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Distribution of ^{3}H-Uridine-5 into Brain RNA Species of Rats Exposed to Various Training Tasks – An Electrophoretic Analysis[1]

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SMITH, J. E. *The distribution of ^{3}H-uridine-5 into brain RNA species of rats exposed to various training tasks – an electrophoretic analysis.* PHARMAC. BIOCHEM. BEHAV. 3(3) 455–461, 1975. – Operant schedules were used to isolate component parts of a training task and specific activities were determine for nuclear and cytoplasmic RNA species separated by polyacrylamide disc gel electrophoresis. Rats exposed to a stimulus or schedule change incorporated more radioactivity into nuclear rRNA and mRNA and cytoplasmic rRNA and tRNA than littermates exposed to no change from baseline training. Rats developing a change in response probability to a stimulus in the environment incorporated more radioactivity into cytoplasmic mRNA.

RNA changes during training Neurochemical correlates of training Rat brain RNA changes
Brain RNA RNA Polyacrylamide gel electrophoresis

SINCE changes in RNA have been correlated with information processing and storage by the central nervous system, there have been attempts to determine the types of RNA that are involved [14,28]. The different species of RNA have different functions and a change in the rate of synthesis, destruction, or cellular location may indicate functional changes that occur in the brain concurrent with behavioral manipulations. The molecular events responsible for changes in response probability (learning) are not known, but if changes occur that involve enzyme induction, synaptogenesis, or nearly any known physiological or biochemical event, then a resulting increase in RNA synthesis is likely. Therefore, changes that occur in RNA species may indicate some of those functional changes that take place.

The different species of RNA not only have different functional roles but also differ in base ratio composition, degree of methylation, molecular weight, and size. These differences have been used as basis for separation and characterization of RNA species [10]. Recent investigations of changes in RNA species during training have used sucrose density gradients [7], RNA-DNA hybridization [27] and base ratio analysis [11]. It has been demonstrated using sucrose density gradients that more radioactivity is incorporated into nuclear and ribosomal RNA (rRNA) in mice given shock avoidance training than in yoked-shocked controls [28]. The greatest increase in labelling was later found to be associated with the polysomes [1,2], and after treatment with RNase the radioactivity was found at the top of the gradient. It was therefore concluded that the labelled species were the messenger RNAs (mRNA) to which the ribosomes were attached since intact ribosomes are resistant to RNase digestion [8].

Early studies using RNA-DNA hybridization techniques reported discovery of a new mRNA species synthesized during shock avoidance training that was different from that of non-shocked controls [22], forced exercise controls [23], or yoked-shocked controls [24]. However, it was then demonstrated that these data may be in error because of incomplete hybridization with control RNA, since insufficient concentration of the control RNA was used to saturate the DNA [27]. More recent attempts using this technique to demonstrate new species of mRNA in animals given visual stimulation compared with those kept in the dark and using adequate levels of control RNA have been negative [6].

The analysis of base ratio changes during training to infer changes in the synthesis or degradation of RNA species in animals during training was one of the first techniques employed in this area. Changes that were found in base ratios of neuronal RNA from the Deiters nucleus of rats trained to climb a wire maintained by food reinforcement are consistent with changes that would be detected if there had been an increase in mRNA concentration [14]. These same changes were observed in glial RNA [15] and

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were present only in the early stages (3–5 days) of training [17]. Whole brain analysis showed an increase in rRNA but no change in tRNA [11]. Similar changes in base composition have been demonstrated in RNA from animals forced to change paws in a change of handedness training task. In the area of the brain, in the hemisphere controlling movements of the paw being changed to, there was a change in base ratios that would be consistent with an increase in mRNA [16] and this change was present only during the early stages of learning [17]. On the other side of the brain in the same area in the same animals and in animals not forced to change paws, the change in base ratios were consistent with an increase in rRNA. This increase in rRNA was also found in the switched animals during late stages of training (6–9 days) [13,18].

These methods of assay of RNA species changes have limitations. Changes in the RNA base ratios are general estimates of changes in RNA species which may not be sensitive to subtle changes or would not detect changes that offset one another. Density gradients suffer from the limitations of existing apparatus. The length of the gradient is an important factor determining resolution; however, existing high speed centrifuge heads do not have sufficient capacity to accommodate long gradients. As a result, the resolution of the RNA into different species is crude compared to column chromatographic techniques [10]. Electrophoresis on polyacrylamide gels is a rapid and versatile technique for separating RNA species yielding good resolution and replicability [5]. This method does not suffer from many of the limitations of density gradients and base ratio analysis.

The changes in RNA species during training that have been reported [8, 14, 16] may not be the results of the specific effects of the change in response probability, but may also be the result of other differences. A training task is a complex environment with stimulus and motor components that may themselves cause changes in RNA synthesis [12,25]. This study was undertaken to use operant training procedures to isolate and measure the effects of some of the component parts of a training task on the rate of incorporation of ^{3}H-uridine-5 into nuclear and cytoplasmic RNA species separated by polyacrylamide disc gel electrophoresis. Changes in nuclear and cytoplasmic RNA fractions of animals trained in these tasks have recently been reported [26]. This study was conducted to determine if those changes are reflected in changes in specific RNA species.

METHOD

Behavioral

Animals. Twelve male Fischer strain F-344, 90–120 day old adult rats (Hilltop Labs., Chatsworth, Calif.) were used in 3 groups of 4 littermates. The rats were maintained at 80% of their free-feeding weights with free access to water in group cages (4 littermates to each cage) in a temperature and humidity controlled environment with the room illuminated continually.

Procedure. In each group of littermates the 4 rats were trained to lever press on a fixed ratio 20 (FR 20) schedule for food reinforcement until stable baselines were obtained (± 5 responses per min). Within each litter the number of responses emitted, number of training sessions, and the number of reinforcements received during training were held constant.

After stable baselines were obtained (approximately 35 sessions), the 4 littermates were lightly anesthetized with ether and injected with 50 μc of ^{3}H-uridine-5 (specific activity 28 c/mM) in 50 μl of saline into the area above each cerebral hemisphere approximately 4 mm lateral to the superior saggital suture, 2 mm anterior to the posterior lateral suture, and 3 mm below the skin with a 27 g needle [4]. Sixty min after administration of the label, the animals were exposed with random assignment to one of the following training conditions:

(a) A multiple 2 min FR 20 2 min extinction schedule (MULT). In this condition the session light that had been present during FR 20 training alternated between being lit 2 min during which the FR 20 component was in effect and being extinguished for 2 min during which the extinction (EXT) component was in effect. The animals exposed to this condition developed a discrimination and responded at a normal rate when the light was on and reinforcement obtained but responded very little when the light was off when no reinforcement was available.

(b) A mixed FR 20 EXT schedule with a random interpolated stimulus change (MIX). In this condition the session light was randomly lit and not lit 50% of the time during each schedule component (FR 20 and EXT). The animals exposed to this condition received the same amount of contingency change (30 min FR 20 and 30 min EXT) and stimulus change (30 min of light and 30 min of no light) overall as the MULT animals, but the stimulus was not correlated with the schedule change. These animals had no cue as to which portion of the schedule was in effect, responded at a high rate all of the time and did not develop a discrimination to the visual stimulus.

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

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

Using these 4 training conditions, neurochemical effects of components of the training task were isolated and measured. The effect of the stimulus change was isolated by comparing the FR 20 Stimulus animal with the FR 20 animal in each litter. The effect of the schedule change (FR 20 to EXT) and discrimination (Contingency effect) were measured by comparing the MULT with the FR 20 Stimulus animal since both were exposed to the same stimulus change. The effect of the discrimination was measured by comparing the MULT with the MIX animals since both were exposed to the same amount of physical stimulus and schedule change.

Biochemical

Immediately after the 60 min exposure to the training condition, the animals were sacrificed by immersion in Freon 12 cooled in liquid nitrogen (-155° C). Stainless steel tubes 2½ in. in dia. were used both as transporting devices during training and as freezing tubes that could easily be lowered into a deware of Freon 12 to minimize the stress effects of the sacrifice procedure. The brains were chiseled out without being allowed to warm using a vise and chisels cooled to -197° C with liquid nitrogen. The olfactory bulbs and cerebellum were discarded and the brains stored at -197° C until used for biochemical analysis.

The brains were separated into nuclear and cytoplasmic

franctions and the RNA extracted using a procedure previously described [26]. The brains were pulverized, homogenized, the nuclei deposited by centrifugation, and the cytoplasmic fraction decanted. The polysomes attached to the perinuclear membrane were released by detergent treatment, the nuclei lysed, the nuclear RNA released from the chromatin by DNase treatment, and the RNA from both nuclear and cytoplasmic fractions extracted with hot phenol-chloroform. The RNA was precipitated, collected by centrifugation, and quantitatively assayed for amount [19] and radioactivity.

The nuclear and cytoplasmic RNA was separated into species with composite agarose-polyacrylamide disc gel electrophoresis using a modified procedure of one previously reported [3]. Gels of 2.25% (w/v) acrylamide and 0.15% (v/v) ethylene diacrylate containing 0.5% agarose were used.

Gel Preparation. Agarose (0.188 g) was dissolved in water (18.7 ml) by heating with continual stirring and cooled to 50°C. A solution of acrylamide-ethylene diacrylate (5.6 ml) (15% acrylamide (w/v)–1% ethylene diacrylate (v/v)) and 3E buffer (12.5 ml) (0.12 m NaAc, 0.006 M EDTA, pH 7.2) was heated to 50°C and added to the agarose solution. The solution was degassed by evacuation in vacuo for 30 sec, TEMED (0.03 ml of N,N,N′,N′-tetramethylethyenediamine) was added and mixed briefly; freshly prepared 10% ammonium persulfate (0.3 ml) was added and the gels rapidly poured into 10 x 0.6 cm glass columns that were precoated with a 4% solution of Kodak Photo Flow 200 and with the bottoms sealed with parafilm. After 30 min the polymerized gels were extruded with gentle air pressure and 0.5 cm was excised from each end (extruded agarose layer). The bottoms were covered with dialysis tubing, several holes were punctured to facilitate fluid flow and the gels were stored overnight at 4°C in E buffer (0.04 M TRIS-HCl, 0.001 M NaAc, 0.02 M EDTA, 0.2% sodium dodecyl sulfate, pH 7.2).

Gel Electrophoresis. The gels were prerun at 5 mA per gel, 65 volts DC, for 2 hr using the E buffer as electrophoresis buffer. The samples were layered on the gels in sample buffer (80% E buffer–20% glycerol) in volumes from 5 to 50 μl in quantities from 40 to 90 μg. The gels were electrophoresed for 135 min at 5 mA per gel and 65 volts DC.

Gel Analysis. The gels were scanned at 260 mμ with a Gilford spectrophotometer equipped with a linear transporter and integrating recorder. The gels were then cut into 1.5 mm sections and each section placed in a separate counting vial and digested overnight with 100 μl of concentrated HNO_3 and 100 μl 30% H_2O_2. The digested gel slices were counted for radioactivity in a Beckman LS-200 liquid scintillation counter using 10 ml of a TLA (Beckman)–70% toluene–30% absolute methanol counting cocktail. A quench curve and automatic external standardization (A.E.S.) were used to correct for efficiency of counting.

RESULTS

Behavioral

A full description of the patterns of responding generated by the contingencies in the 4 training conditions has been previously reported [26]. The animals exposed to the MULT condition developed a discrimination and responded at a high rate when the light was lit and the FR 20 component was in effect and responded very little when the light was extinguished and the EXT component was in effect. The animals exposed to the MIX schedule had no cue to distinguish which protion of the schedule was in effect and therefore, did not develop a discrimination and responded at a high rate during both components of the schedule (FR 20 and EXT). In the FR 20 Stimulus condition the stimulus had no consequences and no control over bar pressing behavior developed. As a result of these contingencies the patterns of responding of the MULT, MIX, and FR 20 Stimulus animals to the stimulus change was markedly different. The response rate ratios (responses during successive 2 min light/2 min no light) in the last 8 min of the training session for the MULT group differ from the MIX (Paired t = 6.79, df = 2, p = 0.01) and the FR 20 Stimulus groups (paired t = 9.35, df = 2, p = 0.01). Log transformations were performed on the response rate ratios to normalize the data and one-tailed t tests were performed since apriori predictions of direction were possible. The stimulus develops control of bar pressing behavior in the MULT group but not in the MIX or FR 20 Stimulus groups.

Biochemical

With the absorbance distribution of the gels at 260 mμ and the distribution of radioactivity in the gels, it was possible to construct a composite of the two for both RNA fractions (nuclear and cytoplasmic) for each animal. Figure 1 is a composite plot of nuclear RNA and Fig. 2 of cytoplasmic RNA from one of the animals exposed to the MULT condition. The sedimentation coefficients (S) of the RNA species have been used for identification of the species and are labelled in Figs. 1 and 2. Ribosomal RNA is composed of the 28S, 18S and 5S components. Messenger RNA activity in brain has been demonstrated in the 12S fraction [20]. Transfer RNA is the 4S species. By measuring the area of the absorbance distributions with a polar planimeter and computing the total DPM for the gel slices from that area, it was possible to compute a specific activity per μg of RNA (absorbance curves of standard solutions of RNA were used to determine concentration). The distributions were divided into three general areas and the specific activity for those determined. The areas treated in this fashion were: (a) 30S through 18S – rRNA; (b) 17S through 6S – mRNA; (c) 5S through 4S – tRNA (even though the 5S species is part of the ribosomal subunit).

The large fraction seen at 7 cm migration distance in nuclear RNA (Fig. 1) is oligodeoxyribonucleotides (degraded DNA) from the DNase digestion of the chromatin material [21]. Because of this large absorbance it was not possible to compute a specific activity for the nuclear tRNA fraction.

Nuclear rRNA. The distribution of radioactivity into this fraction of the trained animals is shown in Fig. 3A. The mean specific activities for the different groups showed that the MULT, MIX, and FR 20 Stimulus groups have very similar specific activities while the FR 20 has less (Table 1). An analysis of the magnitude of the effects of the component parts of the training task showed that there are no significant differences in the specific activity of nuclear rRNA (Table 2).

Nuclear mRNA. The distribution of radioactivity into nuclear mRNA for the trained animals is shown in Fig. 3B. The mean specific activities for the different training conditions show that the MULT group has more radioactivity in

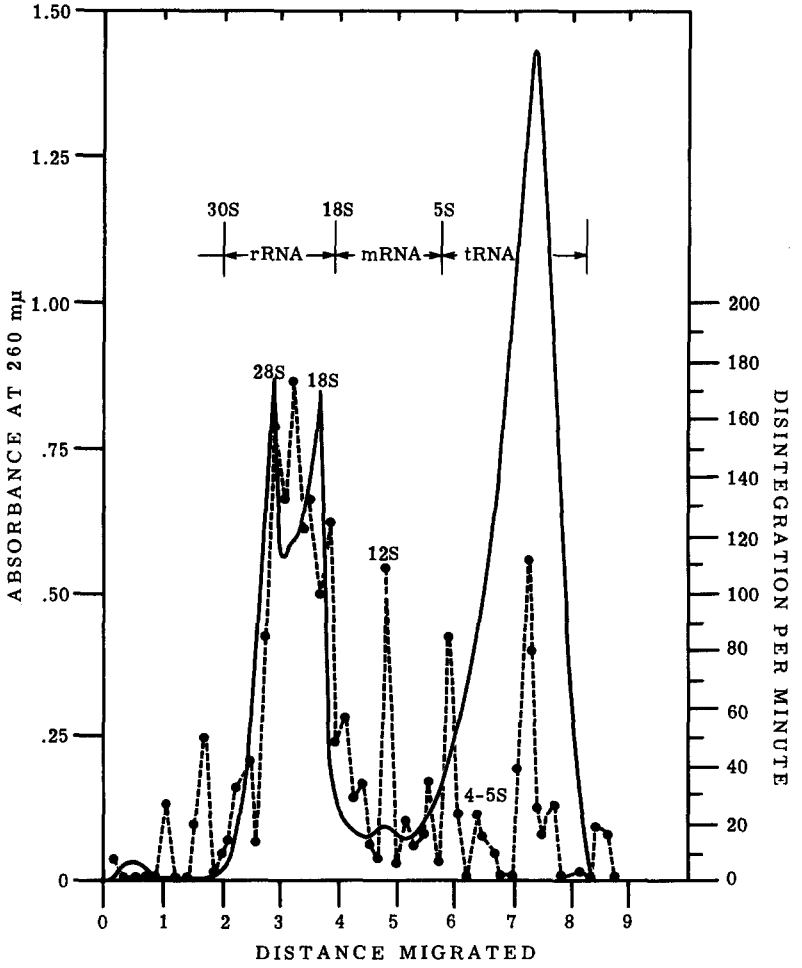

FIG. 1. The distribution of ^{3}H-uridine-5 into nuclear RNA separated by polyacrylamide disc gel electrophoresis from an animal exposed to the MULT condition. The solid line is absorbance at 260 mμ and the dotted line DPM.

this fraction but also has a larger standard error (Table 1). An analysis of the effects of the component parts of the training task showed that there are no significant differences in specific activity of nuclear mRNA (Table 2).

Cytoplasmic rRNA. The specific activities for cytoplasmic rRNA are similar to that of nuclear rRNA but somewhat lower because of the large amount of rRNA in the cytoplasm (Fig. 3C). The mean specific activities of rRNA for the trained animals showed that the MULT, MIX, and FR 20 Stimulus groups have similar specific activities while the FR 20 group has less. An analysis of the effects of the component parts of the training task showed that there are no significant differences in the amount of radioactivity incorporated into cytoplasmic rRNA (Table 2).

Cytoplasmic mRNA. The distribution of radioactivity into cytoplasmic mRNA is similar to nuclear mRNA (Fig. 3D) except the specific activities are lower due to a probable dilution effect in the cytoplasm or the incomplete transport of these species to the cytoplasm. Again the MULT group has a higher specific activity than the other three groups (Table 1). There is a significant difference in the amount of radioactivity incorporated into cytoplasmic mRNA between the MULT and MIX groups (Table 2).

Cytoplasmic tRNA. The specific activity of tRNA is very similar for all the groups (Fig. 3E and Table 1). An analysis of the component parts of the training task showed that there are no significant effects on the level of radioactivity incorporated into cytoplasmic tRNA.

DISCUSSION

The four training conditions were chosen to separate and measure some of the neurochemical effects of the component parts of the total training task. The effect of the stimulus change on the rate of incorporation of radioactivity into nuclear and cytoplasmic RNA species was isolated and measured by comparing the FR 20 Stimulus group with the FR 20 group. The effect of the schedule change plus the discrimination (Contingency Change) was determined by comparing the MULT with the FR 20 Stimulus group since both were exposed to the same stimulus change. The effect of the discrimination in responding was determined by

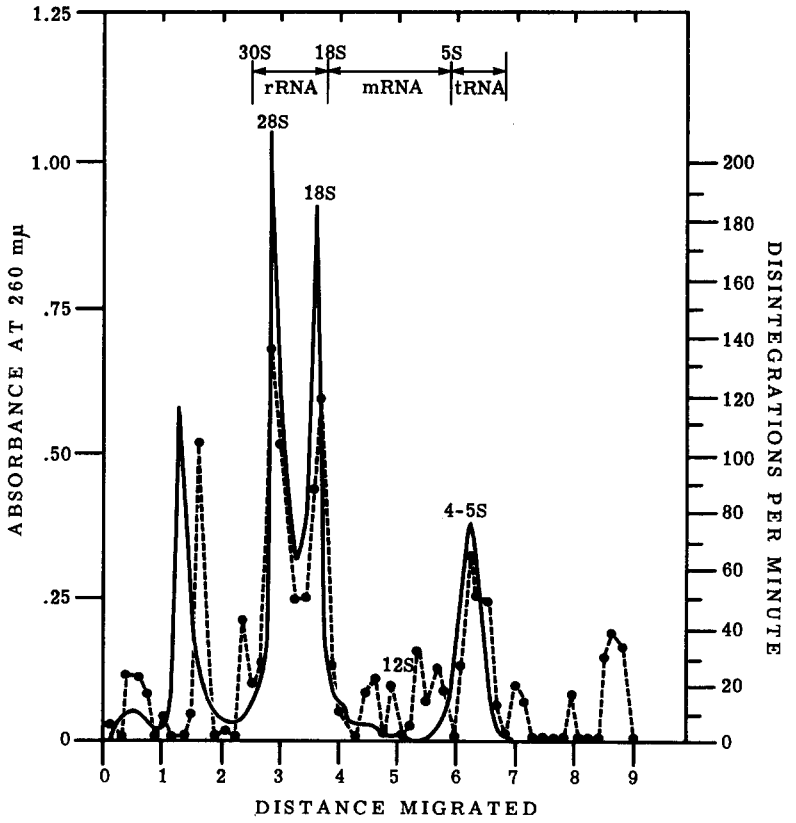

FIG. 2. The distribution of ^{3}H-uridine-5 into cytoplasmic RNA separated by polyacrylamide disc gel electrophoresis for the same animal from Fig. 1. The solid line is absorbance at 260 mμ and the dotted line DPM.

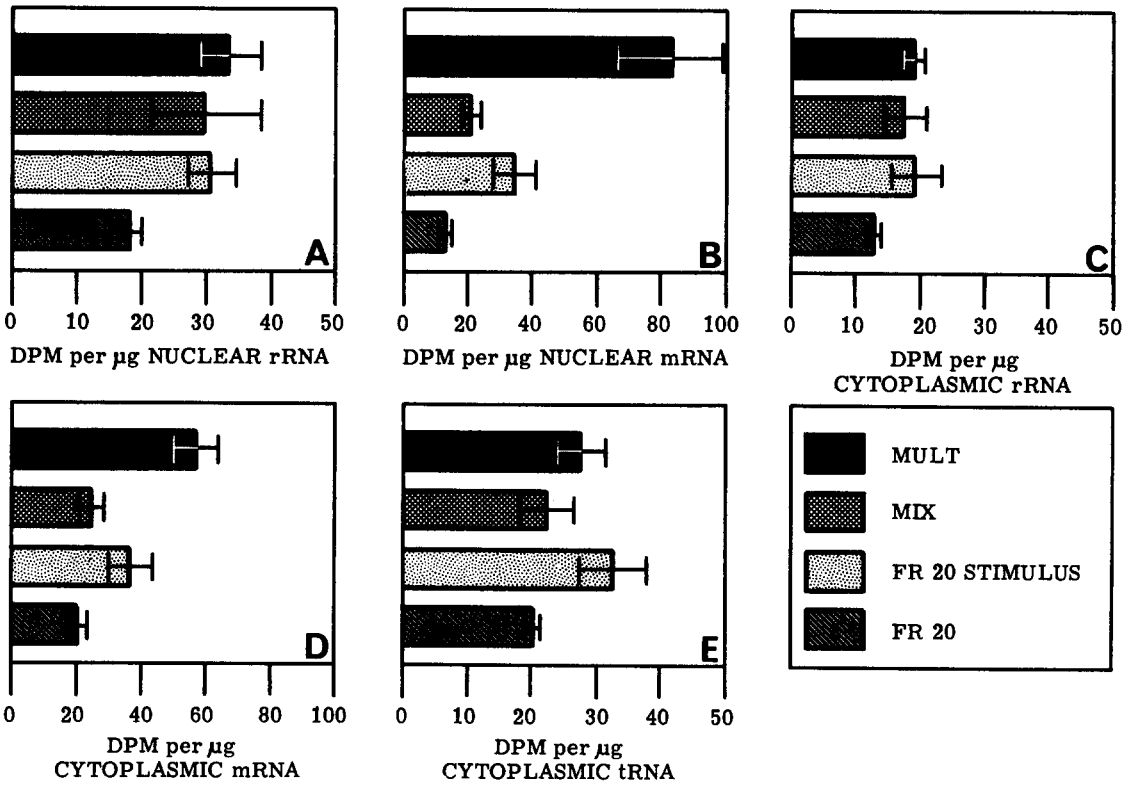

FIG. 3. The specific activity of nuclear rRNA (A) and mRNA (B) and cytoplasmic rRNA (C), mRNA (D), and tRNA (E) from 3 groups of trained animals. The bars are standard error of the means.

TABLE 1

DPM PER μg OF NUCLEAR rRNA AND mRNA AND CYTOPLASMIC rRNA, mRNA, AND tRNA FOR THREE GROUPS OF TRAINED LITTERMATES

	Nuclear		Cytoplasmic		
	rRNA	mRNA	rRNA	mRNA	tRNA
MULT	33.2 ± 9.3	83.0 ± 34.3	19.2 ± 2.6	56.9 ± 13.3	27.5 ± 6.6
MIX	29.8 ± 16.6	20.5 ± 2.6	17.4 ± 6.7	24.6 ± 6.7	22.3 ± 7.9
FR 20 Stimulus	30.8 ± 7.1	34.3 ± 12.5	19.5 ± 7.7	37.3 ± 12.9	32.7 ± 9.8
FR 20	18.4 ± 2.9	13.0 ± 1.5	13.0 ± 1.6	21.0 ± 5.0	19.9 ± 1.3

Values represent means ± SEM (N = 3).

TABLE 2

PAIRED t AND p VALUES FOR THE EFFECTS OF THE COMPONENT PARTS OF THE TRAINING TASK ON THE SPECIFIC ACTIVITY OF BRAIN NUCLEAR AND CYTOPLASMIC RNA SPECIES FROM THREE GROUPS OF TRAINED LITTERMATES

	Nuclear		Cytoplasmic		
	rRNA	mRNA	rRNA	mRNA	tRNA
Stimulus Change	$t = 1.96$	$t = 1.59$	$t = 0.59$	$t = 1.19$	$t = 0.96$
(FR 20 Stimulus vs FR 20)	$p = 0.08$	$p = 0.14$	$p = 0.29$	$p = 0.19$	$p = 0.22$
Contingency Change	$t = 0.81$	$t = 1.21$	$t = -0.03$	$t = 1.07$	$t = 0.43$
(MULT vs FR 20 Stimulus)	$p = 0.25$	$p = 0.17$	$p = 0.49$	$p = 0.19$	$p = 0.34$
Discrimination	$t = 0.11$	$t = 1.59$	$t = 0.17$	$t = 2.99$	$t = 0.30$
(MULT vs MIX)	$p = 0.45$	$p = 0.14$	$p = 0.42$	$p = 0.05$	$p = 0.41$

One-tailed t tests were performed since previous data [26] allowed an apriori prediction of the direction of change.

comparing the MULT with the MIX group since both were exposed to the same amount of stimulus and schedule change. Using this method, changes in nuclear and cytoplasmic RNA species could be investigated and attributed to component parts of the training task.

The FR 20 group, which is exposed to no stimulus or contingency change from baseline training, consistently shows the lowest specific activity of all RNA species investigated. A stimulus change alone (FR 20 Stimulus) produces an increase in the rate of incorporation of radioactivity into all RNA species investigated compared to the FR 20 group. A change in the schedule (EXT) which is not correlated with a corresponding stimulus change (MIX) does not increase the rate of incorporation of radioactivity into these RNA species above that of the stimulus change. Exposure to a schedule change that is correlated with a stimulus change (MULT) that produces a change in response probability (discrimination in responding to the stimulus) produces an increase rate of incorporation of radioactivity into the nuclear and cytoplasmic mRNA fractions. This increase is consistent with previous reports of changes in mRNA during training [8, 14, 16]. However, the changes are more marked in the cytoplasmic fraction which is the proposed site of action of mRNA [9].

The fact that there are no significant differences in the rRNA, tRNA, and nuclear mRNA fractions between the groups is parsimonious with the hypothesized roles of these RNA species [9] (however, the specific activity of nuclear tRNA was not determined). Messenger RNA is believed to act primarily in the cytoplasm where the genetic information is translated into protein synthesis with the aid of the ribosomes and tRNA. Cellular events that are responsible for information processing and storage may require increased levels of rRNA and tRNA that are required for enzyme induction, synaptogenesis, or just about any other physiological or biochemical event. In this experiment the

increased specific activity in nuclear rRNA and cytoplasmic rRNA and tRNA in the groups exposed to a change from baseline training (MULT, MIX, and FR 20 Stimulus) compared to the FR 20 group, indicates that a change in the training environment alone may be enough to produce this increase. However, the changes in the nuclear and cytoplasmic mRNA fractions are much more marked in the animals that develop a change in response probability (MULT) than those exposed to just a change in the environment (MIX and FR 20 Stimulus). This suggests that changes in response probability (learning) may specifically require increases in translation of the genetic information into protein by mRNA.

These data indicate that environmental changes may cause a general increase in all RNA synthesis but that the more specific effects of developing a change in response probability are reflected by an increase in the rate of mRNA synthesis and transport to the cytoplasm and thus an increase in translation of genetic information into protein. These findings are consistent with previously reported changes in RNA species [1, 2, 8, 11, 13, 14, 15, 16, 17, 18, 28].

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