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Protein Changes in the Rat's Prefrontal and "Inferotemporal" Cortex After Exposure to Visual Problems

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MOGENSEN, J. AND O. S. JØRGENSEN. *Protein changes in the rat's prefrontal and "inferotemporal" cortex after exposure to visual problems.* PHARMACOL BIOCHEM BEHAV **26**(1) 89–94, 1987.—The proteins D2 (N-CAM) and D3, both markers for brain neuronal membranes, MM, a marker for mitochondria, and CM, a cytoplasmic marker protein, were studied in the prefrontal (anteromedial) cortex and "inferotemporal" (Te2) cortex of rats by crossed immunoelectrophoresis. Three experimental groups were investigated: Rats trained to criterion in a visual pattern discrimination test (learning), those run as yoked controls and finally, rats kept in individual cages and not subjected to any training (passive). Statistical analysis indicated that behavioral procedures and marker proteins contributed significantly to the variation. Further analysis indicated that the significant changes occurred in the D3- and MM-protein and that both the learning and yoked control groups had significantly increased concentrations of these two proteins when compared to the passive group. Furthermore, the concentration of D3- and MM-protein in the yoked control group was significantly higher than that of the learning group. The results seem to indicate that changes in concentration of these proteins can be more easily related to the activity of "searching for an adequate behavioral strategy" than to the formation of an "engram."

D2-protein "Inferotemporal" cortex Noncontingent reinforcement Prefrontal cortex Rat
Neuronal membrane proteins Visual problems N-CAM D3-protein

IN a previous study we measured the concentration of a number of neuronal proteins in the prefrontal (anteromedial) cortex, occipital cortex and anterior and posterior parts of the neostriatum of three groups of rats: one, which learned spatial delayed alternation, another composed of yoked controls and finally, a passive control group [14]. The only significant change, an 8% decreased concentration of neuronal membrane proteins, was found in the prefrontal cortex of the yoked control group. We suggested that the reduction of the "synaptic mass" in the prefrontal cortical area of the yoked controls could have been caused by the "search" for the correct spatial hypothesis [3,12]. The term "hypothesis frustration" was suggested to describe the cause of the synaptic changes found in the yoked control animals [14].

In our further attempts to elucidate the processes underlying the behavior-related synaptic modifications we have presently employed a visual pattern discrimination task. The design with a learning group, a yoked control group and a passive control group was preserved. In discrimination tasks a stable performance around chance level is usually followed by an abrupt shift to nearly perfect performance. By sacrificing the animals six hours after the occurrence of this shift, we attempted to gain information about the protein relationships at the "moment of learning"—the moment at which the animal "finds" the hypothesis which is in agreement with the experimenter-defined solution [3,12].

Four proteins were measured by quantitative immunoelectrophoresis: the neuronal membrane proteins D2 and D3, the mitochondrial protein MM, and the cytoplasmic protein CM. The D2-protein is found on the outside of the presynaptic membrane [7] and on neurites [1,11]. Although a small amount of D2 may be associated with brain astrocyte membranes [13], D2 belongs to the N-CAM family of neuronal adhesion molecules [4,11] and the concentration of D2-protein may be used as an index for synaptic turnover [9,10]. D3 is localized in the presynaptic membrane and may be a measure of the density of synapses [7, 9, 10]. The mitochondrial marker MM is located to mitochondria whereas the cytoplasmic marker CM is present in neuronal cytoplasm (L. Clemmesen, O. S. Jørgensen, R. Hemmingsen, D. I. Barry and T. G. Bolwig, submitted). MM and CM were studied mainly as controls for the D2- and D3-proteins.

Two structures were chosen for investigations: the prefrontal (anteromedial) cortex and the Te2 cortical area (Fig. 1). The Te2 area [17] has recently been shown to resemble the primate inferotemporal cortex in a number of ways (G. Williams, J. Mogensen, O. Lindvall, A. Björklund and I.

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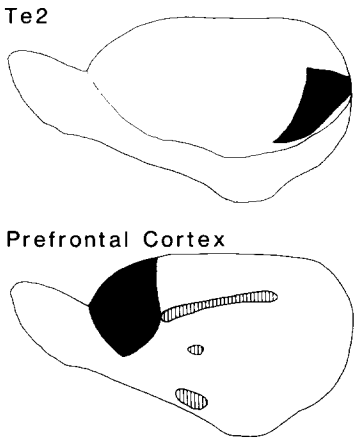

FIG. 1. Schematic illustration of the two cortical areas sampled. Top: Lateral view of the rat brain showing the Te2 ("inferotemporal") area. Bottom: Medial view, showing the anteromedial (prefrontal) area.

Divac, submitted) and may therefore be the cortical "focus" for visual pattern discrimination.

METHOD

Subjects

Thirty-six experimentally naive male Wistar rats weighing approximately 225 g at the beginning of the experiment were housed in single cages with water always available. They were fed commercial rat chow once daily after training and were deprived to and maintained on approximately 90% of ad lib body weight. The rats were divided into 12 squads, each made of 3 animals of approximately equal initial weight. Within each squad the rats were randomly assigned to one of three treatment groups: Visual pattern discrimination learning (VPD), yoked activity control (YAC) or passive control (PAS). Since two squads failed to satisfy the double criterion employed (see the Behavioral Procedure section) and were excluded for this reason, the biochemical analysis was performed only on the 10 remaining squads.

Apparatus

The training was performed in a gray, open, one-unit Grice box, the general floor plan of which has been shown as Fig. 1 in [2]. The start box which was separated from the rest of the apparatus by a transparent guillotine door was 10 cm wide and 21 cm long. The distance from the guillotine door to the choice point (the point at which the choice area met the entrances of the two alleys) was 18.5 cm. Each alley was 44.5 cm long and 17.5 cm wide. Two food cups containing mashed rat chow were always present at the end of each alley and were concealed by two 4 cm high barriers. In each alley a trap-door of the same width and height as the alley was present 14.5 cm from the choice point. These two trap-doors were clearly visible from the entire choice area and were equipped with counter-weights to permit a gradual closing of the doors during the shaping procedure. During the actual discrimination training these trap-doors carried the discriminanda. The discriminanda were × and +, the latter was always the positive discriminandum. The two symbols were constructed to have equal areas of the lines (53 cm^2). All walls of the apparatus were 23 cm high. The apparatus was placed in the middle of a well lit room in which other rats were present during testing.

Behavioral Procedure

Habituation and shaping. All VPD and YAC animals underwent first habituation and shaping. On the first two days they were placed one after another in the maze (from which the guillotine door and the trap-doors had been removed and which was covered to prevent escape). They were left in the maze for 15 min each. During this time the rats had free access to food in both alleys. After this habituation period (on day 3) the guillotine door and the trap-doors were introduced, the maze was no longer covered and all animals were shaped by being repeatedly placed in the start box and released—the trap-doors were left widely open to allow easy access to the alleys. The rats were shaped to enter any of the alleys within 5 sec after their release (too long latencies caused removal from the maze and approximately 15 sec delay to the next trial). When the animals promptly entered one of the alleys when released, the second part of the shaping procedure was initiated. During this period the trap-doors were gradually closed, each step in the closing procedure depended on the rat's readiness to promptly pass through the trap-doors with the particular, larger angle. When the trap-doors were completely closed (positioned vertically) and an animal in this situation in 20 consecutive trials entered either alley within 5 sec after release it was shifted to the next step of training: visual discrimination (VPD animals) or yoked activity control procedure (YAC animals).

Visual discrimination. Each animal was given 40 trials daily. The position of the positive discriminandum on each trial was decided according to a pseudo-randomized series [5]. On each trial the animal was placed in the start box, released and allowed to enter one of the alleys and make an attempt at opening the trap-door. If the correct alley was chosen the trap-door would be found unlocked and the animal could enter the second part of the alley and have 8 sec access to food. If the wrong alley was chosen the rat found the trap-door locked and was immediately removed from the apparatus. Following each trial the animal was placed in a transport cage and left there for approximately 10 sec during which the trap-doors were arranged for the next trial; if no shifting of the trap-doors was required, a "mock shift" was performed to produce noises equivalent to what would occur at an actual shifting. For each day the number of errors in each of the four blocks of 10 trials were counted. The rats were trained until they reached the criterion of making 2 errors or less in the last 10 trials. However, in order to satisfy the double criterion required for the animals which should be subjected to biochemical analysis, a rat would also on the criterion day have to show more than 2 errors in at least one of the three first groups of 10 trials.

Activity control. Each YAC animal was run as a "yoked control" to the VPD rat of the same squad. All parameters of the activity control procedure were the same as for the visual discrimination procedure, but the delivery or non-delivery of reward was noncontingent upon the behavior of the animal. The trials that would be reinforced were predetermined by the correct trials of the yoked VPD animal (e.g., if the VPD animal made errors on trials 2, 12, 18, 19, 22, 31, 33 and 39, the yoked YAC animal would be reinforced on all but trials 2, 12, 18, 19, 22, 31, 33 and 39, regardless of the behavior of the YAC animal). On trials that should be reinforced both

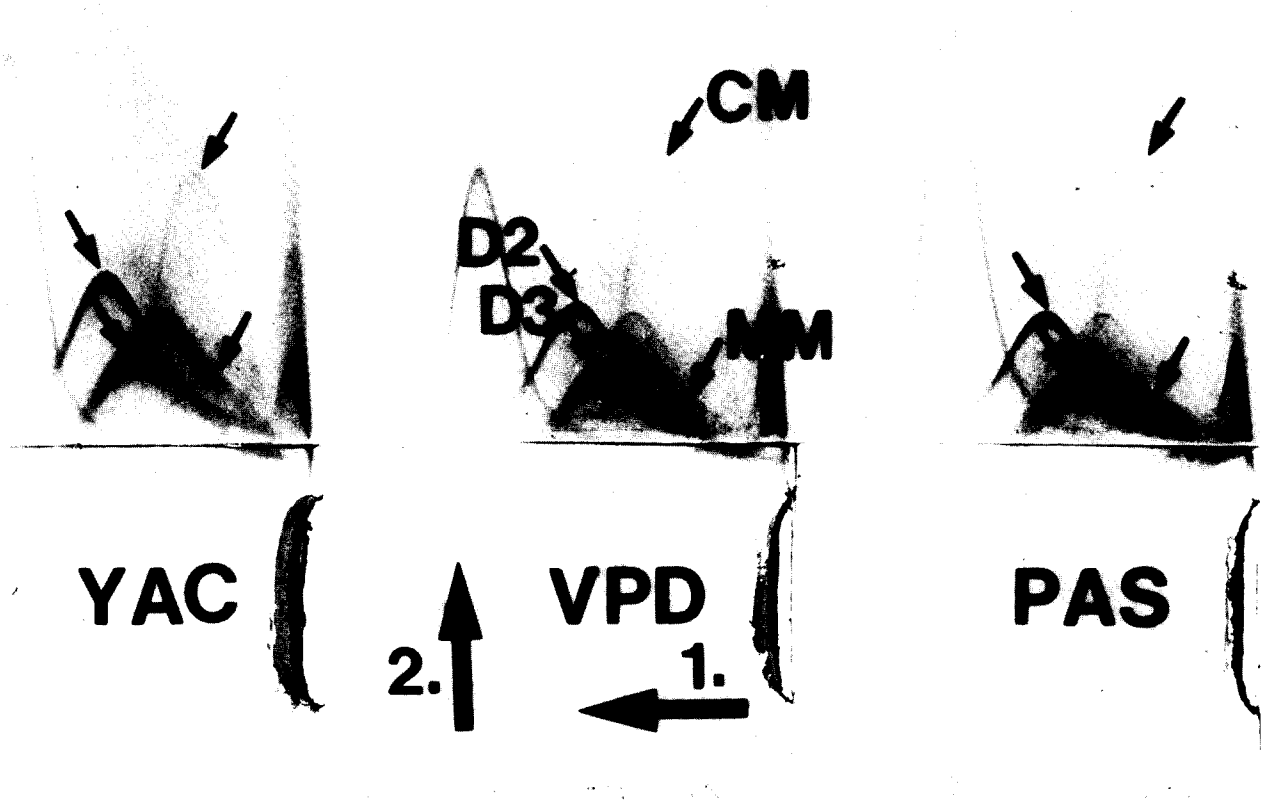

FIG. 2. Crossed immunoelectrophoresis of Te2 cortical samples (72 μg protein) with the antigen precipitates labeled at the summits. The antigens studied are D2 (N-CAM), D3, the mitochondrial marker MM, and the cytoplasmic marker CM. The directions of the first and second dimension electrophoreses are also shown (arrows pointing towards anode).

TABLE 1

THREE-WAY ANALYSIS OF VARIANCE

Source of Variation	Degrees of Freedom	Variance Estimate	F-Ratio Compared to Within Cells	Probability
Behavioral procedures (BP)	2	64.60	52.35	<0.01
Marker proteins (MP)	3	28.38	22.99	<0.01
Brain areas (BA)	1	2.01	1.63	n.s.
BP × MP interaction	6	10.63	8.61	<0.01
BP × BA interaction	2	0.58	0.47	n.s.
MP × BA interaction	3	3.20	2.59	n.s.
BP × MP × BA interaction	6	1.00	0.81	n.s.
Within cells	216	1.23	—	—

trap-doors were left unlocked and on trials that should not be reinforced both trap-doors were locked.

Passive control. During the whole training period the PAS rats were subjected to as little handling as possible and had no maze experience. On the day a VPD rat reached criterion all three rats of that squad were sacrificed. The timing of sacrifice was such that the VPD and YAC rats were sacrificed exactly 6 hours after they had terminated training on that day.

Sampling of Brain Areas

After being anaesthetized by chloroform the rats were killed by decapitation and the brains were dissected out according to a procedure previously described [14]. The present study differed from that previously described by only taking cortical samples and instead of the occipital area the Te2 area was taken. Immediately after a sample had been taken it was placed in a preweighed 1.5 ml conical minivial

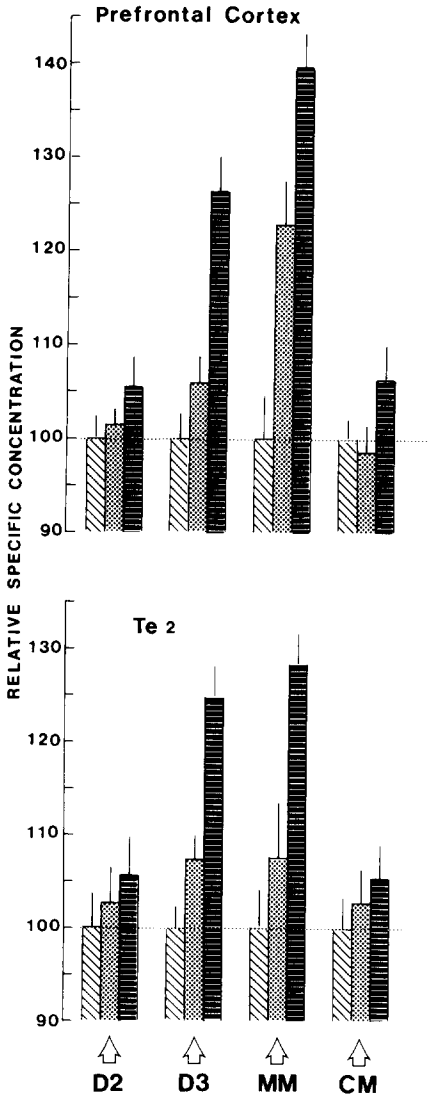

FIG. 3. The normalized relative specific concentrations of the four proteins in the passive control group (PAS, single hatching), the visual pattern discrimination group (VPD, dotted areas) and the yoked activity control group (YAC, horizontal stripes) in the two cortical areas. The values are given as mean with S.E.M.

containing 15 μl saline, the vial was weighed, frozen, and stored at −80°C.

Quantitative Protein Determination

Samples of brain tissue were homogenised by an Ultra-Turrax homogenizer (Ika-Werk, FRG) in 30 volumes of a solubilization medium containing 2.7% (w/v) Triton X-100 (Sigma Chemical Co., MO), 100 U/ml aprotinin protease inhibitor (Bayer, FRG), 73 mM Tris, 24 mM barbital, and 2 mM NaN_3 at pH 8.6 and incubated for 2 hr at 20°C [9]. The protein concentration of the homogenates was measured by a modification of the method of Lowry *et al.* [15].

The concentrations of the specific proteins in the homogenates were measured by crossed immunoelectrophoresis with anti-rat brain synaptosomal membranes (batch anti SPM-0978) as described previously [7] (Fig. 2). Besides D2 and D3, the crossed immunoelectrophoresis also enabled precipitates of the mitochondrial and cytoplasmic markers to be studied (L. Clemmesen, O. S. Jørgensen, R. Hemmingsen, D. I. Barry and T. G. Bolwig, submitted). Homogenate samples containing about 60 μg protein were applied to the gel plates. The first-dimension electrophoresis was run for 30 min at 10 V/cm, the second-dimension immunoelectrophoresis overnight at 2.5 V/cm, both at 15°C. All gels contained 73 mM Tris, 24 mM barbital, 0.6% Triton X-100, 2 mM NaN_3, and 1% agarose HSA (Litex, Denmark) at pH 8.6. The second-dimension gel further contained polyspecific anti-serum against rat brain synaptosomal membranes at 30 μl/cm^2.

The amounts of specific proteins in the samples were quantified by planimetry of the areas under the stained immunoprecipitates on the electrophoresis plates. The specific concentration was calculated as the ratio between the area and the total protein content of the sample. The relative specific concentration was obtained by further dividing this ratio by the corresponding specific concentration in a reference sample of adult rat brain tissue from the same region. Finally, the normalized relative specific concentration was calculated by multiplication with a factor for each marker protein studied in order to achieve a mean concentration of 1.00 in the passive control group (PAS).

RESULTS

The 10 VPD rats that satisfied the double criterion reached this proficiency after a median learning period of 6 days, excluding the shaping phase.

The cortical samples weighed 45±8 mg (prefrontal) and 29±6 mg (Te2) (means with standard deviations) and they contained 108 mg protein/g wet weight.

The marker protein results are shown in Fig. 3 as mean values with standard error of means for the 4 proteins analysed in both cortical areas from the 3 experimental groups. Three way analysis of variance [16] indicated significant effects of behavioral procedures ($p<0.01$) and marker protein specific concentration ($p<0.01$). Also the interaction between behavioral procedure and marker proteins was found to be significant ($p<0.01$) (Table 1). In the subsequent analysis by the Newman-Keuls method [16] (Table 2) the results from the two cortical samples were pooled because no area difference was found in the analysis of variance. The Newman-Keuls test showed the following differences in the average specific concentration of the proteins: In the YAC group the concentrations of both D3 and MM were significantly higher than the corresponding concentrations in both the VPD and PAS groups ($p<0.01$ for all 4 comparisons); the concentration of D3 was significantly higher in the VPD group when compared to the PAS group ($p<0.05$) and the concentration of MM was significantly higher in the VPD group when compared to the PAS group ($p<0.01$).

DISCUSSION

The pattern of protein concentration changes in the group taught a visual pattern discrimination (VPD) and the yoked activity control (YAC) group was identical. The observed increase of specific concentration of the D3-protein may indicate an increased synaptic concentration. Since this increase in D3 was not accompanied by significant changes in the D2-protein concentration it could be hypothesized that the possibly increased synaptic concentration may reflect growth of mature synapses rather than de novo synap-

TABLE 2

ANALYSIS OF MARKER PROTEINS (GIVEN AS MEAN WITH S.E.M.)

Behavioral Treatment	Marker Proteins: Normalised Relative Specific Concentration			
	D2	D3	MM	CM
PAS	100.0 (2.1)	100.0 (1.8)	100.0 (3.2)	100.0 (2.0)
VPD	102.1 (2.1)	106.8 (1.9)*	115.2 (4.1)†	100.7 (2.3)
YAC	105.5 (2.6)	125.7 (2.4)†‡	134.2 (2.8)†‡	105.8 (2.5)

*Significantly ($p<0.05$) higher than PAS.
†Significantly ($p<0.01$) higher than PAS.
‡Significantly ($p<0.01$) higher than VPD.

togenesis, which would be accompanied by an increased concentration of D2-protein as well. The increased concentration of the MM-protein may indicate an increased concentration of mitochondria which reflects a response to increased metabolic demand. The absence of change in the concentration of the CM-protein indicates that the observed changes are selective.

In short, the results seem to indicate an increase in synaptic mass and energy demand in the two investigated cortical structures in both the VPD and YAC animals. Whereas the pattern of changes is the same these increases are significantly greater in the YAC group than in the VPD group.

It seems unlikely that the changes found in the VPD group are directly related to the formation of the "memory trace" for the task learned by the VPD animals because changes in the same direction but of an even higher magnitude were observed in the YAC animals which had no possibility to learn the experimenter-defined task. A more likely explanation is that, like the YAC group, the VPA group was subjected to "hypothesis frustration" [14] which caused the synaptic changes and the increase in metabolic demand. The "hypothesis frustration" of the VPD animals could have occurred in the "presolution period"—the period from the beginning of specific training until the moment the hypothesis chosen for testing is in agreement with the experimenter-defined solution. Since the changes found in the YAC group are significantly higher than those seen in the VPD group the effects of "hypothesis frustration" seem to have been greater in the YAC group than in the presolution period of the VPD group.

One striking difference between the present and the previous study [14] is the direction of the marker changes in the yoked animals. In the previous experiment we observed an 8% decreased concentration of presumably synaptic markers while we presently found an increase in D3 concentration amounting to 26%. Since the task, the number of trials per day and the criterion for sacrifice have been changed between the two studies, a number of factors may account for this difference. Furthermore, since identical changes were found in both investigated cortical structures the present experiment does not tell whether the observed effects are localized to certain cortical areas or may be distributed diffusely through the cerebral cortex.

In a study with a related design, composition changes of RNA-base were determined in the prefrontal and inferotemporal cortical areas of monkeys either trained in delayed alternation or visual discrimination or run as randomly reinforced controls to one of the two tasks [6]. Changes in composition of RNA were found in both randomly reinforced groups, but in the group which was control in the delayed alternation experiment the change was found in the prefrontal but not the inferotemporal area, whereas in the randomly reinforced animals from the visual discrimination task the change was found in both areas.

Since only the relative specific concentrations of the proteins are directly relevant for the purpose of the present study, the absolute concentrations were not determined. However, in a previous study [8] the concentrations of D2 and D3 were found both to be about 2 mg/kg wet weight; the absolute concentrations of CM and MM are unknown.

Although, as indicated above, many questions remain to be answered by future experiments, the present results make it likely that "hypothesis frustration" in tasks other than delayed alternation is able to affect the synaptic mass of the cerebral cortex.

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