Protein Changes in Different Brain Areas as a Function of Intermittent Training

(brain protein/behavior/training/cortex/limbic system)

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ABSTRACT Incorporation of [^3H]leucine into protein of eight different brain areas in rats was determined during 1 month of intermittent training for reversal of handedness. The incorporation values of all areas of the trained animals were lower than those of the controls. This finding was corroborated by autoradiography. After initial training the relative specific activities of the sensory-motor and entorhinal cortex and reticular formation were low compared to those of the hippocampus. With more training and time, these values became inverted.

It is known that changes of neural activity are accompanied by measurable changes in macromolecules of brain cells (1-5). The aim of the present study is to follow protein changes in small, defined areas of the brain as a function of increased animal activity distributed over a period of 1 month. The studies have encompassed such phylogenetically different areas as the reticular formation, the limbic system, and cortical areas. The parameter used has been relative specific activities of μg protein samples, which contain both neurons and glia. The aim is not to investigate correlates to behavior. Instead, the functional correlate can be phrased as increased sensory and motor activity over a period of time.

MATERIALS AND METHODS

38 Female Sprague-Dawley rats were used. They were 65-days old and weighed around 160 g at the onset of the experiment and 230 g at the end (after 35 days). The activity test used was reversal of handedness, which was induced without surgery or tying of limbs. Rats show a preference in handedness, 40% are right-handed, 45% left-handed, and 15% ambidextrous. We have used a revised test originally described by Peterson (6), Wentworth (7), and Buresova (8). Before the training experiments began, all the rats were reversed with respect to day and night for a period of 1 week, and this reversal was maintained during the 1-month period.

The original test arrangement of Peterson has been modified in the following way (9). In 23 out of 25 free-choice reachings, the rats showed whether they were left- or right-handed in reaching into a narrow, downward-tilted glass tube to retrieve, one by one, protein pills 4 mm in diameter with their preferred paw. Half of the glass tube was constantly kept full of protein pills by a special filling arrangement. To induce the rats to reverse handedness, we arranged a glass wall parallel to the glass tube on the side opposite that of the preferred paw. Space then prohibited the use of the preferred paw, and the rats soon began to use the nonpreferred paw. They were given two training periods of 25 min

each per day. After this initial training period, the rats were returned to cages, where they stayed together for an intermission of a fortnight without training. Then they were given 2 days of training, four sessions altogether, after which a renewed intermission without training was allowed. The period was finished with 2 days of training with four sessions altogether. A successful reach was one where the rat quickly grasped a protein pill with the paw and retrieved it. The three persons handling the training had no knowledge of the previous treatment of the rats, and the personnel in the biochemical laboratory received the rats designated only by number. Control rats had the same age and weight and got the same amount of food and water as did the trained animals.

The incorporation of [^3H]leucine into acid-precipitable material after intraperitoneal injection of 1 mCi of [^3H]leucine ([4,5- ^3H]L-leucine, The Radiochemical Centre, Amersham, England, 1 ml per injection was used) was followed in the eight different brain areas from 5 to 65 min after the last leucine injection. The [^3H]leucine was administered as follows: 300 μCi at the start of the training, 300 μCi after 10 min, and 400 μCi after 20 min during the last training session. The training thus went on for a further 5 min after the last injection. The rats were killed 1 hr after training was finished. The brain areas were dissected out under a stereomicroscope after the brain was sectioned at four levels by the description of Craigie (10). The incorporation into acid-insoluble material of the eight areas of the control animals reached a maximum 45-50 min after the last injection and decreased slightly after 60 min, after which the values continuously decreased during the observation time of 4 hr. The corresponding values of the trained animals were maximal after 60 min. There is thus a small phase difference of around 10 min between the two curves. As a compromise, we have therefore compared material from control and trained rats at 60 min after the last injection. In Table 1 is listed the distribution of the specific activity values from the eight brain areas of control and trained rats. The specific activity values, corrected for the concentration of free [^3H]leucine of the left and right hemispheres, did not differ from each other. Control rats, which used only the preferred paw when trained during 4 days (25 min/day twice a day), showed the same specific activity values of the hippocampal total protein as caged control rats with free choice. Therefore, such caged controls were used throughout.

The protein of the area dissected was extracted in a solution of 20 μM sodium thioglycolate, 0.25 M sucrose with 0.1 % Triton X-100, buffered at pH 6.7 by the addition of 0.5 M

Tris-phosphate to a final concentration of 0.06 M. After precipitation with 10% Cl_3CCOOH (4° for 20 min), centrifugation, and repeated rinsing with 5% Cl_3CCOOH , the precipitate was dissolved in 1 N NaOH, and 25 μl was taken for protein determination according to Lowry (11). For activity measurements of the dissolved acid precipitate, 25 μl of the sample and 500 μl of Soluene (Packard Instruments) was kept overnight; 15 ml of the scintillation fluid [11 g of Permablend (Packard Instruments) in 2 liters toluene] was added, and, after cooling, the activity was determined in a Packard Tri-Carb spectrophotometer. 50 μl of the supernatant plus 15 ml scintillation fluid was used for activity determination.

RESULTS

In Table 2, data are presented from cortical areas (sensory-motor cortex anterior to the bregma plus entorhinal cortex) and from the limbic system (the hippocampus and dentate gyrus). The incorporation values (specific activity) of the controls decrease significantly with age, from 65 to 100 days, while the relative specific activities (Ap/As) are fairly constant for the two regions (I and II) during the whole month. This finding is important to keep in mind when control data are used for a comparison with results from trained rats. The incorporation values into acid-insoluble material of both cortex and the limbic system of rats trained for the initial 4 days and also for an additional 2 days are significantly lower than the corresponding values of the controls. The incorporation values of the material obtained after 4 + 2 + 2 days of training with two intermissions of a fortnight each do not differ from the corresponding control values. As will be shown for the detailed analyses to be described below, the decreased incorporation into brain protein is a consistent result of the study. The specific activity of the protein of the sensory-motor and entorhinal cortex during the 35-day period decreased by 45%, and by the same percentage in the limbic system in the controls.

Fig. 1 demonstrates specific activity values (dpm/ μg of acid-insoluble material) for the eight different brain areas in control and trained rats. The areas studied were: sensory-motor cortex (about 3 mm cranially of the bregma), nucleus dorsomedialis thalami, entorhinal cortex, septum, corpus mamillare, nucleus dentatus, the hippocampus (CA3 region),

TABLE 1. Incorporation of [^3H]leucine into acid-insoluble material 60 min after intraperitoneal injection of 1mCi leucine

Area	Specific activity*	
	Controls†	Trained‡
Cortex	42 $\pm$ 2.1	26 $\pm$ 1.4
Thalamus, nucleus dorsomedialis	42 $\pm$ 2.1	17 $\pm$ 1.0
Entorhinalis	46 $\pm$ 2.2	23 $\pm$ 1.1
Septum	33 $\pm$ 1.9	19 $\pm$ 1.0
Corpus mamillare	40 $\pm$ 2.0	20 $\pm$ 1.4
Nucleus dentatus	38 $\pm$ 2.0	19 $\pm$ 1.3
Hippocampus (CA3)	43 $\pm$ 2.3	19 $\pm$ 1.3
Formatio reticularis	32 $\pm$ 1.8	21 $\pm$ 1.4

* Disintegrations per min of acid-insoluble material per μg of acid-insoluble material. Values are $\pm$ SEM.

† Three control rats.

‡ Four trained rats (4 days of training).

TABLE 2. Survey of results from cortical areas, I (sensory-motor plus entorhinal cortex), and from limbic system, II (the hippocampus and dentate gyrus), after 4, 6, and 8 days of training

No. days	Area, Rats	M	Ap	As	Specific activity (Ap/M)	Relative specific activity (Ap/As)
4	I Tr	1617	39374	125061	24.4	0.31
	I C	1244	54148	45867	43.5	1.18
	II Tr	372	7088	14534	19.1	0.49
	II C	294	11933	15672	40.5	0.76
6	I Tr	1612	28454	70814	17.7	0.40
	I C	1026	34341	35847	33.5	0.96
	II Tr	610	10150	28780	16.6	0.35
	II C	389	12681	17016	32.6	0.75
8	I Tr	1320	29801	60472	22.6	0.49
	I C	1688	41752	38384	24.7	1.09
	II Tr	324	6556	15000	20.3	0.44
	II C	392	8578	10784	21.8	0.79

M, μg protein in sample; Ap, number of disintegrations/min of acid-insoluble protein; As, number of disintegrations/min of supernatant; Tr, trained rats; C, control rats. The data represent single measurements on material pooled from four trained and three control rats.

and formatio reticularis. Samples were taken bilaterally. This high-resolution analysis gives result of the same general character as those given in Table 2, that is, the specific activity values of the trained rats are decidedly lower than those of the controls and converge towards the same values after the last training period.

In Table 3 are listed the ratios of radioactivity in the acid precipitate to those in the acid-soluble fraction for calculation of efficiency of brain protein synthesis in the eight different areas. The relative specific activity values of the different areas from the sensory-motor cortex to the reticular formation in the trained rats are 25–90% of the control values. The values of the pyramidal cells in the CA3 area of the hippocampus are the least depressed. The values of the sensory-motor and the entorhinal cortex increase during the 1 month of intermittent training. After the initial 4 days of training, the relative specific activity values of these areas are low in relation to that of the hippocampus. It is interesting that when the cortical values increase with time and training, those of the hippocampus decrease. It is furthermore to be noted that the reticular formation, the thalamus, and the cortical areas follow each other and are higher at the end of the month of intermittent training. Septum, the relay station between subcortical areas and the hippocampus, shows low values throughout the whole training period.

A pertinent question is whether the differences in the incorporation of the labeled precursor into protein and in the relative specific activities are due to changes in the rate of synthesis or in the precursor pool. Some circumstantial evidence indicates that the changes observed reflect changes in protein synthesis.

In order to answer this question, we performed the following experiment. A group of four rats was trained for 4 days as described above. 5 min before the end of the last training

TABLE 3. Relative specific activities $\pm$ SEM (A_p/A_s , see Table 2) of eight different brain areas of controls and rats trained to reverse handedness for 25 min/day twice a day during 4 + 2 + 2 days with 14 days intermission between each of the three training periods

Brain area	Training (days)*			Control†
	4	6	8	
Cortex	0.40 $\pm$ 0.01	0.51 $\pm$ 0.02	0.53 $\pm$ 0.02	1.40 $\pm$ 0.15
Thalamus, nucleus dorsomedialis	0.28 $\pm$ 0.01	0.35 $\pm$ 0.02	0.39 $\pm$ 0.03	0.95 $\pm$ 0.15
Entorhinalis	0.25 $\pm$ 0.002	0.32 $\pm$ 0.01	0.46 $\pm$ 0.02	0.84 $\pm$ 0.02
Septum	0.19 $\pm$ 0.004	0.29 $\pm$ 0.01	0.26 $\pm$ 0.01	0.78 $\pm$ 0.12
Corpus mammillare	0.39 $\pm$ 0.02	0.48 $\pm$ 0.04	0.43 $\pm$ 0.04	0.87 $\pm$ 0.09
Nucleus dentatus	0.39 $\pm$ 0.02	0.34 $\pm$ 0.01	0.41 $\pm$ 0.03	0.81 $\pm$ 0.03
Hippocampus	0.69 $\pm$ 0.04	0.38 $\pm$ 0.03	0.47 $\pm$ 0.03	0.75 $\pm$ 0.06
Formatio reticularis	0.32 $\pm$ 0.01	0.36 $\pm$ 0.03	0.52 $\pm$ 0.03	0.90 $\pm$ 0.03

* Four trained rats were used for each training period.

† Three control rats were used.

period, the animals were injected intraperitoneally with one single injection of 1 ml of [3 H]leucine (1 mCi/ml) and killed immediately after the training. In the same way, three control rats were injected intraperitoneally with the same amount of [3 H]leucine and killed 5 min after the injection. In both cases, the specific activities of the acid-insoluble material from the eight different brain areas and the concentration of the radioactive material (assumed to originate mostly from radioactive leucine) from the acid-soluble material were measured. For all areas the specific activity was lower in the trained animals than in the control animals (average, 3.11 times lower) and the equivalent concentration values were higher (average, 1.83 times higher).

Relative specific activity may be defined as $R = sB/sA$ (12), where sB is the specific activity of the compound B (protein) formed from labeled A (leucine), and sA the specific activity of A at the time of injection ($t = 0$). If $d(sB)/dt$ is measured at $t = 0$, the value of $dR/dt = d(sB)/dt \cdot 1/sA$ would give an approximate value of the turnover rate. From the data cited above, it is clear that $d(sB)/dt$ for the acid-insoluble material is higher (3.11 times) for the control ani-

mals than for the trained rats. If the turnover rate is the same for trained and control rats, the following relation should be valid:

$$[d(sB)/dt \cdot 1/sA] \text{ Trained} = [d(sB)/dt \cdot 1/sA] \text{ Controls}$$

which gives

$$(sA) \text{ Controls}/(sA) \text{ Trained}$$

$$= [d(sB)/dt] \text{ Controls}/[d(sB)/dt] \text{ Trained} = 3.11.$$

If we denote the concentration of radioactive leucine in the trained animals by $1.83n$, it is n in the control animals, and we obtain

$$n/(cL) \text{ Controls}:1.83n/(cL) \text{ Trained} = 3.11$$

where $(cL) \text{ Controls}$ and $(cL) \text{ Trained}$ denote the concentration of total leucine in the control and trained animals, respectively. Thus,

$$(cL) \text{ Trained}/(cL) \text{ Controls} = 5.7$$

which means that the concentration of leucine in the pools of the different brain areas of the trained animals should be about six times higher than that in the pools of the control animals. This seems unlikely. Thus, it cannot be assumed that the turnover rate in the two cases are the same. It is highly probable that the turnover rate is higher in the control ani-

FIG. 1. Specific activity of acid-insoluble material in eight different brain areas of trained (+) and control (O) rats at 4, 6, and 8 days of training, with two intermissions of 14 days each between the three training periods (see text). (1) cortex, (2) thalamus, nucleus dorsomedialis, (3) entorhinalis, (4) septum, (5) corpus mammillare, (6) nucleus dentatus, (7) hippocampus, (8) formatio reticularis.

TABLE 4. Ratio (Tr/C) between incorporation (number of grains/volume unit) of nerve cells in different brain areas of trained (Tr) and control (C) animals

Brain area	Tr/C	$(A_p/A_s) Tr / (A_p/A_s) C$
Cortex	0.155 $\pm$ 0.047	0.29
Thalamus, nucleus dorsomedialis	0.031 $\pm$ 0.009	0.29
Septum	0.071 $\pm$ 0.011	0.24
Hippocampus	0.254 $\pm$ 0.058	0.92
Formatio reticularis	0.050 $\pm$ 0.005	0.36

In the last column are given the ratios between the relative specific activities from trained and control animals taken from Table 3. All data for trained animals refer to 4 days of training.

FIG. 2. (Top) Section through cingulate cortex showing pyramidal cells of trained (*left*) and control (*right*) rats. (Bottom) Section through the hippocampus (CA3 region) showing pyramidal nerve cells of trained (*left*) and control (*right*) rats. Staining with methylene blue. Magnification $\times 1000$. See text.

imals in accordance with the value of relative specific values at 1 hr after injection discussed above.

Banker and Cotman (15) observed that tritiated leucine in supernatants of brain material was rapidly metabolized; however, this would not cause a serious error in our case, since significant differences were found in uncorrected specific activities. Further evidence that the decrease observed in specific activities reflects real protein changes is furnished by autoradiographic results. For the autoradiographic study, two groups of five rats each were used. Three rats in each group were trained as described above for 4 days with two training sessions daily. Two rats were controls. All rats except one control were injected with 1 mCi of [^3H]leucine

(1 mCi/ml) intraperitoneally. The injected rats were killed 15, 60, and 85 min after the injection; brains were immediately taken out, frozen, and sectioned. The sections, 4 mm thick, were fixed with Carnoy's solution, dehydrated, embedded in paraffin, and sectioned in 5- μm sections. These sections contained the eight brain areas described above. On each glass slide to be covered with Kodak K2 emulsion, five sections of each area were placed: control section from an uninjected rat, control section from a rat that had received [^3H]leucine, and three sections from injected rats killed 15, 60, and 85 min after injection. The preparations were exposed for 12, 29, 56, 78, and 140 days, after which they were developed for 4 min in Ilford ID 19, rinsed, and fixed for 7–10 min in 30%

sodium thiosulphate. The sections were counterstained with 1% toluidine blue. The sections were studied and photographed with a Zeiss plane achromate $\times 100$, numerical aperture 1.30.

Each 5- μ m section was photographed on 9 $\times$ 12-cm plates at four different levels of optical depths in order to localize and count all particles contained in the section. Optimal results were obtained with the sections exposed for 56 days. The silver grains contained in each nerve cell studied were registered in the following way. The contours of each cell were drawn on a thin cellulose acetate foil and the grains on the four foils were marked with India ink and counted. The outline of the cell counted was copied on a transparent paper, and the surface of the cell was determined by weighing. In this way, the number of grains per cell volume was obtained.

Table 4 gives the quantitative result from the CA3 region of the hippocampus, septum, the dorsomedial nucleus of the thalamus, the reticular formation, and cortex, expressed as the ratio of number of particles per cell volume between trained and control rats. The numerical value of this ratio is less than one for all brain areas, which means that the control rats have higher incorporation values than the trained animals. The hippocampus has the highest ratio value, and cortex and the other regions a significantly lower one. This agrees well with and supports the biochemical data given above. Fig. 2 shows photographs from cortex and pyramidal cells of the hippocampus of trained and control rats. Note the scarcity of silver grains above the cells of the trained rats.

DISCUSSION

The evidence presented indicates that the protein changes observed in the eight small defined areas of the brain reflect changes in protein synthesis. One of the main observations is the decrease of the relative specific activities of protein in the different areas of the trained rats after intraperitoneal administration of precursor. This phenomenon may mean that part of the protein synthesis has become inhibited. This does not exclude that small amounts of protein—e.g., brain-specific proteins constituting only 0.1–0.2% of the total protein—undergo an extensive synthesis in such areas. That increased neural activity decreases the incorporation of labeled precursors into macromolecules of rat brain has been found also for RNA by Glassman (4), and for protein by Tiplady (13).

The second observation is the changes of relative specific activities as a function of training with time when the eight different areas were observed during 1 month. After the initial training periods, the hippocampus had the highest values, which decreased with more experience and time. The values of the sensory-motor, entorhinal cortex, and the reticular formation were initially low compared to those of the hippocampus, but increased significantly with more training and time. Due to the high resolution of the analytical procedure, it is thus possible to state that there occur differentiated time sequence protein changes during increased neural activity that proceed in opposite direction when the hippocampus and

cortical areas are compared. Septum differs from that pattern and is nonresponsive.

When we discuss our results, it should be emphasized that results from different types of behavioral tests cannot always be compared, and interpretation must be made with caution. The experimental conditions involve various types of sensory and motor activity; the amount of stress varies, in some experiments electric shocks are applied, etc.

It should be noted that results from incorporation experiments with intraperitoneal administration of label cannot be compared with those obtained from intraventricular administration. This fact has been discussed by Bowman and Kottler (5).

Beach *et al.* (14), using another conditioning test, found an increased number of grains over cell nuclei in the CA1 region of the hippocampus and of the septum. We found a decreased number of grains over the nerve cells of septum and over the CA3 hippocampal cells. Our autoradiographic result completely paralleled our biochemical result.

The underlying mechanism of the changes observed is not elucidated by the present study. In speculation, it may be that the behavioral demands induce substances to be released in the nervous tissue which in some areas act as inhibitors of protein synthesis and in other areas induce increase. The depressive effect may serve a selective mechanism, that of letting small amounts of specific proteins be synthesized in a partially inhibited area.

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