Title

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ATTEMPTS TO TRANSFER LEARNING IN RATS WITH EXTRACTS CONTAINING RIBONUCLEIC ACID AND WITH BRAIN HOMOGENATES

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Abstract

This report describes five attempted replications of the "click" experiment described by Babich et al., and of the "click-light" experiment described by Jacobson et al. In the "click-light" experiments, there were not only click-trained donors and light-trained donors, but also naive donors. The chemical isolation procedure followed that described by Babich et al. as closely as possible, except that in one click experiment the "RNA" was isolated by phenol extraction followed by chromatography through Sephadex G-50. Training and testing procedures also followed the published reports closely. Neither the results of the click experiments nor the results of the click-light experiments indicate a transfer of a tendency to approach the food cup with the appropriate stimulus. A sixth experiment attempted transfer with whole brain homogenates. However, many of the rats exhibited uncoordinated behavior as a result of the homogenate injection, and there was no increase in tendency to approach the food cup by the "trained" recipients. Until the conditions necessary to carry out "memory transfer" experiments are adequately and precisely defined, the present positive reports that memory can be transferred by RNA remain an enigma.

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According to two recent reports, when rats were trained to approach a food cup whenever a particular stimulus was given, this training could then be transferred to naive rats by injecting them with ribonucleic acid (RNA) extracted from the brains of the trained group.[1,2] Fjerdingstad et al.[3] have also reported an effect of RNA extracted from the brains of trained rats on learning in naive rats. Our report describes five unsuccessful attempts to replicate the experiments of Babich et al. Three experiments involved click alone as the stimulus, and two required discrimination between click and light as the stimuli. In all of these experiments we have attempted to follow as closely as possible the procedures described by Babich et al.[1] and

Jacobson et al.[2] This was facilitated greatly by additional explicit details provided us by Jacobson and Bubash concerning the training and testing procedures as well as the chemical isolation procedures. In a sixth experiment with click as stimulus, learning transfer was attempted using a brain homogenate; this experiment was also unsuccessful in demonstrating any transfer of the predicted response.

During our attempts to replicate the experiments of Babich et al., other groups have also attempted either replication or extension of this work. Gross and Carey[4] have described their unsuccessful attempts to replicate Babich et al. They concluded that failure to obtain a replication was due to some (unknown) procedural difference rather than the seemingly trivial ones mentioned in their report. Luttges, Johnson, Buck, Holland, and McGaugh[5] have used a wide variety of training and isolation procedures with both mice and rats, and concluded that "the reported 'transfer' effect, if it exists, is either a very limited phenomenon or a very difficult one to reproduce." Personal communications from Leaf, Dutcher, Horovitz, and Carlton, from Wagner and Galambos, and from Corson and Enesco indicate that these workers have also been unsuccessful in demonstrating transfer with "RNA" fractions.

Reports suggesting that fractions other than RNA may be active in transferring learned responses have also appeared recently. Ungar has shown that habituation to sound[6] and a blast of air (personal communication) may be transferred from rats to mice by material believed to be a relatively low molecular weight peptide. Rosenblatt has reported both replication of the Babich click experiments[7] and extension to other behavioral tests and to other brain chemical fractions.[8,9] However, the magnitude of the behavioral difference that he obtained was much smaller than those reported by Babich et al. Rosenblatt interprets his results as suggesting that a low molecular weight protein is the active agent.

Methods

Since small differences in experimental procedure may be important, these details have been described in this report more completely than is customary.

Subjects: Seventeen 240 day old male S_3 rats weighing 300 to 400 g, from the Psychology Department colony, were used for Experiment 1. In the four subsequent experiments, male Sprague-Dawley rats obtained from Adams Caviary, La Puente, California, were used. This is the same strain and supplier as in the experiments of Babich et al. In Experiment 6, male Sprague-Dawley rats were obtained from Berkeley-Pacific Laboratories, Berkeley, California. Rats weighed 150 to 180 g when received. They were housed three to a cage for at least one week prior to the start of any experiment, at which time they averaged 200 g. Only those rats which showed the normal steady weight gain during this holding period were subsequently used. The wire cages were placed on trays filled with wood shavings. In Experiment 4, wire-bottom cages were suspended to accustom the rats to walk on the grid floor of the test apparatus. During this time, the rats were given Simonson S/D chow pellets (Gilroy, California) and water ad lib., and weighed daily. The colony and testing rooms were separate, and the rats were maintained on

an artificial diurnal cycle with 12 hr of light beginning at 7 A.M. every day.

Apparatus: Two Grason-Stadler Skinner boxes (model E 3125 B-100) were used in these experiments. The internal dimensions were 24 cm (w) x 29 cm (d) x 19 cm (h). The goal area adjacent to the food cup marked with an 8 cm black line on each wall was 6.5 cm (w) x 11 cm (d). The right end plate on these boxes had been replaced by one modified as shown in Figure 1 to conform as closely as possible to that used by Babich et al. The food cup was at the left corner of the right end plate, the food lever in the center was jammed so that it was inoperative, and the red and green jewel lights were disconnected. A 7 watt house light, which was partially masked by an aluminum plate, was mounted on the upper right quadrant of the plastic front door. These boxes were placed within a larger box provided with a round glass porthole for viewing the rat, and a fan to ventilate and to provide a masking noise for external sound. In those experiments in which a light blink was used as a stimulus, the house light was blinked three times within one second and the food pellet was dispensed by hand through a funnel and plastic tubing leading into the food cup. In the last three experiments, the Skinner box was placed within an additional large box with sound absorbing material on five sides to further dampen exterior noise. The two Skinner boxes used were designated "A" and "B", and were not used interchangeably for training or testing in case any subtle differences existed between them.

Behavioral Procedures

Assignment and treatment of subjects: At the start of each experiment, the rats were caged singly and assigned randomly (unless otherwise stated) to three groups: Trained Donors, Naive Donors, and Recipients. (There was also a fourth group in Experiment 6.) The assignments are summarized in Table I, and the behavioral procedures are described in detail next.

1) Trained Donor group. This group was further subdivided into an "A" group and a "B" group, according to whether they were to be trained in Skinner box A or in Skinner box B. Figure 1 illustrates the experimental apparatus. The rats were food-deprived for two days prior to the start of training. During training, each rat received 200 food-reinforced trials (one 45 mg Noyes food pellet per trial) each day for four days (5 days in Experiment 6). On the next or final day, each rat received 100 trials within one hour prior to sacrifice and extraction of "RNA" from the brain, as described below. A counter was operated simultaneously with the food dispenser, to record the total number of daily trials.

In each experiment, rats were trained to approach the food cup of the Skinner box on hearing the click produced by the magazine pellet dispenser. Initially, on the first day of training, the pellet dispenser was operated producing a click and delivering a single Noyes pellet when the rat was near the food cup. It generally required several minutes for the rat to find the first few pellets and to start to eat them. As training progressed, the click was withheld until the rat moved progressively further and further from the cup. On the first day of training, the rats responded fairly well to the click after about 30 to 50 stimuli. Whenever the rat returned to the food cup prior to the click and food delivery, it was not rewarded, and by this

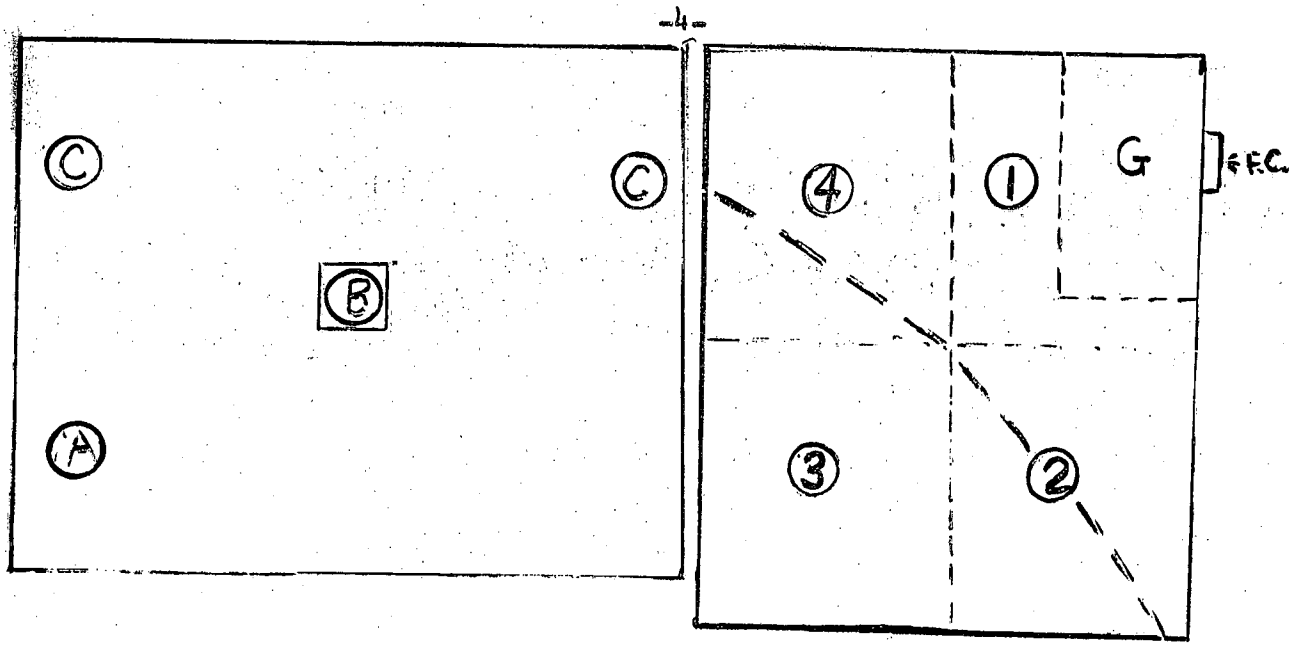

Fig. 1A

Fig. 1B

Fig. 1A. View of modified right end of training box, food cup (A) in lower left corner, and inoperative lever (B) in center. Red jewel lights (C) also inoperative.

Fig. 1B. Quadrants of box used in training, recording activity, and testing. The training area was progressively enlarged from Quadrant 3 in Experiment 1, to the quarter circle in Experiments 2 and 3, and finally included Quadrants 2, 3, and 4. The goal area is delineated by G. None of these areas were marked on the floor of the box.

TABLE I

Summary of Conditions for Six Experiments Attempting to Demonstrate "Memory" Transfer

A. CLICK EXPERIMENTS

Expt. No.	Sacrifice Date	No. of Recipient Rats* and Source of Injected Material	Comment on Training	Time of Sacrifice	Comments on Isolation Procedure	Time of Injection (Hrs. after Sacrifice)	Time of Testing (Hrs. after Injection)
1	10/10/65	5 "Click RNA" 2 "Naive RNA" 3 Saline	Click rats trained to go specifically to Quadrant 3 corner.	0900-1030	"RNA" isolated by phenol-saline extraction after grinding brain in sand. Alcohol ppt. material readily soluble.	5	4, 6, 8, 22, and 24
4	11/6/65	8 "Click RNA" 8 "Naive RNA"	Generalized training--rat trained to respond from anywhere in Quadrants 2, 3, and 4.	1000-1130	"RNA" isolated by Miss Bubash using phenol-saline after grinding brain with glass beads. Alcohol precipitated material slow to dissolve--required overnight at 23°, 1.3 mg RNA and 3.0 mg phenol/sample.	24	4, 8, 10, 22, and 24
5	12/10/65	8 "Click RNA" 4 "Click" homogenate 8 "Naive RNA"	Generalized training as in Expt. 4. Correct area randomized during last two sessions.	1500-1700	"RNA" isolated by Miss Bubash using phenol-saline after grinding brain with glass beads. Aqueous phase passed through Sephadex G-50 column and high molecular weight material used. 1.0 mg RNA/sample. Whole brain homogenate made in saline.	22	6, 10, 12, 20, and 24

TABLE I (continued)

A. CLICK EXPERIMENTS (continued)

Expt. No.	Sacrifice Date	No. of Recipient Rats* and Source of Injected Material	Comment on Training	Time of Sacrifice	Comments on Isolation Procedure	Time of Injection (Hrs. after Sacrifice)	Time of Testing (Hrs. after Injection)
6	1/3/66	8 "Click" homogenate 8 "Naive" homogenate 8 "Yoked" homogenate	Six training sessions. Correct area randomized during last two sessions.	0830-1130	Brain homogenized in saline after rat sacrificed without anesthesia and by decapitation.	5 min.	4, 7, 18, 22, 48, 72, and 96
			B. CLICK-LIGHT EXPERIMENTS				
2	10/23/65	5 "Click RNA" 5 "Light RNA" 5 "Naive RNA"	Click and Light rats trained to respond from anywhere in Quadrant 3.	0830-1030	"RNA" isolated by phenol-saline extraction after grinding brain in sand. Alcohol ppt. material readily soluble. 1/3 mg RNA and 2.0 mg phenol/sample.	5	4, 8, 12, 22, and 24
3	10/31/65	5 "Click RNA" 5 "Light RNA" 5 "Naive RNA"	Click and Light rats trained to respond from anywhere in Quadrant 3 and parts of Quadrants 2 and 4.	0830-1030	"RNA" isolated by phenol-saline extraction after grinding brain with glass beads. Alcohol ppt. material readily soluble.	5	4, 8, 12, 22, and 24

*Male S_3 rats were used in Experiment 1; male Sprague-Dawley rats were used in Experiments 2 through 6.

means it was gradually trained to "hold" or not respond in the absence of stimuli.

On the first day of training, the response rate generally decreased markedly after the rat had eaten 100 to 125 pellets, in which case the rat was returned to the home cage and the remaining trials were given two or three hours later. A total time of about two hours was required for each rat on the first day; on subsequent days, 45 to 75 min was required. Variations in the training procedure will be discussed separately for each experiment below.

In Experiments 2 and 3, an additional group was trained to respond to three rapid blinks of the 7 watt light on the Skinner box door. On the first day, the rats were initially trained to respond to the slight noise produced by the pellet dropping into the food cup simultaneously with the light's blinking. As training progressed, the pellet was withheld more and more frequently until after the rat responded to the light alone. Training rats to respond to light was more difficult than training rats to respond to click, and after the first day, training required from 60 to 90 min.

Variations in training procedures: Slight modification of the training procedures were made in each experiment. In Experiment 1, the rats were trained to return to Quadrant 3 (Fig. 1) and put their head into the corner--that is, the head was as far as possible from the food cup. The rats were also trained to leave the food cup as soon as they had swallowed the food pellet, and soon they learned a rather stereotyped response involving a rapid shuttling back and forth between the diagonal corners.

In Experiment 2, the requirement that the rat have its nose in the corner was relaxed, and the rat was trained to respond to either the click or to light when its body was inside Quadrant 3 and it was facing away from the food cup. In Experiments 3 and 4, the response area was further enlarged to include approximately half of Quadrants 2 and 4 (see Fig. 1). In Experiments 5 and 6, in addition to using the enlarged area, we attempted to randomize the training still further by requiring the rat to be in a randomly preselected quadrant (other than Quadrant 1) before giving the cue. This procedure reduced the more or less mechanical shuttling back and forth of the rat seen in the first four experiments, and increased the "searching" activity of the rat. Training time was increased by about 15 min per session over the other conditions. It appeared to be a more difficult routine for the rats to learn.

In Experiment 3, recipients from the "click" trained rats of Experiment 2 were given click-training, and the recipients from the light-trained rats of Experiment 2 were given light-training. Since these rats were already adapted to the box, training proceeded more rapidly, particularly on the first day.

2) Naive Donor group. Each rat of this group was fed 10 g of Simonson pellets each day except on the morning of sacrifice, when it was given 100 Noyes pellets. The only handling was the daily weighing.

3) Recipient group. This group was further subdivided into an "A" group and a "B" group according to the Skinner box used. Each rat of this group was placed in the appropriate box for 15 min each day for the four days prior to injection, in order to eliminate the startle response rats commonly show to a new stimulus, and so that the box would not be a totally new environment during testing. Each session the rat received two unreinforced stimuli at random during the 15 min. (In the case of the click-light experiment, each recipient received 4 stimuli, 2 click and 2 light during each adaptation session.) Prior to placing these animals in the box, all traces of food were removed from the food cup and the shavings in the tray below the Skinner box bars were replaced with fresh shavings to minimize any chance that the rat would associate any specific area of the box with food. These rats were also maintained on 10 g of Simonson pellets given each day after the adaptation session until the last session. The rats were thus deprived for 36 to 48 hr prior to testing. In Experiments 3 through 6, four extra rats were adapted. During the last adaptation session, an activity record was obtained for each rat of the recipient group. We recorded each rat's movement from quadrant to quadrant of the box during each minute, and any unusual tendency to go to the food cup, bite the cup, to freeze, or to sit in front of the food cup. This information was recorded on a form (Appendix A). The four rats deemed to be most undesirable for subsequent testing based on these observations were eliminated at this time. The source of brain material (trained or naive) subsequently injected into the recipients was made by one of us who had not participated in this screening, and without regard to the rat's behavior during this adaptation period.

The only restriction was that material from rats trained in Box A was injected into a rat adapted in Box A, and material from a rat trained in Box B was injected into a rat adapted in Box B. Testing was then done in the appropriate box.

4) Yoked control group. These rats (used only in Experiment 6) were placed in a Skinner box during the training of the trained donor groups and their food dispenser was operated simultaneously (producing a click), but no food was delivered. Thus, they received the "click" stimulus but no food reward. They received 12 g of Simonson chow pellets after each session.

Chemical Procedures

Phenol extraction procedure: The isolation procedure for Experiments 1 through 4 closely followed that described by Babich et al. The rat, after being recoded so that the chemist was not aware of its prior behavioral treatment, was killed by ether anesthesia. Immediately after respiration had ceased, the brain was exposed and removed. In Experiments 1 through 3, the anterior and posterior ends of the brain were discarded by making a posterior cut on a line joining the superior colliculus with the rostral end of the pons, and an anterior cut just behind the olfactory bulbs. In Experiment 4, the frontal area to about 2 mm posterior to the olfactory bulbs was removed. The tissue was not weighed (estimated weight 1 g) but was dropped immediately into a 12 cm mortar which had been cooled on ice and which contained 5 ml of 0.9% NaCl (0°) and 5 ml of 90% phenol (approximately 12°). In Experiments 1 through 3, 1 g of sand (B and A, washed and ignited) was added (in Experiment 4, 200 mesh pyrex glass beads were used), the mortar was transferred to the

benchtop at room temperature, and grinding was initiated. After approximately 3 min (or 180 "strokes"), the mortar contents were a uniform viscous consistency, and they were transferred to a 50 ml polypropylene centrifuge tube with the aid of a rubber policeman. The samples were stored in ice for 10 to 60 min at this point until a group of 8 was ready for centrifugation.

The samples were centrifuged at 18,000 RPM for 30 min at 2° in the SS-34 head of a Serval RC-2 centrifuge. Following centrifugation, the top aqueous layer was removed with the aid of a 5 ml serological pipette and a Propette rubber bulb. An average of 3.7 ml could be removed from each tube without detectable contamination from the gel-like material at the interface. This sample was pipetted into a clean centrifuge tube which was kept in ice. After a series of samples had been collected, they were made 0.1 M in $MgCl_2$ by the addition of 1.0 M $MgCl_2$ and 2 volumes of cold ethanol were added. The samples were mixed thoroughly by gentle swirling and allowed to stand for 15 min in ice. After centrifugation at 6000 RPM for 15 min in the SS-34 Serval head, the supernatant was gently poured off and the tubes were allowed to drain for two to three minutes at room temperature. Droplets of the supernatant which adhered to the top of the centrifuge tube were dislodged by gentle tapping, or removed with a Kimwipe. The drained tubes were placed in ice and 2.0 ml of cold 0.9% NaCl was added. The samples were gently and briefly swirled to speed the rate of solution, and if necessary, the pellet was broken up by gently stirring with a glass rod. The samples were stored in ice for two to four hours prior to injection. In Experiment 4, the samples which did not dissolve readily were gently shaken up to 18 hr at room temperature and then injected.

Sephadex G-50 procedure: In Experiment 5, the phenol procedure was modified and instead of isolating the "RNA" fraction from the aqueous phase by alcohol precipitation, small molecular weight impurities were removed by gel filtration on a column of Sephadex G-50. The procedure used was modeled after that of Babich et al. (personal communication). The dissection and isolation procedure followed that described for Experiment 4 above, except that brains were pooled into groups of two for the initial centrifugation. Subsequently, the clear portions of the aqueous layers were removed and further pooled into four samples of four brains each, representing either trained or naive rats from Boxes A or B separately. Two Sephadex G-50 columns, 3.5 x 60 cm, were equilibrated as well as eluted with 0.01 M $MgCl_2$, 0.01 M NaH_2PO_4 - Na_2HPO_4, pH 7.2, and 0.134 M NaCl in the coldroom at 3°. One column was used for the extracts from the trained animals (two runs with extensive washing in between), and the other for the extracts from the naive animals. However, the samples were coded so the chemist did not know which samples were from "trained" and which were from "naive" animals. The samples, total volume of each about 11 ml, were placed on the columns and eluted with the $MgCl_2$-sodium phosphate-NaCl solution, and the effluent was monitored at 280 mμ with a Gilson monitor; 2.5 ml fractions were collected. The absorbance at 260 mμ of the initial, high molecular weight peak was subsequently accurately determined, and the eight fractions with an absorbance greater than 0.8 and representing the center of the peak were combined. This combined peak was subsequently administered to four recipient rats.

Experiment 3 was done after extensive discussion and a demonstration of the isolation procedures by Miss Bubash; the isolation of "RNA" in Experiment

4 was done by Miss Bubash, who also isolated the material used in Experiment 5 through the initial stage of the Sephadex column isolation.

Total brain homogenate: In Experiment 6, the animal was sacrificed by decapitation, the brain was removed as in Experiments 1 through 3, and then immediately homogenized in 5 ml of cold 0.9% NaCl, using a Potter Teflon-glass homogenizer. The total homogenate, volume approximately 6 ml, was injected into a recipient rat within 5 min after homogenization.

Estimation of RNA and phenol: RNA and phenol in the samples were estimated by diluting a 25 µl or 50 µl aliquot of the dissolved sample to 1.0 ml and determining the absorbance in a Cary Model 11 spectrophotometer at 258 and 286 mµ at pH 7, and again after the addition of 10 µl of 2 M NaOH to bring the pH to 11. The absorbance of 1 mg/ml of Torula RNA (Calif. Biochem. Corp.) at 258 mµ is 19.9 at pH 7.0, and 21.6 at pH 11.0; at 286 mµ the values are 5.9 (pH 7.0) and 5.4 (pH 11.0). The absorbance of 1 mg/ml phenol at 258 mµ is 7.2 (pH 7.0) and 6.9 (pH 11.0), while at 286 mµ, the values change markedly as a function of pH, increasing from 0.45 (pH 7.0) to 26.2 (pH 11.0). Appropriate simultaneous equations were used to estimate the RNA and phenol. In Experiment 5, only the absorbance at 260 mµ at pH 7 was determined since the phenol had been removed.

In all of these isolation procedures, we were concerned about the possible consequences of ribonuclease contamination, including ribonuclease from hands. However, the precautions taken varied. In each experiment after the first, the chemists used new rubber or plastic gloves, and in Experiments 2 and 3, all containers had either been heated in an oven at 125° for 1 hr, or had been soaked with 15% H_2O_2 for 30 min. Clean glassware was used for each sample at each step of the isolation procedure, with the exception that only four mortars were used; they were rinsed in tap and distilled water and drained between samples.

Administration of "RNA" and brain homogenates: In Experiments 1 through 4, "RNA" samples were isolated from individual "trained" or "naive" rats as described above. Approximately 90% of the isolated material was injected intraperitoneally into the recipient rats, using the xiphoid process as a guide and injecting at an oblique angle laterally to avoid hitting vital organs. Rats were injected in pairs at 10 to 15 min intervals (one for testing Box A and one for Box B) so that subsequent testing could be at closely fixed intervals after injection. The remaining sample was accounted for by loss in the syringe and by that used to estimate the amount of RNA isolated. Disposable new 2 ml plastic syringes with 3/4" 22 gauge needles were used in Experiments 1 through 3: glass syringes were used in subsequent experiments. In Experiments 5 and 6, when homogenates were injected, 5 ml glass syringes with 18 gauge needles were used.

Testing: Prior to testing, each recipient rat was assigned a new code number so that the four testers did not know either the prior behavior of the rat during adaptation or the source of the injected material. The testers only knew in which of the two Skinner boxes (A or B) the recipient was to be tested and the order of testing. The tests were conducted in five sessions given between 4 and 25 hr after injection of the recipients. Prior to testing, the tube from the food dispenser to the food cup was removed and

blocked, the food cup was thoroughly cleaned of any food crumbs, and fresh shavings were placed in the trays below the wire cage. In Experiments 2 through 4, prior to placing each rat in the box, a few Noyes pellets were powdered on the wire grid floor in a generally unsuccessful attempt to increase the rat's exploratory activity.

For testing, each animal was placed in the Skinner box and was allowed at least one minute to decrease its initially rapid exploratory activity before the first stimulus was given. A stimulus was given when the rat was in Quadrant 3 and facing away from the food cup. The animal had to be alert and rearing or grooming. To the best of the tester's ability, test stimuli were not given if it was anticipated that the rat was going to move suddenly into a new quadrant. Stimuli were given at least 30 sec apart, and in some cases, during a session when an animal was too inactive to test, the door was opened briefly or the animal was gently moved to the front of the cage. In such cases, at least one minute was allowed before giving another stimulus. Each test session consisted of five unrewarded clicks (C) in Experiments 1, 4, 5, and 6, and of five unrewarded clicks and five unrewarded light flashes (each consisted of three rapid blinks) (L) in Experiments 2 and 3. The order of presentation of stimuli in these two experiments was LCCLLCCLLC during the first three sessions, and CLLCCLLCCL in the last two sessions.

At the time a stimulus was given, a silent electric timer with small glow lights which went out at 5 and 10 sec was started. A positive response was recorded if within either 5 or 10 sec of the stimulus the rat placed either his head or front paws in the 6.5 x 11 cm rectangular area delineated by the vertical black marks on the box walls and surrounding the food cup. In addition, the tester recorded any other responses such as startle, turn head, turn body, etc. which might have been useful in distinguishing "trained" and "naive" recipients.

At the end of each session, the rat was returned to its home cage in another room. Since the rat had been deprived for 36 to 48 hr prior to testing, it was given 5 to 6 g of Simonson pellets after all animals had completed test session 3. The test schedule was so arranged that no tester tested the same group of rats in two consecutive test sessions, nor did any tester test any group of rats more than twice. Separate score sheets (Appendix A) were used for each rat for each session. For the first three experiments, a less detailed score sheet, also shown in Appendix A, was used. The same form as that used in the adaptation session was used in the subsequent experiments. Results were not summarized or discussed until after the fifth test session.

Results and Discussion

Our initial objective was to repeat either the "click" or "click-light" experiments described by Babich *et al.*[1] and Jacobson *et al.*[2], in which they report the increased tendency of rats injected with RNA from trained rats to approach a food cup when presented with the appropriate stimulus. Five experiments have been done which were essentially replications of those reported by the UCLA group, and the results are summarized in Tables II and III. In a sixth experiment using whole homogenate of rat brain (Fig. 2), no significant indication of a transfer of a "trained response" was noted. In fact, there was a slight difference in the negative direction.

TABLE II

Total Number of Responses of Individual Rats in Three Click Alone Experiments
(5 session of 5 trials/session)

Expt. No.	No. of "Click" Injected Rats	No. of Responses Within 5 sec (upper row) 10 sec (lower row) Individual Rats	$\bar{X}$	S.D.	%	No. of "Naive" Injected Rats	No. of Responses Within 5 sec (upper row) 10 sec (lower row) Individual Rats	$\bar{X}$	S.D.	%
1	5	1.5*,2,2,0,1	1.3	0.7	5.2	2	0,0	0	-	0
						3**	1, 1.5*, 1.5	1.3	0.2	5.2
4	8	0,3,1,2,2,0,0,1	1.1	1.1	4.4	8	3,1,0,1,3,0,1,6	1.9	1.9	6.4
		2,10,2,5,3,1,6,3	4.0	2.7	16		5,4,3,2,9,1,3,9	4.5	2.8	18
5***	8	1,6,1,2,3,1,0,1	1.9	1.0	7.6	7	8,0,0,0,2,2,1	1.9	2.6	7.6
		1,12,5,6,9,2,1,4	5	3.7	20		12,1,3,0,6,3,2	3.9	3.7	15.6
Total:	21		1.4	1.8	5.6	20		1.6	2.1	6.4
	16		4.5	3.2	18	15		4.2	3.3	17

*0.5 indicates a response recorded by one observer but not by the other.

**These three rats were injected with 2.0 ml of 0.9% NaCl instead of with a "naive" brain homogenate.

***The "RNA" was isolated from a Sephadex G-50 column; 5.0 ml of the RNA was injected into each rat. One naive rat was adversely affected by the injection, and could not be tested.

TABLE III

Total Responses of Each Rat in Two Click-Light Experiments
(50 possible responses, 25 click & 25 light)

"RNA" Source	Responses within 5 sec			Responses within 10 sec		
	Click	Light	Click minus Light	Click	Light	Click minus Light
			Experiment 2			
"Click Trained"	4	3	1	13	8	5
	0	0	0	3	3	0
	1	1	0	3	2	1
	4	0	4	9	5	4
	2	1	1	3	1	2
Average	2.2	1.0	1.2	6.2	3.8	2.4
"Light Trained"	0	1	-1	0	3	-3
	0	2	-2	4	5	-1
	0	0	0	3	1	2
	0	0	0	0	0	0
	2	0	2	4	3	1
Average	0.4	0.6	-0.2	2.2	2.4	-0.2
"Naive"	0	0	0	2	0	2
	0	1	-1	1	4	-3
	4	0	4	7	3	4
	0	0	0	1	1	0
	3	0	3	5	2	3
Average	1.4	0.2	1.2	3.2	2.0	1.2
			Experiment 3			
"Click Trained"	1	1	0	8	4	4
	1	1	0	3	2	1
	0	0	0	2	0	2
	3	0	3	5	4	1
	0	0	0	4	0	4
Average	1.0	0.4	0.6	4.4	2.0	2.4
"Light Trained"	1	0	1	2	2	0
	0	0	0	4	2	2
	1	2	-1	2	2	0
	2	0	2	6	4	2
	0	0	0	0	1	-1
Average	0.8	0.4	0.4	2.8	2.2	0.6
"Naive"	1	0	1	2	1	1
	2	0	2	2	0	2
	2	0	2	10	1	9
	sick - not tested			-	-	-
	sick - wouldn't complete tests			-	-	-
Average	0.6	0	0.6	4.7	0.7	4.0

(Combined averages on next page)

TABLE III (continued)

	Average ± S.D. of Experiments 2 and 3 Combined					
"Click"	1.6±1.5	0.7±0.9	0.9±1.4	5.3±3.4	2.9±2.3	2.4±1.6
"Light"	0.6±0.4	0.5±0.8	0.1±1.3	2.5±2.0	2.3±1.5	0.2±1.5
"Naive"	1.5±1.4	0.1±0.3	1.4±1.4	3.7±3.1	1.5±1.3	2.2±3.2

Experiment 1 was carried out primarily to familiarize ourselves with some of the problems that might be encountered in either training or testing the rats; it is the only "click" experiment to show a greater tendency for the "click" injected rat to exceed the control (Table II). The small number of animals used make the results difficult to evaluate. In this experiment, we trained the rats to go to a very specific area of the training box--namely, the front left corner--before the next cue and pellet would be delivered. Subsequently, during testing, we found it difficult to get the injected rat in a comparable position prior to giving the cue (click), and we reasoned that the association of position and cue may not have been precise enough during testing to "trigger" a response from the test rats. The rats used in this experiment were somewhat older and from a different strain than those used by Jacobson et al., and we found them to be relatively inactive in the testing situation, spending a great deal of time grooming or just sitting down. These rats had been adapted only for two brief 15 min sessions prior to testing, and no food was sprinkled onto the bars of the box. These factors may also have contributed to the difficulty of testing.

Experiments 2 and 3 utilized, in addition to the "click" and "light" trained rats described by Jacobson et al.[2], a third "naive" group as donors. We reasoned that the addition of a "naive" group would afford three comparisons--light vs. click, light vs. naive, and click vs. naive. Unfortunately, as discussed below, the long testing session necessitated by this experimental design made these experiments less than satisfactory. The training technique employed in these experiments was more generalized than that used in Experiment 1; in Experiment 2, the rat was taught to respond from any place in Quadrant 3, and in Experiment 4 the response area was further enlarged to include part of Quadrants 2 and 4. The recipient rats were well adapted by four prior 15 min sessions in the training boxes, and they generally remained active for 7 to 10 min. However, in each of these experiments, we found testing to be very laborious and difficult due to the large number (10) of unreinforced stimuli that had to be presented to the rats in each test session. Generally after 5 or 6 stimuli had been given, the rats were not interested in further exploration, even when the bars were liberally coated with powdered Noyes food pellets. The difficulty of the testing in this type of experiment was confirmed by discussion with Miss Bubash. The results of these experiments are summarized in Table II. In Experiment 3, the rats injected with material from "click" trained rats did respond more to the click than to the light stimulus at both 5 and 10 sec, while the converse was true for the "light" injected rats. However, particularly at 5 sec, the "click minus light"

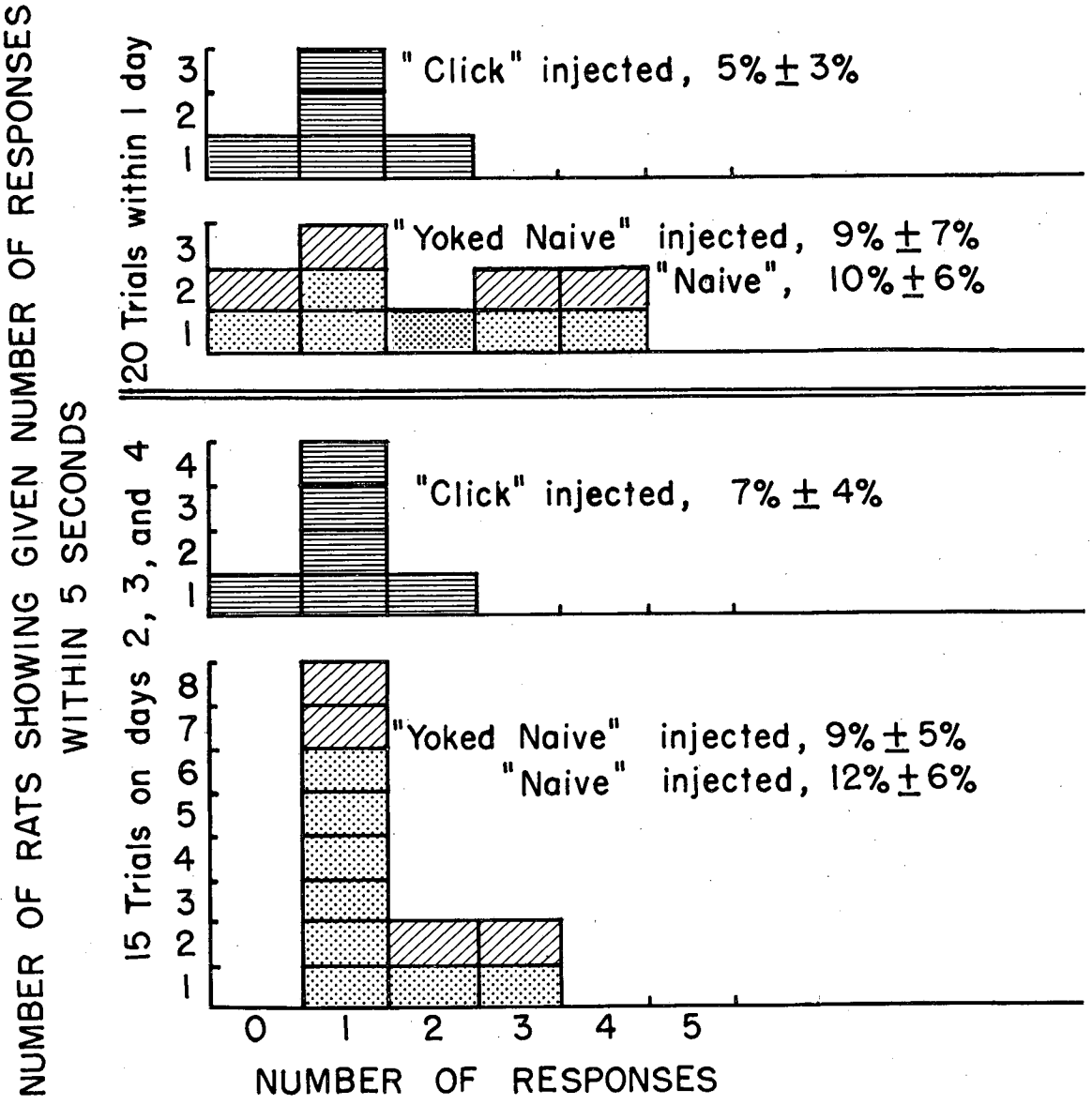

Fig. 2. Comparison of individual responses within 5 seconds of "Click homogenate" injected ≡, "Naive homogenate" injected ////, and "Yoked Naive homogenate" injected ⁙ rats. Four sessions with a total of 20 trials were made within 24 hours, and 3 daily sessions of 5 trials each were made on days 2, 3, and 4. There were 8 animals injected in each group, but tests could not be completed on 2 click injected, 4 naive injected, and 3 yoked naive injected rats.

score for the "click" injected rats was the same as for rats which had received extracts from naive donors. In both experiments, rats injected with material from "light" trained rats did appear to respond less to a click than either "click" or "naive" injected rats, but they also responded less to the light than did the click trained rats. In all cases, the average response rate to either click or light was very low and not comparable to that reported by Jacobson et al., for recipients of material from trained rats.

Experiments 4 and 5, summarized in Table II, again compared the response of animals injected with material isolated from "click" trained and naive rats. A generalized training procedure was used, and in Experiment 5, during the last two training sessions, the behavior of the rat was further randomized by varying the quadrant to which the animal had to go prior to getting the cue and food reward. The RNA isolation of Experiment 4 was done by Miss Bubash; and, as noted in Table I, due to difficulty in dissolving the RNA, injection and testing were started approximately 24 hr after sacrifice. Testing also followed sacrifice by one day in Experiment 5, in which Sephadex isolation procedure was used. In both of these experiments, testing proceeded relatively smoothly, most test sessions requiring only 6 to 10 min per rat. In part, we attribute this to the fewer number (5) of stimuli required, in part to the fact that most tests were done during the day and early evening, and in part to the rejection of several potentially undesirable recipients during the adaptation screening. Nevertheless, as shown in Table II and Figure 3, the rats injected with RNA isolated from "click" trained rats did not respond more frequently than did those injected with material from naive rats. In addition, in Experiment 5, four rats were injected with whole brain homogenates from click-trained rats. Their responses were 2, 3, 1, and 0 at 5 sec (ave. 1.5 ± 1.1), and 4, 6, 6, and 3 (ave. 4.8 ± 1.3) at 10 sec. The results of all three "click" experiments are also averaged at the bottom of Table II, and the lack of a difference between groups is reaffirmed.

Thus, in the four experiments (Experiments 2 through 5) most closely patterned after those described by Jacobson and Babich, no evidence for the transfer of a learned behavioral tendency has been observed.

We have done one experiment in which a total brain homogenate was used to transfer "click" training. The results are summarized in Figure 2. The rats were adversely affected by the homogenate. Although no behavioral differences were noted in the home cages, in the testing box many of the homogenate injected rats displayed an unusual uncoordinated behavior during early test sessions. Four test sessions were carried out within 24 hr, and one session daily on each of the succeeding three days. No increased tendency of "click" recipients as compared to recipients from either naive or yoked controls was noted. In fact, the recipients from "trained" animals had a lower response rate (6%) than either recipients from the naive rats (11%) or from the yoked naive rats (9%).

The contradictory nature of the presently known reports can perhaps be reconciled if one assumes that indeed RNA is not the active transfer agent, but rather another class of compound such as nucleoprotein, protein, lipid, or even carbohydrate, as suggested by the work of Rosenblatt and Ungar. The isolation procedures used by Babich et al. for RNA are not highly specific.

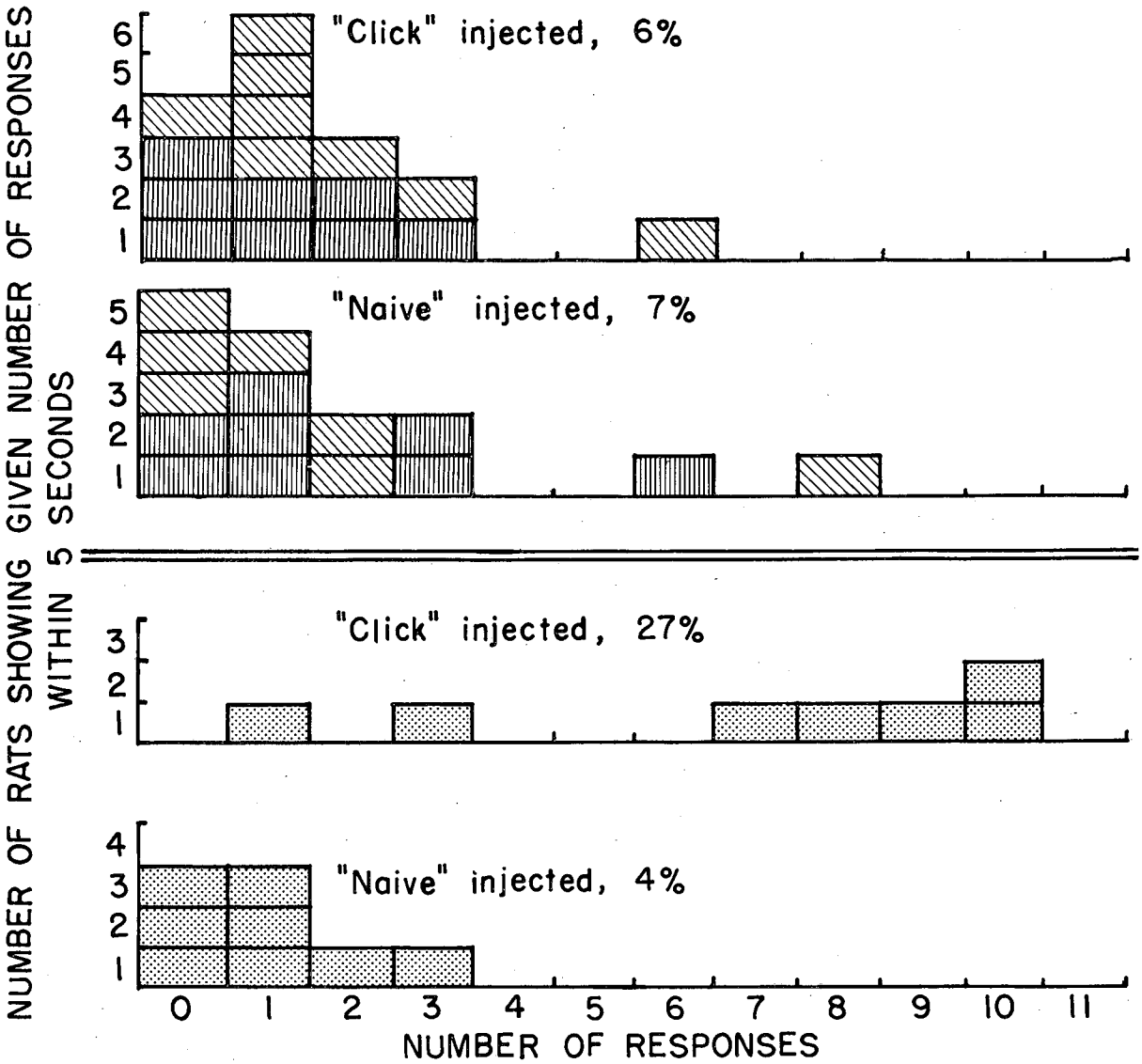

Fig. 3. Comparison of individual responses within 5 seconds of "Click RNA" injected and "Naive RNA" injected rats in Experiments 4 and 5 (above) with those reported by Babich et al. and (below) (1). Twenty-five trials were given to each rat in 5 sessions within 24 hours.

In the absence of appropriate sensitive analyses for the purity of the RNA sample, caution should be exercised in describing its chemical nature. Since phenol was used to isolate the RNA, absorbance at 260 mμ is a comparatively non-specific measure for quantitation of RNA. It was for this reason that we measured the absorbance at several wavelengths, and at two different pH's. Babich et al. have indicated considerable variability in the solubility of the "RNA" which they isolated. We also noted variability; the product isolated in Experiments 2 and 3 was readily soluble, whereas the product isolated in Experiment 4 required overnight for resolution. It should be noted, however, (Table I) that the product isolated in Experiment 4 had considerably more phenol (and presumably ethanol), and it is our impression that the solubility of the "RNA" precipitate is influenced by the amounts of ethanol in the pellet. In attempting to replicate the procedures of Babich et al., it is difficult to assess the precautions to be taken to inhibit ribonuclease. Babich et al. (personal communication) believe that the RNA is very subject to shear and breakage, yet no unusual precautions were described in their reports to inhibit ribonuclease. Conceivably, native RNA might not be able to transfer learning, whereas partially degraded RNA might have the unique properties necessary to penetrate the blood-brain barrier and to transfer information. Until the conditions necessary to carry out "memory transfer" experiments with the aqueous layer of a phenol-saline extract of brain are adequately and precisely defined, the present positive reports of Babich et al. and Jacobson et al. remain an enigma.

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Notes and References

*USPH Special Postdoctoral Fellow from Duke University Medical School.

†Postdoctoral Fellow on leave from Weizmann Institute.

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Appendix A

Form A

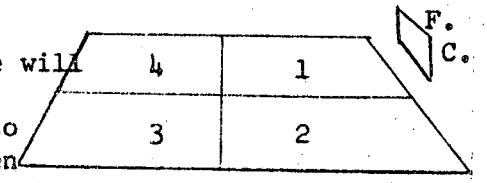
Rat No. ____ Date _____ Time _____ _____
Start End

Session _____ Skinner Box ___ Experimenter _____ Recorder, if not E _____
(4 or 5)

Trial	Stimulus	Response	Comments
1	C		
2	L		
3	L		
4	C		
5	C		
6	L		
7	L		
8	C		
9	C		
10	L		

Form B

Rat No. ____ Date _____ Session ____ Experimenter _____



In order to quantify the rat's activity and the amount of attention it gives to the food cup, we will take the following records: The floor of the Skinner Box will be considered to be divided into quadrants, as shown here. Start a stopwatch when the rat is put into the box, and keep it running throughout the session. Use one row below for each minute. During the minute, mark a tally each time the rat enters a quadrant with its head and forefeet. If the rat stays in the same quadrant for the whole minute, there will be no tally for that minute. Make a separate tally each time it puts its head into the food cup (F.C.). If the rat has to be taken out of its box during a session, keep the watch going and note this under comments. If the session lasts more than 30 min, go to a second sheet, labeling it carefully.

Minute	Quadrants 1	2	3	4	Total for min.	Food Cup	Comments
1							
2 thru 30							