FINAL REPORT

STUDIES OF MEMORY TRANSFER IN RATS

by
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The work accomplished under this contract represents an intermediate phase of a program which had been previously initiated in our laboratory, and is currently being continued under the sponsorship of the National Institutes of Health. In the ONR sponsored program, five tasks were originally specified: (1) To design a standard behavioral test which could be used to assay "transfer effects" obtained by brain extracts; (2) To determine standard conditions for testing for transfer effects; (3) To investigate subcellular localization of the active components, by means of differential centrifugation of brain homogenate; (4) To compare effects obtained in different training situations; (5) To study the feasibility of transfer studies in cats.

Although work is still continuing in most of these areas, preliminary results on each of the above problems were obtained during the contract period.

(1) A standard behavioral test was developed, consisting of a right-left discrimination problem, in which a rat is required to select one of two bars in a modified Skinner Box, for a food reward. Recipient rats are first trained to operate a single-lever box, then given two pretests, with bilateral reinforcement (for either response) in the two-lever box, and are then injected intravenously with brain extract. The injections are followed by six five-minute test sessions (two per day) in the two-bar box, with bilateral reinforcement to keep the responses from extinguishing. This technique has been published in a paper by Rosenblatt and Miller, "Behavioral Assay Procedures for Transfer of Learned Behavior by Brain Extracts", Proc. Nat. Acad. Sci., 56 (1966) pp. 1423-1430 and 1683-1688. It has also been described in an informal report by Rosenblatt, Helms, Othmer, and Rosen, which is included as a supplement to this report. Statistical techniques and computer programs for evaluation of data obtained by this procedure have now also been completed.

(2) An intensive study of concommitant variables, and their effect on performance of rats in transfer tasks was conducted during the past year. The variables which appear to be most important to control include the activity level, or speed of learning, of the recipient rat (measured by the number of responses in the one-bar box on the third preconditioning session); the degree

of starvation, and the preliminary right or left bias of the recipients. In all tests run during this period donors were trained for ten days; future experiments will investigate the duration and other aspects of the donor training procedure as contributing factors. Statistical programs for control of these concommitant variables by analysis of covariance and related regression techniques have been designed, but at present we recommend the more conservative procedure of running large numbers of rats and controlling the principal variables by excluding from the sample any rats who are not within preassigned bounds. The effects of varying dosage have been under the most intensive investigation, and it now appears that the dose-response curve shows a number of independent effects which are superimposed on one another, possibly attributable to distinct chemical factors. Some of these effects lead to a "positive transfer" phenomenon in which the recipients show the same side bias as that which the donors have learned, while others lead to a "negative transfer", in which the side bias is reversed (left-trained extract producing right side preferences, and vice versa). The analysis of each of these effects, and its dependence on dosage is most complicated, and will require considerably more time to disentangle in detail. Significant effects have been found, however, over a dosage range from 1/80 brain to 4.0 brains per recipient rat, and it is possible that even smaller doses may produce significant effects with increasingly refined extracts.

(3) A single pilot study was carried out on subcellular localization, which seemed to indicate no activity in nuclear, myelin, and cytoplasmic fractions, some activity in the microsomal fraction, and maximum activity in the mitochondrial fraction (which would have contained membrane fragments and other debris, in addition to mitochondria). A report on this study will be published after replications have been completed.

(4) Alternative training situations tested have been discussed in the Rosenblatt-Miller paper referred to above. These included maze tests and boxes in which the rat was required to choose between a right and left door. Additional experiments included a test of savings methods for evaluating transfer, which seemed to have no advantage over the other methods in use, and a box with two feeders and a single bar, where the feeder (left or right)

first approached by the rat was recorded. This latter design also seemed to offer no clear advantages. After several unsuccessful attempts at training rats in a box with visual stimuli to identify the correct lever, an experiment on a white-black visual discrimination with a Lashley jumping stand was completed, with a significant positive transfer effect in a pilot run. A set of semi-automated jumping stands have now been built, and visual discrimination experiments will be continued with these.

(5) A pilot study on transfer in cats has been completed by Rodman G. Miller, employing a Y-maze in which donor animals were trained to turn right or left for a food reward. The procedure was equivalent to that employed for rats with a similar Y-maze. A slight but inconclusive indication of a negative transfer effect was found, with low dosages, with a small number of cats in the experiment. The effect seemed to have about the same magnitude as might have been expected from rats, under analogous conditions. While this should ultimately be reexamined, we believe that further studies with cats are premature at this stage.

With the completion of most of the requirements for an assay procedure (albeit this is still crude and demands large numbers of test animals for reliable results) our attention has shifted to purification and refinement of the extract, and most of our work under the new NIH grant is expected to be concentrated in this direction.

SUPPLEMENT TO

STUDIES OF MEMORY TRANSFER IN RATS FINAL REPORT

PROTOCOL FOR BEHAVIOR INDUCTION EXPERIMENTS

By Frank Rosenblatt, Karen Helms, Susan Othmer, and Steven Rosen

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OUTLINE OF EXPERIMENTAL DESIGN

The procedure described here is an outgrowth and refinement of behavioral assay procedures described by Rosenblatt and Miller in PNAS, 56 (Nov. and Dec. 1966) 1423-1430 and 1683-1688. Basically, the procedure is as follows:

1. One group of donor rats is trained (10 days, 15 min. per day) to push the left-hand bar of a pair of bars at one end of a training box, for a food reward. A second group is trained to push the right-hand bar for food reward. No negative reinforcement is used. The foodcup is at the opposite end of the box from the levers.

2. The brains of the left trained rats are combined in a pooled homogenate, from which an extract is obtained by procedures detailed in the following pages. A second extract is prepared by the identical procedure from the brains of the right trained rats.

3. A group of recipient rats is preconditioned for 5 daily sessions of 15 minutes in a box with a bar at one end and a foodcup at the opposite end, being taught to press the bar for a food reward.

4. The recipient rats are pretested (2 5-minute sessions) on the sixth day in the two-bar box, being fed for either bar, so as not to train them for a particular side bias.

5. The recipient rats are assigned at random to two or more subgroups, each of which is injected intravenously (via the external jugular vein) with one dosage of one of the two extracts. It is recommended that several subgroups (consisting of at least eight rats per subgroup) should be used, with at least three dosages for each of the two extracts. Recommended dosages are 0.5, 1.0, and 2.0 donor brains per recipient. This distribution of dosages is important since there appear to be effects which mimic the behavior of the donor rats at intermediate, and possibly at high dosages, and effects which invert the behavior of the donor rats at low dosages, particularly with recipients who are in the top 30 percent on activity as measured in the third preconditioning session. (See the above PNAS reference for details of this effect.)

6. Beginning the day after injection, the recipients are given two five-minute test sessions per day in the two-bar box, being fed regardless of which bar they push. With a properly preconditioned rat, this guarantees a high yield of responses in each test session, without extinction through the test series which is continued for three days. Test sessions are identical with pretest sessions in every detail. Test sessions on any one day are spaced about six hours apart.

7. The number of times that a rat chooses the left bar after a trip to the food cup, and the number of times the rat chooses the right bar after a trip to the food cup are recorded. In the one-bar boxes, the number of cycles between foodcup and bar is recorded. A floor pedal in each box indicates to the counting equipment when the rat has completed a cycle from the lever end of the box to the foodcup. The rat obtains at most one pellet (45 mg Noyes grain pellets) for each trip from the cup to the bars, and only the first bar-press after going to the foodcup is recorded. The resulting choices are recorded by automatic counters as the number of "right decisions", the number of "left decisions", or simply the number of cycles in the one-bar boxes. Records are also kept of the weight of each rat, which is reduced to 80 percent of the initial weight by starving the rat except for reinforcement until the weight falls to this level, and then feeding it one large chow pellet per day, with an additional pellet for each additional 5 grams below the "target weight". Rats are weighed and fed at the end of each day's test and training sessions.

8. The percent left responses is computed for each rat for each test and pretest session. The mean of this percentage for all six test sessions is also computed. The effect of an extract is generally measured by the difference between the mean of these left percentages for the group of left-injected rats at a given dosage and the mean of the left percentages for the group of right-injected rats at that same dosage. The significance of this difference is measured by a U-test (see PNAS reference for further details). High correlations (.60 to .80, typically) are found between the percent left on the second pretest and percent left on subsequent post-injection tests. An analysis of covariance technique is recommended to control for the effects of this correlation. Due to the strong tendency of active rats to invert at low dosages, while inactive

rats tend to give "positive" transfer at the same dosages, it is recommended that sufficient rats should be run to permit separate analyses for rats giving over 30 cycles (reinforced responses) in their third preconditioning session, and rats with 30 or less responses in the third preconditioning session. There is also a tendency for less starved rats to invert more than hungry rats, which may also be controlled by covariance methods if weight data are recorded. The percentage weight loss at the time of injection has been used in our studies as a measure of starvation.

Additional measures of performance which have been found to yield significant results with large numbers of recipients have included the number of "extreme rats", whose mean percentage left responses over the six tests is greater than 90 or less than 10, and a contingency table showing the numbers of rats whose percent left responses is greater than the mean or less than the mean, in the left-injected and right-injected groups. We have found, for example, that in a group of several hundred rats accumulated over a large number of repetitions of this basic design, about 88 percent of the "active" rats (over 30 preconditioning cycles in the third session) who were injected with left-trained extract at dosages less than one brain per rat were inverted (went to the right) while the majority of active right-injected rats (at the same dosages) went to the left. This inversion tendency is either reduced or converted to a positive tendency for the inactive rats, and tends to disappear at higher dosages. There is some indication from our most recent experiments, however, that very high dosages may again lead to a diminution or possibly a reversal of transfer effects. A similar result has also been reported to us informally by several other investigators.

The rats used in our current series of experiments are all Holtzman females, obtained at 60 days old. On arrival the rats are kept for several days to several weeks in large colony cages before being used in an experiment. Older rats tend to give markedly lower activity levels. The day before starting training or preconditioning, rats are placed in individual cages, weighed, and started on the reduced feeding schedule. Care is taken to maintain an ample water supply at all times.

The one-bar and two-bar boxes are modifications of Scientific Prototype box Type A-100, with the cup centered at one end and the bars symmetrically

mounted at the opposite end. There are no signal lights, water feeders, or other special components present, the box deliberately being stripped of all distracting elements. We have found that any extraneous devices or even drill holes in the box have a pronounced effect in reducing bar-pressing activity. At present we use six boxes in parallel, with interchangeable 1-bar and 2-bar boxes which can be installed in soundproof chambers as shown in figs. E through H, in the accompanying photographs. Feeders are standard Scientific Prototype pellet dispensers, and the soundproof chambers are built by the same company.

Photographs A through D show details of the injection procedure. The rat is anesthetized lightly with ether, and fastened to a board with protected aligator clips held by rubber bands (A). A wad of cotton wetted with ether is used to maintain the anesthesia. After swabbing the area over the right jugular vein with ethanol, an incision is made as shown in (B) and (C). The injection is made with the needle held as in (D), entering the vein through the margin of the overlying muscle, which stabilizes the vein and needle. The incision is then dusted lightly with sulfathiazole, and closed with a single wound clip. If a vein is dammaged during the operation, the opposite vein may be used, but with a practised operator this should rarely happen.

In handling the 90 percent acetone fraction (see following section) extreme care must be taken in injection, which is done very slowly with artificial respiration if the rat shows any sign of respiratory arrest. This fraction and the 60 percent fraction appear to be the two most active ones in our experiments.

CHEMICAL EXTRACTION PROCEDURE

A. General

The extraction procedure used in our lab was determined through a series of practice extractions-- using dry weight, Ultraviolet spectra, total organic assay, and the Folin-Ciocalteau protein assay to measure the extent of extraction; and through the series of actual experiments with trained rats. The method most recently developed and primarily used in our later experiments, consists of fractionation of a water extract of the brain by a series of acetone concentrations and subsequent re-extraction of the precipitated material with water. This procedure takes between eleven and fourteen days to complete depending on the amount of brain to be extracted. Temperature is controlled to 0°-10°C. Special attention is paid to temperature control during the actual acetone fractionation and ranges from 0°-4°C. All centrifuging is done on a Spinco L-2 preprative ultra centrifuge using either the Type 21 or Type 19 rotor with a speed setting of 15,000 rpm. Homogenizing is done with a Sorvall omnimixer, speed setting 4 for one minute. The Burrel wrist action shaker is used for all shaking.

A. General

B. Removal of Brains

1. Rats decapitated without anaesthetic
2. Brains removed within 30 seconds
3. Groups of 30 brains homogenized in 3 times the wet weight of the brains (3W) glass distilled water (0-4°C), (ca. 1.6 g/brain)

C. Water Extraction of Brains

1. Shake homogenate 1 hour (0-10°C)
2. Centrifuge 1 hour (21 rotor, 15,000 rpm 0-4°C)
3. Supernatant (S_1) stored (0-4°C)
4. Pellet rehomoginized in 3W H_2O
5. Shake 2 hours
6. Centrifuge 1 hour
7. Supernatant (S_2) stored
8. Pellet rehomogenized in 3W H_2O
9. Shake 12-15 hours
10. Centrifuge 1 hour

D. Acetone Fractionation

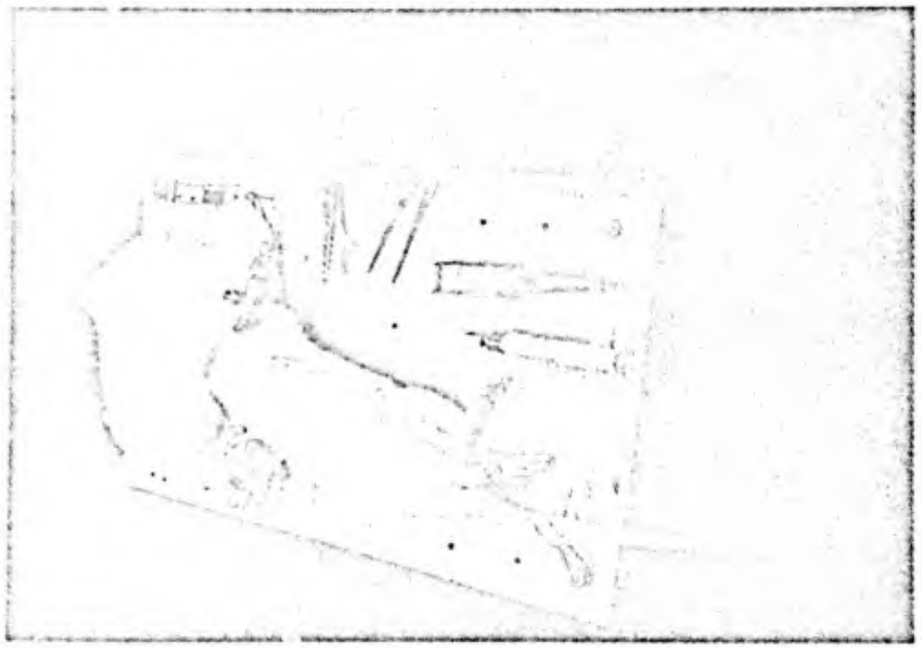
1. Supernatant (S_1, S_2, S_3) combined and buffered at pH7.4 with .03M phosphate buffer
2. Acetone added to 10% final concentration (100% acetone was added from a graduated cylinder with hand stirring. Practice extracts indicate that slower additions (10-15 ml/min) produces a cleaner fractionation)
3. 24 hour precepitations (0-4°C)
4. Centrifuge 30 minutes (19 or 21 rotor, 15,000 rpm, 0-4°C)
5. Pellet homogenized in 1.5W H_2O and lyophilized to dryness

6. Acetone added to supernatant to 40% final concentration repeat (3) - (5)
7. Acetone added to supernatant to 60% final concentration repeat (3) - (5)
8. Acetone added to supernatant to 90% final concentration repeat (3) - (5)

E. Water Extraction of Acetone Fractions

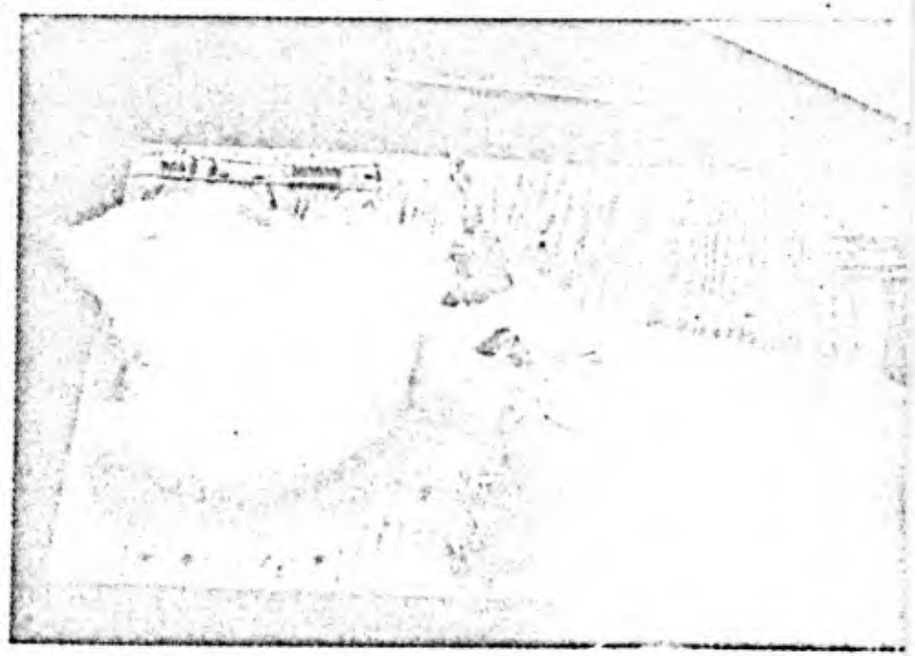
1. Homogenize each dried acetone precipitate in 1.5W H_2O (based on total wet weight)
2. Shake 24 hours (0-10°C)
3. Centrifuge 1 hour (21 rotor, 15,000 rpm. 0-4°C)
4. Supernatant (AS_1) stored (0-4°C)
5. Rehomogenize pellet in 1.5W H_2O repeat (2) - (4)
6. Rehomogenize pellet in 1.5W H_2O repeat (2) - (4)
7. AS_1, AS_2, AS_3 combined and lyophilized to final injection volume
8. Centrifuge 5 minutes (21 rotor, 3,000 rpm, 0-4°C) to remove suspended material

A.



a

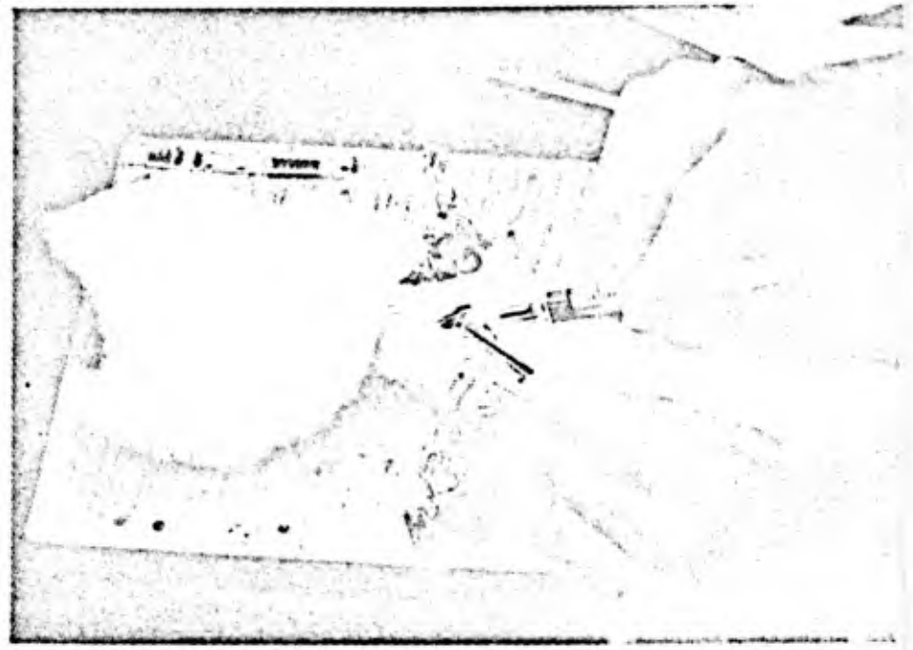
C.



B.

b

D.



E.

a

G.

c

F.

b

H.

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13. ABSTRACT

Experiments on transfer of behavioral phenomena from trained to untrained rats by means of brain extracts are described. These experiments cover standardization of test procedures, concommitant variables, subcellular localization of activity, and a pilot study with cats.

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