Pharmacology Biochemistry and Behavior, Vol. 3, pp. 463–470. ANKHO International Inc., 1975. Printed in the U.S.A.

Distribution of ^{3}H-Uridine-5 in Rat Brain Areas After Exposure to Various Training Tasks – An Autoradiographic Analysis[1]

JAMES E. SMITH[2]

Psychiatry Research, University of Minnesota, Minneapolis, MN 55455

(Received 25 November 1974)

SMITH, J. E. *Distribution of ^{3}H-uridine-5 in rat brain areas after exposure to various training tasks – an autoradiographic analysis.* PHARMAC. BIOCHEM. BEHAV. 3(3) 463–470, 1975. – Operant schedules were used to isolate components of a training task and the distribution of radioactivity into various brain areas was studied using high resolution autoradiography. Rats exposed to a visual stimulus change showed more labelling in hippocampal area CA1 than littermates exposed to no stimulus change. Rats exposed to a non-cued contingency change showed higher labelling in hippocampal areas CA2 and CA4 than littermates exposed to a cued contingency change. The results suggest that hippocampal changes in RNA synthesis may be related more to the aversive conditions of the training task than to specific learning changes.

RNA changes during training Neurochemical correlates of training Neurochemical correlates of behavior
Autoradiography

MANY studies of neurochemical correlates of training have attempted to assign functions to specific brain areas. Three general research techniques have been used to investigate RNA changes in brain areas during training: dividing the brain into subunits and studying changes in those subunits [1, 7, 14, 15, 21, 23]; microdissection of brain areas and individual cells and investigating changes therein [9]; and autoradiography [13, 16, 17].

With dissection of the brain into large subunits, it has been demonstrated that absolute levels of RNA increase in the hippocampus and neocortex of rats given shock avoidance training [15] and in the cerebrum and brain stem of shock avoidance trained rats exposed to extinction [1]. Radioactive precursors to RNA were incorporated at an increased rate, compared to controls, in the hippocampus of rats exposed to reversal training in a Y maze [3], in the diencephalon of mice given shock avoidance training [23], and in the forebrain roof on the imprinted side of the brain of split brain chicks [8].

In studies using microdissection of small cell samples, increases were found in the absolute level of RNA in neurons [10] and glial cells [11] of the lateral vestibular nucleus of rats trained to climb a wire to obtain food. In rats forced to change paws used to retrieve food pellets from a narrow tube, increases in RNA content were found in neurons from the area of the brain controlling movement of that paw [12].

Autoradiography has been used to study changes in the distribution of radioactive precursors to RNA in brain areas of rats given shock avoidance training. Compared to yoked-shocked controls, increased incorporation of radioactivity has been found in the hippocampus [13] and hippocampal areas CA1, CA2, CA3, and CA4, the dendate gyrus, the deep cortex of the visual areas, the cortex cigularus [16], the cytoplasm of pyramidal cells in hippocampal areas CA1, CA2, and CA3 and granular cells [17].

That these changes in RNA are the result of the observed changes in response probability (learning) – and not non-specific stimulation and stress differences – has not been demonstrated. Training tasks are complex environments that contain many stimuli that may themselves cause changes in RNA synthesis [6]. In the present study, operant training procedures and high resolution autoradiography have been used to isolate the effects of some of the component parts of a training task on the distribution of ^{3}H-uridine-5 in various brain areas of trained rats. The distribution of radioactive precursors to RNA in various brain areas may reflect changes in RNA synthesis in these areas. Changes in the distribution of ^{3}H-uridine into RNA [19] and RNA species [20] of animals trained in these

[1] This research was funded by USPHS Training Grant 5–TI–MH–8565–05.

[2] Current address: Institute of Psychiatric Research, Indiana University School of Medicine, 1100 W. Michigan Street, Indianapolis, Indiana 46202.

tasks have previously been demonstrated. This study was undertaken to determine if those changes are accompanied by changes in specific brain areas.

METHOD

Animals

Twelve male Fisher F-344 strain 90–120 day old rats (Hilltop Labs, Chatsworth, Calif.) in 3 groups of 4 littermates were used. Littermates were housed 4 to a cage, maintained at 80% of their free feeding weight, and given free access to water in a continually illuminated, temperature and humidity controlled environment.

Procedure

Littermates from this highly inbred strain were used to minimize genetic differences that might affect the background level in neurochemical pathways being studied. Each group of littermates was trained to lever press on a fixed ratio 20 (FR 20) schedule for food reinforcement (45 mg Noyes pellets) until stable baselines were obtained (±5 responses per minute), one training session per day, seven sessions per week. To minimize differences due to differential experience in the training environment, an FR 20 baseline training schedule was used. This produced similar response rates, with which the number of reinforcements obtained the number of responses emitted, and the number of training sessions administered, were held constant within each experimental group (litter).

After stable baselines were obtained (approximately 35 sessions), the 4 littermates were injected intraperitoneally with 100 μc of ^{3}H-uridine-5 (specific activity 28 C/mM) 60 min prior to exposure to 60 min of one of the following training conditions:

(a) A multiple 2-min FR 20 2-minute extinction schedule (MULT). This condition consisted of two alternating schedules of reinforcement – FR 20 and extinction with a different stimulus present during each. With this schedule the session light that had been present during FR 20 training alternated between being lit for two minutes (during which the FR 20 component was in effect and food was available) and being extinguished for two minutes (during which the extinction (EXT) component was in effect and food was withheld). The animals exposed to this condition developed a discrimination and responded at a normal rate when the light was lit and reinforcement was obtained but responded very little when the light was extinguished and no reinforcement was available.

(b) A mixed FR 20 EXT schedule with a randomly interpolated stimulus change (MIX). In this condition reinforcement was programmed by the same two schedules (FR 20 and EXT) alternating at random without stimuli correlated with the change of schedule. The session light was randomly lit and not lit 50% of the time during each schedule component. These animals received the same amount of schedule change (30 min FR 20 and 30 min EXT) and stimulus change (30 min of light and 30 min of no light) as the MULT animal, but the stimulus change was not correlated with the schedule change. These animals had no cue as to which portion of the schedule was present and responded at a high rate during both components (FR 20 and EXT).

(c) FR 20 stimulus change schedule (FR 20 Stimulus). In this condition the session light was lit 2 min and extinguished 2 min in the same alternating sequence as in the MULT condition but there was no schedule change. The stimulus presentation in this condition was exactly the same as the stimulus presentation in the MULT condition.

(d) FR 20 schedule. These animals were exposed to no change from the original training condition.

With these four training conditions it was possible to separate and measure the affects of component parts of the total training task. In each litter the effects of the stimulus change were determined by comparing the FR 20 Stimulus animal with the FR 20 animal. The effect of the contingency change (schedule change plus the discrimination in responding) was determined by comparing the MULT animal with the FR 20 Stimulus animal since both were exposed to the same stimulus change. The effect of the discrimination was determined by comparing the MULT with the MIX animal since both were exposed to the same amount of physical stimulus and schedule change.

Immediately after completion of the 60 min treatment session, the animals were sacrificed by immersion in Freon 12 cooled in liquid nitrogen (-155°C). Stainless steel tubes were used both as transporting devices during training and as freezing tubes that were easily lowered into a Dewar flask of Freon 12. The brains were chiseled out with a vise and chisels cooled in liquid nitrogen (-197°C). The frozen brains (-197°C) were mounted on tissue carriers with a 5% gelatin solution that were immediately immersed in liquid nitrogen for rapid solidification and to prevent warming. Four-micron coronal sections of the brains were made at -30°C with an International custom rotary microtome mounted in a cryostat (that was equipped with arm ports and a clear plastic window). The sections were picked up under safe lighting (Wratten No. 2 filter, Eastman Kodak Co.) on microscope slides precoated with NTB-2 nuclear track emulsion (Eastman Kodak Co.) by gentle pressure with the slides also at -30°C. The autoradiographs were exposed for four weeks in light tight boxes containing dessicant in the cryostat at -30°C. The autoradiographs were then developed 2 min at 18°C with Kodak Dektol developer, fixed 3 min in Kodak acid fix, washed 10 sec in gently circulating tap water, fixed in neutral buffered Formalin for 4 min, washed 5 min in gently circulating tap water, stained 5 min in methyl green-pyronine, air dried, cleared in xylene, coverslipped, and grain counted under dark field illumination at 1000 dia. with an American Optical Model 10 Microstar monocular microscope. Controls for negative and positive chemography were processed with each group [18]. Sixteen brain areas were counted from each animal (Fig. 1). A sample of 5 counts of a 2500 μ^2 grid area was taken from each of these areas and a background sample of five counts taken from the emulsion adjacent to the tissue sections. These background counts were subtracted from the counts obtained from the tissue sections. The counts from the hippocampal cell layers and visual areas were summed to produce the individual area counts.

RESULTS

Behavior

The patterns of lever press responding during the 60 min training session are different for the different training conditions. Cumulative records for the day before and the day of exposure for one group of littermates are shown in

BRAIN AREAS COUNTED

I. CORTEX

Motor Area 4

Visual Areas 18
17
18a

II. HIPPOCAMPUS

CA-1 Pyramidal Layer
Molecular Layer
Polymorphic Layer

CA-2 Pyramidal Layer
Molecular Layer
Polymorphic Layer

CA-3 Pyramidal Layer
Molecular Layer
Polymorphic Layer

CA-4 Pyramidal Layer
Molecualr Layer
Polymorphic Layer

FIG. 1. The brain areas from which five 2500 μ^2 counts were made from autoradiographs of trained rats.

Fig. 2. The animal exposed to the MULT schedule developed a discrimination and responded at different rates during the two stimulus conditions. The different patterns of responding are more evident in Fig. 3 which shows the response rate ratios (ratio of responses during successive 2 min of light to responses during 2 min of no light) for the same group of animals. The ratios are large for the MULT animal which responds primarily in the presence of the stimulus light when reinforcement is present and responds little when the light is out and no food is available. For the MIX and FR 20 Stimulus animals, the response rates are the same during both stimulus conditions and thus the ratios are about 1.0. In the last eight minutes of the training session (2 four-min segments) the response rate ratios of the MULT animals differ significantly from the MIX (paired t = 3.44, df = 2, p = 0.03) and the FR 20 Stimulus (paired t = 3.51, df = 2, p = 0.03) animals (Log transformations were performed to normalize the data and one-tailed confidence levels used since apriori prediction of direction was possible). The Stimulus develops control of lever pressing behavior of the MULT animals but not the MIX or FR 20 Stimulus animals.

Autoradiography

The autoradiographs were counted under dark field illumination with which it was possible to get accurate counts of single silver grains since the 0.5 micron grains appear as bright white dots on a dark background [18]. The cell nuclei appear as red and yellow areas and the extranuclear space as pink. Parallel processed controls for posi-

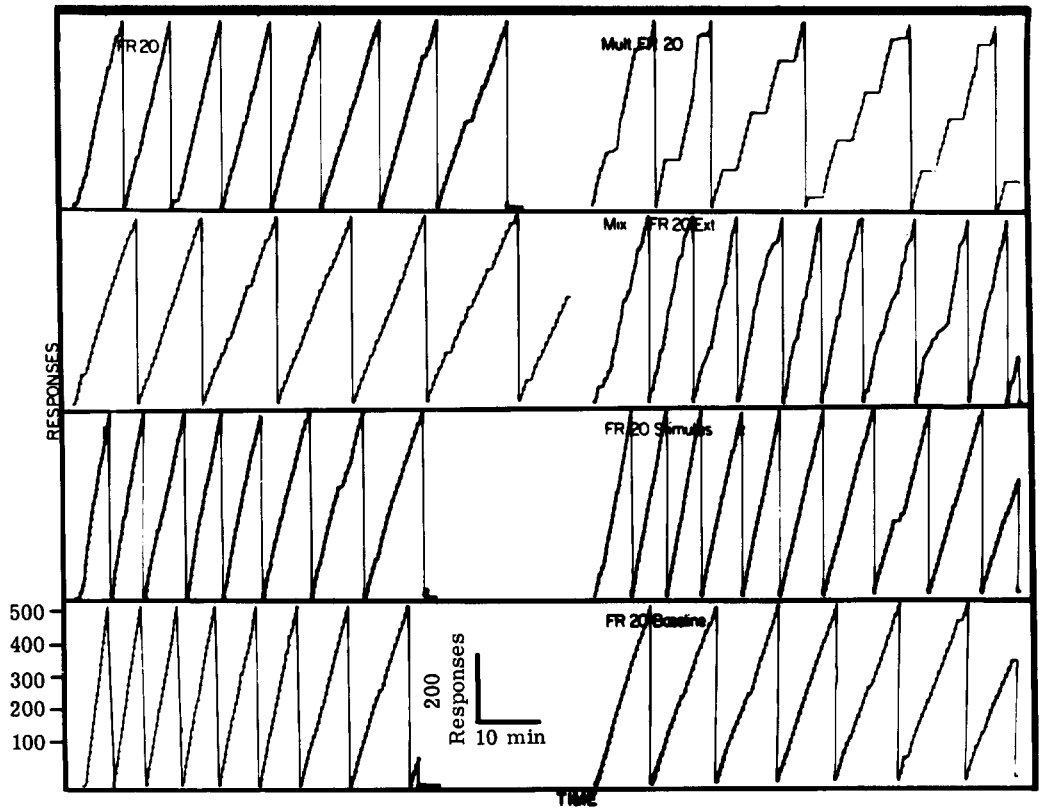

FIG. 2. Cumulative records of individual animals' lever pressing behavior the day before (left and the day of exposure to the 60 min training task (right) for one group of littermates used in this experiment.

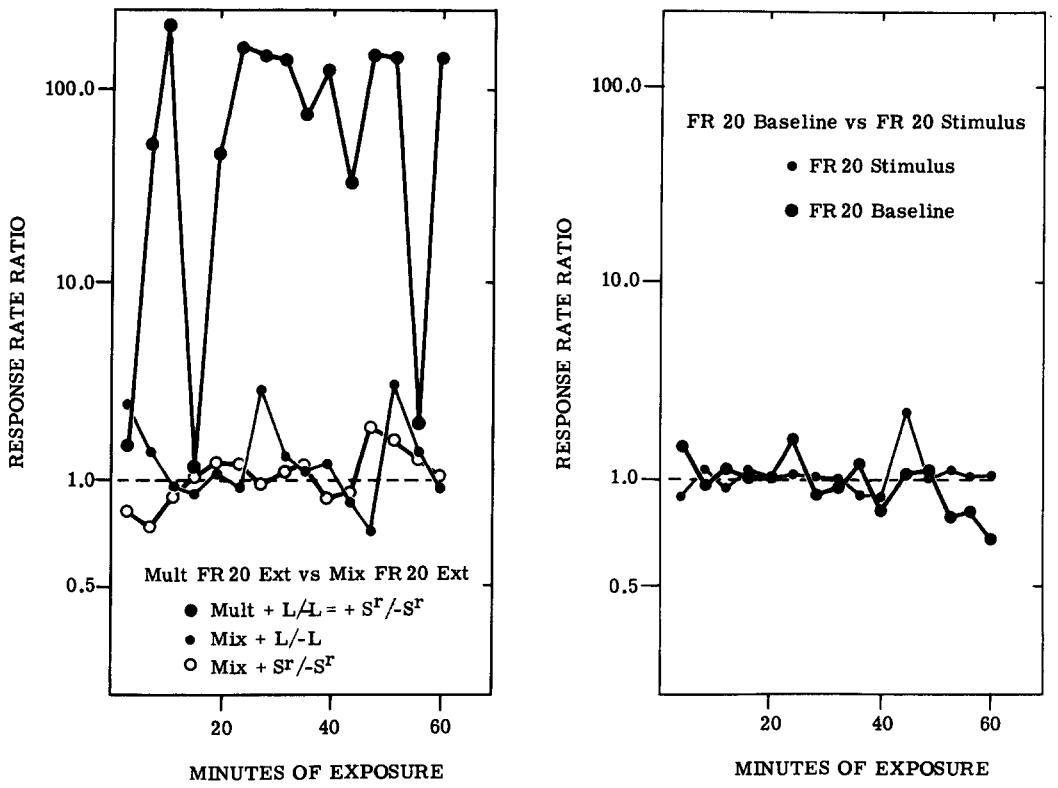

FIG. 3. Response rate ratios for the 60 min training session for the group of littermates from Fig. 2 (+L = stimulus light on; -L = stimulus light off; $+S^r$ = with food = FR 20; $-S^r$ = without food = EXT). Response rate ratios were calculated by computing the ratio of responses during successive 2 min periods of light to 2 min of no light for the MULT, MIX and FR 20 stimulus groups and the ratio of responses during a corresponding period for the FR 20 animal since there was no stimulus change. In this way fifteen ratios are computed for each animal for the 60 min training session.

tive and negative chemography were negative. In brain areas of some of the animals the tissue counts were slightly below background. These were recorded and processed as negative numbers rather than zero to allow statistical evaluations to be performed. This prevents tests of significance from being deceptively significant because of the decreased error term that would be obtained if the negative values were treated as zeros.

The mean net grain counts for motor area 4 are shown in Fig. 4A. The MULT group has a higher grain density than the MIX, FR 20 Stimulus or FR 20 groups (Table 1). There are no significant differences for the component parts of the training task (Table 2).

Paired *t* analyses have been used to test the effects of the component parts of the training tasks throughout this experiment. The discrimination and stimulus effects are independent comparisons. However, the contingency change effect may not be an independent comparison, and must be recognized as such. If the stimulus produced an effect in the opposite direction of the effect produced by exposure to the MULT condition, then this comparison would not be independent. However, previous data [19,20] indicates that the change is in the same direction and therefore this comparison is likely to be independent.

The mean net grain counts for the visual areas are shown in Fig. 4B. The MULT group has less mean counts than the MIX, FR 20 Stimulus or FR 20 groups (Table 1). An analysis of the effects of the component parts of the training task on the net mean grain counts in the visual cortex showed no significant difference (Table 2).

The mean net grains in the hippocampus area CA1 are shown in Fig. 4C. The MIX group has the highest density with the FR 20 Stimulus group next, and the MULT and FR 20 groups having the least (Table 1). An analysis of the effects of the component parts of the training task on the grain density in area CA1 showed that significantly more radioactivity is found in area CA1 in animals exposed to a visual stimulus change (FR 20 Stimulus) that animals not exposed to a stimulus change (FR 20) ($p = 0.01$) or than animals that develop a new pattern of responding to the visual stimulus change (MULT) ($p = 0.05$) (Table 2).

The net mean grains in Area CA2 are shown in Fig. 4D. Again the MIX group has the highest mean value, the FR 20 Stimulus group the next highest, and the MULT and FR 20 groups the least (Table 1). An analysis of the effects of the component parts of the training task showed that the animals exposed to the non-signalled contingency change (MIX) have more ($p = 0.04$) radioactivity in area CA2 than animals exposed to the signalled contingency change (MULT) (Table 2).

The MIX group shows the highest grain density in area CA3, the FR 20 Stimulus group is next, followed by the

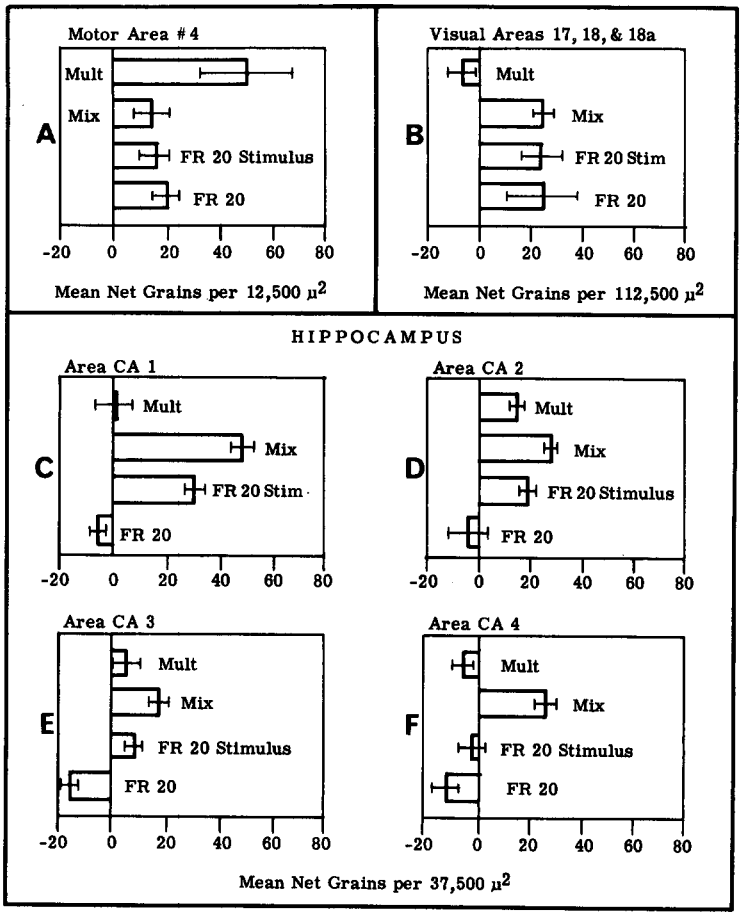

FIG. 4. The net mean grain densities for the trained rats for the motor cortex area 4 (A); visual cortex areas 17, 18 and 18a (B); and the hippocampus area CA1 (C), CA2 (D), CA3 (E), and CA4 (F).

TABLE 1

DISTRIBUTION OF SILVER GRAINS IN AUTORADIOGRAPHS OF BRAIN AREAS OF THREE SETS OF TRAINED LITTERMATES*

	Motor Area 4	Visual Areas 17, 18, 18a	Hippocampal Areas CA 1	CA 2	CA 3	CA 4
MULT	49.7 ± 42.0	−6.0 ± 10.2	1.7 ± 13.9	12.7 ± 5.4	6.3 ± 8.2	−12.2 ± 11.2
MIX	13.5 ± 15.3	25.0 ± 0.5	51.3 ± 10.5	24.8 ± 3.6	20.0 ± 8.2	25.3 ± 10.3
FR 20 Stimulus	15.5 ± 9.8	23.5 ± 17.8	33.3 ± 6.9	16.3 ± 4.4	10.3 ± 6.8	−2.0 ± 12.5
FR 20	20.0 ± 11.0	25.0 ± 40.8	−2.5 ± 5.1	−5.0 ± 18.1	−13.2 ± 5.5	−12.2 ± 11.2

*Values are means ± SEM (N = 3 per group).

TABLE 2

PAIRED t VALUES AND p VALUES FOR THE EFFECTS OF COMPONENT PARTS OF THE TRAINING TASK ON THE DISTRIBUTION OF SILVER GRAINS IN BRAIN AREAS OF THREE SETS OF TRAINED LITTERMATES

	Motor Area 4	Visual Areas 17, 18, 18a	Hippocampal Areas CA 1	CA 2	CA 3	CA 4
Stimulus Change	$t = -0.41$	$t = -0.04$	$t = 18.64$	$t = 1.22$	$t = 3.49$	$t = 1.08$
(FR 20 Stimulus vs FR 20)	$p = 0.74$	$p = 0.97$	$p = 0.01$*	$p = 0.38$	$p = 0.08$	$p = 0.42$
Contingency Change	$t = 0.74$	$t = -1.28$	$t = -4.54$	$t = -0.50$	$t = -0.27$	$t = -0.37$
(MULT vs FR 20 Stimulus)	$p = 0.54$	$p = 0.38$	$p = 0.05$*	$p = 0.66$	$p = 0.82$	$p = 0.76$
Discrimination	$t = -0.70$	$t = -3.00$	$t = -2.07$	$t = -4.88$	$t = -2.95$	$t = -9.09$
(MULT vs MIX)	$p = 0.56$	$p = 0.10$	$p = 0.18$	$p = 0.04$*	$p = 0.10$	$p = 0.01$*

Two-tailed confidence levels are used since apriori predictions of direction were incorrect.
*Significant effects

MULT and FR 20 groups (Fig. 4E and Table 1). An analysis of the effects of the component parts of the training task showed no significant differences (Table 2).

The MIX group is the only group that shows a level of labelling above background in area CA4. All other groups are less than background (Fig. 4F and Table 1). An analysis of the effects of the component parts of the training task on the distribution of radioactivity in area CA4 showed that the animals exposed to the non-signalled contingency change (MIX) have significantly ($p = 0.01$) more silver grains in area CA4 than the animals exposed to the signalled contingency change (MULT) (Table 2).

DISCUSSION

Autoradiography has been used by three other groups looking for changes in RNA during training. All three studies used shock avoidance training and a perfusion technique for tissue preparation. Increased incorporation of labelled uridine into the hippocampus of shock avoidance trained rats compared to yoked-shocked controls has been demonstrated [13]. With fifteen minute postsession subarachnoidal injection of ^{3}H-uridine, an increased proportion of radioactivity was found in the cytoplasm of pyramidal cells of the hippocampus in areas CA1 and CA2 and in the dendate gyrus CA3 granular cells of shock avoidance trained rats compared to yoked-shocked and untrained controls [17]. In shock avoidance trained rats, ^{3}H-uridine was incorporated at an increased rate into areas CA1, CA2, CA3, and CA4 of the hippocampus, the granular areas of the dendate gyrus, the deep cortex of the visual areas and the cortex cingulara compared to shocked and untrained controls [16]. These studies used a perfusion technique and parafin infiltration which subjects the tissue to extreme anoxic and ischemic conditions. Induction and a resulting increase in RNA synthesis are possible results of such tissue changes. In this study the animals were sacrificed by immersion in liquid nitrogen and the tissue not allowed to warm above -30°C until after the autoradiographs were exposed for four weeks and ready for development and fixation. The silver grains represent total radioactivity – precursor and products – in rats exposed to minimal anoxic and ischemic conditions. The specific changes due to training may be subtle and thus easily masked by more diverse stimuli such as ischemia and anoxia.

It has previously been demonstrated with the training tasks used in this experiment that ^{3}H-uridine is absorbed into the brain and incorporated into total and cytoplasmic RNA to a greater degree in rats exposed to the MULT condition than in littermates exposed to the stimulus change only [19]. When the nuclear and cytoplasmic RNA was electrophoresed on polyacrylamide gels, the cytoplasmic mRNA of the MULT group showed a significantly higher specific activity than that of littermates exposed to the MIX schedule [20]. These findings are consistent with previously reported data [12]. However, the results of the high resolution autoradiographs in this experiment appears to be contrary to existing data that reports increased RNA synthesis in the hippocampus of animals during training [2, 3, 13–17].

It has been suggested that events in the hippocampus are directly related to the learning process. However, the MULT animals' hippocampal labelling is no different from the FR 20 group (no change from baseline) and the MIX animals' is significiantly higher. All but one of the studies that have reported an increased rate of RNA synthesis in the hippocampus during training have used shock avoidance training procedures [13, 15, 16, 17] which are stressful procedures; that study [3] used reversal training for water reinforcement in a Y-maze. The hippocampus is an integral part of the limbic system which has been implicated in emotional behavior. It is possible that increased rates of RNA synthesis in the hippocampus reflect the degree of stress or aversiveness in the training task and is not a specific learning effect. It has been demonstrated that one IP or ten daily IP injections of saline produce an increase in the absolute levels of RNA in the hippocampus [15]. It has also been demonstrated that exposure to the goal box alone in the Y maze, or to water drinking in the home cage or the goal box, produces increased rates of incorporation of a radioactive precursor into hippocampal RNA [2]. A novel

environment or stress alone may be adequate stimuli to bring about hippocampal RNA changes.

The bulk of the data showing changes in hippocampal RNA during training have used shock avoidance which are stressful procedures. Control groups are usually animals presented with non-contingent shock. It is possible that non-contingent shock presentation and avoidance are not comparable procedures in that the non-specific effects of the procedures upon behaving organisms are not comparable. Differences in autonomic responses have been reported in monkeys given contingent and non-contingent shock [4]. These autonomic responses suggest that animals exposed to the contingent shock show more stress effects [22] than animals given non-contingent shock. Hippocampal differences in RNA synthesis from controls may be due to stress differences in the shock avoidance procedure.

Data indicate that pigeons prefer MULT schedules over MIX schedules in a concurrent situation [5]. In a two-key concurrent option situation, pigeons respond almost completely in the MULT component even if the MIX component has a higher density of reinforcement. This result is explained as a preference of the animals for contingency changes with correlated discriminative stimuli rather than contingency changes without correlated stimuli. Contingency changes with uncorrelated stimuli are relatively more aversive (less preferred) than contingency changes with correlated discriminative stimuli.

It is possible that the MIX schedule is more aversive than the MULT schedule because of the contingency change with the uncorrelated stimulus. The increased labelling in the hippocampus of the MIX group could reflect the relative degree of aversiveness of that experimental condition. Rats given reversal training in a Y maze are exposed to a contingency change with no correlated stimulus similar to the MIX animals in this experiment. The differences demonstrated between the reversed and the nonreversed groups [3] may reflect the relative degree of aversiveness in the training of these two groups and not "learning" specific changes.

The training task used in this experiment has allowed the separation and measurement of component parts of the training task. The effect of the stimulus change (FR 20 Stimulus versus FR 20) was an increased level of radioactivity in Area CA1 of the hippocampus. The effect of the contingency change (MULT versus FR 20 Stimulus) was a decreased level of radioactivity in area CA1 of the hippocampus. The animals to whom the stimulus change had no consequence and thus was novel (FR 20 Stimulus) showed more labelling in Area CA1 of the hippocampus than animals to whom the stimulus had consequences or informational properties (MULT). The effect of the discrimination in responding (MULT versus MIX) was the opposite of the acquisition of the change in response probability. The MIX group which was exposed to the non-signalled schedule change showed more radioactivity in areas CA2 and CA4 of the hippocampus. This may be the result of the greater degree of aversiveness that is associated with the MIX compared to the MULT condition. These differences in hippocampal areas may be related to the functional role of these areas in behavior.

The MULT group which develops a change in response probability to the stimulus change showed an increased absorbtion of radioactive precursor to RNA into the brain, an increased percentage of precursor incorporated into RNA, increased specific activity of total and cytoplasmic RNA [19], and a higher specific activity cytoplasmic mRNA [20], but in the hippocampus, the MULT group showed a level of radioactivity that was no different from baseline training (FR 20 group). The increased RNA synthesis in the hippocampus that has been previously reported in trained animals may be the result of the stress factors in these training tasks, and not the result of specific learning changes.

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