MASTER

NEUROBIOLOGY PARADIGM

THE TRANSFER OF MAGAZINE TRAINING BY INJECTION OF
BRAIN RNA IN RATS

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ABSTRACT

Food-deprived rats were trained to approach a milk dipper in an operant conditioning chamber in response to a distinct "click" made by the dipper when it was elevated into the chamber. An RNA extract was prepared from the brains of these animals, as well as from the brains of control animals that had received no such training. The RNA was injected into the brains (sub-arachnoid space) of naive recipients, who were then required to learn to press either of the two bars in the chamber in order to elevate the dipper and get milk. Recipients injected with RNA from trained donors typically learned the problem within 5 days. Recipients that received RNA from untrained donors typically did not learn the problem until after 5 days, if at all.

INTRODUCTION

Since 1965, more than 30 "memory transfer" experiments with rats have been performed in this laboratory, the majority of which yielded successful results. Of the various types of experimental procedures we have tested, the one described below seems the most promising and hence the most suitable for replication in other laboratories. We have now performed enough parametric studies to be fairly sure of what most of the critical variables are that must be controlled if the experiment is to yield positive transfer results.

In the experimental paradigm described below, we made an attempt to transfer the tendency to approach a food cup, NOT the tendency to press a lever in an operant conditioning chamber. We picked this situation for several reasons.

It was our feeling that the simpler the habit-to-be-transferred, the more likely it was that we would obtain positive results. Second, we wanted a situation in which the recording of data, and to a great extent the training of both donor and recipient animals, could be handled automatically, thus freeing us from criticisms concerning possible experimenter bias. Third, we wanted a situation in which we could maximize the possible differences between experimental and control recipients. The magazine-approach paradigm seemed to satisfy all three criteria.

Picture a rat in a Skinner box. Typically there is a lever near to which is mounted a food cup. The rat must learn to press the lever in order to get food pellets which drop into the cup. The closer the cup is to the lever, the more rapidly the learning takes place. If one mounts the food cup on the wall opposite to the lever, the rat learns very slowly, but still it will usually learn. To retard the speed of learning even further, one may substitute a milk dipper for the food cup. In such a situation, a dipper automatically elevates a small portion of milk into the chamber for a few seconds after each bar-press, but the milk is withdrawn if the rat does not drink it immediately. In order to associate the milk with pressing the bar, the rat must cross the chamber to the food magazine within a few seconds after it presses the bar. Rats usually learn such a task only with great difficulty. In order to train a rat under these conditions, one must break the training up in two parts. During the first part of the training, the rat is put into the box and the dipper is elevated automatically at regular intervals no matter what the animal is doing (the lever is disconnected from the dipper during this part of the experiment). Since the solenoid operating the dipper makes a distinct "click" when it elevates, the rat soon learns that the sound of the click means that food is available and approaches the food magazine each time it hears the click. Once the animal has made the "food-click" association, it learns the "bar-press-click-food" association

rapidly.

It was our plan to give our experimental donors just the first part of the training (so-called "magazine training") during which they learned to approach the milk dipper at the sound of the click. RNA would be extracted from their brains and injected into the brains of untrained experimental recipients. Control recipients would receive RNA injections from donors not given the magazine training. Both groups of recipients would then be required to learn to press the bar to get the milk WITHOUT first being given magazine training. If the "food-click" association could be transferred biochemically, the experimental recipients could be expected to show fairly rapid learning of the bar-press habit, while control recipients would be expected to learn the task slowly, if at all.

We have made use of this paradigm in some ten different experiments and have always achieved success so long as (a) the amount of RNA injected was at least equivalent to the amount extracted from about one-third of a donor brain, (b) the recipient animals were properly gentled and deprived prior to injection, and (c) the experimental and control group recipients were balanced (in terms of their pre-injection response rates) prior to receiving the RNA.

EXPERIMENTAL PROCEDURE

Treatment of Subjects:

The subjects, both donors and recipients, were experimentally naive male hooded rats of the Long Evans strain (obtained from Maxfield Animal Supply Co., Cincinnati, Ohio; and Blue Spruce Farm Inc., Altamont, New York) having an average body weight of 250 gm. (range: 240 - 260), and were 50 - 60 days old at the beginning of the handling and deprivation schedules. The animals were

maintained in group cages (metallic, 7 x 14 x 20 in.), 5 per cage, and received food (Purina Lab Chow) and water ad lib. for 5 days.

Eighty per cent of their ad lib. feeding body weights were determined based on the average of their weight during the last 3 days. On the 6th day after arrival, each animal was moved to a metal individual cage (7 x 8 x 10 in.). As maintained, the animals could not see each other but were exposed to normal laboratory conditions. Once daily each animal was gently handled for 3 min.; the handling was followed by weighing and feeding. Each animal was given 10 gm. of food for that day when its body weight was 80% of its ad lib. feeding weight. This weighing and feeding procedure was continued throughout the experiment for both donors and recipients. The handling schedule was maintained for more than 10 days in the donors before their training began and for more than 20 days in the recipients before pre-injection testing.

Apparatus for Training and Testing.

All training (donors) and testing (recipients) was conducted in two identical wooden two-bar operant conditioning chambers (Fig. 1) with a 16 x 14 in. mesh floor and with 7 in. high walls one of which, a sliding door, consisted of a one-way glass. The chambers were housed in separate sound-insulated enclosures covered with fiber glass. The interior walls and floors of the chambers were painted flat black. Ceilings consisted of glass through which the inside of the chambers were illuminated by 14-W cool white bulbs (fluorescent).

The manipulanda, two square bars (1 x 1 x 1/4 in.) protruded, 8 in. apart, into the chambers through vertical slits in one of the walls 2 in. above the floor. A vertical force on each bar of approximately 24 gm. triggered a condenser discharge into an AC motor that activated a metal dipper which delivered 0.08 ml of

Fig. 1. Apparatus used in the magazine training and the pre- and post-injection tests. (S = solenoid; D = dipper; D. A. = dipper area; B = bar.)

milk through the hole in the bottom of the dipper area. A cavity was made 2 in. above the floor so as to form the dipper area (2 1/2 in. in diameter, 1 1/4 in. deep) in the wall opposite to the one in which the bars were set. A solenoid, placed behind the dipper, generated a distinct click (which was much louder than usual clicks in conventional Skinner boxes) and lifted up the dipper through the hole. A milk dish which was placed directly under the dipper area was sufficiently large to contain enough milk for 180 continuous reinforcements and deep enough to prevent the recipients from stealing the milk by stretching their forelegs through the hole.

In our later experiments, a photo-cell device was attached to the dipper area so as to record the number of times the animal stuck its nose inside the dipper area.

A jet of air was introduced into the sound-insulated enclosure through a small hole (1/2 in. in diameter) made in an upper part of a wall. Another small hole was connected with a vacuum device. These two attachments provided cool fresh air, constant white noise, and removed warm stale air from the inside of the chamber throughout the experiment. Temperature of the inside of the chambers was kept at approximately 70 - 75° F.

The milk dish, dipper and the dipper area (inside and outside of the chamber), bars and bar area were cleaned and fresh sawdust was placed in the tray under the mesh floor of the chamber whenever necessary (usually every 4 hours). Condensed milk to which was added 10% sucrose was used as a reinforcer.

During the donor training, both bars were disconnected from the dipper mechanism so that a bar press did not activate the click noise or dipper. During the recipient testing, the bars were connected to the dipper mechanism so that each bar press activated the dipper (which made a "click" as it elevated into place).

Training and testing of both donors and recipients proceeded automatically throughout all experiments. The animals were put into the chamber when it was dark. Immediately after the door was closed and the house light was turned on, the first dipper presentation occurred (in donor training).

Training of Donors.

Each donor animal was trained to approach the dipper under a VI-45 sec. continuous reinforcement (CRF) schedule. The animal was given 60 trials per session for 5 days (plus one extra session at the start for hand-shaping). During the first two days, the dipper was elevated for 20 seconds during each training trial. On day 3, the time was cut to 10 seconds for each presentation, to 7.5 seconds on day 4, and to 5 seconds on the final day of training. The donors were maintained at 80 per cent body weight and were trained when 23 hours deprived. After each training session, the animal was weighed and given enough Purina Lab Chow to bring it back up to 80 per cent of normal body weight.

During the preliminary shaping session, the dipper (with milk in it) was held up by E's hand from the beginning of the session until the animal found it and consumed the milk. The animal was given the milk 10 times in this manner whenever it approached the dipper. No click was sounded at this stage of shaping. Next, the click was sounded while the animal was sticking its nose inside the dipper area deep enough so that it could very easily find the dipper with milk immediately after the click in spite of any emotional response the animal might evince to the onset of the click. This click-dipper presentation was repeated successively more than 10 times until the rat consumed the milk within 20 sec. 10 times successively. Then, the click-dipper presentation was withheld until the animal moved away from the dipper area an inch or two. After the click-dipper presentation was repeated for

5 - 10 times under this condition, the inter-trial interval was gradually increased up to 60 sec., and then it was gradually shifted to the VI-45 sec. CRF schedule. Total number of dipper presentations was approximately 60. Almost all animals established dipper approach response during this shaping session.

Untrained donor animals were treated similarly to the trained donors outside of the chamber situation. These animals had been handled and deprived and maintained at 80 per cent body weight throughout. During the last 6 days prior to the sacrifice, untrained donors were given extra handling equivalent in time to the trained donors and weighed, fed, and maintained at 80% body weight. The untrained donors did not experience the chamber situation, however, and were not fed milk.

Trained and untrained donors were sacrificed in a paired fashion.

Preparation of Brain Nucleic Acid Mix (RNA)

Each of the trained and untrained donor animals was sacrificed by decapitation, using a guillotine device, at 8 hours after the final training session or the final treatment (handling, weighing and feeding) in a manner which minimized emotional disturbances. The brain was removed without damage and was cut on a line joining the superior colliculi and the rostral end of the pons, and cut at the roots of the olfactory bulbs so as to secure the entire cerebrum. Each cerebrum was frozen in a beaker placed on the powdered dry ice within 1 - 2 min. after the decapitation. The cerebrums were kept in a freezer overnight (or approximately for 8 - 16 hours). The frozen cerebrum was homogenized in a 30 ml glass homogenizer with a teflon pestle with 10 vol. of cold saline (0.154 M) for approximately 3 min. at 208 rpm. The homogenate was poured into a cold 100 ml beaker placed on ice, 5 vol. of cold liquefied phenol (88%) was added to this homogenate, and the mixture was stirred for 30 min. at 4° C (in a refrigerator). The mixture was poured into 15 ml.

corex centrifuge tubes and then centrifuged at 20,000 x G for one hour at 2° C in a Sorvall centrifuge with an SS-34 rotor. The clear supernatant was carefully removed with a pasteur pipette; contamination with phenol or with the interface materials was avoided. RNA was precipitated from this supernatant by successively adding sufficient 1 M $MgCl_2$ to bring up the supernatant to a concentration of 0.1 M $MgCl_2$; 2.5 vol. of cold ethanol (95%) was then added. The mixture was shaken for approximately 30 sec. In most of the cases the RNA precipitated out at this stage gradually as a filamentous cottony precipitate. The tubes were put back onto ice immediately and were stored in a freezer over night (approximately for 15 hours) to further increase the precipitation. The RNA was collected and packed in the tip of the tubes by centrifuging this suspension at 2500 rpm for 30 min. at 4° C, using an International centrifuge. The aqueous layer was decanted, and the tubes were kept upside down in the refrigerator for ca. 3 min. Five ml of 75% ethanol was added to each pellet (amount equivalent to the RNA mix from one cerebrum), and the pellet was completely dissolved in the ethanol. The suspension was centrifuged at 2500 rpm for 30 min. at 4° C. This washing procedure was repeated at least 3 times to complete the removal of remaining phenol. The remaining ethanol was evaporated off by blowing an air jet across the solution the ca. one min. on ice. The dry pellet equivalent to the amount of RNA from one cerebrum was dissolved in 0.8 ml saline. (The yields of the RNA were in the range of 0.8 - 1.2 mg per gm. of cerebrum as determined from the optical density at 260 mp).

Each preparation was stored in a freezer until the time of injection (one to 3 days).

The entire process of extraction was carried out at 2 - 4° C.

Injection into the Subarachnoid Space of the Brain.

At 10 hours after the pre-injection test, after which each animal did not

receive food (so that the animal was under 34 hours food deprivation), the recipient animal was injected with RNA from a trained or untrained donor. The injection was made into the space between the olfactory bulbs so as to facilitate rapid diffusion of the injected materials into the subarachnoid space of the brain while the animal was under pentobarbital anaesthesia.

Prior to the injection, recipient animals were assigned to groups by equating their scores during the pre-injection response test. Each of the trained and untrained RNA preparations was given a code number and injected blind. The recipient animals were tested blind as well.

Prior to injection of the RNA, each animal was anaesthetized with an intra-peritoneal injection of sodium-pentobarbital in a manner that minimized emotional disturbance. A cut was made along the line of suture sagittalis between the animal's eye balls with a sharp knife, remaining muscles were removed and the skull was exposed. The incision was thoroughly sterilized (with zephiran-chloride: ca. 0.2%). A dental drill with a one mm wide tip was used to make a hole in the suture sagittalis directly between the eye balls (i. e. , above the space between the olfactory bulbs). The drill tip was placed on the suture sagittalis so as to form an angle of 45° with the frontal plane (Fig. 2).

The hole was drilled gently only about 2/3 of the way through the skull, to prevent mechanical leakage of the RNA solution after injection. A 27-gauge needle was placed in the hole and was rotated several times under moderate pressure for 10-15 sec. to slightly pierce the remaining bone. This needle was then removed, and a 250 µl tuberculin syringe with a 30-gauge 1/4 in. long needle was used to inject the RNA. This syringe had been kept in a refrigerator and loaded with a 200 µl of cool RNA solution immediately before the injection. The tip of the needle was introduced by rotation under minimum pressure. The needle was kept at a 45° angle

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The Turnover of Radioactive Phosphate Injected into the Subarachnoid Space of the Brain of the Rat

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In the literature little information is given on the rate of phosphorylations in the brain, and even the few studies which have been made in this field are without real value because of unsatisfactory experimental conditions and methods. Perhaps the main reason for this neglect is that many acidic substances hardly penetrate into the brain at all from the remainder of the organism. The 'blood-brain barrier' acts as an extremely effective obstacle to any exchange of such substances between the central nervous system and the rest of the organism, and thus makes the former with respect to permeability a virtually 'closed' system. Hevesy (1939a, b) and Hevesy & Hahn (1940) have shown that even a fairly long time after subcutaneous or intravenous injection of ^{32}P only a very small fraction of the substance could be found in the brain and a number of workers have, erroneously, interpreted these results as implying that the brain must have a very low turnover rate (Borell & Öström, 1947; Reiss, Badrick & Halkerston, 1949). This, however, by no means follows from the experimental findings.

Bakay & Lindberg (1949), in their investigations on the role of the cerebrospinal fluid in the metabolism of the brain, showed that ^{32}P injected intracisternally spread very rapidly into different parts of the rabbit's brain, and that after a relatively short time a considerable part of the newly introduced phosphate could be found in organic linkage. But these experiments did not yield a detailed picture of the metabolic conditions. The object of the present study was to investigate the extent and the rate of incorporation of the newly introduced phosphate into the various acid-soluble phosphorus fractions and also the turnover rate of these fractions inside the barrier.

EXPERIMENTAL

Operative procedure. White rats (100-150 g.) were injected with about 0.1 ml. of a solution containing approx. 6.1 mc. ^{32}P and a negligible amount of phosphate. The injections were made without anaesthetic as follows: the injection needle was placed in the sutura sagittalis on a level with the connecting line between the two eyes and forming an angle of about 45° with the frontal plane (see Fig. 1) and

Fig. 1. Intracranial injection on rats.

was introduced by rotation under moderate pressure. The injected solution thus enters the space between the bulbi olfactorii, whence it can spread easily in the subarachnoid space without injuring important motor or vegetative centres. Following the injection the condition of the animals was observed. Usually no change was noted. In the cases in which convulsions, paralysis, or other symptoms of relatively severe brain injury were observed, however, the animals were either rejected or used for longer experimental periods.

Preparation of samples. The animals were killed by drowning in acetone cooled to -60 to -70° with CO_2 , in which they died instantly. They were taken out about 15 min. after death and kept in solid CO_2 until required.

The brains were removed under continuous cooling with solid CO_2 with the aid of a hammer and chisel (see LePage, 1946). A small portion of the medulla oblongata (1-5 mg.) was placed directly in the electron counter to see whether the labelled phosphate had entered and spread in the brain.

*Is the same thing true
with case of
macromolecules
RNA?*

with the frontal plane, 2 mm deep from the outside surface of the skull. Two hundred ul of the cool RNA solution were injected into each recipient over a 5 min. period.

The needle was withdrawn slowly as the injection was made so that leakage of the injected solution was minimized. After complete withdrawal of the needle, the incision was sterilized and the epidermal tissues were dried slightly with cotton for 30 sec. The incision was closed without suturing by pressing the skin edges together with moderate pressure from a pair of forceps.

Testing of Recipients.

(A) Pre-Injection Test.

Each recipient animal was tested 10 hours before injection (i. e. , 14 hours before the first session of the post-injection test) under the same experimental conditions as obtained during the post-injection test. Animals were assigned to experimental or control groups according to baseline response tendencies during the pre-injection test.

The pre-injection test lasted for 60 minutes, during which time a bar-press elevated the dipper for 20 sec. It was typically the case that few if any animals actually obtained the milk during the pre-injection test, however.

In several of our early experiments in this series, we found a significant correlation between the number of bar-presses the rat made during pre-injection testing (regardless of whether or not it obtained reinforcement) and the probability that it would eventually learn to press the bar to obtain the milk. In general, it appears that animals with a high pre-injection response rate (more than 10 bar-presses during the hour's test) will learn the task no matter what one injects them with, while animals with a very low response rate (0-2 bar-presses) fail to learn

no matter what one injects them with. The reasons for this fact are obvious and have nothing to do with "memory transfer" per se. Rats with a very high initial response rate are active animals, hence much more likely to explore the box immediately after a bar press, hence more likely to discover the connection between the milk and pressing the bar. Rats that are (for some reason) low responders tend to be frightened animals that cower in the corner of the box and do not explore; this high emotionality would very likely overwhelm the effects of injection of trained material.

It is important to note, then, that the "transfer effect" can best be demonstrated when one uses animals with a "medium" pre-injection response rate (2 - 10 bar-presses) and when one makes sure that experimental and control groups are balanced in terms of this factor. Any experiment that does not employ such balanced, pre-tested groups must be held suspect no matter what its results.

It has been our experience that the average pre-injection response rate varies widely in animals obtained from the same supplier at different times. We find it expedient to order at least twice as many recipient animals as we plan to use so that we can discard the very high and low responders prior to injection.

(B) Post-Injection Test

Following injection, each animal was weighed but was not fed; thus when it was given its first post-injection test 14 hours after the injection, the animal had been food-deprived for 48 hours. The post-injection testing procedure was the same as was used for the pre-injection test. The dipper was elevated for 20 sec. after each bar-press, the inter-session interval was 24 hours, the animals were food-deprived for 23 hours (after the first session) and were maintained at 80% of normal body weight across sessions.

Criterion for learning was specified as being 60 "correct" bar-press

responses within 30 minutes. "Correct" bar-press responses were defined as those responses that actually actuated the dipper. Any responses the animal made during the 20 sec. dipper-holding period (after a "correct" response) were thus not counted. The animals were tested for 10 sessions, or until they met the criterion.

RESULTS

The results of three experiments are shown in Tables I, II, and III. The results of three other experiments in this series are not presented because they merely duplicate the three shown in the tables. In other experiments, we have tested various factors that influence the transfer phenomenon and have found that the experimental animals should be given at least the equivalent of the amount of RNA extracted from one-third of a donor brain, that the recipient animals must be properly gentled and deprived prior to injection, and that the experimental and control group recipients must be balanced (in terms of their pre-injection response rates) prior to receiving the RNA.

In very recent studies we have added a photo-cell to the milk dipper that allows us to measure the number of times that the recipients poke their noses into the dipper well during both the pre-injection and the post-injection tests. Our data suggest that the change in dipper-approaches following injection is at least as sensitive an index of the transfer effect as is the speed at which the rats reach the bar-press criterion. Almost without exception, the number of magazine entries (i.e., dipper-approaches) increases in animals injected with RNA from trained brains, while the number of magazine entries decreases in animals injected with RNA from untrained brains.

Day	Rat #	Injected With RNA From Brains of Trained Donors					Injected With RNA From Brains of Untrained Donors				
		1	2	5	7	10	3	4	6	8	9
1		11	6	26	9	2	2	2	4	2	0
2		55	13	55	2	46	0	3	0	0	1
3		60	60	60	5	60	3	3	2	1	4
4		60*	34	60	3	60	1	1	0	1	6
5			60*	60*	5	60*	1	4	2	2	4
6					28		1	4	1	0	0
7					60		1	4	2	0	0
8					60		0	4	2	0	0
9					60*		0	1	8	0	2
10							0	3	6	1	4

*Criterion is 60 responses in less than 30 minutes.

TABLE I. Number of bar-press responses during each one-hour daily test session for naive rats injected with RNA from trained and untrained donors.

	Group A				Group B				Group C				Group D			
	Injected With RNA From Brains of Trained Donors				Injected With Hydrolyzed RNA From Brains of Trained Donors				Injected With RNA From Brains of Untrained Donors				Injected With Hydrolyzed RNA From Brains of Untrained Donors			
Rat #	14	9	6	2	13	10	17	16	15	11	3	12	1	7	4	5
Pre- Injection Responses	2	2	4	7	1	2	4	8	1	2	6	6	0	3	4	9
Day																
1	5	8	5	4	0	1	0	4	0	2	3	1	0	1	1	0
2	8	7	3	22	1	2	0	5	0	3	0	3	1	0	1	2
3	60	15	3	60	0	0	0	4	0	1	1	6	1	1	3	16
4	60*	60	60	60*	0	0	0	8	1	2	2	47	0	2	3	19
5		60*	60*		0	1	0	24	0	2	1	60	1	44	4	51

*Criterion is 60 responses in less than 30 minutes.

TABLE II. Number of bar-press responses before injection and during each one-hour daily test session for naive rats injected with hydrolyzed or unhydrolyzed RNA from trained and untrained donors.

Injected With RNA
From Brains of
Trained Donors

Injected With RNA
From Brains of
Untrained Donors

Rat #	1	4	5	6	8	11	2	3	7	9	10
Pre- Injection Responses	4	6	7	7	5	6	4	6	4	5	5
Day											
1	0	32	2	4	33	42	3	6	2	1	1
2	1	60	5	3	60	60	1	2	1	1	0
3	1	60*	60	53	60*	60	4	1	0	2	3
4	0		60*	55		60	5	0	2	1	2
5	2			60*		60*	7	1	1	0	2
6	1						2	1	1	0	2
7	0						4	3	1	2	2
8	0						5	1	1	15	5
9	0						1	1	1	60	60
10	0						1	1	2	60	60*

*Criterion is 60 responses in less than 30 minutes.

TABLE III. Number of bar press responses during each one hour daily session for naive rats injected with RNA from magazine trained and untrained donors.

In the experiment whose results are shown in Table II, we tested the recipients both before and after injection for gross activity. No significant increase in activity level was found in either experimental or control animals after RNA injection.

DISCUSSION

All in all, the paradigm described in this paper seems an excellent one in which to test the "memory transfer" effect, although we admit that it requires much more elaborate equipment than has been used in many other such studies.

We cannot emphasize too greatly that our procedure must be followed ~~ex~~actly if one expects to obtain positive results. In particular, the recipient animals must be well-gentled and well-deprived, the injection should be made exactly as described herein, the amount of RNA injected must be as indicated, and above all, the experimental and control groups MUST be balanced in terms of their pre-injection response rates. The transfer effect simply cannot be shown in very low responders or very high responders. It is our belief that the single most critical variable in all transfer experiments is the behavior pattern of the recipients prior to injection, yet it is a variable that many experimenters have completely ignored in past studies.