designated "correct" for the particular mouse. There was no side preference; half the mice first entered the left arm and half first entered the right arm. It should be added that the sequences of consecutive left or right responses gave a cumulative distribution associated with a bivariate, random, independent variable (Hays 1965). The potential confounding effect of olfactory cues was therefore not considered to be a problem. The apparatus was cleaned with absolute alcohol and allowed to dry after each 10 mice, then washed at the end of each day. At the end of each trial, the mouse was removed from the goal box by lifting the plastic liner and carefully returning the mouse to its home cage. A new trial began by placing the mouse in the start box, sounding the buzzer, and raising the guillotine door, with footshock beginning 5 s later if the mouse had not moved into its correct goal box.

As training proceeded, a mouse could make one of two types of responses. A response latency of 5 s or less was considered an avoidance since the mouse did not receive footshock. A response latency longer than 5 s was recorded as the escape latency from the applied footshock. The mice were weakly trained on T-maze active avoidance by giving them only three training trials, with a 45 s interval between trials. This reliably ensured that few saline control mice (25%–30%) would be classed as remembering the original training. Thus, drug-induced improvement in retention could readily be detected. Two exclusion criteria were applied to all groups to reduce learning variability among mice, as follows. On the first training trial, any mouse with an escape latency greater than 20 s was discarded. Mice not having at least one errorless escape latency between 1.5–3.5 s on training trial 2 or 3 were also excluded since failure to escape the shock within 1.5–3.5 s indicates little or no learning had occurred. Mice with escape latencies of 1 s or less have good retention test performance without drug administration, thus making it difficult to test for improved retention due to drug administration. Less than 15% of the subjects were discarded by these exclusions.

One week after training and drug administration, T-maze training was resumed until each mouse made its first avoidance response. Two measures of retention were ana-

Table 2. Effects of two-drug combinations on retention test performance (Dunnett's *t* test was used to determine if the difference between the mean trials to first avoidance for the optimal drug dose and control was significant)

A: ARE + TAC									
ARE (μg/mouse)	0	0.35	0.88	1.75	3.5	7.0			
TAC (μg/mouse)	0	0.035	0.088	0.18	0.35	0.7			
Mean trials to first avoidance	4.28	3.45	3.00[a]	3.10	2.95	3.60			
SEM	0.18	0.27	0.28	0.26	0.23	0.28			
Recall score (%)	25	45	70	75	70	45			
B: ARE + EDR									
ARE (μg/mouse)	0	0.88	1.31	1.75	3.5	7.0	14.0	21.0[b]	
EDR (μg/mouse)	0	3.85	5.70	7.70	15.4	30.8	61.6	92.4[b]	
Mean trials to first avoidance	4.40	4.25	3.25	2.30[a]	2.75	2.20	2.45	3.65	
SEM	0.19	0.31	0.34	0.28	0.23	0.19	0.31	0.37	
Recall score (%)	25	35	60	85	75	65	55	40	
C: EDR + TAC									
EDR (μg/mouse)	0	1.75	3.50	7.00	17.50	35.00	70.00[b]		
TAC (μg/mouse)	0	0.088	0.18	0.35	0.88	1.75	3.50		
Mean trials to first avoidance	4.08	4.05	2.90	3.50	2.90[a]	3.50	3.35		
SEM	0.19	0.27	0.27	0.26	0.21	0.30	0.26		
Recall score (%)	30	45	65	50	75	65	60		
D: EDR + OXO									
EDR (μg/mouse)	0	2.45	4.9	9.8	19.6	39.2	78.4[b]		
OXO (μg/mouse)	0	0.88	1.75	3.5	7.0	14.0	28.0[b]		
Mean trials to first avoidance	4.03	4.15	3.25	2.30[a]	2.75	2.90	2.95		
SEM	0.20	0.30	0.31	0.30	0.25	0.23	0.31		
Recall score (%)	25	30	55	75	80	75	65		
E: TAC + OXO									
TAC (μg/mouse)	0	0.09	0.18	0.35	0.88	1.75	3.50	8.75	17.50[b]
OXO (μg/mouse)	0	0.0018	0.0036	0.007	0.018	0.035	0.07	0.175	0.35
Mean trials to first avoidance	4.15	3.80	3.90	3.25	3.30	3.15	2.90[a]	3.00	2.90
SEM	0.17	0.25	0.32	0.25	0.25	0.28	0.28	0.25	0.28
Recall score (%)	27.5	35	45	60	55	65	65	70	60
F: ARE + OXO									
ARE (μg/mouse)	0	3.5	7.0	14.0	17.5[b]				
OXO (μg/mouse)	0	0.44	0.88	1.75	2.19				
Mean trials to first avoidance	4.40	3.85	3.55	3.10	2.90[a]				
SEM	0.24	0.33	0.27	0.32	0.23				
Recall score (%)	25	35	55	70	70				

[a] Significant at $P < 0.01$

[b] The stepwise increase in drug dosage was discontinued here because the next increase would yield a recall score of 60% or higher for this drug alone (compare Table 1)

lyzed. The first measure was the mean number of trials to the first avoidance response for all subjects within a group.

The second measure (percent recall score) was derived to better visualize the effects of drug treatments on foot-shock avoidance performance and to correspond with usual reporting practice. Each mouse making its first avoidance in three trials or less was classed as remembering the original training. This criterion was adopted because it provided optimal separation between the retention test performance of naive subjects (with no T-maze training) and well-trained subjects (Flood et al. 1975). A criterion of only one avoidance response was used because in previous experiments we found that when a mouse is removed from the goal box without hand contact (using the plastic liner previously described) it continues to make at least six avoidance responses in succession before it makes another escape response. In a current study employing the same training task, out of 200 mice, 68% reached criterion with no shock, 31% received one shock after making the first avoidance

and 1% received two shocks. Thus, training to a criterion higher than one avoidance response would not provide a better measure of retention test performance and would significantly increase the time and cost of conducting these experiments.

Statistical evaluation. The overall significance of each drug treatment effect was determined by a one-way analysis of variance of the common logarithmic transform of the recall scores to insure a normal distribution of data and to eliminate covariance between means and variance (Keppel 1973; Winer 1971). Dunnett's t test was used to test the significance of all drug group means against the control group mean (Winer 1971); only the comparison yielding the largest t value is reported. Critical table values were obtained for k equal to the number of groups with the degrees of freedom associated with the error term of the ANOVA.

Drugs. Mice received a 0.35 ml SC injection of saline or drug solution within 2 min after training. The dose of drug, expressed as micrograms mouse, is given for each experiment. All solutions were blind-coded to eliminate bias. Training and testing of control and drug groups were essentially random, since the experimenter did not know what treatment was to be given after training or had been given prior to testing. The drugs were obtained from the following sources. Edrophonium chloride (EDR, Tensilon, FW 201.7) was a generous gift from Hoffman La-Roche, courtesy of Dr. W. Scott. Arecoline hydrobromide (ARE, FW 236.1), oxotremorine sesquifumarate (OXO, FW 380.4), and tetrahydroaminoacridine hydrochloride sesquihydrate (TAC, tacrine, FW 261.7) were purchased from Sigma Chemical. Doses are expressed as micrograms of the salt but are referred to by the name or acronym (ARE, EDR, OXO, TAC) of the base. Drug solutions were prepared fresh daily.

Results

Experiment 1. Effect of subcutaneous administration of cholinergic agonists and anticholinesterases on retention test performance

The purpose of this experiment was to determine: (1) if cholinergic drugs that increase the activity of cholinergic receptors directly (ACh agonists: ARE, OXO) or that delay the hydrolysis of endogenous acetylcholine (anticholinesterases: EDR, TAC) would improve retention test performance when administered SC and (2) the shape of the dose-response curves. Mice were trained and injected as described above. The mice received either ARE, EDR, OXO, TAC, or saline. The drug dosages are given in Table 1. Retention was tested 1 week after training and drug administration.

As expected under the given training conditions, the saline-injected control groups had poor avoidance performance (25%–30% recall) on the retention test. Averaging all groups, most mice (85%) in all groups remembered the correct arm for escape from shock. All drugs yielded an inverted U-shaped dose-response curve, with optimal retention test performance occurring with 44 μg ARE, 245 μg EDR, 87.5 μg OXO or 52.5 μg TAC (Table 1). The F values of the ANOVAs were 5.31 (*df* 8,191); 5.15 (*df* 9,210); 6.16 (*df* 8,191); and 6.60 (*df* 7,172), respectively.

Table 3. Comparison of the total drug dose in the two-drug combinations expressed in terms of the equipotent dose of arecoline (EP−ARE) for maximal retention test performance so that the dose of different drugs can be added to estimate the total dose administered

Treatment	Optimal dose (EP−ARE)		Percent dose reduction from dose of ARE given alone (186.4 nmol/mouse)	Cholinergic mechanism
	nmol/mouse	μg/kg		
ARE+TAC	8.1	54	95.7	Agonist+AChE
ARE+EDR	13.3	89	92.9	Agonist+AChE
EDR+TAC	16.4	110	91.2	AChE+AChE
EDR+OXO	29.8	200	84.0	AChE+Agonist
TAC+OXO	31.4	211	83.2	AChE+Agonist
ARE+OXO	63.0	422	66.2	Agonist+Agonist
ARE	186.4	1,250	–	Agonist
OXO	186.4	1,250	–	Agonist
TAC	186.4	1,250	–	AChE
EDR	186.4	1,250	–	AChE

Experiment 2. Effect of two-drug cholinergic combinations on retention test performance

The purpose of this experiment was to determine to what extent pairs of the drugs used in experiment 1 would potentiate each other's effect on retention test performance. We explored both the dose of each drug in the combination and the ratio of the two drugs. In preliminary studies, depending on the pair of drugs being administered, an appropriate total dose at each ratio tested had a maximal effect on performance but only a few ratios yielded recall scores of 70% or greater (Flood et al. in preparation). Each ratio used in experiment 2 is that which yielded both the highest recall score and the greatest potentiation of each drug in the combination. Mice were trained, injected, and tested as in experiment 1. A dose-response curve based on a fixed ratio of drugs in a combination was determined for each of the six possible two-drug combinations: ARE+EDR, ARE+OXO, ARE+TAC, EDR+OXO, EDR+TAC, and TAC+OXO. The specific doses are given in Table 2. The optimal dose is defined as the lowest dose that resulted in the highest recall score. For some of the combinations the dose was not extended to higher levels if the resulting dose of either drug alone would have improved retention test performance (as determined from the dose-response data of experiment 1); higher doses would not have permitted detection of drug supra-additivity.

The footshock avoidance performance of the saline control groups was poor, as intended, with only 25%–30% recall. All optimal doses of the two-drug combinations improved footshock avoidance performance relative to the saline-injected control groups. The F values of the ANOVAs were 4.85 (*df* 5,134); 7.61 (*df* 7,172); 4.56 (*df* 6,153); 9.03 (*df* 6,153); 4.04 (*df* 8,191); and 6.67 (*df* 4,115) for groups A–E, respectively, in Table 2. The optimal dose of each drug in a combination (Table 2) was far less than the optimal dose for the drug given alone; the minimal reductions from the optimal single-drug doses ranged from 66.2%–95.7% (Table 3).

In Table 3, the EP−ARE is the equipotent dose of ARE for maximal retention test performance. EP−ARE is used

so that the doses of different drugs can be added to estimate the total dose administered. The choice of ARE was arbitrary; the same results would be obtained if any of the other drugs were chosen as the basis for comparison. To quantify the overall extent of dose reduction, we proceeded as follows. To compare a single-drug dose with the total dose of a two-drug combination, we first converted all dosages to the molar basis; thus all comparisons refer to the free base of each drug. Each dose, expressed as nanomoles of drug per mouse, was converted to the equipotent ARE dose (EP−ARE). In experiment 1, 1,215 nmol (245 μg) of EDR has essentially the same effect on retention as did 186.4 nmol (44 μg) of ARE. Similarly, 230 nmol of OXO and 201 nmols of TAC are equipotent with 186.4 nmol of ARE. In the two-drug groups, the total dose of drugs administered together can, therefore, be expressed as the sum of the EP+ARE of each drug. For example, in the ARE+EDR group, 7.4 nmol of ARE and 38.2 nmol of EDR (5.8 nmol EP−ARE) were administered together; the total dose expressed in terms of ARE is 7.4+5.8=13.2 nmol. The dose (μg/kg) is based on a median body weight of 35 g. The percent dose reduction is based on EP−ARE. The percent reduction is a measure of relative effectiveness of the drug combinations in relation to the single-drug effects obtained in experiment 1 and indicates that these combinations had greater than additive effects on memory retention.

Experiment 3. Test of proactive drug effects on retention test performance

The purposes of this experiment were to determine if a delayed injection would facilitate memory and whether any prolonged effects of drug administration would facilitate performance on the retention test rather than memory processing. The subjects and training were as for the previous experiments. The subjects were divided into 10 drug groups with 10 mice per group. These groups received an injection of the optimal drug doses indicated in experiments 1 and 2, but 24 h after training rather than immediately after training. A last group of 20 mice received an injection of saline 24 h after training. As in experiments 1 and 2, a retention test was given 1 week after training. Since most studies indicate that memory processing is susceptible to drug manipulation for only a few hours after training, we would expect that the 24-h delay in drug administration would not alter memory processes. Thus, if a drug group showed improved retention test performance, it would indicate a proactive effect of the drug treatment on performance, not on retention.

The results were clear; none of the 10 drug treatments facilitated retention when administered 24 h after training and 6 days prior to testing retention. The recall score for the control group was 20% with mean trials to first avoidance of 4.2. The recall scores ranged from 10%–30% across drug groups with mean trials to first avoidance response ranging from 4.0 to 4.9, which are within the range of control group means to first avoidance in experiments 1 and 2 (Tables 1 and 2).

Discussion

These experiments confirm that single cholinergic agonists or anticholinesterases improve retention test performance when administered SC in appropriate doses, in agreement with previous reports (Bartus et al. 1981; Flood et al. 1983; Sitaram et al. 1978; Strong et al. 1980). The new finding is that SC administration of two drugs in combination improves retention at substantially reduced doses of each drug.

Table 4. A comparison of the total drug dose administered intracerebroventricularly (ICV) or SC needed to achieve optimal retention test performance, expressed in terms of the equipotent dose of arecoline (EP−ARE)

Drug treatment	EP−ARE (nmol/mouse)		
	ICV	SC	Ratio (SC/ICV)[a]
ARE	0.424	186.4	440
ARE+EDR	0.0230	13.2	578
Reduction from ARE alone (%)	94.6	92.9	–
EDR+OXO	0.0190	29.8	1,590
Reduction from ARE alone (%)	95.5	84.0	–
ARE+OXO	0.0172	63.0	3,670
Reduction from ARE alone (%)	95.9	66.2	–

[a] The corresponding ratio for EDR itself (not EP−ARE) was 2,450 and for OXO itself was 8,750

Table 3 shows the EP−ARE for the total amount of drug administered to each subject in all two-drug groups. The percent dose reduction indicates how much less drug was required in the two-drug combinations than for a single drug to achieve equal effects on retention.

Another way of testing for supra-additive effects, a term which is preferred to "potentiation" in this case (Fingl and Woodbury 1975), is to estimate the theoretical optimal dose if the drugs in a two-drug combination acted only additively to improve memory retention. The EP−ARE for each drug is 186.4 nmols. If the interaction between two drugs, say EDR+TAC, were simply additive, then one-half of the optimal dose of EDR (93.2 nmol, EP−ARE) plus one-half of the optimal dose of TAC (93.2 nmol, EP−ARE) should represent the optimal dose of the combination, i.e., 186.4 nmols, EP−ARE. The observed optimal EP−ARE for the two-drug combinations ranged from 63.0 to 8.1 nmol (Table 3), a reduction of 66.2%–95.7% from the theoretical additive doses. Thus, the observed interactions are clearly supra-additive, by factors of 3.0–23.0.

Previously, we reported (Flood et al. 1983) that combinations of ARE, EDR and OXO showed supra-additive effects on retention test performance when injected intracerebroventricularly (ICV); TAC was not included in that experiment. The dose-response curves for single drugs and for two-drug combinations are inverted U-shapes, whether administration is central (ICV) or peripheral (SC). Comparing the results of these experiments with previous experiments using ICV administration, it appears that single drugs administered centrally or peripherally show dose-response curves with steep slopes on each side of the curve; this is more pronounced with centrally administered drugs (Flood et al. 1981, 1983). Two-drug combinations injected peripherally tend to induce maximal memory retention over

a wider range of doses than those administered centrally; this has the clinically desirable effect of broadening the therapeutic window. Table 4 compares the optimal dose of drugs given ICV with those given SC. Three of the two-drug combinations run in experiment 2 were also studied using the ICV route of administration under comparable training conditions. For the purpose of establishing how much of a dose reduction occurred with these combinations under each route of drug administration, all drug doses were converted to EP−ARE and the total amount of drug administered in the combinations was compared to the appropriate optimal dose of ARE given alone.

In Table 4, it is clear that as would be expected SC doses are consistently larger than ICV doses; the ratios of EP-ARE doses were 440–3,670 (SC/ICV). The percent dose reduction in the two-drug combinations relative to ARE alone was more uniform for ICV administration (94.6%–95.9%) than for SC administration (66.2%–92.9%). This difference may be due to pharmacokinetic factors, which are minimized by ICV administration. Overall, both routes of administration (SC and ICV) demonstrate marked supra-additive effects of two-drug combinations upon retention.

It is generally considered that the blood-brain barrier excludes systemically administered EDR from the central nervous system (CNS). Our results with SC injection indicate that sufficient EDR penetrates the barrier to produce the observed effects or else the effects are related to peripheral interactions. The optimal SC dose of EDR (245 μg per mouse) exceeds the optimal ICV dose (0.10 μg per mouse) by a factor of 2,450.

If toxicity were potentiated to the same degree as memory retention, then very little would be gained by the use of combination drug therapy. TAC and OXO improve memory retention when given alone in spite of the observed tremors. However, tremors were not observed in any of the drug combinations at doses that improved memory retention.

In preliminary studies of drug effects on accelerating roto-rod performance, we have found that the least toxic dose for disrupting roto-rod performance was 2.3 times higher for ARE+TAC than for ARE or TAC alone and 5.1 times higher for TAC+OXO than for TAC or OXO alone. Thus these combination are less toxic than any of the single drug treatments. Clearly it is the reduction in drug dosage in combinations and the absence of toxic interactions, that result in decreased toxicity in combinations at doses that improve memory.

In a study of OXO-induced hypothermia, it was found that the optimal dose for improvement of memory induced a 3.5° C drop in rectal temperature, which was blocked by simultaneous administration of ARE. The combination of TAC+OXO, which so effectively blocked impairment of roto-rod performance by both drugs, did not block hypothermia. Of the combinations we have studied thus far and for the behavioral toxicity tests conducted, including LD_{50}'s, toxicity is at most additive and in a few cases infra-additive.

Our interpretation of the data assumes that the limited amount of training that the mice received did not result in a sufficient amount of ACh receptor activity for adequate long-term memory to be formed. Improved retention test performance in mice injected with the receptor agonists, ARE and OXO, or the anticholinesterases, EDR and TAC, could improve memory processing by prolonging receptor activity initiated during training. Evidence that prolonging the duration of cholinergic receptor activity results in better memory retention has been reported in a study in which a noneffective dose of ARE (50 ng) was injected ICV at each of three 90-min intervals starting immediately after training. The group receiving three successive injections of ARE had significantly better retention test performance than saline-injected controls (Flood et al. 1984). Appropriate controls showed no enhancing effect on retention when 150 ng was injected 180 min after training, indicating that enhanced retention was not due solely to the total amount of drug received.

In Table 3, the column head "Cholinergic mechanism of action" indicates the generally accepted mode of action on the CNS. This is not necessarily the mechanism of action on retention test performance since primary stimulation of the cholinergic system could have secondary effects upon other transmitter systems, which directly or in interactions with other physiological systems could alter memory processing by nonspecific mechanisms.

The cholinergic system is known to be important in physiological functions throughout the organism, in addition to its role in the CNS. Manipulation of the cholinergic system also affects function of the cardiovascular, respiratory, thermoregulatory, gastrointestinal, urinary and skeletal muscle systems, and could in turn affect performance on memory retention tests. Evaluation of the effects of combinations of cholinergic drugs upon these non-CNS systems would therefore be of interest in reaching an understanding of the mechanisms of the effects we observed. In addition, the nature of the paradigm leads to the conclusion that memory processing was in fact affected by the drug treatments. Since the mice were injected *after* training, the drug treatments could not have affected acquisition. Proactive effects upon retention test performance per se are unlikely in view of the 1-week interval between drug injection and retention testing. Experiment 3 confirmed this conclusion. Thus, the drug treatments appear to alter processes occurring after training, and before retention testing, in a manner which leads to improved memory retention.

Supra-additivity was found both for combinations of a receptor agonist and an anticholinesterase (e.g., ARE+EDR, ARE+TAC, OXO+EDR, OXO+TAC) and for combinations of drugs having the same mechanism of action (e.g., ARE+OXO, EDR+TAC). The first type of interaction could be interpreted as reflecting the synergistic interaction between the exogenous agonist and the prolonged action of endogenous acetylcholine that would result from its slower hydrolysis by inhibiting acetylcholinesterase.

The interpretation is more difficult for the second type of interaction. One possibility is that the pharmacokinetics of the drug combinations provided a prolonged increase of ACh receptor activity that favors memory processing. This could be reflected in differential rates of peripheral degradation and speed of entering the CNS as well as differential action on receptor subtypes.

For the ARE+OXO combination, supra-additivity could reflect the differential action of the two drugs on subtypes of central ACh receptors. Briggs et al. (1982) have described two muscarinic agonist receptor subtypes in rat brain. For the EDR+TAC combination, the interaction of anticholinesterases could be interpreted as differential

affinity of the two drugs for multiple forms of acetylcholinesterase; at least four molecular forms have been identified (Massoulie and Bon 1982).

Specific classes of cholinergic drugs may have a greater effect upon the memory retention of animals than of man and dose-response may vary as a function of age. Nevertheless, the limited animal data available suggest that similar effects on retention can be obtained with drugs that increase cholinergic system activity, despite differences in some cholinergic brain functions between animals and man. The weak training that our mice received may mimic the apparent memory impairment associated with the altered cholinergic system of the aged. If the results of these studies can eventually be generalized to aged humans, at least in principle, they suggest that carefully selected combinations of drugs might provide a useful approach to providing the degree of enhancement needed for "meaningful" improvement in some individuals with failing memory, while reducing drug dosage and undesirable side effects.

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