

ANALYSIS OF PROTEIN LEVELS AND SYNTHESIS AFTER LEARNING IN THE SPLIT-BRAIN PIGEON

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SUMMARY

A series of experiments were performed to study the effects of learning upon proteins in the split-brain pigeon. With this preparation, one side of the brain was trained in a color discrimination task while the other side served as a naive control. Proteins from both sides of the brain were separated by one- and two-dimensional polyacrylamide gel electrophoresis and analyzed to measure protein levels or protein synthesis. No differences were found between trained and naive telencephalic hemispheres or between trained and naive optic lobes in the steady-state protein levels or in protein synthesis.

INTRODUCTION

Since Halstead's proposal in 1948 suggesting a role for proteins in learning, an extensive literature has grown on the subject^{9,11,22,24}. Changes in the concentration and rate of synthesis of specific brain proteins and glycoproteins have been reported to occur with training^{2,4,13,25,26}. On the other hand, other workers have been unable to find such differences^{1,8,19,21}. Thus, the literature is controversial and there is neither general agreement regarding the number and nature of the proteins involved nor, indeed, agreement as to whether proteins are involved at all. These conflicting results may arise from differences in the types of learning experiments, animal species, or the different biochemical methods employed to analyze proteins. However, they may well also arise from a serious shortcoming common to all these approaches and that is the

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difficulty in devising adequate controls for the training situations^{3,9}, despite great ingenuity on the part of some workers.

One system which seemed to us to satisfy the requirement for an appropriate control is the split-brain pigeon preparation. In this animal, all optic neurons cross over at the chiasma. Transection of the supraoptic decussation prevents interocular transfer of color and pattern discriminations and confines to the trained hemisphere what is normally transferred to the other^{5,6}. Since the experimental and control hemispheres reside in the same animal, any biochemical changes occurring from factors not directly related to learning (stress, arousal, etc.) will occur equally in both hemispheres.

After a monocular color discrimination task, protein levels and synthesis were monitored by one-dimensional gel electrophoresis, high-resolution two-dimensional gel electrophoresis and fluorography. Protein synthesis was measured in a brain slice system while protein levels were measured both in the slice system and in selected regions of freshly extirpated brain. Within the limits of our methodology, no differences were found between trained and untrained hemispheres either with respect to optic lobes or telencephalic hemispheres.

MATERIALS AND METHODS

Surgical procedure

Transection of the supraoptic decussation was performed using Meier's procedure²⁰. White Carneaux pigeons (Palmetto Pigeon Plant, S.C., U.S.A.) were anesthetized by an intramuscular injection of 0.8 ml ketamine hydrochloride (100 mg/ml) and the head fixed in a stereotaxic apparatus. An incision was made in the skin of the cranium and a small area of bone (approximately 4×5 mm) removed with a dental drill. The dura was cut lengthwise on one side of the superior sagittal sinus and drawn over the median sagittal plane to allow introduction of the stereotaxic knife. The supraoptic decussation was transected according to the coordinates in the atlas of Karten and Hodos¹⁵. After the knife was withdrawn, the operative field was covered with Gel Foam and the skin sutured.

Behavioral paradigm

The three-key pigeon test chamber and the simultaneous color discrimination task used have been described previously¹⁰. The split-brain pigeons, maintained at 80% of their free-feeding weights, were goggled so that either eye could be occluded by attaching an opaque disc. The pigeons were autoshaped to peck a white center key using both eyes and subsequently shaped to peck the key employing either eye.

For the learning session, one eye was occluded and the pigeon presented with a completely novel situation. After pecking the white key, it had to learn to simultaneously discriminate between red and green side keys which became lit when the white key was pecked. To control for color preference, the first 20 trials were programmed so that either color would produce positive reinforcement. On all subsequent trials following the initial 20, only the non-preferred key was programmed to produce a

reward when pecked. A response to the correct color was rewarded by food and an incorrect response was followed by a 10 sec blackout. Split-brain pigeons consistently learn this discrimination in approximately 200 trials in an elapsed time of about 1 h. Each training session was run until the criterion was reached of 5 successive successful sessions, a successful session being considered to be at least 18 out of 20 correct responses.

Protein synthesis in tissue slices

Immediately after the training session the brain was removed and the telencephalic hemispheres and optic lobes were dissected from the trained and naive sides of the brain and were chilled in the incubation medium of Elliott⁷ at 4 °C. Tissue slices approximately 300 μ m thick were cut manually from each of the two dissected parts of the brain and were then incubated in 5 ml (optic lobe) or 8 ml (telencephalon) of the above incubation medium containing 100 μ Ci L-[4,5-³H]leucine (62 Ci/mmol) in a 95% O₂-5% CO₂ atmosphere. The incubation was performed in sealed flasks for 1 h at 37 °C in a water bath with shaking. The reaction was terminated by chilling the flasks in ice, after which the slices were briefly centrifuged at 4 °C and washed with 0.32 M sucrose solution to remove the added salts.

Tissue fractionation. Prior to further analysis either for content or synthesis of various proteins, the incubated slices or the freshly extirpated tissue was somewhat arbitrarily separated into a membrane-mitochondrial pellet (pellet fraction) and a post-mitochondrial supernatant fraction (supernate). This was accomplished by homogenizing the tissue in ice-cold 0.32 M sucrose at 900 rpm with a motor driven teflon-glass homogenizer. After centrifuging the homogenate at $750 \times g$ for 5 min, the pellet was discarded and the supernate was then centrifuged at $10,000 \times g$ for 20 min. After resuspending the pellet in 3 ml of 0.32 M sucrose, both pellet and supernatant fractions were further purified by another centrifugation step at $10,000 \times g$ for 20 min. In preparation for electrophoresis, the supernate was dialyzed against H₂O overnight and was then freeze-dried. The $10,000 \times g$ pellet was washed with no more than 1 ml of H₂O, recentrifuged, and dissolved in the standard electrophoresis sample buffers for one-dimensional¹⁶ or two-dimensional²³ electrophoresis.

Synaptic plasma membranes (SPM) were prepared from the freshly excised tissue using the combined flotation-sedimentation density gradient procedure of Jones and Matus¹⁴. The purity of the synaptic plasma membranes was monitored by electron microscopy after fixation in 2.5% glutaraldehyde, post-fixation in 2% OsO₄, block-staining with aqueous 2% uranyl acetate, embedding in Epon-Araldite, and post-staining with 1% aqueous uranyl acetate and Reynold's lead citrate¹².

Analytical procedures. One-dimensional SDS polyacrylamide gel electrophoresis (10% polyacrylamide) was performed on a vertical slab gel apparatus using the Tris-glycine system described by Laemmli¹⁶ and two-dimensional gel electrophoresis was performed according to O'Farrell²³. Both one- and two-dimensional gels were stained with Coomassie blue and destained with 7% acetic acid. For detection of radioactive label, fluorography was performed using 2,5-diphenyloxazole-impregnated gels and Kodak X-Omat XR-1 film¹⁷. Quantitation of stained one-dimensional gels

was done directly using a Shimadzu microdensitometer while fluorographs of such gels were scanned with a Joyce Loeb microdensitometer. Two-dimensional gel patterns were analyzed qualitatively for differences. This was achieved by visual inspection of the two gels side by side for changes in spot size or density or for the appearance or disappearance of spots. These inspections were routinely made by a number of laboratory workers some of whom possessed no knowledge of the nature of the experiment. Because the two-dimensional gel electrophoresis method is prone to artifacts (such as streaking, poor separation in the first dimension, etc.) it was necessary to run each sample in duplicate and sometimes in triplicate. The naive and trained hemispheres from eight separate experiments were analyzed.

RESULTS

Evidence for disruption of interhemispheric transfer

A critical control in the present study was to confirm the observation by other workers^{5,6,20} that transection of the supraoptic decussation prevents interocular transfer of monocularly learned color discriminations. This was achieved by monocularly training split-brain pigeons on the simultaneous color discrimination task and then testing the other eye. The results (not shown) demonstrated that performance by the non-trained hemisphere was at chance, indicating that disruption of interhemispheric transfer had occurred.

Comparison of protein levels by one-dimensional SDS gel electrophoresis

The effect of the training program on the levels of various proteins of the trained and the naive brain hemispheres was tested. In the experiments in this section, as well as in all other experiments described here, the optic lobes and the telencephalic hemispheres were separated for this purpose.

The tissues were fractionated into supernatant and pellet fractions. The results of one-dimensional SDS gel electrophoresis of the supernatant proteins of the optic lobes are shown in Fig. 1A. About 45 bands are visible and no qualitative or quantitative differences between the patterns derived from the trained and the naive hemispheres can be discerned. The failure to find a difference was confirmed by scanning densitometry of the gels (data not shown), although less peaks are resolved by this method than by visual observation.

Similar analyses were done on the supernates from the telencephalon (Fig. 1B). It will be noted that the one-dimensional electrophoresis gel pattern is remarkably similar to that of the optic lobe. Again, however, no differences between experimental and control hemispheres could be detected either by visual inspection of the stained gels (Fig. 1B) or by microdensitometry (data not shown).

The results of analysis of the pellet fractions of the two brain areas are shown in Fig. 2A and B. About 57 bands are visible in each pattern with no differences discernible, either visually or by instrument, between naive and trained hemispheres. The results presented in this section are typical of those obtained from 4 replicate experiments.



Fig. 1. One-dimensional SDS polyacrylamide gel electrophoresis of supernatant protein from naive and trained hemispheres. A: naive (N) and trained (T) optic lobes. B: naive and trained telencephalic hemispheres.

Because of the possibility that learning might be mediated at the synaptic level, at least in part, the effect of our discrimination task on the proteins of the synaptic plasma membrane was also tested using one-dimensional gel electrophoresis. The results, both in the case of the optic lobes and the telencephalon, again do not reveal any significant differences in the protein levels (data not shown).

Comparison of protein levels by two-dimensional gel electrophoresis

In addition to the one-dimensional electrophoresis experiments already described, a higher resolution two-dimensional system, which separates proteins according to their isoelectric point in the first dimension and their molecular weight in the second dimension, was employed to study the effects of training on protein levels.

Representative two-dimensional gel electrophoretograms of the pellet fraction proteins from the trained and naive optic lobes are shown in Fig. 3. Inspection of the

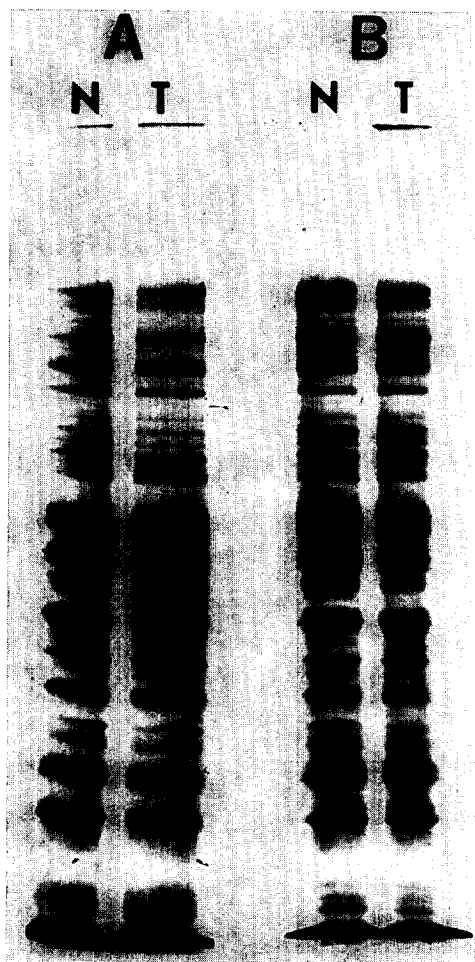


Fig. 2. One-dimensional SDS polyacrylamide gel electrophoresis of pellet fraction proteins from trained and naive hemispheres. A: naive (N) and trained (T) optic lobes. B: naive and trained telencephalic hemispheres.

two gels shows that the patterns are qualitatively very similar. No reproducible changes in the pattern of spots between the naive and trained sides of the brain were detected; that is, no spots appeared or disappeared as a result of training. This conclusion was drawn from analysis of these two gels, their replicates, and from 7 other separate experiments. The two-dimensional patterns from the telencephalic hemispheres were similar to those obtained with the optic lobes and no differences between the pellet fraction proteins from the trained and naive telencephalic hemispheres were detected. Two-dimensional gel electrophoresis of the pellet fraction proteins resolved approximately 200 distinct spots after staining with Coomassie brilliant blue.

The high resolution two-dimensional electrophoresis system was also utilized to compare the supernatant proteins from the naive and trained sides of the brain. No

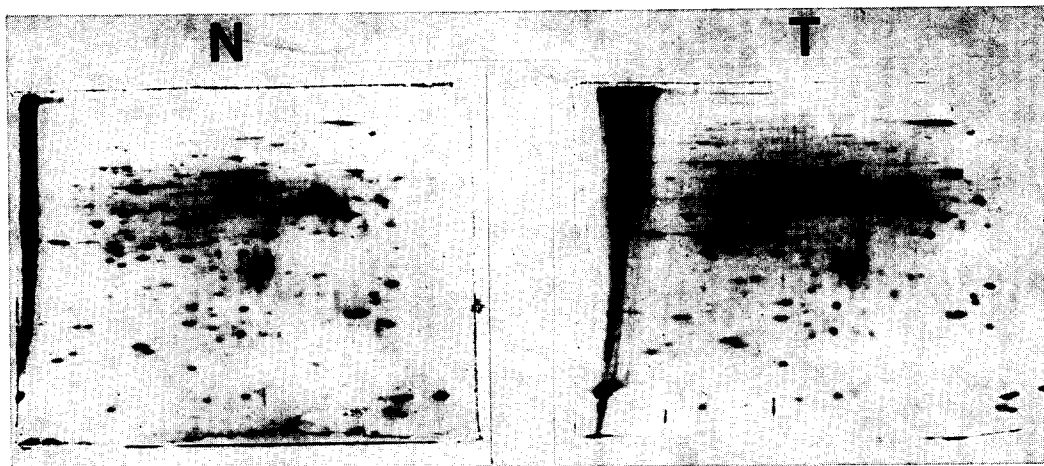


Fig. 3. Two-dimensional slab gel electrophoresis of pellet proteins from naive (N) and trained (T) optic lobes.

reproducible differences in the pattern of spots between the naive and trained optic lobes or between the naive and trained telencephalic hemispheres were detected. No spots appeared (or disappeared) with training and no obvious changes in the densities of any proteins were reproducibly observed. In Fig. 4, the supernatant protein patterns, again one of many replicate experiments, from the naive and trained telencephalic hemispheres are shown.

Effects of training on protein synthesis

It is conceivable that changes in protein levels were too small to detect against the large steady-state protein background. A more sensitive method is to monitor protein synthesis. Our first attempts were in vivo studies in which up to 2 mCi of either [^3H]leucine or a mixture of [^3H]amino acids was administered by brachial vein



Fig. 4. Two-dimensional slab gel electrophoresis of supernatant proteins from naive (N) and trained (T) telencephalic hemispheres.

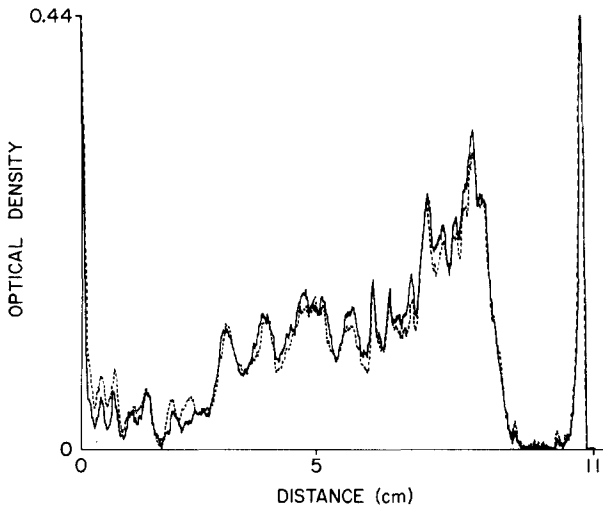


Fig. 5. Comparison of protein synthesis in trained (----) and naive (—) sides of the brain by densitometric scanning of fluorograms. Pellet protein fractions from telencephalic hemispheres are shown.

injection. However, the resulting protein specific activity was too low for subsequent analysis. We then turned to the tissue slice technique and these experiments are described below.

After training, the brain slices from the trained and naive sides of the brain (optic lobes and telencephalic hemispheres) were incubated with labeled leucine and the pellet fractions and supernatant fractions prepared. The proteins were separated by one-dimensional SDS gel electrophoresis and the level of radioactivity was detected by fluorography. Inspection of the fluorographs from the pellet fraction (naive optic lobe vs trained optic lobe, naive telencephalic hemisphere vs trained telencephalic

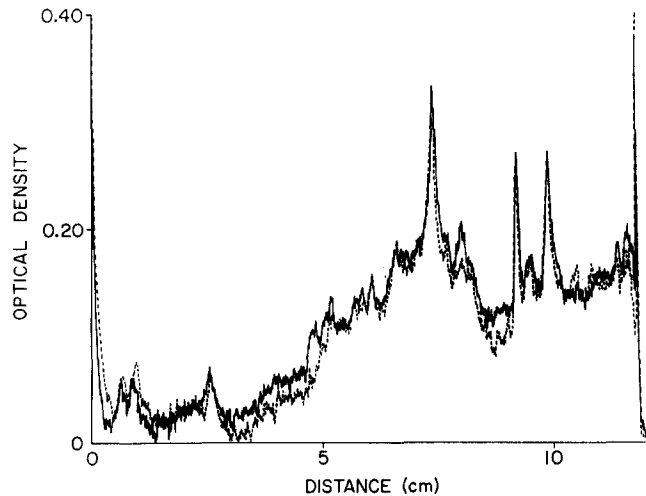


Fig. 6. Comparison of protein synthesis in trained (----) and naive (—) sides of the brain by densitometric scanning of fluorograms. Supernatant protein fractions from optic lobes.

hemisphere) showed the patterns to be remarkably similar. The identity of the profiles was confirmed by quantitative densitometry of the fluorographs. For example, the densitometric scans for the naive and trained telencephalic hemispheres are shown in Fig. 5.

Protein synthesis for the supernatant fractions from the naive and trained sides of the brain was also evaluated as described for the pellet fractions. Once again the patterns were very similar and no reproducible differences were detected between the trained and naive profiles. In Fig. 6 the densitometric scans from the supernatant naive optic and trained optic are presented. The small variations found in the patterns of protein synthesis between the two optic lobes are within the limits of experimental error.

DISCUSSION

We report here the use of split-brain pigeons to study the biochemical correlates of learning. With this color discrimination paradigm no differences were found in the levels of proteins between the trained and naive hemispheres in any of the fractions analyzed. This was the case using one-dimensional electrophoresis which resolved approximately 50 protein bands and two-dimensional electrophoresis which yielded more than 200 distinct protein spots.

Behavioral-related changes in protein concentration have been reported previously. Bogoch found increases in the concentration of particular glycoprotein fractions in response to operant conditioning of pigeons⁴. Two of the changes observed by Bogoch were subsequently confirmed by Barraco and Irwin², who trained pigeons in a visual discrimination task. In another study protein levels were found to change in cortical synaptic membranes of rats after handedness-transfer training¹³. In contrast to these results and in agreement with our results, protein concentrations were reported to remain the same in experimental and control rats⁸ and in experimental and control goldfish¹⁹ after shock avoidance conditioning training.

The synthesis of proteins during training from naive and trained hemispheres of the split-brain pigeon were compared by fluorography. We did not detect any reproducible variation in the [³H]leucine labeling pattern between the experimental and control parts of the brain. In a study closely related to ours, Metzger et al. investigated the effect of monocular visual stimulation and conditioning in the split-brain monkey²¹. After injection of tritiated water the relative rates of bulk protein synthesis were the same in subfractions of the contralateral and ipsilateral hemispheres. Also, Lim et al., using one-dimensional electrophoresis and autoradiography, did not detect any alterations in protein radioactivities in shock-avoidance-trained goldfish¹⁹.

Other workers, however, have found that the rate of incorporation of radioactive amino acids into brain proteins is different in trained and control animals. For example, Hyden recently reported that recurring reversal training resulted in an increase in synthesis of synaptosomal membrane proteins with molecular weights 35,000–45,000 and 60,000 and 100,000 in trained animals compared to active controls¹³. Also, using the transfer of handedness in rats, Levitan et al. showed that

training alters the incorporation of radioactive amino acids into synaptosomal membrane proteins¹⁸. However, the change did not appear to be specific for any particular protein, but rather increased proportionately in all the bands separated by electrophoresis¹⁸. Shashoua identified three cytoplasmic proteins from the goldfish brain that consistently incorporated more labeled amino acid in animals that acquired a new pattern of swimming behavior²⁵.

Our failure to detect alterations in protein concentrations or synthesis after learning is subject to a number of interpretations. With the split-brain system, any biochemical changes resulting from factors not directly related to learning would effectively cancel out in both hemispheres. Therefore, one possible explanation of our results is that we are not able to detect the small biochemical changes, resulting solely from learning, against the large background of normal cell metabolism. This interpretation lends support to the general criticism^{3,9}, that in some studies observed biochemical changes resulted from side-effects of the training procedure, such as stress, arousal and motor activity. Alternatively, our negative findings might simply result from certain factors in our experimental design. Nevertheless, others have reported changes using the same precursor, a similar time course, similar learning paradigms and comparable biochemical methods. We recognize that our findings might also provide a basis for the contention that proteins do not participate in the learning process. However, we feel it is more likely that such changes escape detection by our analytical methods.

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