Identification of Specific Changes in the Pattern of Brain Protein Synthesis After Training

Abstract. *Double labeling studies with [^{3}H]valine and [^{14}C]valine were used to investigate the pattern of protein synthesis in the brains of goldfish. The protein fractions in three bands (α, β, and γ) on sodium dodecyl sulfate–polyacrylamide gels indicate that more valine was incorporated in the brains of goldfish that had been trained in a vestibular conditioning task than in the brains of untrained fish or fish trained in a variety of control behavioral situations. Changes in the pattern of labeling were localized in the cytoplasmic fraction of the brain; no increases in labeling occurred in either the nuclear or synaptosomal components. The results suggest that a specific change occurs in the pattern of protein synthesis in the brain after the acquisition of a new behavior.*

Several investigations have suggested that brain protein metabolism can be influenced by the acquisition of new patterns of behavior. It has been found that (i) inhibitors of protein synthesis can prevent the formation of long-term memories (*1*), (ii) a general increase in the incorporation of amino acids into labeled proteins occurs after training (*2*), (iii) certain proteins become modified by phosphorylation during training (*3*), and (iv) a change in the relative amounts (*4*) of two forms of the S-100 protein occurs after learning (*5*). These results, however, give no indication that there are any specific brain proteins whose pattern of synthesis or rates of turnover, or both, are altered by the training procedures used. I have used a double labeling procedure to investigate this possibility.

The work reported here was based on previous findings that specific changes occur in the RNA of the goldfish brain during training (*6*). Groups of goldfish were trained in a vestibulomotor conditioning task and then injected with either [^{3}H]valine or [^{14}C]valine (*7*) at the time when the concentration of the RNA molecules that increase with learning would be at a maximum in the cytoplasmic fraction, that is, 5 hours after training. One to four hours later the pattern of protein synthesis was analyzed by electrophoresis on sodium dodecyl sulfate (SDS)–polyacrylamide gels and a ratio obtained for the amount of labeled valine incorporated by trained goldfish to the amount incorporated by untrained goldfish. The results of this study suggest that the incorporation of valine into three particular protein bands was consistently increased after the animals learned a new vestibulomotor conditioning task (*8*).

Goldfish (Comet variety from Ozark Fisheries) were conditioned and trained by the float-training procedure previously described (*6*, *8*). In this method, a polystyrene foam float (0.7-cm cube) is sutured to the ventral midline of each animal at a position 1 mm caudal to the base of its pectoral fins. Each goldfish, initially suspended in an upside-down position by the float, goes through a reproducible series of stages in its attempt to swim upright in a horizontal posture.

A total of 200 goldfish were used for the biochemical experiments and 70 for the behavioral investigations. The animals were of two sizes: 6 to 7 g in weight (about 1 year old) or 12 to 15 g (more than 2 years old). The float size used for each animal was the maximum that it could handle: 0.6 to 0.7 cm for the small fish and 0.8 to 0.9 cm for the large fish. The initial and final performance score for each animal was determined at 10 minutes and 4 hours after attachment of the float. Since the magnitude of error in the behavioral testing procedures (*6*) was about 15 percent, all individual goldfish which showed increased performance scores by greater than 15 percent were considered learners (L); while those demonstrating less than a 15 percent change were designated nonlearners (NL). Groups of L animals when tested on day 3 or day 11 after the first trial achieved the same score as they did at the end of trial 1, in about 5 minutes or 10 minutes, respectively. Compared to the 3 or 4 hours required for the initial training on day 1, these data suggest that the animals can "remember" the skill.

In each experiment, seven goldfish of the same size were trained for 5 hours and then injected with 50 μc of [^{3}H]valine (specific activity, 2 c/mmole). In a separate tank, seven control animals of the same size received no training but were injected with [^{14}C]valine (specific activity, 200 mc/mmole). The labeled valine was injected into the fourth ventricle of the goldfish brain as a solution in 10 μl of 0.14*N* NaCl (*8*). After 1 to 4 hours (see Table 1) the brains from a ^{3}H-labeled experimental group and from a ^{14}C-labeled control untrained group were dissected and homogenized together in 8 ml of 0.32*M* sucrose containing 1 m*M* calcium

Table 1. Gel electrophoresis data for the patterns of incorporation of [^{3}H]valine and [^{14}C]valine. Results are expressed as percentage increases in the ratios of labeled valine in experimental (trained) animals to the labeled valine in control (untrained) animals. For each experiment brain fractions from seven experimental animals were compared with fractions from seven control animals. The training scores are for 10 minutes after the attachment of the floats and for 4 hours after the initiation of training. For the training score criteria, see (*16*). The learner-forgetters could not remember the skill when tested 3 days after the initial training; NC denotes no protein changes.

Experiment No.	Training score		Time after injection (hours)	Increase at bands (%)			R_F values*		
	10 minutes	4 hours		α	β	γ	α	β	γ
	Experimental animals received ^{3}H; controls (untrained) received ^{14}C								
1	32	64	4	60	80	100	.46	.49	.57
2	26	64	4	60	78	100	.44	.51	.60
3	11	87	4	41	47	91	.46	.50	.58
4	21	86	1	37	44	43	.45	.55	.60
5	25	55	1½	22	26	31	.44	.52	.58
6	11	81	1½	35	30	40	.46	.50	.56
	Experimental animals received ^{14}C; controls (untrained) received ^{3}H								
7	12	81	1½	15	20	30	.46	.52	.60
8	25	80	1	17	25	18	.42	.49	.57
	Passive nonlearners received ^{3}H; controls (untrained) received ^{14}C								
9	2	12	4	NC	NC	NC			
	Active nonlearners received ^{3}H; controls (untrained) received ^{14}C								
10	29	33	4	NC	NC	NC			
11	46	55	4	NC	NC	NC			
	Learner-forgetters received ^{3}H; controls (untrained) received ^{14}C								
12	24	93	4	NC	NC	NC			
13	22	68	4	NC	NC	NC			

*The R_F values represent the migration distances for proteins separating at the α, β, and γ bands on the SDS-polyacrylamide gels relative to the bromophenol blue dye front. For each experiment (1 to 13) a parallel C/C ratio was determined in order to make certain that the double labeling method was valid for the study.

acetate and 1 m*M* valine (*9*). The homogenates were then separated into nuclear, myelin, synaptosomal, microsomal, and cytoplasmic (90,000*g* supernatant) fractions (*10*). Only the nuclear, synaptosomal, and cytoplasmic fractions had sufficient labeled products for subsequent analyses. In two experiments the isotopes were reversed, the trained experimental animals being injected with [^{14}C]valine and the untrained control animals with [^{3}H]valine (see experiments 7 and 8, Table 1). The labeled products were then dissolved in BPS (brain protein solvent, containing 15 percent urea, 1 percent SDS, and 0.04*M* EDTA in 0.01*M* phosphate buffer, *p*H 8). The BPS solutions were purified to remove lipids and to decompose the labeled aminoacyl transfer RNA (*11*) by several dialyses against phosphate (*p*H 10) for a total of 24 hours followed by several changes of distilled water; they were finally reconstituted into BPS (50 μg of protein per 100 μl of BPS). The samples were then heated for 10 minutes at 95°C in the presence of mercaptoethanol (100 μl/ml) prior to electrophoretic separations on 10 percent SDS-polyacrylamide gels cross-linked with ethylene diacrylate (4 μl/ml) (*12*). Good separations with more than 20 distinct bands staining with Coomassie blue were obtained after 4 hours at a current level of 7 ma per gel.

The gels were frozen and sliced (1 mm), and then put into vials and hydrolyzed with concentrated ammonia. The solution was dried, dissolved in 1 ml of water, mixed with 10 ml of Bray's liquid (*13*), and the radioactivity was measured in a Beckman liquid scintillation counter. The ^{14}C and ^{3}H content of each sample was determined after corrections for isotope spillover and quenching.

Figure 1 shows data obtained in the double labeling experiments. Graph a in Fig. 1A gives ^{3}H/^{14}C ratios for the cytoplasmic proteins from an experiment with an untrained control versus another untrained control (C/C). A constant value of 3.4 was obtained for all regions of the gel. The data in the graph, normalized to 3.4 to give a relative labeling ratio of one, indicate that no technical artifacts were introduced during the homogenization, fractionation, and sample handling methods, and that the double labeling method was valid for the study. The nuclear fraction from the same experiment also gave a ratio of 3.4, indicating that the amino acid precursor pools for the nuclear and cytoplasmic fractions were equivalent for all the labeled proteins, and that the results for the nuclear fraction might be used as an internal index for assessing the differential labeling of the metabolic pool which in turn labels proteins in the two fractions. This is especially important for the analysis of the data, because the ^{3}H or ^{14}C is introduced as a single injection into the brain and is a variable factor during the time course of incorporation (*14*).

Graph f in Fig. 1A shows that the patterns of synthesis of most of the brain proteins did not change. At three protein bands (see α, β, and γ in graph e), however, the incorporation of valine increased in the experimental (E) groups. The relative labeling ratios in graph e were normalized to the constant value of 2.3, obtained for the ^{3}H/^{14}C ratio of the nuclear fraction from the same experiment. Similarly, the ^{14}C data in graph f were normalized by multiplying the results by 2.3. The majority of the proteins in the cytoplasmic fraction had the same ^{3}H/^{14}C ratio as that obtained for all the proteins in the nuclear fraction, suggesting that the amino acid precursors had no special features that might have resulted in the preferential labeling of the cytoplasmic proteins. Thus changes in the pattern of incorporation into the cytoplasmic proteins separating at α, β,

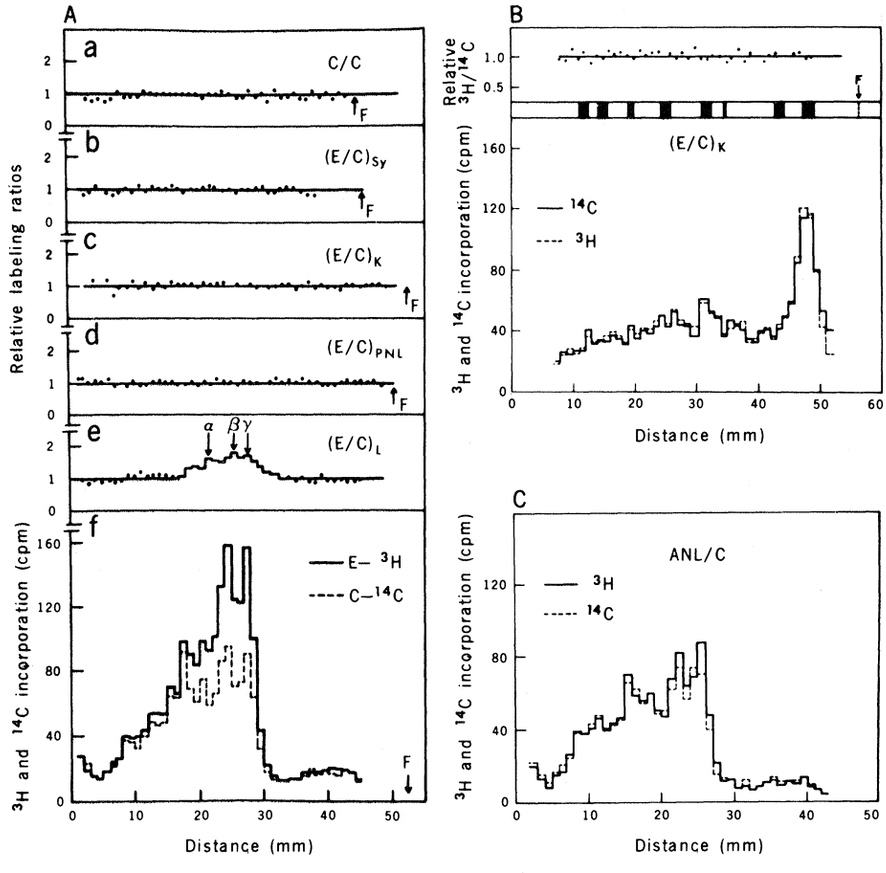

Fig. 1. Gel electrophoretic analysis of goldfish brain proteins. All samples are from homogenates of seven experimental (trained, *E*) animals injected with [^{3}H]valine and seven control (untrained, *C*) animals injected with [^{14}C]valine. The migration position of the dye front on the SDS-polyacrylamide gels, after 4 hours at 7 ma per gel, is shown by *F*. The gels were sliced into 1-mm segments to obtain sufficient counts in each slice. This results in a broadening of the pattern of the labeled peaks and the ratios. (A) Graphs a to e show the relative ^{3}H/^{14}C ratios. Graphs a, d, and e give the relative ratios for the cytoplasmic fraction to the constant ratios obtained for the nuclear fractions from each experiment. Graphs b and c are the normalized ratios for the brain synaptosomal (*Sy*) and the kidney (*K*) cytoplasmic proteins for an experimental (trained) group versus a control (untrained). The ^{14}C data for the controls were multiplied by a factor of 2.3, the constant ratio of ^{3}H to ^{14}C obtained for all the proteins of the nuclear fraction from the same experiment. The same factor, 2.3, was used to normalize the data in graph e. In graph d, *PNL* signifies passive nonlearners; in graph e, *L* signifies learners. (B) Gel electrophoresis data for the cytoplasmic proteins from the kidneys of experimental (trained) animals injected with [^{3}H]valine versus control (untrained) animals injected with [^{14}C]valine. (C) Labeling pattern of an SDS-polyacrylamide gel for the cytoplasmic proteins from an active nonlearner (*ANL*) group injected with [^{3}H]valine. The data for the control group were multiplied by a factor of 3 so that they could be plotted on the same scale.

and γ may be regarded as an indication of increased valine incorporation. This aspect of the data represents a type of internal control. Additional internal controls for this experiment are provided by the data for the synaptosomal fraction (graph b) and the kidney cytoplasmic fraction (graph c) showing no observable changes. Some increased labeling occurred in a band with proteins that migrated at a rate slower than those at α, but this was not always reproducible.

In Table 1, experiments 1 to 6 indicate that there is a consistent increase in the labeling of the α, β, and γ proteins for the goldfish, injected with [^{3}H]valine, that mastered the training. Similar increases were obtained for trained animals injected with [^{14}C]valine (Table 1, experiments 7 and 8). The average increases in incorporation were 36, 41, and 57 percent for the bands with average R_F values of 0.45, 0.51, and 0.59, respectively. The migration of these proteins on the SDS gels indicates that they have molecular weights of approximately 37,000, 32,000, and 26,000, respectively. A comparison of the isotope ratios for ten gel slices at the positions of α, β, and γ with ten slices on either side of the peaks in a Student's t-test gave P values of less than .005, indicating that the changes are statistically significant. Figure 1B also shows the data for the labeling pattern of the kidneys of trained versus control goldfish. Here the constant ^{3}H/^{14}C ratios obtained indicate that in one other organ of the same animals there are no measurable protein changes.

Consistent increases in the pattern of amino acid incorporation at three bands on an acrylamide gel (*15*) do not prove that the changes are related to the training of the goldfish. They could be due to such factors as recovery from stress of the training experience, the physical exercise involved in learning the new swimming skill, or some other effect. Control experiments were conducted to test some of these possibilities.

The animals that were nonlearners were separated from the learners and studied as groups in comparison to untrained controls. They showed no changes in their nuclear, synaptosomal, or cytoplasmic proteins, even though they had been exposed to the same stimulus and had been present in the same tank as learners. Table 1 shows data for two types of groups of nonlearners: passive nonlearners (PNL) and active nonlearners (ANL). The PNL's start the training with a low group score and end with a score less than 15 percent higher (graph d in Fig. 1A; experiment 9 in Table 1); they show no protein changes. The ANL's (Table 1, experiments 10 and 11) have a high score at the beginning of training, and this score remains high throughout training but does not increase (that is, less than 15 percent). The ANL's work hard at performing the task but do not exhibit any protein changes (see Fig. 1C). Thus the physiological activity and the complex movements required to carry out the behavior cannot be considered as major determinants of the protein changes.

A behavioral control for the effects of stress was devised from studies of the influence of age on learning and memory in the goldfish. Animals that were 1 year old or less (6 to 7 g in weight) could remember how to swim upright with the float after 3 and 11 days. The older goldfish (12 to 15 g) could learn the skill but did not remember it 3 days after the training. Thus the older goldfish, because they had no long-term memory of the training, could be used to determine the effects of recovery from stress experienced during the learning. Table 1 (experiments 12 and 13) shows the results for such learning and forgetting groups (LF). The older animals showed no protein changes even though substantial learning occurred (group scores changed from 24 to 93 percent and 22 to 68 percent, respectively). If stress was a major factor for producing the protein changes then both the L and LF groups would be recovering from its effects during the labeling period. The fact that no such changes were obtained for the older goldfish suggests that stress is not a major determinant for the increases in radioactivity obtained in the α, β, and γ bands. There is, of course, the possibility that the task is easier and less stressful for the older animals, but they appear to acquire the new swimming skill at about the same rate as the younger animals, and do not "remember" it.

These experiments indicate that the increased labeling of the proteins in the α, β, and γ bands is related in some way to the acquisition of the new behavior. It is important to note, however, that the double labeling method indicates only that changes occur in specific proteins, it does not provide any evidence for any general increases in brain protein metabolism that might occur. In addition, the appearance of three bands on the SDS-polyacrylamide gels does not necessarily mean that each band is a single protein. The apparent molecular weights and subcellular distribution properties of the proteins separating at α, β, and γ rule out the possibility that they include any of the previously identified brain-specific proteins, such as S-100 (*5*). At present no clearly defined mechanism can be postulated for the way in which soluble cytoplasmic proteins might be involved in information processing. The fact that no synaptosomal protein changes are obtained suggests that the direct use of the proteins separating at α, β, and γ in any membrane structures cannot take place without further modification of the proteins. One possibility is that the proteins could play a role in stimulating growth of specific neuronal structures. Clearly, further experiments are required. The results, nevertheless, provide some evidence for a specific change occurring in the pattern of protein synthesis after the acquisition of a new behavior.

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References and Notes

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