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Altered Learning by Recipients of Brain Extracts from Trained and Retrained Donors[1]

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WEBSTER, J. C. AND K. A. FOX. *Altered learning by recipients of brain extracts from trained and retrained donors.* PHARMAC. BIOCHEM. BEHAV. 2(2) 209–213, 1974. – Male C57Bl/6J mice were trained to make either left or right turns in a Y maze to receive a food reward. One group was trained to criterion in one day; another group was similarly trained and maintained at that level of training for four additional days. The water-soluble fraction of the donor brains was transferred by subdural injection to recipient animals of the same strain. Recipients trained to the same side as their donors required fewer trials to reach criterion than did recipients trained to the opposite side. There was no effect due to maintenance of donor training. In addition, donor and recipient performance was negatively correlated.

Behavioral bioassay (interanimal transfer) Approach learning Y maze C57Bl/6J mice

THE DISCOVERY of macromolecular changes associated with learning and memory has stimulated investigation along several parameters. One of these is the influence of the amount of training. Data on this parameter has come from experiments investigating the incorporation of radioactive label [1,5], the effect of antimetabolites on memory [6], and the use of brain material from trained donors in recipients [2, 3, 10]. The latter studies demonstrated that when donors are given ten trials per day, the maximal effect on recipients is found after donors are trained for ten days, with the effect being independent of the dose [4].

An inherent difficulty in interpreting these data is that animals trained by equal numbers of trials may differ in the degree of training achieved. Moreover, the decay of memory from day to day is not known and may also differ among individual animals. In order to circumvent the disadvantages of this procedure, all experimental donors in the present study were trained to the same criterion. A portion of these were retrained for each of four additional days and thus were maintained at the criterion level of training for five days. This procedure makes it more convenient to draw inferences about the differential macromolecular synthesis of the various donor groups. In particular, it provides indirect evidence for the hypothesis that restoration of the decayed memory on days two through five may be due to restoration of degraded macromolecules.

Additionally, donor brains were prepared individually for injection so that the correlation between donor's and recipient's performance could be assessed. A negative correlation has previously been reported [7].

METHOD

Animals

Six week old C57Bl/6J mice (Jackson Laboratories, Bar Harbor, Maine) were housed individually and maintained for three weeks on a feeding schedule during which a dish containing four g of powdered Purina Lab Chow was presented at 9 a.m. and removed at 3 p.m. Water was provided ad lib.

Apparatus

A Y maze was constructed with each of the three arms equal in all respects so that each end box could be used both as a goal box and as the start box of the next trial. The angle between arms was 120°. The sides and bottom were constructed of 1/4 in. clear Plexiglas and were blackened on the outside. The removable top was of 1/8 in. clear Plexiglas. The end boxes were 10 × 15 cm with a plastic food dish 4.5 × 2.5 × 0.5 cm on placed against the far wall of each. Between each end box and the connecting stem was a manually operated guillotine door of 1/8 in. clear Plexiglas. The distance from the door to the center of the maze was 32 cm and the stem width was 5 cm. The height of the maze was 10 cm. Illumination was by two overhead table lamps 45 cm from the top of the maze which contained 40 W bulbs.

Donor Procedures

Donors were not fed for 48 hr prior to the first day of

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training. On Day one, donors were given 20 nonreinforced trials in the maze to acclimate them to the apparatus and to assess their turning preference. Animals showing a marked preference (16/20 turns to one side) were not used. All animals were trained opposite to the preference shown in the pretest, until the criterion of seven consecutive correct turns was achieved. The reward for each correct response was a 20 mg food pellet (Noyes Co., Lancaster, NH). A trial was begun when the door to the start box was raised and ended when the door to the goal box was lowered. Although a wrong turn at the choice point was technically the criterion for an incorrect response, in practice there were no incidents of direction reversal in the stem. This was probably due to the narrowness of the stem. Approximately one-half of all donors were trained to turn left and one-half to turn right. The donors which were to be given retraining trials on the following four days were maintained on the feeding schedule. On each of Days 2 through 5 these animals were retrained to the criterion of seven consecutive correct responses. Control donors were handled and maintained on the feeding schedule but were given no maze experience.

Donors were sacrificed by cervical dislocation 1 hr after the end of the last training session. Within one minute the brain was removed, the olfactory bulbs and cerebellum disattached and discarded, and the remainder rapidly frozen on dry ice in a glass finger bowl. Brains were stored at −20° C until homogenized.

Biochemical Procedures

The brains were individually homogenized in 3 ml of 10^{-2} M potassium phosphate buffer, pH 7.6, containing 10^{-4} M EDTA. Three ml of saturated phenol was then added and the mixture stirred for four hr at 4° C. The mixture was centrifuged for 30 min at 25,000 g and the top aqueous phase removed, frozen and lyophilized. The powdered product was stored in closed vials at −20° C until rehydrated for injection.

Pretesting of Recipients

In the week prior to injection, the preference of each potential recipient was determined in the same manner as had been the donors'. Again, those animals with a marked preference (16/20 turns to one side) were not used. On the basis of the pretest, recipients were paired so that the two recipients receiving material from a common donor would have approximately equal and opposite turning preferences. Animals were also assigned to groups so that the same range of preferences was represented in recipients of donors trained one day and in recipients of donors trained over five days.

Injection of Recipients

Animals were anesthetized with ether and a 0.5 cm incision was made in the forehead. A hole was drilled in the anterior portion of the parietal bone two mm left lateral to the sagital suture to avoid excessive bleeding from the suture. The drill diameter was chosen to secure a snug fit for the 26 ga injection needle. A 50 μl fixed needle Hamilton syringe was used. The needle was shortened to the thickness of the mouse skull at the point of injection. The dried brain extract was dissolved in 40 μl of distilled water. Twenty μl was injected over a 2.5 min period into each member of the recipient pair; each recipient received 0.5 brain equivalent. The liquid was observed to spread over the area of the forebrain equally on both sides of the sagital suture. The biochemical preparation, injection and testing procedures were all performed in a double blind fashion.

Testing of Recipients

The first testing session was approximately 24 hr after the time of injection. Recipients were tested daily for five days. The animals were not fed on the day of injection but were fed for 3 hr after each testing session. On each of the five days, the recipients were trained to the criterion of seven consecutive correct trials. Correct responses were reinforced with 20 mg food pellets. Table 1 describes the groups.

RESULTS

Figure 1 shows the mean trials to criterion on each of the five days of testing for each of the five recipient groups. A repeated measures analysis of variance showed the difference between the five recipient groups to be highly significant, ($F(4, 84) = 4.40$, $p<0.01$, as was the difference over days, ($F(4, 336) = 13.97$, $p<0.001$. There was no groups-by-days interaction effect. Homogeneity of variance ($p<0.01$) was ascertained by Hartley's test [11]. A posteriori comparisons [11] were performed on pairs of groups for the results collapsed over days. The differences between the following pairs were found to be statistically significant ($p<0.01$): 5−0/5−S, 5−0/1−S, 1−0/5−S, 1−0/1−S, C/1−S.

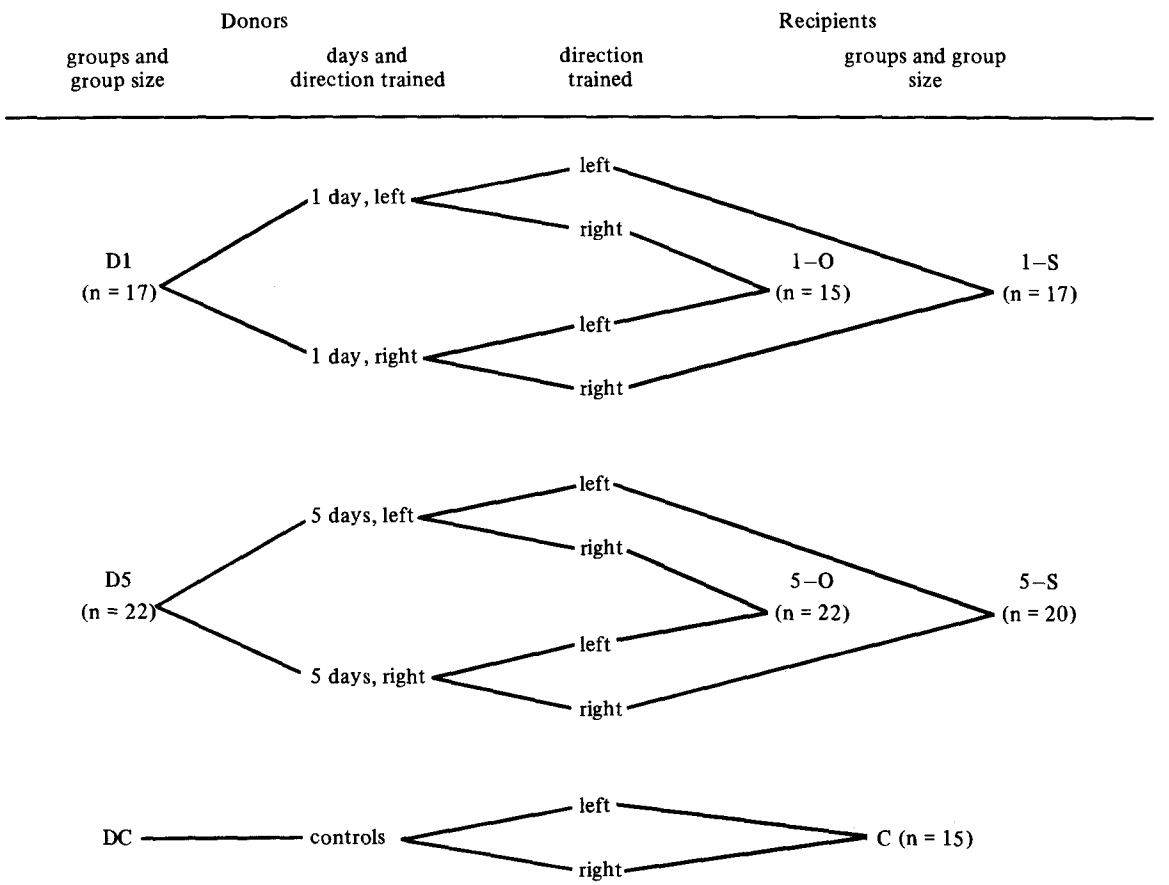
A number of selected correlations between donor and recipient performance were made for the first day and five-day total results. Two correlations were found to be significant. These were: D5 (first day) vs 5−0 (five day total), $r = -.487$, $p<0.05$; and D5 (five day total) vs 5−0 (first day), $r = -.608$, $p<0.01$. Of the 12 correlations computed, 11 were negative, although only the two cited were significant. The binomial probability (Sign Test) of 11/12 is sufficiently small ($p = 0.003$) to suggest a general negative correlation between donor and recipient performance.

DISCUSSION

It can be seen from Fig. 1 that the behavioral bioassay was successful. The recipients trained to turn the same direction as their donors required the fewest trials to reach criterion. The recipients of control material required an intermediate number of trials and those trained to turn in the opposite direction required the most trials to reach the criterion. If the control material can be considered neutral, then relative to the control recipients the performance of the 1−S and 5−S groups was facilitated and that of the 1−0 and 5−0 groups inhibited. This conclusion can only be stated tentatively since, even though the 1−S and 5−S groups are statistically different from the 1−0 and 5−0 groups, only the 1−S group is statistically different from the C group. Thus it can only be stated that there is at least either facilitation or inhibition and the results of the present study favor facilitation. The importance of the internal control (same vs opposite instead of experimental vs control), along with the need for improved methodology is therefore indicated. One improvement should be to increase the specific activity of the active ingredient through a superior isolation procedure. Perhaps then both facilitation and inhibition can be demonstrated. It should

TABLE 1

DERIVATION OF DONOR AND RECIPIENT GROUPS



D1: donors trained to criterion on only one day
D5: donors given four additional days of training
DC: handled control donors, no maze experience
1–S: recipients trained to the same side as one-day-trained-donors
1–O: recipients trained to the opposite side as one-day-trained-donors
5–S: recipients trained to the same side as five-day-trained-donors
5–O: recipients trained to the opposite side as five-day-trained-donors
C: recipients of control donor material

be noted that there is no need to compare the recipient groups to the donor groups since the C recipient group was pretested and tested in the same manner as the donors and additionally received the surgical insult and injection.

The behavioral bioassay, erroneously called transfer of information in the past, is still looked upon by many investigators with skepticism. One argument forwarded by the skeptics is the inability by some people to replicate the basic results. Ungar [8] has compared the procedures used by successful and by unsuccessful laboratories in an attempt to resolve this contention.

In comparing the results of this study to those cited above [2, 3, 10] it can be seen that despite the highly symmetrical paradigm employed, there was no inversion effect such as is frequently found when symmetrical designs are used. Also, although the largest difference among recipient groups was observed on the first testing day, the level of significance for the difference among groups was greater for the data collapsed over days. Thus, the warmup effect previously seen [3,10] did not appear when the present design was used. Some researchers may object to comparison of studies in which different strains of animals, different tasks, etc., have been employed. However, it is likely that the symmetry of the training procedure and the spacing of trials made the largest contributions to the results cited by those studies. The extraction procedure used has also caused questions to be raised as to the nature of the active ingredient and to the effect of the contaminat-

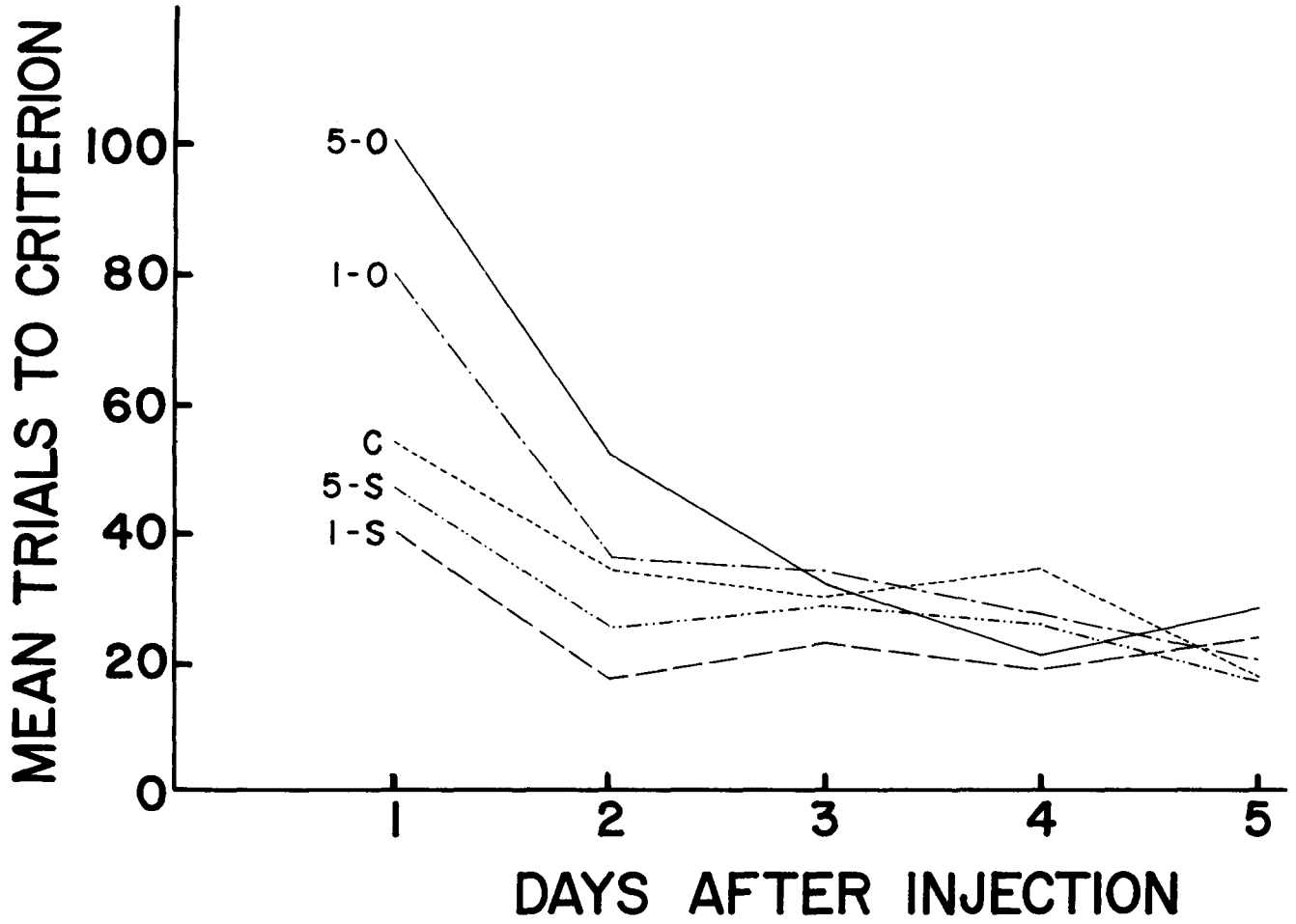


FIG. 1. Mean trials to criterion on each of the five days of testing for each of the five recipient groups.

ing phenol. Although the cold phenol procedure is a method for extracting RNA, the aqueous phase is not free of other material. Indeed, spectrophotometric analysis using the A_{280}/A_{260} ratio showed the existence of proteinaceous material in our extract. The phenol extraction is used by all labs currently using behavioral bioassay and has been used by most laboratories citing positive results in the past. Ungar [9] used the cold phenol extraction as the first step in his isolation of the dark–avoidance producing pentadecapeptide scotophobin. Regarding the phenol contamination, it is true that phenol is soluble in water. However, all recipients received the same account with no apparent ill effects. Dialysis was not used to eliminate the phenol because scotophobin and other behavior-modifying macromolecules have been found to be dialyzable.

The negative correlation between donor and recipient performance is an agreement with previous observations [7]. When recipients are trained in the same direction as their donors, animals receiving material from donors which required few trials to reach the criterion are found to require many trials to learn the task. Recipients of brain material from donors which required many trials seem to need fewer trials to learn the same task. The converse is true for recipients trained in the opposite direction. The following inference are based on the theory that the facilitation or inhibition of recipient learning or responding patterns is solely due to macromolecules they have received and which control recipients have not received. These macromolecules represent a portion of those synthesized by donors under the stimulus of training, control donors not having synthesized these particular molecules. From the correlation data, one can infer that fast learning donors have synthesized less material when criterion is met than slower learning donors which thereupon have more active material to transfer (assuming proportionate losses due to the extraction procedure). The average recipient (trained in the same direction as its donor) of the slow material needs to manufacture less material of its own to reach its own criterion level of the active macromolecule. The converse would be true for recipients trained in the opposite direction as their donor.

The second major inference drawn from this study

concerns the coincidental decay of memory and degradation of macromolecules between learning sessions. This could not be examined in the previous studies since number of trials and not degree of learning was kept constant. In the present study, each animal was trained to the same criterion in one day's session. One group was sacrificed. The other group's performance was brought back up to criterion on each of four additional days. Under the present hypothesis, each animal had synthesized a maximal number of active macromolecules after the first day. For those in the retrained group, any loss due to degradation was replaced in the next session; i.e., the performance and the active macromolecules were both brought back up to maximum. Since the same average number of active molecules existed in the one-day-trained and the retrained donors at the time of sacrifice, the facilitation (or inhibition) of their respective recipients should be equivalent. This is indeed what was observed. Thus we have added indirect evidence that memory of a learned task is proportional to the synthesis of specific macromolecules.

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