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Overview

ROLE OF RNA AND PROTEIN SYNTHESIS IN MEMORY FORMATION

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A brief review is given of experiments which are concerned with the hypothesis that brain RNA and protein synthesis are directly involved in the establishment of long-term memory. It is concluded that these experiments neither support or refute this hypothesis. A convincing demonstration is lacking of interanimal memory transfer by injection of macromolecular extracts. The majority of experiments which attempt to correlate increased macromolecular synthesis with learning use radioactive precursor methods and these studies do not exclude possible changes in precursor specific activity as the cause of the increased labeling. Although some studies find directly observable changes in brain macromolecules in response to training, their relationship to memory formation is unclear. It is possible that these changes represent only an enhanced production of constitutive macromolecules in response to an increase in cerebral metabolism during training, rather than molecular changes that are directly involved with modifying synaptic connectivity. Inhibitors of cerebral protein synthesis block memory formation, but these drugs are not pharmacologically specific and this complicates the interpretation of these studies.

INTRODUCTION

Although the suggestion that brain RNA and protein synthesis might participate directly in long-term memory formation goes back at least 30

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years (1) and has been pursued experimentally for nearly the past 20 years (2), the role that macromolecules play in memory fixation is still obscure. Much of the difficulty lies in the fact that little is known about the neuronal basis for memory, although some interesting speculations exist (3, 4). Most of these are based on Hebb's postulate that memory formation involves functional changes in the synapses of neurons that undergo synchronous activity (5). There is currently no evidence that either supports or refutes Hebb's postulate. In the following, the evidence supporting a direct role for RNA and protein synthesis in memory establishment is considered. Explicit statements are few, but many investigators suggest that memory formation may be dependent on the synthesis of proteins which would then modify existing synaptic connections, and that the subsequent long-term maintenance of this memory requires gene activation (6, 7). There are two main lines of investigation which suggest that macromolecular synthesis is necessary for memory formation. These are that training induces the appearance of new macromolecules and that inhibitors of brain protein synthesis block memory formation.

MEMORY TRANSFER

One source of evidence that brain macromolecules are involved in memory processes comes from observations that extracts of the brain of a trained animal induces memory of this training when injected into a naive animal. Although there are many reports of memory transfer by chemical extracts (8), a convincing demonstration is still lacking. Few of these studies offer evidence that the brain extract produces a behavioral change in the recipient that is specific to the task the donor learns and particular experiments cannot be repeated (9, 10).

Much of the recent interest in memory transfer has centered around the claims of Ungar's group that the memory of conditioned dark-avoidance learning can be induced in naive recipients by injection of scotophobin, a 15-amino acid peptide, which they have isolated and synthesized (11). Although scotophobin is probably not a specific memory code word, it appears that synthetic rat scotophobin has behavioral activity of some kind. Malin and Guttman (12) reproduced Ungar's finding that synthetic rat scotophobin reduced the amount of time naive mice spent in a dark-box. However, this was not observed by Miller et al (13), who also followed Ungar's testing procedure, although these workers found that synthetic scotophobin facilitated the acquisition of dark-avoidance learning in mice. This agrees with the findings of DeWied et al. (14) who, in addition, observed that desacetyl-scotophobin inhibited the extinction of a pole-jumping task in rats. These results suggest that synthetic sco-

tophobin may have general facilitating effects on memory storage that could account for its activity in promoting dark-avoidance learning. However, a more pertinent question is whether a natural scotophobin exists. A very careful study failed to confirm Ungar's finding that a crude brain extract could transfer dark-avoidance learning to naive animals (10). In view of this and similar failures, it is crucial that proponents of memory transfer devise experiments that clearly demonstrate the transfer of specific learning and then detail the procedures used so that the observations can be corroborated in other laboratories instead of extending previous equivocal results (15, 16).

MACROMOLECULAR CHANGES IN RESPONSE TO LEARNING AND MEMORY

Although memories may not be stored in specific macromolecules, numerous studies indicate that brain RNA and protein undergo change in response to learning. These studies show that brain macromolecular changes occur when animals learn to perform certain tasks and are absent when animals are exposed to only the nonspecific aspects of the training. As mentioned before, the rationale behind these experiments is that learning may induce the synthesis of macromolecules, which would modify synaptic connections in some unknown way, to establish the memory for the learning. These experiments have been recently summarized in detail by other reviewers (17, 18).

As Rose and his colleagues have mentioned (19, 20), these experiments are best exemplified by the studies conducted by Glassman and his coworkers and those studies of Hydén and his associates. In Glassman's experiments, mice that were trained in a jump-avoidance task showed greater incorporation of radiouridine into brain RNA than yoked or quiet controls. Yoked controls received the same amount of footshock as the trained mice but could not learn to avoid the shock as their training apparatus lacked the escape platform. Mice were injected intracranially with the RNA precursor 30 minutes before training and were sacrificed at the conclusion of training which lasted for 15 minutes. A 30–40% increase in radioactivity was observed in both nuclear and polysomal RNA extracted from the brains of trained mice compared to brain RNA from yoked controls (21, 22). The increase in labeling was localized mainly to RNA from the diencephalon, and autoradiography revealed that only neurons and ependymal cells in this area were consistently labeled (23, 24). Additional evidence that the increase in labeling was related to learning came from experiments in which radiouridine incorporation was determined when prior trained or previously yoked animals

were trained. In these studies, only the prior yoked animals showed increased incorporation into RNA, although previously trained mice showed greater labeling during extinction (22, 25).

In a few experiments, Glassman and his colleagues have also examined the effects of training in the same task on amino acid incorporation into protein (26), but the relationship to learning is not as clear as with the RNA studies. They observed increases in [^{3}H]lysine incorporation into brain protein in trained mice but also in yoked animals relative to unstimulated controls. Similar changes were also seen in the livers of trained and yoked mice. In a recent study, Glassman's group found that training in a food-reward task increased [^{3}H]lysine incorporation into brain nuclear protein by 36% relative to mice that were exposed to the apparatus without receiving any training (27).

Glassman's experiments are supported by a number of similar studies in which different RNA precursors and appetitive training paradigms were used (28, 29). As other reviewers point out, including Glassman (30), it is not clear in any of these experiments that the rate of macromolecular synthesis actually increases as it is not known whether training alters the specific radioactivity of the precursor over the incorporation period. Localized increases in cerebral blood flow have been observed to occur in chicks during training (31). If more radioactive precursor were delivered by the blood to the brains of trained mice than to yoked controls, then the RNA from trained mice would show more labeling but there would be no actual change in the rate of RNA synthesis. Similarly, if training decreased the amount of endogenous uridine nucleotide, there would be no change in the rate of RNA synthesis even though there might be more incorporation of radioactivity.

It is also necessary to know whether the mean specific radioactivity of the immediate precursor changes during training in order to interpret the significance of any incorporation increase. Glassman's group made a rough correction for precursor pool changes by dividing the radioactivity found in RNA by the radioactivity in the uridine monophosphate (UMP) precursor at the end of training. This correction not only neglected the mean precursor specific activity, but the immediate precursor of RNA synthesis is uridine triphosphate and it is not known whether it always parallels changes in UMP.

These criticisms also apply to Glassman's studies on increased amino acid incorporation or to any experiment that uses radioactive precursors to study macromolecular changes during training. However, there is nothing inherently wrong with the radioactive-precursor approach as long as possible changes in the mean specific radioactivity of the immediate precursor are considered. Hambly et al. (32) found greater [^{14}C]lysine

incorporation into protein in the anterior forebrain roof of chicks during imprinting to a visual stimulus and observed no difference in total tissue lysine specific radioactivity between exposed and control birds. This is the only study to date in which possible changes in specific radioactivity during training were considered. However, as this group has noted (33), the immediate precursor for protein synthesis is aminoacyl tRNA, and it is unclear how total brain specific radioactivity is related to this.

Hydén, by contrast, has largely avoided the use of radioactive precursors and their problems and has instead studied directly observable changes in brain macromolecules during learning. In early studies, Hydén found changes in base ratios and a 10% increase in the RNA content of neurons from Deiter's nucleus when rats were trained to perform a wire-balancing task (34). The base ratio changes were not observed in control rats subjected to passive vestibular stimulation. Similar changes were observed in nerve cells from the right sensory mortor cortex in right-handed rats that were trained to reach for food with the left paw. After four days of training with two 25-minute training sessions per day, cortical neurons from the right side contained 30% more RNA (10 pg) than sensory motor neurons taken from the left untrained side. The G + C/A + U ratio was 20% lower in RNA taken from the trained side (35). The changes in RNA in these studies were determined by pooling microdissected cells and analyzing base composition and amount by electrophoresis and spectrophotometry.

In more recent work, Hydén has examined the response of the brain-specific S-100 protein to training in the transfer of handness paradigm. Pyramidal neurons from the CA_3 region of hippocampus from trained rats contained 10% more S-100 protein than comparable neurons taken from control animals which performed the same task with their preferred paw (36). A new protein band was also observed close to S-100 in polyacrylamide gel fractions from trained animals. This was regarded as S-100 of a different conformation, possibly due to the known interaction of S-100 with calcium ions which also increase in the CA_3 region during training (37).

Hydén's studies are significant in that they show that RNA and protein changes actually occur during learning and are not an artifact of precursor pool changes. Only a few additional studies show alterations in brain macromolecules that are specific to training without using in vivo labeling techniques. Uphouse et al. (38) found a 30% increase in the brain polyribosome/monosome ratio after avoidance training in mice compared to the same ratio in yoked controls. Using a specific radioimmunoassay, Zomzely-Neurath and coworkers determined that brain levels in rats of the neuron-specific 14-3-2 protein were doubled 18 hr after appetitive T-

maze training, compared to levels of the protein in brain areas of activity controls. These workers also observed similar increases in the levels of S-100 after training, but these changes were localized to only the brain stem and medulla of trained rats whereas cortical levels of 14-3-2 were also increased (39, 40).

Although Hydén's studies and the other experiments mentioned establish that RNA and protein changes can actually occur during learning, it is unclear whether these changes have anything directly to do with memory formation. One objection has been raised by Rose and his colleagues (19, 20). This is that trained and control animals probably differ in other behavioral ways, besides the fact that one learns something while the other does not, and that consequently the observed macromolecular differences may not be due solely to learning. In Hydén's experiments on RNA changes in response to vestibular learning, no alterations in RNA were seen in the brains of animals that were passively rotated, so the RNA changes in the trained rats cannot be attributed to just "dizzyness." It is conceivable, however, that the trained rats were more aroused or excited than the controls so that differences in emotional behavior or motor activity could have accounted for some or all of the RNA changes that were attributed solely to learning.

Rose and his coworkers have tried to respond to this criticism by quantifying a variety of behaviors in addition to learning and showing that there is a better correlation between the extent of [^{3}H]uracil incorporation into brain RNA and the degree of learning than between uracil incorporation and any other behavior (41). However, this does not exclude the possibility that a combination of behaviors, say, motor activity and attention, could show the same correlation with uracil incorporation as learning.

A second objection is more fundamental in that it explains why increases in arousal or motor activity might stimulate macromolecular synthesis. It is possible that learning requires a higher level of CNS metabolic activity than passive observation or receiving noncontingent punishment or reward. Perhaps more neurons fire during learning than during noncontingent punishment or some neurons fire at a much greater rate. A higher level of neuronal metabolism would probably require an increase in the synthesis of macromolecules to sustain it. This is supported by studies showing that direct stimulation of neurons increases precursor incorporation into RNA and protein (42, 43). Arousal and motor activity could increase macromolecular synthesis by increasing the firing of neurons that mediate attention or control movements. It is conceivable then that most of the observed changes in RNA and protein synthesis during learning are due to increases in the kind of macromol-

ecules necessary to sustain increased metabolic activity rather than in the production of specific macromolecules that modify synaptic connectivity.

This criticism of correlation studies has been noted by Dunn (18), who found that training in the jump-avoidance task of Glassman did not increase the uptake of [$U^{-14}C$]glucose by the brain relative to yoked and quiet controls (44). The interpretation of this study was that no general increase in brain metabolism was seen under conditions in which greater uridine incorporation occurs. However, it appears that [$U^{-14}C$]glucose is unsuitable for studies of CNS functional metabolism (45). Radioactive glucose is converted quickly to CO_2 which is rapidly lost from cerebral tissue. It would be interesting to repeat this study with [^{14}C]deoxyglucose which, after the initial phosphorylation by hexokinase, is essentially trapped in cerebral tissues for the duration of measurement (45).

Although no studies of this kind with the 2-deoxyglucose method exist, the increase in cerebral blood flow during the training mentioned above indicates that neuronal metabolism increases during training (31). Probably the only way to resolve whether the macromolecules produced during learning are of the general or specific kind would be to isolate them and determine their role in neuronal function. This has only been accomplished to date for the neuron-specific 14-3-2 protein which appears to be identical with a brain-specific enolase (46, 47). Enolase is found in virtually all living cells, and its biochemical function is the conversion of 2-phosphoglycerate to phosphoenol pyruvate. It appears that at least one brain protein which increases during training is of the general metabolic kind, although 14-3-2 may have additional functions besides enolase activity.

In an interesting series of studies, Shashoua (48, 49) has identified and isolated proteins in goldfish brain that incorporated more radiovaline during training in a vestibulomotor task. Three cytoplasmic proteins (α, β, γ) were purified by gel electrophoresis. These had molecular weights of 37,000, 32,000, and 26,000, respectively, and consistently showed 30–100% more labeling during training than during various control procedures for stress and motor activity. Rabbit antiserum against the β-protein was used in a immunohistofluorescence study of its anatomical location. Approximately 15,000 cells in goldfish brain show positive staining for β, with 60% of the cells found in the ependymal zone beneath the optic tectum and vagal lobes. These cells appear to be nonneuronal. The same antiserum caused a 50% loss of memory when injected intraventricularly into goldfish 3–8 hr after training. Control sera, including one that cross-reacted with other goldfish brain proteins, has no effect on retention.

Shashoua has recently found that β is secreted into the CSF of

goldfish brain (50). This is consistent with its location in nonneuronal ependymal cells which appear to be morphologically specialized for secretion. It also suggests that β may act as a neurohumoral factor that participates in memory formation. This is similar to the role pituitary peptides may play in establishing memory in rodents (51) and would also explain how the intraventricularly injected antiserum could interfere with the action of a cytoplasmic protein since presumably it would only slightly penetrate the intracellular space.

Shashoua's isolation studies and those on the 14-3-2 protein indicate that much insight can be gained into the function of macromolecules that change during training. However, in neither case does it appear that these molecules are directly involved in the modification of synaptic connections. It would probably be desirable to study biochemical changes that are correlated better with changes in synaptic connectivity than macromolecular synthesis. The recently developed techniques for labeling neurotransmitter receptors (52) offer the opportunity to determine whether learning directly influences synaptic connectivity. Rose and Steward (53) have recently shown that exposure of dark-reared rats to light induced a transient increase in the binding of a ^{3}H-labeled muscarinic acetylcholine agonist to visual cortex homogenate but not to homogenate of motor cortex. The receptor labeling methods provide an excellent way to relate experience-induced change of various kinds to change in synaptic connectivity and offer wide application for future studies of learning and plasticity.

PROTEIN-SYNTHESIS INHIBITORS AND MEMORY

The remaining support for the notion that long-term memory formation is dependent on macromolecular synthesis comes from studies with cerebral protein-synthesis inhibitors. Although RNA-synthesis inhibitors produce amnesia, the most widely used inhibitor, actinomycin D, causes cerebral damage in rodents and some investigators believe it is not very useful for evaluating the role of RNA synthesis in memory formation (54). A few studies show that less toxic RNA-synthesis inhibitors produce amnesia (55, 56) and more work with these drugs would be valuable to establish the biochemical and behavioral specificities of their effects. When given shortly before or after training, protein-synthesis inhibitors block memory formation in rodents, fish, or birds for a variety of training tasks (57). Most commonly, the drugs used are the antibiotics cycloheximide or anisomycin which can be given peripherally and inhibit cerebral protein synthesis by 85–95% in amnesic doses.

By showing that the absence of protein synthesis leads to amnesia, these studies serve as a complement to correlation experiments in that they address the criticism of whether changes in proteins are directly relevant to memory formation. In most experiments, drug-induced amnesia for a training situation is regarded as poorer performance at retention testing relative to untreated controls. It generally appears that this is due to a specific loss of memory for the training situation rather than to some nonspecific toxic effect of the drug which could impair performance. This is nicely demonstrated in a recent series of studies by Quatermain and his coworkers (58).

Although there is a consensus among investigators that the performance deficit after inhibition of protein synthesis represents amnesia, there is disagreement as to whether the loss of memory is permanent. A number of studies show that spontaneous recovery can occur from amnesia induced by protein-synthesis inhibitors (59, 60). There are a larger number of studies that find no spontaneous return of memory (61, 62) but, even under conditions where memory does not return spontaneously, reversal of amnesia can be produced by "reminder stimuli" (63) or by adrenergic stimulants (64). While some protein-synthesis inhibitor amnesias may be permanent, recovery of memory weakens the notion that memory formation is dependent on protein synthesis because it implies that memories can be stored in the CNS in spite of severe inhibition of protein synthesis. At best, it suggests that the ability to retrieve a memory at some later date depends on intact protein synthesis when the animal was trained.

Flood and Jarvik (65) and Davis et al. (66) suggested that memory may recover after cerebral protein-synthesis inhibition because the initial disruption of consolidation was incomplete. Although they found that anisomycin-induced amnesia for a passive avoidance task was reversed by pretest injection of amphetamine or reexposure to the training apparatus, they presented evidence that recovery only occurred in mice that showed some memory of training in a pretest. No recovery was seen in animals that had very short step-through latencies in the pretest. However, this may only mean that these animals were more refractory to reminder stimuli than animals with longer step-through latencies. Perhaps larger doses of amphetamine or greater reexposure to the training apparatus were needed to recover memory in these mice.

Even granting that under certain circumstances protein-synthesis inhibitors may cause permanent amnesia, there is the question of whether this is due to inhibition of protein synthesis or to side-effects of the drugs. To the extent that these drugs are pharmacologically specific, it is possible to say that protein synthesis plays an important part in establishing some aspect of memory and possibly even a direct role in the modification

of synaptic connections. However, if protein-synthesis inhibitors have effects on other aspects of cerebral metabolism, then it becomes more difficult to attribute the loss of memory to simply inhibition of protein synthesis.

There is much evidence that protein-synthesis inhibitors have many pharmacological side-effects, but even if they were perfectly specific, there would still be the question of whether the loss of memory was due to a reduction in proteins directly involved in changing synaptic connectivity. It is well established that treatments which generally impair neuronal function, such as electroconvulsive shock or hypoxia, are effective amnesic agents (67). If protein-synthesis inhibitors deplete ordinary proteins which are necessary for normal cellular function, it is not surprising that they cause amnesia. This is similar to the argument raised in the last section, where greater macromolecular synthesis during training might only represent an increased production of constitutive molecules in response to greater functional activity. It is also reasonable to suppose that a reduction in general metabolic proteins might be sufficient to cause amnesia.

This possibility has actually been considered for the case of average brain proteins which have half-lives in the range of days (68) or for a hypothetical constitutive protein with a half-life as short as 10 min. In neither case does it appear that depletion of constitutive proteins could account for the amnesia. Injection of intracerebral acetoxycycloheximide 18 hr before training, which produced 90–95% inhibition of brain protein synthesis for many hours and 50–60% inhibition at the time of training, did not affect memory (69). Yet, this procedure would have caused a much greater loss of protein with a half-life of days than injection of an amnesic dose of cycloheximide given 30 min before training. Similarly, if depletion of a constitutive protein with a half-life of 10 min were responsible for the amnesia, cycloheximide given 2 hr before training would reduce levels of this protein much more than cycloheximide given only 5 min before training. Yet only the latter procedure caused amnesia (70).

If protein-synthesis inhibitors were specific, it appears that loss of memory would be due to either depletion of constitutive protein with a half-life less than 10 min or to loss of protein whose synthesis was induced by training, with the interesting possibility that these proteins were specifically involved in memory formation. However, protein-synthesis inhibitors in amnesic doses have a large number of side-effects that are potentially relevant to their amnesic effects. At 30 min after injection when animals are typically trained, amnesic doses of cycloheximide or anisomycin severely inhibit brain catecholamine synthesis from

circulating tyrosine and at the same time elevate or conserve brain catecholamine levels, implying that some other aspect of catecholamine metabolism is changed (71, 72). Cycloheximide and anisomycin elevate brain tyrosine levels (71, 73) and also raise the levels of other brain amino acids including putative amino acid neurotransmitters (74). They also severely inhibit corticosterone synthesis (75) and inhibit brain acetylcholinesterase in vitro using an approximation of the in vivo concentration (76). Cycloheximide also inhibits in vitro tryptophan hydroxylase activity when given intracisternally to rats (77) and causes abnormal electrical activity in the parietal cortex, midbrain reticular formation, and dorsal hippocampus of freely moving mice (78).

Probably many other aspects of cerebral metabolism are also disrupted. It appears as if protein-synthesis inhibitors produce gross abnormalities in brain function at a time when animals are typically trained. As mentioned above, this condition itself, in the absence of protein-synthesis inhibition, often results in amnesia. It is also possible that one or more of the side-effects is sufficient in itself to produce amnesia. Quatermain and Botwinick (79) found that drugs which inhibited catecholamine synthesis to approximately the same extent as cycloheximide produced amnesia with similar characteristics. Rainbow and Flexner (80, 81) observed the same findings in mice treated with the selective catecholaminergic neurotoxin, 6-hydroxydopamine. Najakima (82) suggested that interference with corticosteroids could account for protein-synthesis-inhibitor amnesia.

Other studies have failed to find that individual side-affects are amnesic. Squire et al. (83) found that inhibition of corticosterone synthesis by protein synthesis inhibitors was not sufficient to account for their amnesic effects. Aminoglutethimide depleted plasma corticosterone as much as cycloheximide but did not cause amnesia. Similarly, elevation of cerebral tyrosine levels by itself could not explain protein-synthesis-inhibitor amnesia: a dose of tyrosine that produced a greater increase in brain tyrosine than cycloheximide did not affect memory (73). However, side-effects of protein-synthesis inhibitors occur together in the brain, and it is probably invalid to draw conclusions from studies in which their effects on memory are considered separately. For example, neither lesions of the dorsal noradrenergic bundle nor adrenalectomy alone caused amnesia in rats, but both procedures together impair memory (84).

When all the side-effects of protein synthesis are viewed together, it is apparent that these drugs produce fairly severe distortions in cerebral metabolism which in themselves may well be amnesic. This explanation for the amnesic effects of protein-synthesis inhibitors is a viable alternative to the hypothesis that loss of memory is due solely to a reduction

in the synthesis of memory-related proteins. It would be useful to isolate the rapidly labeling proteins that are affected by the inhibitors and to try to determine their role in neuronal function. It may be that the proteins affected by drugs are ones that could conceivably modify synaptic connectivity. However, as it now stands, there is as much evidence that protein-synthesis inhibitors cause loss of memory by a general disruption of cerebral metabolism as by interference with specific proteins involved in memory storage. It is interesting that in spite of the severe disruption of cerebral metabolism, memory loss after protein-synthesis inhibitors is sometimes only temporary and can often be reversed.

CONCLUDING REMARKS

Although none of the evidence and arguments offered above excludes a direct role for macromolecules in memory storage, the evidence for such an involvement is equivocal at the present time. It would be very helpful if specific molecules that change during learning could be isolated and their role in cellular function determined. This may not be easy as the S-100 protein was first isolated in 1965 and its function is still elusive, although many promising leads exist (47). The cause of amnesia produced by protein-synthesis inhibitors may never be known with certainty due to their multiple side-effects.

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