Brain Polysomes: Response to Environmental Stimulation

Abstract. *Polysomes have been isolated from rat brain and characterized by their appearance in the electron microscope and by their sedimentation in sucrose density gradients. Rats were isolated for 3 days in the dark and were then returned to the light for 15 minutes. The polysomes in brain, but not in liver, decreased in rats deprived of light and increased in those stimulated with light. These findings together with an increased capacity for protein synthesis in the brain in vitro and in vivo suggest that an increase in the activity of messenger RNA in the brain may result from environmental changes.*

Recent experiments have associated brain RNA with information processing and storage. By histochemical and microchemical techniques the concentration of RNA within neurons has been shown to increase with stimulation and to decrease with deprivation (*1*); the base ratios have been demonstrated to change under conditions of "learning" (*2*). Similarly, protein concentrations and specific activity increase in response to visual stimulation (*3*). The mechanism by which such processes are regulated is unknown. However, by analogy with bacterial systems, the availability of messenger RNA may serve as a critical intermediate. In bacteria, mammalian liver, and reticulocyte tissues, such populations of messenger RNA may be defined by their capacity to promote ribosomal aggregation and to stimulate in vitro the incorporation of amino acid into protein (*4*). Our report provides evidence that a similar characterization is possible with brain tissues (*5*), and that with environmental stimulation and deprivation changes occur in the messenger RNA population.

Brain polysomes were characterized by spectrophotometric and electron microscopic techniques and by their capacity for protein synthesis in vitro. Wistar rats (30-day-old) were decapitated; their cortices were separated from underlying white matter and homogenized in 0.25*M* sucrose containing 0.05 mole of tris(hydroxymethyl)aminomethane (tris) buffer, *p*H 7.6; 0.005 mole of magnesium chloride; and 0.025 mole of potassium chloride per liter. The homogenate was centrifuged at 20,000*g* for 30 minutes. Ribosomes were prepared from the supernatant fraction by addition of 0.1 volume of 20 percent deoxycholate (DOC) and centrifugation for 3 hours at 105,000*g* through a 0.5 to 2.0*M* sucrose discontinuous zone gradient. Either the resuspended ribosomal pellet or, alternatively, the supernatant from the second centrifugation was layered over 10 to 50 percent continuous sucrose gradients and centrifuged for 150 minutes at 25,000 rev/min. The tubes were punctured, and the effluent was passed through a recording spectrophotometer. Figure 1 demonstrates the polysome profile, fraction No. 18 represents the peak of single ribosomes (80*S*) (*6*), and those to the left of it represent heavier aggregates that are readily disrupted by ribonuclease. The profile is essentially unchanged when the homogenate is incubated with trypsin, chymotrypsin, or deoxyribonuclease before its treatment with DOC and centrifugation. Omission of DOC results in ribosomal yields of from 80 to 90 percent of that in preparations to which DOC is added. This may indicate that the majority of brain ribosomes are free rather than bound to endoplasmic reticulum.

When checked by electron microscopy, the fractions were found to contain the anticipated ribosomal aggregates arrayed about a linear messenger RNA (Fig. 2). The state of aggregation

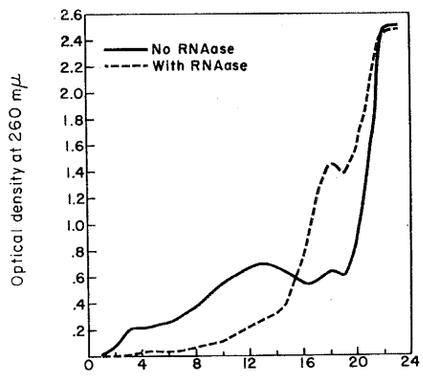

Fig. 1. Brain polysomes were prepared as described in the text. The ribosomal pellet was resuspended in original buffer and incubated for 10 minutes at 37°C with 5 μg of ribonuclease. An untreated fraction and an equivalent sample treated with ribonuclease were centrifuged through a continuous sucrose gradient; the effluent was then passed through a recording spectrophotometer. The abscissa represents the tube number (approximately 1.1 ml per tube). Fractions of heavy ribosomal aggregates are found in tubes with lower numbers.

in the various fractions corresponded to that predicted from the spectrophotometric profile; the aggregates were sensitive to ribonuclease, but insensitive to trypsin, chymotrypsin, and deoxyribonuclease.

To assess the effects of environmental stimulation, we housed 30-day-old rats in individual cages for 3 days in the dark. The experimental group was then removed from the dark and exposed to ambient laboratory light and sound for 15 minutes, while the control population remained in the dark. The animals were decapitated, and their occipital cortices were separated from the remaining cortices; ribosomes were isolated as described. Figure 3 represents the profile, in sucrose gradient densities, of the ribosomes isolated from the occipital cortex of environmentally deprived animals compared to that of ribosomes isolated from rats stimulated with light. The total amounts of RNA in the two groups were not detectably different. However, in each experiment an increase of from 46 to 220 percent in polysomes with variable decreases in single ribosomes was found. The average increase for nine experiments was 82 percent. The overall concentration of polysomes obtained from deprived or light-stimulated animals (Fig. 3) is lower than that of the controls (Fig. 1). Results of experiments with sucrose gradient densities were not specific for ribosomes isolated from the occipital cortex of animals exposed to light. Changes of equal magnitude were seen in profiles of ribosomes isolated from the remainder of the cortex, although similar results were not seen with ribosomes isolated from livers of the two groups of animals.

Since comparable quantities of total ribosomal RNA were used in the gradient, the significant diminution of polysomes associated with environmental deprivation and the increase associated with stimulation reflect changes in the aggregating capacities of the ribosome. Studies with rat liver have shown that polysomes are formed by attachment of single ribosomes to messenger RNA's of various lengths (*7*). In accord with this finding, our own electron microscopic studies indicate that brain ribosomes also consist of aggregates of various sizes which can be reduced to a uniform population of single and double ribosomes by the action of ribonuclease. Since the presence of such polysomal aggregates is a reflection of the presence

of messenger RNA, it follows that the noted increase in polysome content with stimulation may well reflect an increase in messenger RNA.

As in other mammalian tissues studied in vitro (*7*), the polysomal fraction of brain ribosomes will promote greater protein synthesis than single ribosomes will (*8*). As an additional confirmation of the presence of increased amounts of messenger RNA, the protein synthesizing capacity of the ribosomes extracted from the cortices of environmentally deprived and environmentally stimulated animals was compared. The incorporation of radioactive leucine into protein was greater in the presence of ribosomes from stimulated rats than in the presence of those from deprived animals (Table 1). The increase, noted in several experiments, ranged from 60 to 100 percent. In all such experiments either a fraction at *p*H 5 or the supernatant of control homogenates centrifuged at 105,000*g* was used with ribosomes from both experimental groups. When the formation of valyl-transfer RNA or the concentration of activating enzymes was assayed in the supernatant fractions of control and experimental animals, no consistent differences were noted.

The presence of an increased concentration of polysomes and an increased capacity for protein synthesis indicate the presence of additional functioning messenger RNA. At present, we do not have sufficient evidence from experiments with radioactive uridine to determine whether synthesis of new messenger RNA has occurred; it is possible that the aggregation takes place about messenger RNA strands that were synthesized previously. However, the rapid turnover of most messenger RNA's in the brain would make new synthesis likely (*9*).

The stimulation of protein synthesis associated with environmental stimulation was also assessed in vivo. Animals were inoculated intracerebrally with radioactive leucine (1 μc in 10 μl) 1 hour before they were killed. Forty-five minutes after they were inoculated, the experimental animals were brought into the light for 15 minutes, while the control group remained in the dark. Thereafter the animals were decapitated and their cortical tissues were processed for radioactive material soluble in trichloracetic acid and for proteins. Over several experiments an average of 980 count/min was incorporated per milligram of protein in the group exposed to light; 694 count/min per milligram of protein was incorporated in the controls. The range of stimulation in individual experiments was from 20 to 80 percent. The enhancement of incorporation of radioactivity in animals exposed to light was similar for occipital cortical and the remaining cortical proteins. In all experiments, the amounts of radioactivity in acid-soluble materials from control and experimental groups were not essentially different.

Despite the large number of variables, such as the blood-brain barrier, permeability changes, amino acid flux, turnover of amino acid pools, and availability of RNA moieties which may contribute to the final result, the stimulation of protein synthesis in vivo was comparable to that noted in vitro. In the experiments in vitro, conditions were designed so that messenger RNA would be limiting. The comparability of data from experiments performed in vivo therefore suggests that under our conditions the availability of messenger RNA is rate limiting for protein syn-

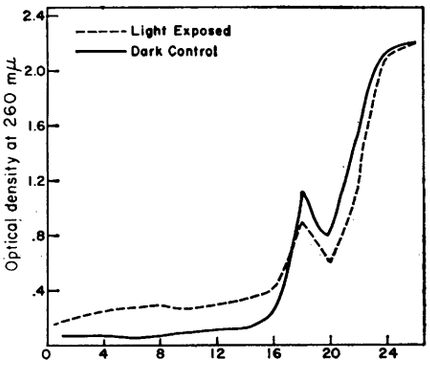

Fig. 3. Response of brain polysomes to environmental stimulation. The conditions are described in the text, the methods are described in the legend to Fig. 1. Equivalent concentrations of RNA were layered over the gradients. The consistent findings are the increased polysomal aggregates and the decreased single ribosomes.

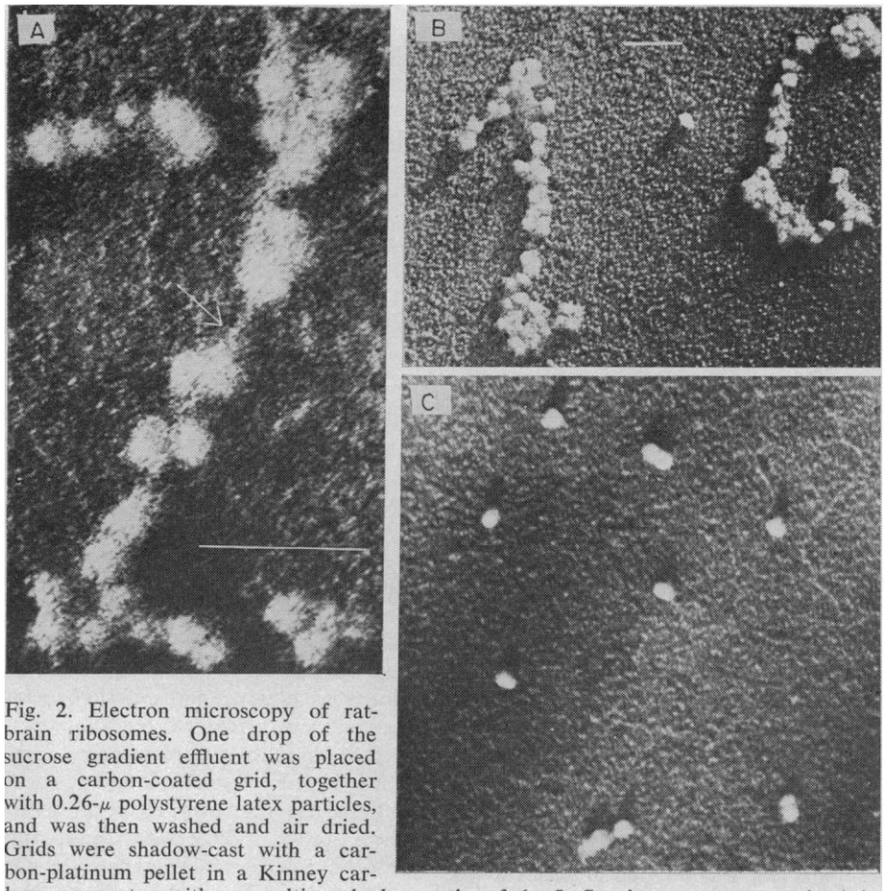

Fig. 2. Electron microscopy of rat-brain ribosomes. One drop of the sucrose gradient effluent was placed on a carbon-coated grid, together with 0.26-μ polystyrene latex particles, and was then washed and air dried. Grids were shadow-cast with a carbon-platinum pellet in a Kinney carbon evaporator with a resulting shadow ratio of 1 : 5. Specimens were examined in Phillips EM 100B and 200 microscopes. (A) Polysomes from tube No. 3 of the gradient. Ribosomes are arrayed about a linear strand of messenger RNA. Marker equals 1000 Å. (B) Heavy aggregated polysomes at lower magnification. (C) A similar fraction treated with ribonuclease prior to examination. There are no aggregated structures, only single and double ribosomes.

Table 1. Cell-free protein synthesis with brain polysomes from animals exposed to dark and light. Incubation was at 37°C for 30 minutes in 0.2 ml containing 5 μmole of tris-Cl, 0.3 mg of ribosomal protein, 0.6 mg of supernatant protein, 2.0 μmole of adenosinetriphosphate, 2.0 μmole of Mg^{++}, 2.0 μmole of phosphoenolpyruvate, 0.5 μmole of guanosinetriphosphate, 10 μg of phosphoenolpyruvate kinase, 20 μmole of NH_4Cl, .02 μmole each of 19 unlabeled amino acids, and 0.25 μc of leucine C^{14} (168 μc/μmole). Portions (0.1 ml) of the reaction mixture were transferred to filter paper disks and processed according to the technique of Mans and Novelli (*14*). The counting efficiency was 40 percent. Each value represents an average of three determinations.

Conditions	Radioactivity (count/min per mg protein)
Complete, light-exposed ribosomes	3010
Complete, dark-exposed ribosomes	1470
Minus ribosomes	80
Light-exposed ribosomes minus supernatant	33
Dark-exposed ribosomes minus supernatant	72
Complete, light-exposed ribosomes plus RNAase	104
Complete, dark-exposed ribosomes plus RNAase	190

thesis in vivo as well as in vitro. This hypothesis is also supported by the fact that decreased amino acid incorporation into protein in the brains of older rats in vivo and in vitro is associated with smaller populations of polysomes (and consequently less functioning messenger RNA) (*8, 10*).

Previous investigations have characterized polyribosomes by their sedimentation behavior in sucrose density gradients, their appearance in the electron microscope, their susceptibility to digestion by ribonuclease, their resistance to digestion by deoxyribonuclease and proteolytic enzymes, and their ability to incorporate labeled amino acid into polypeptide chains. The brain polysomes isolated from young rats possess characteristics which differ only slightly from those of liver preparations. Heavier sucrose is required for resolution of brain ribosomes, possibly because larger aggregates are present and the peptides have, on the average, greater molecular weights. Also, concentrations of ribonuclease greater than those used in similar experiments on liver ribosomes are needed for digestion (5 μg compared to 0.5 μg).

It is not clear from our experiments how environmental stimuli were translated into macromolecular changes. It is tempting to assume that the local release of neurotransmitter from a presynaptic terminal results in postsynaptic macromolecular changes, analogous to the effect of hydrocortisone on the liver (*11*). However, no data are available on this matter; and it is equally plausible that circulating hormones or other factors mediate the response in brain but not in liver.

The response of brain messenger RNA to environmental stimulation not necessarily correlated with "learning" that can be assayed raises an interesting problem. If stimulation alone may evoke this important macromolecular response, we must question the specificity of the participation of RNA in the memory process as has been suggested by the experiments of Zemp *et al.* (*12*) and Hyden (*13*). Our data suggest that template RNA's are involved in any enhanced participation of neural cells within a communal response, but that increases or decreases in messenger RNA may not, per se, specify the extent and duration of the neural information storage.

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References and Notes

1. H. Hyden and H. Hartelius, *Acta Psychiat. Neurol. Scand.*, Kbh Supp. **48** (1948); S. Brattgard, *Acta Radiol. Supp.* **96** (1952).
2. H. Hyden and E. Egyhazi, *Proc. Nat. Acad. Sci. U.S.* **48**, 1366 (1962).
3. G. P. Talwar, S. P. Chopra, B. K. Goel, B. D'Monte, *J. Neurochem.* **13**, 109 (1966).
4. S. H. Barondes and M. W. Nirenberg, *Science* **138**, 813 (1962); T. Staehelin, F. O. Wettstein, H. Noll, *ibid.* **140**, 180 (1963); J. R. Warner and A. Rich, *J. Mol. Biol.* **10**, 202 (1964).
5. While this manuscript was in preparation a detailed characterization of brain polysomes, including their electron microscopic and sedimentation characteristics, appeared (A. T. Campagnoni and H. R. Mahler, *Biochemistry* **6**, 956 1967).
6. We thank Dr. Walter Guild for assistance in performing the sedimentation analysis of the single ribosome peak.
7. H. Noll, T. Staehelin, F. O. Wettstein, *Nature* **198**, 632 (1963).
8. S. H. Appel, unpublished observation.
9. ———, *Nature* **213**, 1253 (1967).
10. R. V. Murthy, *Biochim. Biophys. Acta* **119**, 599 (1966).
11. G. M. Tomkins and E. S. Maxwell, *Annu. Rev. Biochem.* **32**, 677 (1963).
12. J. W. Zemp, J. E. Wilson, K. Schlesinger, W. O. Boggan, E. Glassman, *Proc. Nat. Acad. Sci. U.S.* **55**, 1423 (1966).
13. H. Hyden, *Prog. Nucleic Acid. Res.* **6**, 187 (1967).
14. R. J. Mans and B. D. Novelli, *Arch. Biochem. Biophys.* **94**, 48 (1961).
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Drinking Induced by Carbachol: Thirst Circuit or Ventricular Modification?

Levitt and Fisher (*1*) reported that carbachol applied to a variety of subcortical-limbic sites caused the animal to drink. If carbachol was applied to any one of these sites and atropine to any other such site, drinking elicited by application of carbachol was reduced. Control tests showed that application of sodium chloride at one site did not reduce drinking evoked by application of carbachol. Since the reduction of carbachol-induced drinking caused by application of atropine at any two subcortical sites was always reciprocal, these results may support their view that the "neural basis for the thirst drive consists of complex alternative and reciprocal pathways of neurons susceptible to activation by cholinergic stimulation." I now suggest an alternative to the Levitt–Fisher view which may, in addition, be of some value in interpreting certain data not clearly understood at present.

In a recent experiment (*2*) we stimulated the caudate nucleus of rats, and noticed that three of ten treated rats consistently drank more water than controls during a 10-minute observation period. In cases where the animals drank, probes tended to locate in medial caudate regions (Fig. 1). Why then did chemical stimulation of the caudate nucleus induce drinking? One possible answer is that stimulation is, in fact, affecting the adjacent ventricle. In a recent report on drinking induced by application of carbachol (*3*) it was noted that histological results "indicate a localization of carbachol-induced responding close to the midline, with effectiveness falling off with increasing distance laterally. . . . This large region of the hypothalamus affected by carbachol does not coincide with any obvious anatomical structure." Part of the drinking effect induced by the application of carbachol may be mediated through the ventricle, and such drinking may be caused by modification of the milieu of the ventricle, or by stimulation of receptors that may line the wall of the ventricle, or by both (*4*). The results of Levitt and Fisher, while possibly interpretable in their view on the basis of redundant neural circuitry, might alternatively be understood within the framework of the present hypothesis. Thus, whatever drinking mechanism carbachol activates by way of