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Transfer of Information with Brain Extracts from Donors to Recipients in Passive-Avoidance Behavior

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TOZZI, W., P. SALE AND L. ANGELUCCI. *Transfer of information with brain extracts from donors to recipients in passive-avoidance behavior.* PHARMAC. BIOCHEM. BEHAV. **12**(1) 7–21, 1980.—Experiments were conducted in a step-through, one-trial, passive-avoidance situation, in order to study the effect of crude or 10,000 dalton-ultrafiltered brain extracts, from trained donor rats, on the learning of the same behavior in naive or undertrained recipient rats. A positive transfer effect was consistently detectable in the latter, apparently related to consolidated learning, but not to the level of avoidance performance or of general activation in the donors. Temporal and cognitive requirements, for such an effect to occur, have been established with regard to donor-training and recipient-managing procedures. A tentative explanation of the transfer effect in the passive-avoidance behavior cannot disregard the possibility of material transmission of information pertaining to a response elicited by primary or secondary reinforcement. Such a response, although quite distinct from the somatomotor response, would be necessary to its acquisition but not to its expression.

One-trial passive-avoidance Step-through Rats Brain extracts

STUDIES on the chemical transfer of information by way of brain extracts have often adopted the passive avoidance in the rat as a model of learned behavior. The "step-through" [7, 21, 22, 23, 24] and the "step-down" [11,22] were the main procedures where the possibility of transfer was thought to have been demonstrated. A number of criticisms have been raised on the "positive" findings obtained so, both with regard to training of donors and testing of recipients. Ungar and co-workers [22] used a multi-trial, multi-session procedure with an electric stimulus far greater than required to learn aversion from natural dark-preference. Goldstein [8] remarked that in this procedure only the first trial was actually necessary for learning: all other subsequent trials to which the animal was forcibly exposed, were merely generating an intense degree of stress. The critical value of a one-trial procedure in donor training has been rightly emphasized by Mitchell *et al.* [17]. In fact, Smith *et al.* [21] had already been able to show that this procedure could produce a transfer effect. With reference to Ungar's work, Goldstein [8] also remarked that in the testing of recipients for the transferred dark-avoidance, what really should matter is the increase in latency to perform the averted behavior (entering the dark), and not the decrease in time spent by the rat in the natural preference. The latter might simply reflect a transferred general activation in the state of the recipient and depend more on the stress in donors than on the learning. This possibility cannot be rejected, considering that Ungar and co-workers did omit adequate controls, and that a transferable effect can be obtained from stressed [6] or activated [5] animals. Ungar and his colleagues [24], assuming that dark-preference was a general and invariable constant, used repeated unreinforced trials. Wojcik *et al.* [25] criticized this recipient-testing procedure, demonstrating that dark-preference attenuated with repeated trials. In this connection, Miller *et al.* in mice [16] and de Wied in rats [4] showed that Ungar's scotophobin only had an effect in weakly reinforced recipients.

We have reappraised whether brain extracts from passive-avoidance-trained rats can transfer a pertinent behavioral information to naive or weakly reinforced recipients.

Considering the above criticisms, the following points were focussed because of their importance in the transfer effect: adequacy of the one-trial procedure to generate learning in donors and to assess a learning effect of extracts in recipients; bearing of the stress component in the donor training, engendering a transferable state of general activation; preliminary assessment of the natural dark-preference behavior in recipients.

The results are presented in different parts according to the different basic variable which appeared critical for the occurrence of a real transfer effect.

GENERAL METHOD

Male rats of 150 g, maintained at 24 ± 2°C and 50% constant humidity, with a 12 hr light-dark cycle, were used.

The step-through, passive-avoidance apparatus was exactly as described by Ader *et al.* [1]. It consists of a black-walled, dark box equipped with an electrifiable grid-floor and accessible through a guillotine door from an ele-

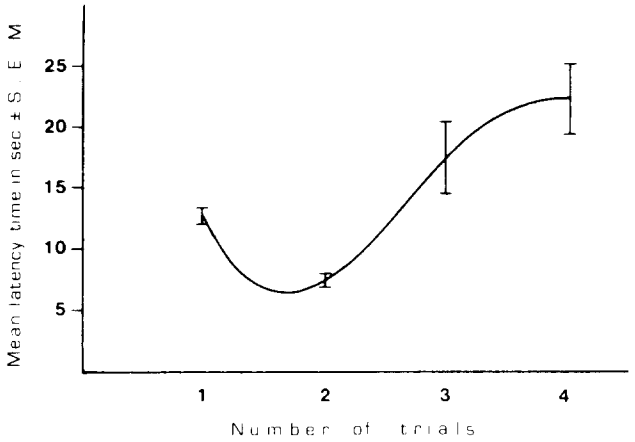


FIG. 1. Regression analysis of latency time values obtained in consecutive, daily, one-trial sessions in naive rats: n=141. Equation: $y = 53.82 - 65.56x + 27.75x^2 - 3.33x^3$.

vated and illuminated runway. Electrification of the grid is given by a scrambler, producing a continuous background noise. The apparatus is placed in an acoustically isolated, shaded room. The naive rat enters the dark box a few seconds after it has been put on the runway. However, if the rat has been foot-shocked immediately after entering the dark compartment (learning trial), an increase in latency to enter is noticed when the rat is placed on the runway again (retention test).

An identical cage, except for the fact that it was lighted and had transparent walls (transparent box), was also available. Crude extracts were prepared by homogenizing brains in distilled water (2.5 ml per brain) with a blender-ultrasonicator (Polytron mod. 10 Brinkmann). After centrifuging at 50,000 g and 4°C for 2 hours, the supernatant was immediately lyophilized and stored at −30°C.

Sometimes the homegenate was agitated overnight at 4°C; however, omission of this step did not appear to appreciably affect the activity of extracts. In some experiments, refined extracts were prepared according to Schreiber and Santos [20], with some slight modifications. Brains were immediately frozen in liquid nitrogen, lyophilized and pulverized in a mortar with dry ice. After sublimation of the dry ice in a −30°C refrigerator, the powder was extracted twice with 1 M acetic acid (1 ml per brain) for two hours. After centrifuging for 1 hour at 6,000 rpm and 4°C, the combined supernatant was immediately lyophilized, the dry residue redissolved in distilled water (1 g in 50 ml), centrifuged as above and ultrafiltered through an Amicon PM 10 membrane (10,000 dalton nominal cut-off) at 4°C. The filtrate (refined extract) was lyophilized and stored at −30°C until use.

On day of treatment, recipients were injected intraperitoneally with 1 ml saline or extract of two brains redissolved in the same volume of distilled water.

EFFECT OF BRAIN EXTRACTS FROM TRAINED DONORS ON NAIVE RECIPIENTS

A *preliminary study* verified the type and consistency of the innate behavior in the passive-avoidance apparatus of rats submitted to consecutive trials without aversive reinforcement, in order to devise the most appropriate model of bioassay of a transfer effect.

TABLE 1

THE EFFECT OF BRAIN EXTRACT FROM PASSIVE-AVOIDANCE-TRAINED DONORS ON LATENCY TIME* IN NAIVE RECIPIENTS

Treatment	48 hr post-treatment single trial		
Saline (59)	12.58 ± 0.90	n.s.	n.s.
Sham-trained-brain (42)	12.20 ± 1.07	n.s.	
Trained-brain (38)	11.50 ± 0.89		

*Mean values ± SEM in sec. Student *t*-test.
( ) Number of animals.

Method

Rats were taken in a single trial per day for 4 consecutive days, measuring latency time to enter the dark box and leaving the rat inside for 10 sec without experiencing any foot-shock.

Results and Discussion

Latency times varied in the successive trials, $F(3,560)=7.34, p<0.001$; a reduction in latency was regularly exhibited in the second trial. As shown in Fig. 1, this phenomenon was adequately described by a cubic regression. This indicates that the natural dark-preference is not a stable feature, at least in this behavioral model. Consequently, the bioassay model could be useful to reliably demonstrate a transfer effect in naive recipients, only resorting to procedures centered on a single post-treatment trial or two trials with treatment between.

FIRST EXPERIMENT

This study intended to ascertain a possible transfer effect in recipients subjected to a single trial after the administration of the trained-brain extract, comparing their latency times with those of control-brain extract or saline recipients.

Method

Donor training consisted of giving: on Day 1, a trial for familiarization (acquaintance trial); on Day 2, a single trial followed by a foot-shock (1 mA per 3 sec) immediately after entering the dark box (learning trial); on both Days 3 and 4 (24 and 48 hr after shock, respectively), one trial for retention (retention test). In each trial, latency time to enter the dark box was recorded up to a maximum of 300 sec, while total time inside was invariably limited to 10 sec. Donor brains, collected by decapitation 72 hr after shock, were immediately frozen in dry ice. Crude extracts were also prepared from control donors, run exactly in parallel to trained donors, except that they were never shocked (sham-trained donors). Forty-eight hours after treatment (trained- or sham-trained-brain extract or saline), recipient rats were tested for acquired passive avoidance in a single trial, measuring their latency times up to a maximum of 300 sec.

This 48 hr interval was chosen to allow for recovery from after-treatment malaise, previously reported when using crude brain homogenates [19].

Results and Discussion

The brain extract from trained donors with maximal performance (latency = 300 sec) was unable to affect latency time to enter the dark box in naive recipients, as shown in Table 1. No significant differences were found among trained-, sham-trained-brain extract and saline groups. Notice that the innate behavior of recipient rats in the passive-avoidance apparatus was unchanged, notwithstanding the manipulation and the unspecific effects of the treatment. In fact, no differences in latency times were found between these injected rats and the untreated rats of Figure 1 in the first trial.

SECOND EXPERIMENT (REPLICATES A, B, C)

It was intended to ascertain a possible transfer effect in recipients which were given two trials with interposed treatment. A paired comparison between the two trials, and a comparison among trained-, sham-trained-brain extract and saline groups in each of the two trials were carried out.

Method

Trained and sham-trained donors, and brain extracts were prepared as in the first experiment. Recipient rats received two trials: 24 hr before and 48 hr after treatment (brain extracts or saline).

Results and Discussion

As shown in Table 2, in each of the three replicates, the

TABLE 2

THE EFFECT OF BRAIN EXTRACT FROM PASSIVE-AVOIDANCE-TRAINED DONORS ON LATENCY TIME* IN NAIVE RECIPIENTS

Treatment	24 hr pre-treatment trial		48 hr post-treatment trial					
Replicate A								
Saline (6)	n.s.	n.s.	8.58 ± 0.65	<0.05	5.33 ± 0.81	n.s.	n.s.	
Sham-trained-brain (14)			12.60 ± 1.64	<0.001	5.61 ± 0.71	n.s.		
Trained-brain (14)		n.s.	9.82 ± 0.78	n.s.	10.71 ± 2.63	n.s.		
Replicate B								
Saline (15)	n.s.	n.s.	11.47 ± 1.53	n.s.	8.07 ± 0.99	n.s.	n.s.	
Sham-trained-brain (13)			16.61 ± 3.76	<0.01	6.35 ± 1.75	n.s.		
Trained-brain (13)		n.s.	10.35 ± 1.15	n.s.	8.61 ± 1.84	n.s.		
Replicate C								
Saline (8)	n.s.	n.s.	8.75 ± 1.93	n.s.	4.44 ± 0.68	n.s.	n.s.	
Sham-trained-brain (20)			8.07 ± 0.77	n.s.	7.67 ± 1.44	n.s.		
Trained-brain (11)		n.s.	7.91 ± 0.75	n.s.	12.82 ± 4.79	n.s.		
A, B, C cumulated data								
Saline (29)	n.s.	n.s.	10.12 ± 0.97	<0.002	6.50 ± 0.64	n.s.	<0.03	n.s. <0.01
					6.54 ± 1.35†			
Sham-trained brain (47)			11.79 ± 1.27	<0.0003	6.69 ± 0.72	<0.05		<0.02
					6.59 ± 1.07†			
Trained-brain (38)		n.s.	9.45 ± 0.54	n.s.	10.60 ± 1.77			
					10.70 ± 1.19†			

*Mean values ± SEM in sec. Student *t*-test for horizontal (paired data) and vertical comparisons.
†Corrected means (see text).
( ) Number of animals.

brain extract from trained donors was unable to produce a difference between pre- and post-treatment latency times in naive recipients. However, there was a tendency to an inter-trial reduction in sham-trained-brain extract groups (significant in replicates A and B) and in saline groups (significant in replicate A), whereas no such tendency was found in trained-brain extract groups in replicate A and C. At no time was there a difference among the groups registered. It must be noticed that this experiment confirmed, as found in the preliminary study, the decrease in latency time from first to second trial, at least in control groups. This resulted more evidently, with high statistical significance, from the analysis of cumulated data of the three replicates (Table 2). Moreover, the analysis of post-treatment latency times made apparent a significant difference between trained- and sham-trained-brain extract groups, as well as between the former and the saline group. No difference was found among pre-treatment latency times. The co-variance analysis for a possible effect of regression of the post-treatment latency value on the pre-treatment one produced a common angular coefficient significant at a 5 percent probability level. On this basis, the corrected means of the three treatments were calculated and compared (Table 2): the difference between the trained- and the sham-trained-brain extract or saline group appeared more accentuated. With reference to the single replicates, the effect of trained-brain extract appeared to counteract the natural decrease, rather than produce an absolute increase, in latency time from first to second trial. The cumulated data analysis stressed the scarce sensitivity and reproducibility of this experimental procedure and its low reliability to demonstrate the existence of a transfer effect. So, the necessity emerged to adopt a more rigorous and discriminative experimental design. For this purpose, an aversive reinforcement was introduced into the recipient test to amplify the possible transfer effect of the trained-brain extract.

EFFECT OF TRAINED-BRAIN EXTRACT ON UNDER-TRAINED RECIPIENTS

THIRD EXPERIMENT

This experiment aimed at verifying the effect of trained-brain extract on the acquisition of the same step-through avoidance in "undertrained" recipients. These were rats shocked in the same sequence as for donors, but at less intensity and duration, so as to engender, by itself, a modest, although reproducible degree of avoidance behavior. The application of the aversive reinforcement in a recipient, whose behavior might have already been imperceivably orientated by the treatment with trained-brain extract, could have exerted a "reminder" [10] or a "booster" effect [3] on the transferred behavior. Using undertrained recipients, the transfer phenomenon might have resulted in a learning enhancement, manifesting in an optimal increase in the latency time following a suboptimal aversive reinforcement.

Method

Animals were trained as in the first experiment, then divided into "rich" and "poor" donors, according to the latency time exhibited in their first retention test ($\leq$300 sec). Sham-trained donors and brain extracts were prepared as in previous experiments. Recipient rats were treated with brain extracts or saline and, 48 hr later, given a trial, immediately followed by a comparatively weak aversive reinforcement (0.75 mA per 1 sec). Animals undertrained like this were repeatedly tested for passive avoidance, measuring latency time 48, 96 and 192 hr post-shock. A non-parametric method of statistical analysis appeared to be appropriate because of the actual presence in this experiment of limit values (300 sec).

Results and Discussion

Undertrained recipients of trained-brain showed a highly significant increase in latency time in comparison with both sham-trained-brain and saline undertrained recipients. This effect was present in all retention tests, as shown in Table 3. So, it was clearly evident that brain extracts from trained donors could potentiate the effect of a low-reinforcing stimulus in recipients. This stimulus per se induced a modest degree of the passive-avoidance behavior (cf. saline and sham-trained-brain extract groups). Furthermore, control measurements in an electrifiable grid-floor cage showed that the above treatment did not appear to modify pain-threshold at the moment of application of the footshock aversive stimulus. Therefore, the effect of the trained-brain extracts might be regarded as increasing the "teaching potency" of such stimulus. The activity of the extracts was not related to performance level of the respective donors ("rich" and "poor"), which differed largely in both retention tests ($p<0.0001$). In fact, "rich" brain recipients performed exactly like their donors, while "poor" brain recipients surprisingly outdid their donors to a highly significant degree, in spite of the weaker aversive stimulus given. It is also to be noticed that in this experiment no extinction of the acquired avoidance was encountered in any recipient group, even at latest times (192 hr-retention test). We also observed that full avoidance was retained up to 30 days after shock in rats given weekly retention tests.

The capacity of trained-brain extracts to enhance learning in recipients, clearly had depended on a transferable biochemical factor induced by training in donors. This factor might have either specifically connected the learned behavior in donors to the to-be-learned behavior in recipients, or, more simply, non-specifically generated an arousal state in the latter, facilitating learning.

FOURTH EXPERIMENT

This study aimed to individuate potential sources of arousal in donors, intrinsic to their training, and capable of simulating a transfer of learning by the transmission of a material arousing undertrained recipients. Therefore, previously reported [21] manipulations of the training procedure were introduced, such as disruption of temporal and local contingency between the aversive stimulus and the natural response of the rat to enter the dark box.

Method

Trained and sham-trained donors were run as in the first experiment. Four donor groups were further introduced: (1) only-shocked group; rats never submitted to trials were shocked immediately after being put into the dark compartment, and then left for 7 sec. This group was used to check the importance of shock per se in donors, as generator of an arousal factor capable of interfering, in undertrained recipients, with the natural tendency to enter the dark box. The following groups differed from the trained one with regard to the learning trial.

TABLE 3

THE EFFECT OF BRAIN EXTRACT FROM PASSIVE-AVOIDANCE-TRAINED DONORS ON LATENCY TIME* IN UNDERTRAINED RECIPIENTS

Treatment	48 hr post-treatment pre-shock trial					Successive retention tests 48 hr					96 hr			192 hr		
Saline (9)	10					28					120			90		
	9–12					12–240					17–300			19–180		
		n.s.				<0.02	n.s.				<0.01	n.s.		<0.01	n.s.	
Sham-trained-brain (25)	10					73					109		<0.04	65		<0.01
9†	7–16					7–300					17–300			8–278		
			n.s.			<0.01		<0.01			<0.01	<0.015		<0.01	<0.003	
Trained-brain (16)		n.s.			n.s.					<0.025	300			300		
				n.s.			<0.015				246–300			163–300		
									<0.03		<0.01			<0.01		
"Poor" donors (8)	14					300										
90†	7–14					300–300										
		n.s.				<0.01	n.s.									
"Rich" donors (8)	9					300										
300†	7–12					270–300										
						<0.01										

*Median values and interquartile intervals in sec.

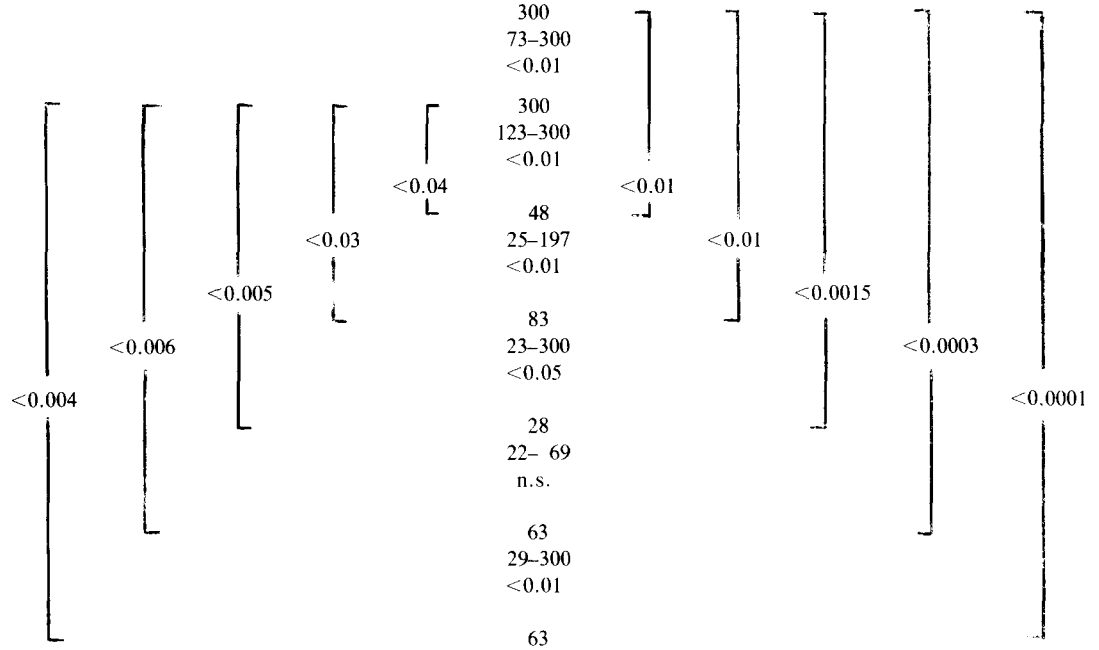
†Median latency times at second retention test in donors (see text). Horizontal comparisons: Wilcoxon matched pairs signed ranks test; vertical comparisons: two-sided Mann-Whitney U test.

( ) Number of animals.

TABLE 4

THE EFFECT OF BRAIN EXTRACT FROM PASSIVE-AVOIDANCE-TRAINED DONORS ON LATENCY TIME* IN UNDERTRAINED RECIPIENTS

Treatment	48 hr
Trained-brain (43)	300
300†	73–300
	<0.01
Mistrained-brain A (22)	300
16†	123–300
	<0.01
Mistrained-brain B (8)	48
7†	25–197
	<0.01
Mistrained-brain C (16)	83
4†	23–300
	<0.05
Shocked-brain (7)	28
	22– 69
	n.s.
Sham-trained-brain (54)	63
7†	29–300
	<0.01
Saline (58)	63
	19–300
	<0.01



*Median values and interquartile intervals in sec.

†Median latency times at second retention test in donors (see text). Comparison versus pre-shock trial (latency times not shown): Wilcoxon matched pairs signed ranks test; vertical comparisons: two-sided Mann-Whitney U test.

( ) Number of animals.

(2) Mistrained group A; rats were put into the dark compartment and immediately shocked. Seven sec later, the animal was placed on the runway and allowed to perform the usual trial. Upon entry, it was removed from cage. This group was used to check the importance of temporal contingency between step-through response and shock in donor-training procedure, in order to generate the transfer effect in undertrained recipients.

(3) Mistrained group B; immediately after entering the dark compartment, the animal was removed to the transparent box, where it was shocked at once and then left for 7 sec. This group was used to check the importance of local contingency between step-through response and shock.

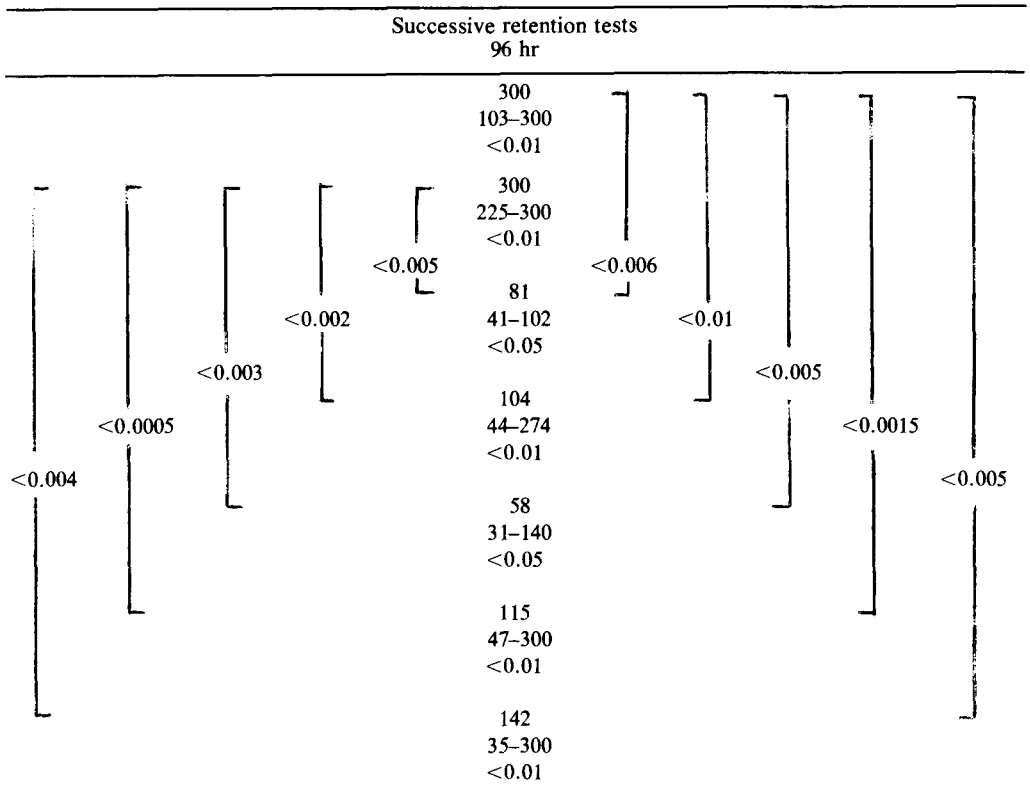
(4) Mistrained group C; rats were put into the transparent box and immediately shocked. Seven sec later the animal was removed to the runway of the step-through cage, where it was allowed to perform the usual trial. Upon entry, it was removed from the cage. This group was used to check the importance of both local and temporal contingency between step-through response and shock.

Brain extracts were prepared as in the previous experiments. After treatment with saline or brain extracts, undertrained recipients were run as in the third experiment.

Results and Discussion

As shown in Table 4, the trained group showed a high avoidance performance and, concomitantly, their brain extract was endowed with a clear-cut transfer activity. The mistrained groups B and C did not show avoidance performance at all, and, concomitantly, their brain extracts were totally devoid of transfer activity. The mistrained group A showed very little avoidance performance, when compared with the trained group. Some learning was present, at any rate, as demonstrated by their significantly increased latency time in the 48 hr-retention test in comparison with each of the other control groups: $p<0.001$ vs sham-trained, $p<0.002$ vs mistrained group B, $p<0.0001$ vs mistrained group C. Concomitantly, brain extract from mistrained group A was endowed with a clear-cut transfer activity, as in the case of trained-brain extract. Such results, on one hand, confirmed the existence of a positive, reproducible transfer effect by brain extract from trained donors. On the other, they demonstrated that an inverted trial-shock sequence, while dimming acquisition of passive avoidance in donors, still allowed their brain extract to enhance avoidance learning in undertrained recipients. This experiment definitely excluded that the transfer effect could coincide with the transmission of an arousal factor, generated in donors by some elements of the training and capable of affecting avoidance performance in undertrained recipients. On the contrary, the transfer effect appeared to be strictly dependent on the totality and correctness of donor-training procedure, with the exception of the right trial-shock sequence. This puzzling finding un-

TABLE 4 CONTINUED



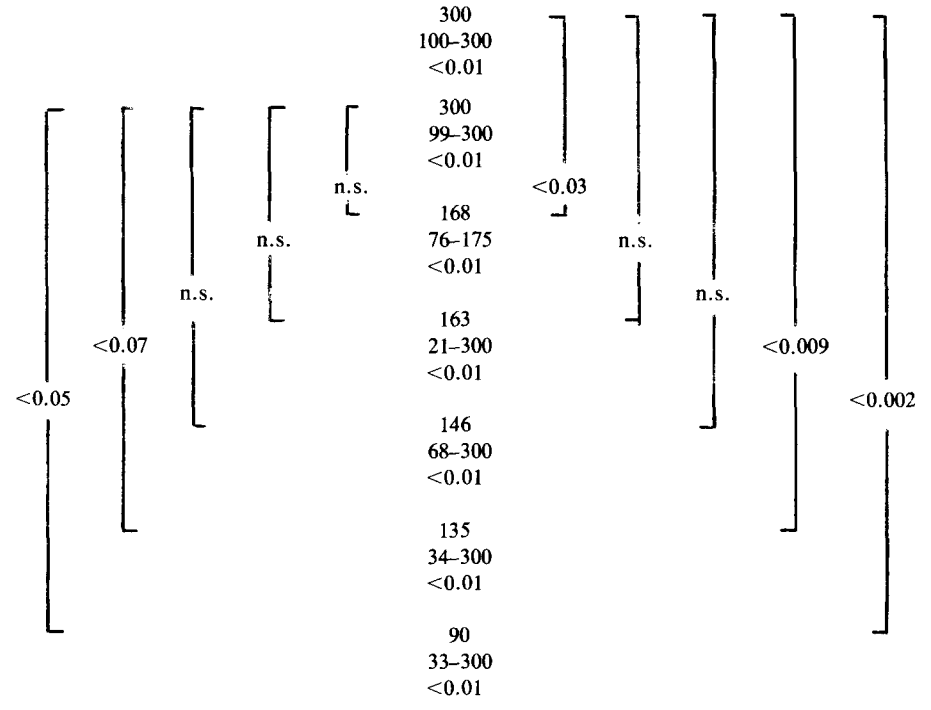


TABLE 5

THE EFFECT OF BRAIN EXTRACT FROM DONORS TRAINED IN PASSIVE AVOIDANCE, WITH DIFFERENT INTERVALS BETWEEN SHOCK AND RETENTION TEST, ON LATENCY TIME* IN UNDERTRAINED RECIPIENTS

	Donors: Single retention test immediately followed by sacrifice at:		Recipients: 48 hr post-shock retention test		
A	0 hr	34 9–300 (27)	n.s.	50 21–196 (9)	n.s.†
B	24 hr	300 170–300 (24)	n.s.	300 67–300 (9)	<0.03†
C	48 hr	300 300–300 (62)	<0.001	58 20–210 (30)	n.s.†
D	72 hr	58 18–236 (37)	n.s.	28 17–300 (11)	n.s.†

<0.001 (A vs C); <0.001 (B vs C); <0.04 (B vs D); n.s. (A vs B); n.s. (A vs D); n.s. (C vs D)

*Median values and interquartile intervals in sec. Two-sided Mann-Whitney U test; one-sided in the comparison† between trained- and sham-trained-brain recipients. Latency times of the latter are not shown and no difference was present among them.

( ) Number of animals.

derlined the dissociation between degree of avoidance performance in donors and transfer effect in recipients, as encountered in the third experiment. Moreover, it indicated that some information might be transferred, pertaining to the experimental apparatus and not to actual donor performance, nor to its visible parameter, the somatomotor response.

CRITICAL REQUIREMENTS OF DONOR TRAINING FOR A TRANSFER EFFECT ON RECIPIENTS

FIFTH EXPERIMENT

The reliability of the bioassay for a transfer effect on the learning of passive avoidance in undertrained recipients made an assessment possible of the critical requisites in donor-training procedure for such effect to occur. Obviously, among various requisites, life span of the learnt behavior in donors needed to be considered first. To evaluate its influence, independently from the interaction among successive retention tests, a single test was adopted in donor-training procedure.

Method

Four groups of trained donors were prepared as in the first experiment, except that they were given one retention test only, 0, 24, 48 or 72 hr after foot-shock and immediately sacrificed. Temporally-parallel, sham-trained groups were also prepared. Brain extracts and undertrained recipient rats were prepared as in previous experiments.

Results and Discussion

As shown in Table 5, the capacity of trained-brain extract to produce a transfer effect, appeared dependent on the time between shock and retention test. The extract prepared from trained rats sacrificed immediately after the 24 hr-retention test, was fully endowed with the transfer effect in undertrained recipients, in presence of high avoidance performance in donors. The extracts, prepared from trained rats sacrificed immediately after the 0 hr-retention test, did not enhance passive avoidance in undertrained recipients, in absence of a clear-cut avoidance performance in donors. This would indicate that an interval between the experience of foot-shock and sacrifice was necessary for the formation of a stable mnemonic trace in donors. The same result had been found in mice [15], which showed no retention of the aversive experience when tested immediately after the learning trial in a similar passive-avoidance situation. Extracts obtained from donors with a retention test 48 or 72 hr after shock had no transfer effect, even though in the former a maximum avoidance performance was registered. This indicated that too long an interval (72 hr) had allowed for the decay of the transfer activity, together with the decay of the mnemonic trace in donors. On the other hand 48 hr-tested donors performed as well as the 24 hr ones, whereas the extract from their brains was practically devoid of transfer effect. This was just the opposite of what was observed in the third and fourth experiment. There, a poor performance in donors was compatible with a positive transfer effect in recipients. Here, a high performance in donors was not accompanied by a transfer effect. This showed even more that a relationship is not necessary between performance in donors and transfer effect of their brain extracts in recipients.

SIXTH EXPERIMENT

The brain extract from donors, given two retention tests

TABLE 6

REDUCTION OF LATENCY TIME* IN PASSIVE-AVOIDANCE-TRAINED DONORS AND OF EFFECT OF THEIR BRAIN EXTRACT ON LATENCY TIME* IN UNDERTRAINED RECIPIENTS INDUCED BY A RETENTION TEST IMMEDIATELY AFTER SHOCK

	Donors										Recipients	
	Successive retention tests†										48 hr post-shock	
	0 hr			24 hr		48 hr		72 hr			retention test	
A	no	(18)		300							300	<0.04‡
				159–300							113–300	
			n.s.						<0.05		(8)	
B	yes	(20)		180							86	n.s.‡
				76–300							9–290	
											(10)	
C	no	(20)		yes		300					300	<0.03‡
						300–300					105–300	
					<0.001				<0.03		(8)	
D	yes	(30)		yes		189					66	n.s.‡
						33–300					46–233	
											(9)	
E	no	(28)		yes		yes		300			300	<0.03‡
								300–300			62–300	
							n.s.		n.s.		(11)	
F	yes	(16)		yes		yes		300			259	<0.05‡
								167–300			115–300	
											(8)	

*Median values and interquartile intervals in sec.

†Last retention test in donors was immediately followed by sacrifice. Two-sided Mann-Whitney U test; one-sided in the comparison‡ between trained- and sham-trained brain recipients. Latency times of the latter are not shown and no difference was present among them.

( ) Number of animals.

(24 and 48 hr after shock, third and fourth experiment) and sacrificed 72 hr after shock, exerted a clear-cut transfer effect. This was at variance with the brain extract from donors similarly sacrificed at 72 hr, but given a single retention test immediately before sacrifice (fifth experiment). So, the present experiment was intended to ascertain the influence on the transfer effect exerted by repeated retention tests in the course of donor training.

Method

Paired groups of trained donors were prepared according to different schedules of retention testing (see Table 6) after the acquaintance and learning trials. They were sacrificed immediately after the last retention test. In each pair of groups, one was given the first retention test immediately after the shock, the other 24 hr later. Temporally-parallel, sham-trained groups were also prepared. Latency times in the last retention test were assumed as indexes of avoidance performance in donors. Undertrained recipients and brain extracts were prepared as in previous experiments.

Results and Discussion

A retention test, given immediately after the shock, reduced the degree of learning exhibited by donors in the retention tests which followed. As shown in Table 6, the difference was clear-cut between 0 hr-tested and -untested donors in the 24 and 48 hr-retention test. The difference tended to be attenuated in the 72 hr-retention test, when the disturbing action of the 0 hr-retention test was probably counteracted by the positive effect of repeated tests (see below). Failure in performance of 0 hr-tested donors was accompanied by failure in giving transfer-active extracts. In fact, among the 0 hr-tested groups, only the 72 hr-retested one gave an extract enhancing learning in recipients. So, the 0 hr-retention test resulted in having generally impaired both learning in donors and transfer effect of their brain extracts.

Latency times of 0 hr-untested donor groups C and E and respective recipients in Table 6 were compared with those of donor groups C and D and respective recipients in Table 5. It was clearly evident that repeated retention tests in donors could reinforce their avoidance behavior and increase transfer activity of their brain extracts.

Performance of donors tested at only 48 hr was indistinguishable from that of donors tested at both 24 and 48 hr, but only brain extract from the latter gave a transfer effect in recipients.

The level of performance in donors tested at only 72 hr

TABLE 7

THE INFLUENCE OF A 24 HR INCUBATION PERIOD AFTER A RETENTION TEST IN PASSIVE-AVOIDANCE-TRAINED DONORS ON THE EFFECT OF THEIR BRAIN EXTRACTS ON LATENCY TIME* IN UNDERTRAINED RECIPIENTS

Donors 24 hr-delayed sacrifice after a single retention test at:					Recipients 48 hr post-shock retention test				
0 hr			88 30–300 (20)	n.s.	77 13–273 (10)				n.s.†
		<0.003				<0.05			
24 hr	<0.05		300 300–300 (16)	n.s.	300 166–300 (6)		<0.05		<0.03†
	n.s.							n.s.	
48 hr			300 145–300 (10)	n.s.	300 269–300 (5)				<0.05†
72 hr			88 21–300 (16)	n.s.	65 22–288 (8)				n.s.†

*Median values and interquartile intervals in sec. Two-sided Mann-Whitney U test; one-sided in the comparison† between trained- and sham-trained brain recipients. Latency times of the latter are not shown and no difference was present among them.

( ) Number of animals.

was very poor in comparison with that of donors tested at 24, 48 and 72 hr, and a transfer effect was exerted only by brain extract from the latter ones.

From the above, it appeared that: (1) a 24 hr interval between shock and retention test was essential for the occurrence of both learnt avoidance in donors and transfer effect of their brain extracts in undertrained recipients; (2) a 48 hr interval between shock and the first retention test was still compatible with the occurrence of learnt avoidance in donors, but not with the occurrence of a transfer effect; (3) a 72 hr interval was incompatible with the occurrence of learnt avoidance and a transfer effect; (4) both these failures were prevented by giving retention tests in a 24 hr cycle, which acted as an aversive reinforcer, in the same way as the shock.

From the findings of this experiment, the relative independence of the transfer effect was again pointed out in undertrained recipients from the level of avoidance performance in donors.

SEVENTH EXPERIMENT

It was intended to study in detail the requisites for the donor retention test to act as a reinforcer, the existence of which had been indicated by the previous experiments: the level of performance, and the interval between shock and retention test. Given that a 24 hr interval was a sufficient incubation period for the shock to act as an aversive reinforcer, it was hypothesized that the same incubation period would have been sufficient for the retention test to act in the same way.

Method

Groups of trained donors were prepared as in the fifth experiment, except that sacrifice was delayed 24 hr after the single retention test. Temporally-parallel, sham-trained groups were also prepared as in previous experiments.

Results and Discussion

Brain extracts from donors tested for retention 24 and 48 hr after shock exerted a transfer effect in undertrained recipients. This effect was absent in the case of 0 hr- and 72 hr-tested groups, as shown in Table 7.

The comparison of these findings with those of the fifth experiment (Table 5) shows that there was no difference in the 24 hr delay in sacrifice after 0, 24 or 72 hr post-shock retention test, with regard to a transfer effect in undertrained recipients. However, a difference was present in the case of the 48 hr-retention test: it could act as generator of a transfer effect when followed by a 24 hr incubation period.

Furthermore, results from the above and the fifth experiment were compared with those from the third and fourth (Tables 7, 5 and 3, 4, respectively).

Latency times of trained donors tested once at either 48 or 72 hr after shock (Experiments 5 and 7) were compared with those of saline undertrained recipients tested at both 48 and 96 hr after shock (Experiments 3 and 4). In the 48 hr-retention test, performance in recipients was significantly very much lower than that in donors, because of the lower reinforcement given (0.75 mA per 1 sec versus 1 mA per 3 sec). On the contrary, the same recipients in the second test, at 96 hr, performed significantly better than donors in the single test at 72 hr, despite the longer shock-test interval. This would indicate that retention tests given in a 48 hr cycle might still exert a reinforcing action on the learnt avoidance.

Moreover, trained donors tested once at 72 hr and sacrificed immediately after (Experiment 5) performed better

TABLE 8

THE EFFECT OF REFINED BRAIN EXTRACT FROM PASSIVE-AVOIDANCE-TRAINED DONORS ON LATENCY TIME* IN NAIVE RECIPIENTS†

Treatment	Single post-treatment trial 6 hr	12 hr	24 hr	48 hr
Saline	5.23 ± 0.92 (6)	4.37 ± 0.56 (6)	6.85 ± 0.98 (23)	6.71 ± 1.21 (24)
Sham-trained-brain	5.14 ± 0.85 (7)	5.21 ± 0.52 (7)	6.16 ± 1.52 (10)	7.98 ± 1.40 (18)
Trained-brain	8.80 ± 0.85 (7)	6.68 ± 0.54 (7)	7.88 ± 1.01 (22)	8.14 ± 1.20 (24)

Saline vs. Sham-trained-brain: 6 hr n.s.; 12 hr <0.01; 24 hr n.s.; 48 hr n.s.
Saline vs. Trained-brain: 6 hr <0.01; 12 hr <0.07; 24 hr n.s.; 48 hr n.s.
Sham-trained-brain vs. Trained-brain: 6 hr <0.01; 12 hr n.s.; 24 hr n.s.; 48 hr n.s.

*Corrected (see text) mean value ± SEM in sec. Student *t*-test.

†Values (not shown) in 24 hr pre-treatment trial were generally higher than in post-treatment trial, as expected from findings of preliminary study.

( ) Number of animals.

than mistrained-A donors, tested at both 24 and 48 hr and sacrificed 72 hr after shock (Experiment 4), but only the brain extracts from the latter gave a positive effect on recipients. On the other hand, performance of the above 72 hr-tested donors was quite similar to that of "poor" donors, tested at both 24 and 48 hr, and sacrificed 72 hr after shock (Experiment 3), but, also in this case, only the brain extracts from the latter gave a positive effect on recipients. So, the transfer effect appeared once again independent from avoidance performance in donors but dependent on their retention tests, repeated at 24 hr intervals. The importance of an incubation period in donors, in order to have a transfer effect of their brain extract in recipients, has been already demonstrated [9].

The critical value of an adequately temporally-paced retention test (followed by a suitable incubation period) in generating a transfer effect, would be very difficult to explain, when considering a necessary correlation between the avoidance response actually retained in donors and that exhibited by recipients. A unitary interpretation of our findings may be attempted, assuming that the information transferred in the recipient was a conditioned response to environmental cues, apprehended and performed by the donor in the learning trial (which excluded any retention test). Such information was distinct and independent from an actual avoidance response. In fact, this response was performed by the donor only in a successive phase of the training course (retention tests). The two kinds of donor response might have mnemonically evolved in different ways, and the transfer effect possibly have resulted from their complex interaction. This would be suggested by brain extracts from 0 hr-tested donors exerting no transfer activity. Brain extracts from 24 hr-tested donors exerted such an activity in the case of both immediate and 24 hr-delayed sacrifice. Brain extracts from 48 hr-tested donors exerted no activity (despite the presence of a high avoidance performance) unless sacrifice was delayed.

TEMPORAL REQUIREMENTS FOR THE MANIFESTATION OF THE TRANSFER RESPONSE IN RECIPIENTS

EIGHTH EXPERIMENT

In all previous experiments, crude brain extracts were usually administered 48 hr before the test, to allow for the recovery from debilitating effects. We considered that the use of refined extracts, apparently devoid of side-effects, would have permitted the study of the effect of brain extracts at shorter intervals after administration.

Method

Trained and sham-trained donors were prepared as in the first experiment, and their refined brain extracts obtained and administered as described in General Method. The effect of trained-brain extract was studied in groups of naive animals treated 24 hr after the acquaintance trial. A second trial was given 6, 12, 24 or 48 hr after treatment. Saline and sham-trained-brain control recipients were run. The possible transfer effect was measured and evaluated as in the second experiment. The same animals were undertrained with a shock immediately after the second trial and tested for retention 24 hr later. To magnify the possible effect of trained-brain extract (expected to be active in its full potentiality, because of its refinement), intensity of the aversive stimulus in recipients was reduced to 0.275 mA per 2 sec. The possible transfer effect at this stage was evaluated as in the third experiment.

Results and Discussion

Refined brain extracts from trained donors were able, as shown in Table 8, to increase latency times of naive recipients in the 6 or 12 hr but not in the 24 or 48 hr post-

TABLE 9

INDEPENDENCE OF THE EFFECT OF REFINED BRAIN EXTRACT FROM PASSIVE-AVOIDANCE-TRAINED DONORS ON LATENCY TIME* IN UNDERTRAINED RECIPIENTS FROM THE INTERVAL BETWEEN TREATMENT AND AVERSIVE STIMULATION

Treatment	24 hr post-shock retention test 30 hr		36 hr		48 hr		72 hr
Saline	61		32		90		43
	56–236		21– 64		39–148		23–140
	(6)		(6)		(23)		(24)
	n.s.		n.s.		n.s.		n.s.
Sham-trained-brain	41		76		53		62
	23– 58	<0.05	9– 82	<0.05	37–300	<0.02	33–184	<0.01
	(7)		(7)		(10)		(18)
	<0.01		<0.08		<0.05		<0.02
Trained-brain	207		176		250		177
	125–300		61–213		73–300		70–300
	(7)		(7)		(22)		(24)

*Median values and interquartile intervals in sec. One-sided Mann-Whitney U test.
( ) Number of animals.

TABLE 10

THE EFFECT OF BRAIN EXTRACT FROM PASSIVE-AVOIDANCE-TRAINED DONORS ON EXTINCTION OF AVOIDANCE BEHAVIOR IN UNDERTRAINED RECIPIENTS TREATED AT DIFFERENT TIMES AFTER APPLICATION OF THE AVERSIVE STIMULUS

	48 hr post-shock treatment			72 hr post-shock treatment		
	Pre-shock trial		48 hr post-treatment retention test	Pre-shock trial		48 hr post-treatment retention test
Saline	8		96	5		38
	4–11	<0.05	86–300	4–8	<0.01	10–162
		(5)			(12)	
Sham-trained-brain	5		140	5		25
	4– 9	<0.02	75–300	3–9	<0.01	9–119
		(7)			(8)	
Trained-brain	8		135	4		28
	6–13	<0.02	34–300	4–8	<0.01	8–60
		(7)			(10)	

*Median latency times and interquartile intervals in sec. Horizontal comparisons: Wilcoxon matched pairs signed ranks test; vertical comparions: one-sided Mann-Whitney U test. Vertical comparisons among treatments in no case showed a difference.
( ) Number of animals.

treatment trial. However, all groups, as shown in Table 9, exhibited an increase in latency time 24 hr after undertraining. The increase was partially evident in the one, shocked 12 hr after treatment. This would show that trained-brain extract operated a modification of naive recipients, so as to delay the natural behavior of stepping into the dark box. It is to be expected that such reduction of drive experienced by naive recipients in the second trial, might have amplified the passive-avoidance response following the aversive stimulus, so enhancing learning.

It was evident that the net enhancing result was largely independent from the length of interval between treatment and presentation of the aversive stimulus in the undertrained recipient.

EFFECT OF TRAINED-BRAIN EXTRACT ON THE EXTINCTION OF PASSIVE AVOIDANCE IN UNDERTRAINED RECIPIENTS

NINTH EXPERIMENT

The fifth experiment had shown that passive avoidance, in trained donors tested once only, decreased between 48 and 72 hr after the application of the aversive stimulus. Assuming that avoidance behavior in undertrained animals followed a similar course, we studied the effect of trained-brain extract on the process of extinction in undertrained recipients. Treatment was given either 48 or 72 hr after application of the aversive stimulus, expecting a positive effect to

result in a delay of extinction, in the first case, or in a recovery of optimal performance, in the second one.

Method

Trained and sham-trained donors and crude brain extracts were prepared as in the first experiment. Undertrained recipients were prepared as in the third experiment, except that treatment with extracts or saline was postponed 48 or 72 hr after shock. A single retention test was given 48 hr after treatment.

Results and Discussion

Treatment with trained-brain extract was totally unable, as shown in Table 10, to modify latency times in undertrained recipients, when given in presence, presumingly, of an already well-acquired passive avoidance or during the extinction phase. Consequently, it was evident that the factor transmitted with trained-brain extract was able to specifically enhance learning of the passive avoidance in recipients. This selectively affected the experience of the aversive stimulation (acquisition) but not its aftermath (retention).

Our finding of the lack of any effect on the extinction process of the acquired passive-avoidance behavior agrees with the previous demonstration [11], which had a similar behavioral model. This means that brain extracts from trained donors had no reminder effect in comparatively undertrained recipients. The 96 hr-tested recipients of the present experiment (one test only, Table 10) tended to behave (independently from the treatment) as sham-trained-brain and saline recipients, correspondingly tested, of the third experiment (two tests, Table 3). This indicated that the injecting procedure alone (48 hr after shock) might have a non-specific reinforcing effect equivalent to the specific one of a retention test at the same time. The occurrence of this effect was critically time-dependent, since it was absent in all recipients injected 72 hr after shock.

GENERAL DISCUSSION

The present experiments demonstrated a positive effect of the brain extract from donors trained in a passive-avoidance task on the learning of the same task in recipients.

This effect appeared specifically dependent on the experience of the learning trial by donors, provided a local contingency was warranted between trial and aversive stimulus in the experimental apparatus. Non-specific association, bound to the experimental situation, could be ascertained not to play a role in the transfer effect.

The effect was consistent and reproducible in undertrained recipients, treated with the extract before, not after, the supraliminal aversive stimulus. On the contrary, a transfer effect was inconsistent in naive recipients, and therefore, it was not certain that the enhanced learning in undertrained recipients could be attributed to information, conveying a cognitive behavioral content, transferred with the extract.

Enhanced learning in undertrained recipients was not dependent on the level of performance in donors, albeit specifically bound to their state of learning. In fact, "poor" and "rich", as well as mistrained-A donor brain extracts gave similar enhancing effects (Experiments 3 and 4).

The dissociation between potency of the extracts in recipients and degree of performance in their donors may appear incompatible with a transfer effect consisting of transfer of a behavioral response as such. It would also be incompatible with the weak aversive stimulus in recipients acting as a trigger of the transferred response. Compatibility would be assured only by the donor performance reflecting, and the retention test measuring, not the transferable avoidance learning, but another parameter affecting the expression of a learnt behavior, for instance retrieval capacity. This distinction of different factors contributing to the actual performance in a learned behavior has been proposed by Quartermain *et al.* [18] to explain the partial inhibition by cycloheximide in mice or ECS in rats of fixation of mnemonic traces of a passive-avoidance response. These authors ascribed the deficit in performance more to a failure of retrieval than to a disruption of consolidation process.

Brain extracts from high-performance donors tested for retention 48 hr after shock did not enhance performance in undertrained recipients, at variance with donors tested 24 hr after shock (Experiment 5). On the other hand, an enhancing effect was obtained with brain extracts from donors reinforced with a retention test (Experiment 7) and from mistrained-A donors (Experiment 4). In both cases, the animals were sacrificed 24 hr after the last retention test. Generalizing, one can say that an enhancing effect, independent from the donor performance, was only obtained if a 24 hr period were interposed between aversive stimulus and sacrifice, or between sacrifice and a retention test sufficiently close to the shock to act as a reinforcer.

Altogether, our findings indicate that neither a preformed behavior, nor a general activation aroused by training-stress (sham-trained-, mistrained-B-, mistrained-C-, only-shocked-brain extracts were totally ineffective) were likely to be transferred in recipients. Instead, it seemed probable that the transfer of information pertinent to a specific component in donor-training process, increased the "teaching efficiency" of the aversive stimulus in recipients, so resulting in enhanced learning. The equal degree of transfer effect from "poor" and "rich", as well as from mistrained-A donors, and its absence from high-performance donors, tested once at 48 hr after shock, would not regard the consolidated somatomotor response, but another responding component of the total apprehensible passive-avoidance behavior.

There are many experimental findings in favor of the existence of a component, emotional in nature, acquired in the passive-avoidance training, together with the somatomotor response, but consolidated independently from it.

Hine and Paolino [12] demonstrated that the passive-avoidance response is concomitant with an emotional response measurable as a modification in cardiac rate. According to the authors, the conditional autonomic response instaurates more rapidly than the behavioral response (inhibition of step-through) evoked by specific environmental cues. In fact, it is only the latter one that can be abolished by post-training amnesia-inducing procedures. Bohus [2] found that occurrence of passive-avoidance behavior is accompanied by tonic and phasic responses of cardiac rhythm. The former (bradycardia) is the emotional correlate of a generalized, conditioned, behavioral change, and is evoked in the aversively experienced animal by the avoidance apparatus. The latter (abrupt decrease in heart rate or arrhythmia) is viewed as specific sign of a highly discriminative fear response related to approach-avoidance movements in the conflict situation.

The emotional component in the total passive-avoidance behavior would explain the paradox that animals, taken in

repeated (unreinforced) retention tests, did not show any extinction. It was as if the repeated exposure to the situation, where the aversive experience had been made, had acted as a reinforcer by itself. This would not be surprising, since in the training for a passive-avoidance task the environment becomes an aversive cue [12]. In this context, it is to be noticed that "poor" and "rich", as well as mistrained-A donors had two retention tests: this common feature might have been responsible for the equal activity of their brain extracts, despite the wide range of their somatomotor responses. Our findings, in the hypothesis that the effect of brain extracts may consist of a transfer of a conditioned, emotional response acquired by donors in the avoidance training, should be examined by ignoring the level of donor performance per se. Rather, the interaction in donors should be taken into account between reinforcements (shock, primary, and retention test, secondary) and incubation time (reinforcement-sacrifice interval), as the limiting factor in the appearance of a transfer activity. It has to be considered that emotional and somatomotor response in avoidance behavior might evolve differently depending on their mnemonic fixation and extinction. This possibility is suggested by the fifth experiment. Brain extracts from donors tested for retention immediately after shock were devoid of transfer activity. In the case of instant sacrifice, these donors had had no time to consolidate any response. In the case of 24 hr-delayed sacrifice, the last experience to be memorized was not that of shock but of the shock-immediate-retention-test complex. In donors tested and sacrificed 24 hr after shock, the emotional response might already be present, as shown by the transfer activity of their brain extract (see also Experiment 6). The retention test could reinforce such response, fostering the permanence of the transfer activity in case of 24 hr-delayed sacrifice. Donors tested and sacrificed 48 hr after shock gave totally ineffective brain extract, despite their high performance: the emotional response to the old aversive experience in the learning trial might have extinguished at this moment. However, the new aversive experience in the retention test could reactivate such response and reinstate the transfer activity in the case of 24 hr-delayed sacrifice. In donors tested and sacrificed 72 hr after shock, both emotional and somatomotor response had extinguished. No transfer activity remained in brain extract, and the retention test was unable to reactivate both the responses, and to reinstate transfer activity.

The present findings speak in favor of the existence of a chemical transfer of information from trained donors into recipients taken in the same passive-avoidance task. It is impossible at this moment to make any definite statement on the content of the transferred information, because of the aberrant situation where we had to consider a variable, the emotional response, the assessment of which had not been planned. At any rate, it would appear that the transfer effect in the avoidance behavior concerns the former more than the somatomotor response.

This interpretation might be supported by some reports, according to which an increased level of emotionality was found in brain-extract recipients, when exposed to the same experimental apparatus, where their donors were shocked [7,17]. This phenomenon was also observed in scotophobin-treated animals, specifically related to the experimental situation in which scotophobin was originally obtained [14].

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