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Electroconvulsive Stimulations, Learning, and Protein Changes in the Rat Brain

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MOGENSEN, J., S. L. PEDERSEN AND O. S. JØRGENSEN. *Electroconvulsive stimulations, learning, and protein changes in the rat brain.* PHARMACOL BIOCHEM BEHAV 47(3) 647-657, 1994.—Two groups of rats were subjected to 17 training sessions on an operant task demanding the sequential operation of two manipulanda, while two other groups were left with no training experience. Within both the trained and passive groups one was exposed to a series of 12 electroconvulsive stimulations. The series of training and stimulation sessions were concurrent but arranged in such a way that at least 24 h always separated training and stimulation. Upon completion of the behavioural part of the experiment the concentrations of the marker proteins neural cell adhesion molecule (NCAM), D3, synaptophysin, and S100 were estimated in the prefrontal and occipital parts of the cortex, the hippocampus, and in the total forebrain. The electroconvulsively stimulated animals demonstrated severe impairment of learning. The pattern of marker protein concentrations indicated that acquisition and/or performance of the task and exposure to electroconvulsive stimulation were both accompanied by similar patterns of synaptic changes: an increased concentration of small synaptic vesicles in both the prefrontal cortex and the total forebrain and an increased synaptic remodulation in the prefrontal cortex.

Electroconvulsive stimulation (ECS) Sequential behaviour Learning Synaptic remodelling
NCAM D3 S100 Synaptophysin

ELECTROCONVULSIVE therapy (ECT) is still considered the most efficacious treatment of psychotic depression (4,12,27,44) and even to be valuable against other types of disease including psychoses of schizophrenic origin (27). However, ECT is known to exert a negative influence on cognitive functions, and amnesia is a common side effect to ECT (42). In animals, electroconvulsive stimulation (ECS) has been demonstrated to impair memory [e.g., (29,32,45)]. However, ECS-induced amnesia has been found to depend critically on both the type of material to be remembered and the timing of electroconvulsive stimulation [e.g., (29-32,40,43)].

In the present study we addressed whether a series of 12 ECSs distributed over a four-week period would influence the concurrent acquisition of a "sequential," operant task. We included studies of changes in synaptic marker protein concentrations to gain information about some of the synaptic mechanisms accompanying the task acquisition. Previously, we have used a similar approach to clarify some of the synaptic mechanisms associated with learning and problem-solving behaviour (34,35). Our studies have indicated that both successful and unsuccessful attempts to solve a number of behavioural tasks are associated with regionally localized synaptic marker changes.

With the aim of evaluating both the contributions of ECS and task acquisition together with their interactions with respect to possible synaptic modifications, we studied four groups of animals: two groups that were trained on the sequential, operant task and two "passive" groups that never received any specific training. One of the trained groups and one of the nontrained groups received a series of ECSs spaced over the period of learning, while the two remaining groups were only subjected to a sham procedure.

The three neuronal marker proteins studied were NCAM, the neural cell adhesion molecule (5,17,19,24); D3, a marker of synaptic membranes (17-19); and synaptophysin, an intrinsic membrane protein of small synaptic vesicles (26,46,47). Additionally, the glial marker protein S100, a soluble cytoplasmic protein, was studied. S100 has been found to demonstrate learning-associated concentration changes (11,14-16) and to possess neurotrophic activities (25). The ratio between NCAM and D3 can be taken as an indicator of synaptic sprouting (22,24).

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Three forebrain structures were chosen for investigation: 1) the prefrontal (anteromedial) cortex (found to be one of the "foci" for mediation of the presently studied sequential, operant task [(33), J. Mogensen & S. Holm, in preparation]), 2) the occipital cortex (chosen as a neocortical "control" area that is not believed to be specifically involved in mediation of the presently studied task), and 3) the hippocampus (frequently speculated to be critically involved in forms of learning and memory [e.g., (38)]). Also, the "residual forebrain" (all forebrain tissue not included in the three aforementioned structures) was sampled for analysis.

METHODS

Subjects

Forty experimentally naive male Wistar albino rats served as subjects. They weighed approximately 400 g at the beginning of the experiment. All animals were housed in single cages with water always available in a colony room with a 12-h light–dark cycle (on 0600, off 1800). They were fed commercial rat chow once daily (after the training of the two "training groups") and maintained on approximately 90% of their ad lib body weights. The rats were divided into four experimental groups: training on the "sequential," operant task and exposure to electroconvulsive stimulation (SEQ/E, $n = 11$); training on the "sequential," operant task and exposure to "sham"-ECS (SEQ/C, $n = 10$); exposure to ECS without any concurrent training (PAS/E, $n = 9$); and exposure to sham-ECS without any concurrent training (PAS/C, $n = 10$). The subjects were weighed before the distribution into experimental groups and the distribution ensured that the groups were balanced according to body weights.

Apparatus

The operant chambers used—the "Student Research Model" of Ralph Gerbrands Company (Arlington, MA)—were identical to those used in previous studies on prefrontal cortical mediation of sequential behaviour (33). The chamber contained a bar mounted on one wall, a rod protruding into the chamber from its ceiling, a signal lamp near the bar, and a reinforcement magazine into which 45-mg pellets were delivered accompanied by an audible click. Relevant responses were made either by pressing the bar or by displacing the rod from the vertical. The chambers were enclosed in sound-shielded boxes. Solid state equipment controlled the experiment and recorded the bar presses and rod responses.

Behavioural Procedure

On day 1 the animals of the SEQ/E and SEQ/C groups were allowed a 30-min session of habituation followed (on day 2) by a 15-min session of magazine training. The rats were then—on day 3—shaped to bar press and after shaping immediately given a 15-min session of continuous reinforcement (CRF). On days 4 and 5 the animals were exposed to one daily 15-min session of CRF training. During habituation, magazine training, shaping, and CRF training the signal lamp was constantly lit. The sequential task was introduced on day 6. In the sequential task a bar press would only be reinforced if preceded by a rod response, and only the first bar press following a rod response would be reinforced. The first bar press of a session was reinforced regardless of whether or not it had been preceded by a rod response. When a session began the signal lamp was lit and remained lit until the first bar press. Each rod response lit the lamp and each reinforced bar press turned the lamp off. Thus, the light signaled that the next bar press would be reinforced. One daily 60-min session of this task was given until the animal within a session received five or more reinforcements. Upon reaching this criterion the animal would be subjected on the next day to a 10-min session of the sequential task and 24 h later subjected to the first electroconvulsive stimulation (or sham-ECS procedure). The remaining 16 training sessions of the experiment were all of 10-min duration, and designated sessions I–XVI.

Electroconvulsive Stimulation

The ECS was given via electrodes in the earholes, using a stimulus of 50 mA for 0.9 s (a stimulus able to cause clonic/tonic convulsions). Animals from the two sham-ECS groups were subjected to a procedure similar to administration of ECS except for the absence of any electric stimulus.

Distribution of Electroconvulsive Stimulations and Training Sessions

The day of the first electroconvulsive stimulation constituted the first day of the 28-day period during which the 12 electroconvulsive stimulations and 16 last training sessions (sessions I–XVI) were distributed. Each day the animals were exposed to either an electroconvulsive stimulation or a 10-min training session. Care was taken to arrange the timing of stimulations and training sessions in such a way that the training sessions and stimulations were always separated by at least 24 h ("poststimulation pause" = "prestimulation pause" = 24 h). ECSs (or sham-ECSs) were administered on days 1, 3, 6, 8, 11, 13, 16, 18, 21, 23, 26, and 28 of the 28-day period, while training sessions were given on all other days. During training sessions animals of the PAS/E and PAS/C groups remained without handling in their home cages in the colony room.

Within 24 h after the final ECS (on the 28th day) all animals were sacrificed.

Sampling of Brain Areas

After being anaesthetized by chloroform the rats were killed by decapitation, and the brains were dissected out according to the following procedure: After the occipitoparietal bones had been removed, a coronal cut, perpendicular to the dorsal surface of the brain, was made just behind the posterior edge of the frontal bones at bregma. Such a cut passes close to the midline portion of the anterior commissure. The two blocks of the brain were further divided by a sagittal cut into four quadrants. From the two frontal blocks the prefrontal samples (the entire medial cortical area) were taken according to the procedures of our previous studies (34,35). From the posterior quadrants the occipital cortex was sampled according to the procedure used by Jørgensen and Bock (20) and Mogensen et al. (35). Then, by blunt dissection the remaining neocortex and hippocampus of the posterior blocks were deflected laterally, and the hippocampi were sampled under direct visual guidance. All forebrain tissue that had not been sampled as "prefrontal cortex," "occipical cortex," or "hippocampus" was combined into the sample "remaining forebrain." Immediately after a sample had been taken it was placed in a preweighed 0.5-ml conical minivial containing 15 μl saline, and the vial was weighted, frozen, and stored at −80°C.

Quantitative Protein Determination

The thawed tissue was homogenized by sonication in solubilization buffer comprising 4% Triton X-100, 73 mM Tris, 24 mM barbital, 2 mM NaN_3, pH 8.6, and 100 U/ml aprotinin (Novo, Denmark), to a final concentration of 3–4% w/v. The protein concentration in the solubilized homogenates was estimated by the modified Lowry method (39). After solubilization for 20 h at 4°C the amounts of NCAM and D3 protein were estimated by quantitative crossed immunoelectrophoresis using about 60–100 μg solubilized protein precipitated with a polyvalent rabbit antiserum to rat brain synaptosomal membranes (17,23). Synaptophysin (synaptin) was estimated by crossed immunoelectrophoresis against a polyvalent antiserum to rat synaptic vesicles (1,6). The tissue samples were analyzed in blocks comprising a representative from each treatment group. S100 was estimated by radial immunodiffusion of solubilized tissue samples as has been previously described (23). Planimetry was used to estimate the areas below immunoprecipitates formed in immunoelectrophoresis or the areas enclosed by the precipitate formed in immunodiffusion. Linear relationships between such areas and the protein concentration in the brain homogenate were demonstrated for all antigens studied.

Calculations

From the area measurements the specific concentrations of the antigens were calculated relative to the protein concentration in the homogenates. The values were expressed in arbitrary units either as a percentage compared to the mean of corresponding tissue from control rats (PAS/C) or as the ratio of concentrations for two protein markers, normalized to 1.00 for PAS/C control rats.

Statistics

The behavioural data were analysed by nonparametric statistical methods (41), since such a material is unlikely to be normally distributed. Similar problems were absent in the case of the results from the biochemical analysis, and consequently we subjected this material to parametric statistical tests (48), which were able to provide a more detailed analysis, including multidimensional analysis of variance (ANOVA) with measures of interactions. Because the tissue samples had been analyzed in blocks, the ANOVA included a block-related covariate factor to correct for the significant plate-to-plate variation in immunoelectrophoresis. Group differences among samples were tested for each protein marker by one-way ANOVA, followed by Newman-Keuls procedure for multiple comparisons of means (48). Studentized range standard errors of means were calculated from the within-group residual mean sum of squares in the relevant ANOVA.

RESULTS

The outcome of the behavioural parts of the experiment is shown in Fig. 1. For rats subjected to the training procedure the behavioural performance on each of the sessions I–XVI was evaluated based on three parameters: 1) the number of reinforcements obtained during the session, 2) the number of bar presses during the session, and 3) the number of rod responses during the session.

After completion of the experiment it was brought to our attention that for a 5-day period (days 20–24 of the experiment) the colony room had been managed by a different and less experienced staff. Inspection of the behavioural results

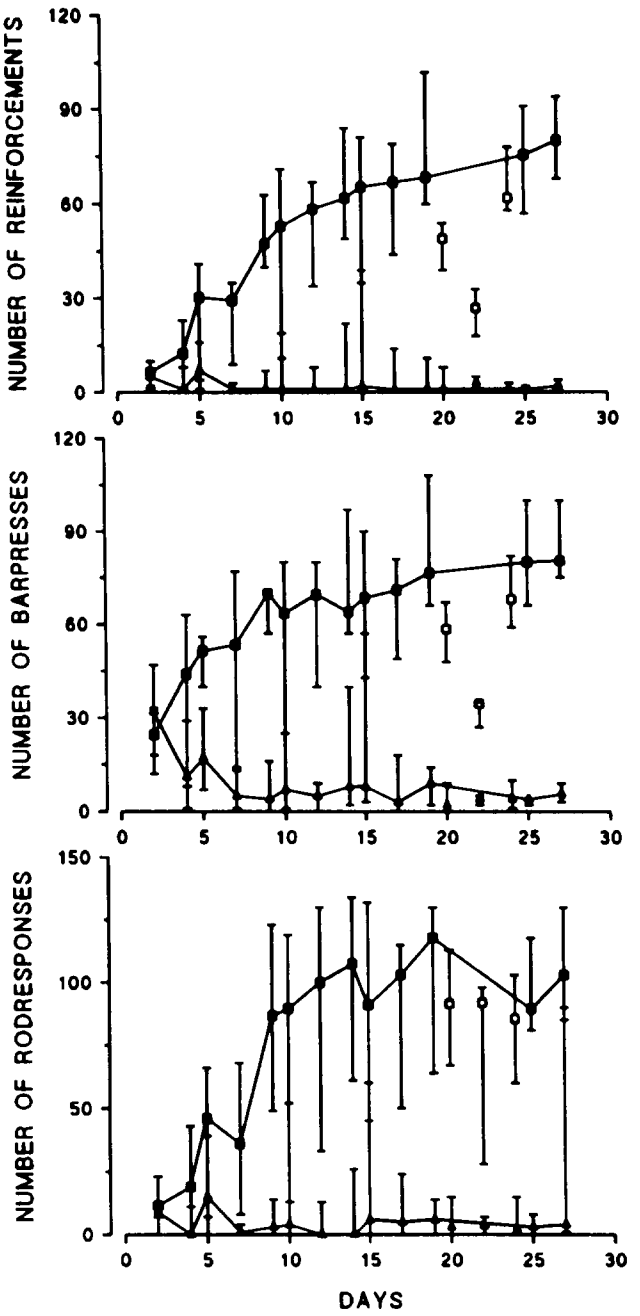

FIG. 1. Behavioural performance during the training period of the SEQ/E (▲) and SEQ/C (■) groups. Median values are given with the range between 25 and 75% fractiles. As described in the Results section nonexperimental factors interfere with the behavioural results obtained during the day 20–24 period of the experiment and consequently data from this period (sessions XII, XIII, and XIV) were excluded from statistical analysis and are here shown by open symbols.

from the three training sessions (sessions XII, XIII, and XIV) occurring during this period revealed in animals of a high task proficiency a temporary behavioural impairment (see Fig. 1) which can not be explained by any experimental factor. Consequently, it was decided that for all animals the behavioural

data from sessions XII, XIII, and XIV would be excluded from the statistical analysis.

On all sessions the behavioural results were compared across the two treatment groups (SEQ/E and SEQ/C). For these analyses the Mann-Whitney U test, two-tailed (41), was utilized. On sessions I–IV no significant group differences were found. On session V the number of bar presses of the two groups differed significantly ($p < 0.05$). On session VI no parameter revealed significant group differences. On session VII and all subsequent sessions all three parameters showed significant group differences ($p < 0.05$).

Additionally, we wanted to evaluate whether any significant—presumably learning-related—development occurred across sessions in the behavioural parameters of the impaired SEQ/E animals (as well as the control SEQ/C group). Consequently, the potential session-to-session changes in behavioural performance were analysed separately for the SEQ/E and SEQ/C animals by the Spearman rank-order correlation test (41).

The Spearman rank-order correlation test demonstrated that no significant session-to-session differences were found on any behavioural parameter in the SEQ/E animals. As expected, significant ($p < 0.001$) session-to-session differences were found on all three behavioural parameters of the SEQ/C animals.

Average wet weights of the samples were 50 mg (prefrontal cortex), 64 mg (occipital cortex), 80 mg (hippocampus), and 1125 mg (residual forebrain). By combination of the values obtained from the four sampled "brain structures" in each rat a fifth set of biochemical data was calculated which represented the "total forebrain" with 85% contribution from residual forebrain. The focus of the following analysis will consequently be on the prefrontal and occipital cortices, the hippocampus, and the forebrain in its entirety.

The marker protein results are shown in Table 1 as mean values with standard errors of means for the four proteins analysed in the four studied cerebral regions of the four experimental groups. These results are illustrated in Figs. 2–5.

Parametric two-way ANOVA (across the two dimensions [A] presence or absence of ECS and [B] presence or absence of exposure to the learning paradigm) was performed separately for each of the four studied antigens in each of the four "structures." The results of the ANOVA are shown in Table 2. In the prefrontal cortex significant effects were found on the relative specific concentrations of both NCAM (effects of ECS [$p < 0.001$] and interaction between ECS and learning [$p < 0.01$]) and synaptophysin (effects of ECS [$p < 0.05$] and interaction between ECS and learning [$p < 0.01$]). In the occipital cortex significant group differences were revealed in NCAM (effect of ECS [$p < 0.01$]), synaptophysin (effect of ECS [$p < 0.01$]), and S100 (interaction between ECS and learning [$p < 0.05$]). In the hippocampus no significant group differences were found. For the total forebrain, significant group differences appeared on the relative specific concentrations of D3 (effect of ECS [$p < 0.01$]), synaptophysin (effects of ECS [$p < 0.01$] and learning [$p < 0.001$]), and S100 (effect of learning [$p < 0.01$]).

In a subsequent analysis by the Newman-Keuls method, comparisons were made between the individual group results along those dimensions that in the ANOVA had been found to contain significant group differences. The outcome of that analysis is illustrated in Figs. 2–5. In the prefrontal cortex the relative specific concentrations of NCAM contained significant differences between the PAS/C group and all other

TABLE 1

RELATIVE SPECIFIC CONCENTRATIONS OF THE FOUR MARKER PROTEINS (IN ARBITRARY UNITS) IN THE FOUR "STRUCTURES" OF THE FOUR BEHAVIOURAL GROUPS

	MARKER PROTEINS			
Group	NCAM	D3	SYN	S100
Prefrontal cortex				
SEQ/E	106.2 ± 2.0	102.8 ± 1.3	104.7 ± 1.7	103.5 ± 3.4
SEQ/C	104.9 ± 2.0	102.6 ± 1.2	105.9 ± 1.8	97.2 ± 3.1
PAS/E	109.3 ± 2.1	102.3 ± 1.3	107.2 ± 1.4	94.9 ± 4.5
PAS/C	100.0 ± 2.7	100.0 ± 2.2	100.0 ± 1.4	100.0 ± 5.4
Occipital cortex				
SEQ/E	103.5 ± 2.6	98.2 ± 2.0	107.3 ± 3.2	105.2 ± 2.2
SEQ/C	96.7 ± 2.7	97.8 ± 2.6	99.4 ± 2.5	98.9 ± 1.3
PAS/E	101.9 ± 2.8	100.6 ± 3.1	101.7 ± 1.6	95.4 ± 3.0
PAS/C	100.0 ± 3.2	100.0 ± 3.1	100.0 ± 2.5	100.0 ± 2.9
Hippocampus				
SEQ/E	102.9 ± 2.4	104.3 ± 3.1	103.7 ± 3.3	99.5 ± 2.6
SEQ/C	101.8 ± 2.3	101.2 ± 2.9	104.4 ± 4.2	99.9 ± 2.3
PAS/E	103.9 ± 2.6	100.8 ± 2.7	98.3 ± 3.6	96.7 ± 1.2
PAS/C	100.0 ± 2.4	100.0 ± 2.7	100.0 ± 3.1	100.0 ± 1.8
Total forebrain				
SEQ/E	103.3 ± 1.7	104.6 ± 1.4	106.1 ± 1.3	106.2 ± 2.5
SEQ/C	100.3 ± 1.6	100.1 ± 1.5	104.0 ± 1.1	103.4 ± 1.7
PAS/E	98.8 ± 2.6	100.5 ± 1.6	103.5 ± 1.4	97.5 ± 1.4
PAS/C	100.0 ± 2.0	100.0 ± 1.2	100.0 ± 0.5	100.0 ± 1.6

For descriptions, see Methods. Concentrations are given as means ± SEMs.

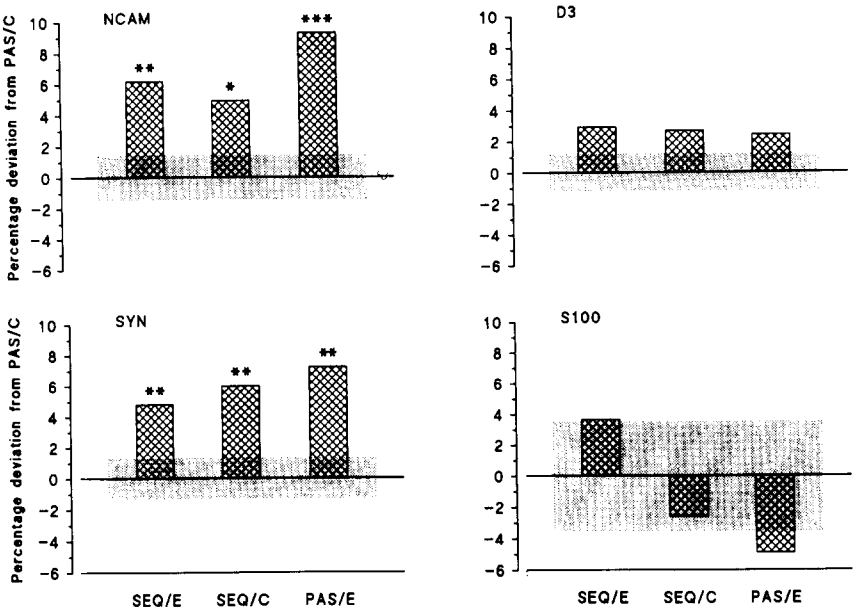

FIG. 2. Relative specific concentrations of the four marker proteins in the prefrontal cortex of the SEQ/E, SEQ/C, and PAS/E groups as percentage deviations from the relative specific concentrations of the same marker proteins in the PAS/C group (for details see the Methods section). The Studentized range standard error of means was calculated from the one-way ANOVA, corrected for significant plate-to-plate variation in immunoelectrophoresis, and is indicated by shading. Significant differences between the PAS/C group and other groups are indicated above the bars, while other significant group differences are indicated below (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

groups. The relative specific concentrations of synaptophysin in the prefrontal cortex revealed significant differences between the PAS/C group and all other groups. In the occipital cortex the concentrations of NCAM revealed a significant difference between the SEQ/E and SEQ/C groups. The concentrations of synaptophysin in the occipital cortex contained two significant group differences: The SEQ/E group differed significantly from both the PAS/C group and the SEQ/C group. The relative specific concentrations of S100 in the occipital cortex demonstrated a significant difference between the SEQ/E and PAS/E groups. The relative specific concentrations of D3 in the total forebrain revealed two group differences: The SEQ/E group differed significantly from both the PAS/C group and the SEQ/C group. For the relative specific concentrations of synaptophysin in the total forebrain, the PAS/C group differed significantly from all other groups. Three significant group differences were found in the S100 values of the total forebrain: The SEQ/E group differed significantly from both the PAS/E group and the PAS/C group. The SEQ/C group differed significantly from the PAS/E group.

To obtain estimates of potential neuronal sprouting in the four "structures" of the four groups we calculated the ratios between NCAM and D3 as well as between NCAM and synaptophysin. The parametric ANOVA (48) revealed that in the prefrontal cortex both the ratio between NCAM and D3 and the ratio between NCAM and synaptophysin contained significant group differences ($p < 0.001$ for NCAM/D3 and $p < 0.01$ for NCAM/SYN). Subsequent analysis by the Newman-Keuls method (48) demonstrated that the NCAM/D3 ratio of the prefrontal cortex contained the following significant group differences: The PAS/C group differed from the SEQ/E group ($p < 0.01$), the SEQ/C group ($p < 0.05$), and the PAS/E group ($p < 0.001$), and the PAS/E group differed from both the SEQ/E ($p < 0.01$) and SEQ/C ($p < 0.001$) groups (see Fig. 6). Similar analysis of the NCAM/SYN ratio in the prefrontal cortex demonstrated that the PAS/E group differed significantly ($p < 0.01$) from both the PAS/C and SEQ/C groups (see Fig. 7).

DISCUSSION

In the present study a clear difference could be seen between the acquisition of a sequential, operant task by animals subjected to a concurrent series of ECSs and the acquisition of the same task by those rats who instead of ECS only received a "sham" procedure. As can be seen from Fig. 1 and the statistical analyses described in the Results section, the animals subjected to the control procedure demonstrated significant session-to-session differences on all three measures of performance and clearly showed a pattern of gradual and significant improvement of their task performance. The animals subjected to electroconvulsive stimulation did not reveal such a pattern on any behavioural parameter.

While none of the three behavioural parameters showed significant differences between the two acquisition groups on session I, significant differences could be demonstrated during and at the end of training. While a clear task acquisition was apparent in the animals subjected to sham electroconvulsive procedures, the animals receiving ECS showed no sign of task acquisition.

It seems safe to conclude that at least as far as the presently

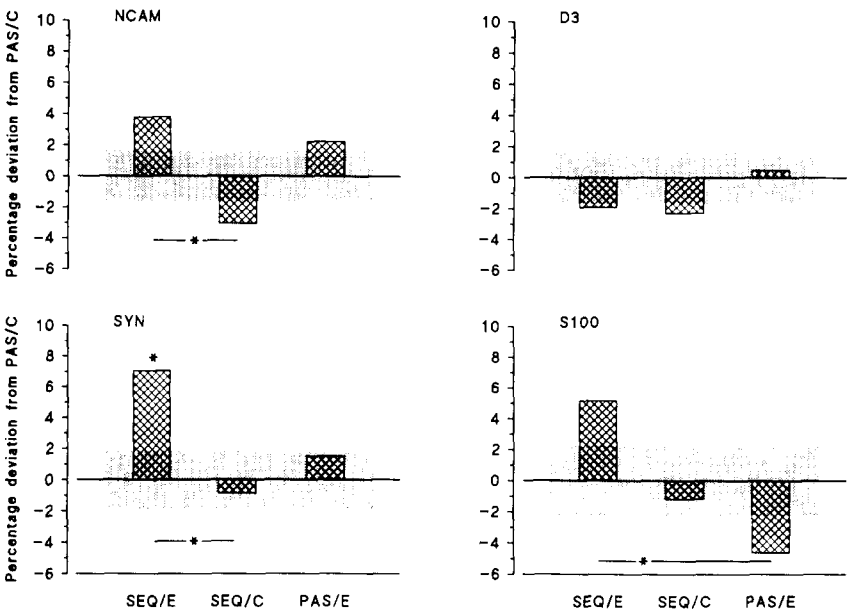

FIG. 3. Relative specific concentrations of the four marker proteins in the occipital cortices of the SEQ/E, SEQ/C, and PAS/E groups as percentage deviations from the relative specific concentrations of the same marker proteins in the PAS/C group. The Studentized range standard error of means was calculated from the one-way ANOVA, corrected for significant plate-to-plate variation in immunoelectrophoresis, and is indicated by shading. Significant differences between the PAS/C group and other groups are indicated above the bars, while other significant group differences are indicated below (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

used task is concerned the acquisition process of the rat is significantly impaired by a series of 12 ECSs—even if the stimulations are arranged in such a way that sessions of stimulation and training are always separated by at least 24 h. Future studies will be needed to clarify the generality of these findings. For instance, it could be speculated that the presently observed impairment is partly secondary to a stimulation-induced impairment in the exploratory activities. A reduced exploration would impair early phases of the acquisition process during which parts of the (directly or indirectly reinforced) activities of the animal are of an exploratory nature.

In most behavioural tasks the retrograde amnesia induced by ECS is most prominent if stimulation is administered immediately upon training sessions [e.g., (29,32,45); for exceptions see (30,31,40,43)]. The stimulation is frequently assumed to induce retrograde amnesia by interference with aspects of "consolidation" [e.g., (32)]. Since in the present study all electroconvulsive influences—retrograde or anterograde—on the task performance would have to span a period of at least 24 h, the marked behavioural impairment of stimulated animals must be the consequence of 1) stimulation-induced modification of a memory trace ("engram") that already had been consolidating for 24 h, 2) proactive effects of stimulation on neural processes essential to formation and consolidation of engrams, or 3) proactive stimulation effects on neural processes of only indirect importance to learning and memory (e.g., processes subserving exploration). If the ECS of the present experiment exerted its effects in a proactive manner it had to do so over a time span of at least 24 h. Although the exact duration of the consolidation process is unknown, the use of nootropic substances has provided results which seem to indicate that aspects of consolidation may be modified even 24 h after the learning trial (36). In the present study we focused on a number of synaptic marker proteins which seem to be influenced by ECS (2,21) and may reflect the creation and/or consolidation of the engrams (34,35).

The regional analysis of synaptic marker proteins (Tables 1 and 2 and Figs. 2–7) revealed that the relative specific concentrations of the studied marker proteins as well as the ratios between NCAM and D3 and between NCAM and synaptophysin marker proteins were regionally affected by ECS; acquisition and/or performance of the operant, sequential task; and the interaction between ECS and behaviour.

Significant effects of ECS only were found in the prefrontal cortex (significant increases in the concentrations of NCAM and synaptophysin and the NCAM/D3 as well as NCAM/SYN ratios) and the total forebrain, where the concentration of synaptophysin was significantly increased. ECS of animals subjected to the training procedure was associated with a significant increase in the occipital cortex concentration of both NCAM and synaptophysin and in a significant total forebrain increase in the concentration of the D3 protein. These results indicate that the number of small synaptic vesicles increased in both the prefrontal cortex and other forebrain regions, while the synaptic remodelling increased selectively in the prefrontal cortex (as reflected in the increased concentration of NCAM and the increased ratios between NCAM and the D3 and synaptophysin proteins). If animals were subjected both to the training procedure and to ECS, otherwise nonsignificant changes in protein concentration reached the level of significance: In the occipital cortex an increased concentration of synaptophysin indicated increased

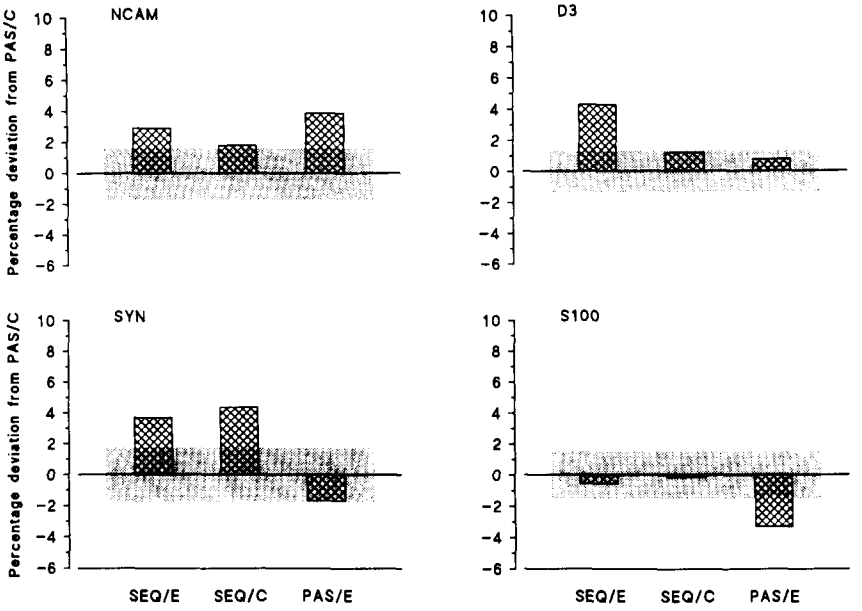

FIG. 4. Relative specific concentrations of the four marker proteins in the hippocampi of the SEQ/E, SEQ/C, and PAS/E groups as percentage deviations from the relative specific concentrations of the same marker proteins in the PAS/C group. The Studentized range standard error of means was calculated from the one-way ANOVA, corrected for significant plate-to-plate variation in immunoelectrophoresis, and is indicated by shading. Significant differences between the PAS/C group and other groups are indicated above the bars, while other significant group differences are indicated below (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

number of small synaptic vesicles and in the total forebrain a significantly increased concentration of the D3 protein, combined with a similar but not significantly increased NCAM, indicated an increase in the total synaptic mass.

Analysis of synaptic marker proteins in regions of rat brains has previously been utilized in two studies addressing the question of the neural consequences of ECS (2,21). The present findings are in general agreement with the results of these studies. The major discrepancies between the outcomes of previous and present experiments are found in the outcome of analysis of occipital cortical samples. The animals serving as subjects in the two first studies of the effects of ECS on the concentration of synaptic marker proteins (2,21) were young rats (35 days of age at the beginning of the experiment). The subjects of the present study were approximately 80 days old at the beginning of the experiment and were consequently substantially older than the animals previously studied. The factor of age may be of importance when the results obtained in previous and present studies are compared (e.g., the previous findings of synaptic changes in the occipital cortex have been interpreted as reflecting a delay in the development of this cortical area). It might be speculated that the more pronounced discrepancy between past and present results of the occipital cortex analysis primarily reflects the age difference between the subjects of the studies in question. ECS-induced increases in synaptic remodelling have recently been demonstrated in the piriform cortex of rats (J. Kragh, T. G. Bolwig, D. P. D. Woldbye, and O. S. Jørgensen, in preparation).

Significant effects of exposure to the operant, sequential task (as expressed in a significant difference between the SEQ/C and PAS/C groups) were found in the prefrontal cortex (a significant increase in concentration of NCAM, synaptophysin, and the ratio between NCAM and D3) and in the total forebrain (significantly increased concentration of synaptophysin). These results indicate that the process of acquiring and/or performing the operant, sequential task was associated with at least two forms of synaptic change: In both the prefrontal cortex and other forebrain structures the numbers of small synaptic vesicles increased significantly (as reflected in significantly increased concentrations of synaptophysin), and in the prefrontal cortex the synaptic remodelling increased significantly as indicated by the significantly increased concentration of NCAM and the significantly increased ratio between NCAM and D3.

In the animals that received ECS, exposure to the behavioural task was associated with increased concentrations of the S100 protein in both the occipital cortex and in the total forebrain. Although such a finding is hard to interpret, it should be noted that the S100 protein seems to possess neurotrophic activities (25), and hippocampal as well as cortical concentrations of this protein have been found to change in animals learning a behavioural task (11,14–16). Furthermore, intraventricular application of antibodies against S100 seems to abolish at least some types of learning (15).

It can be concluded that aspects of the behavioural procedure were associated with increased synaptic remodelling in the prefrontal cortex and increased number of small synaptic vesicles in the prefrontal cortex as well as other forebrain structures. It should be remembered, however, that in the present design the comparison is between synaptic processes in the "learning" group and a control group which (although food-deprived on a schedule similar to that of the experimen-

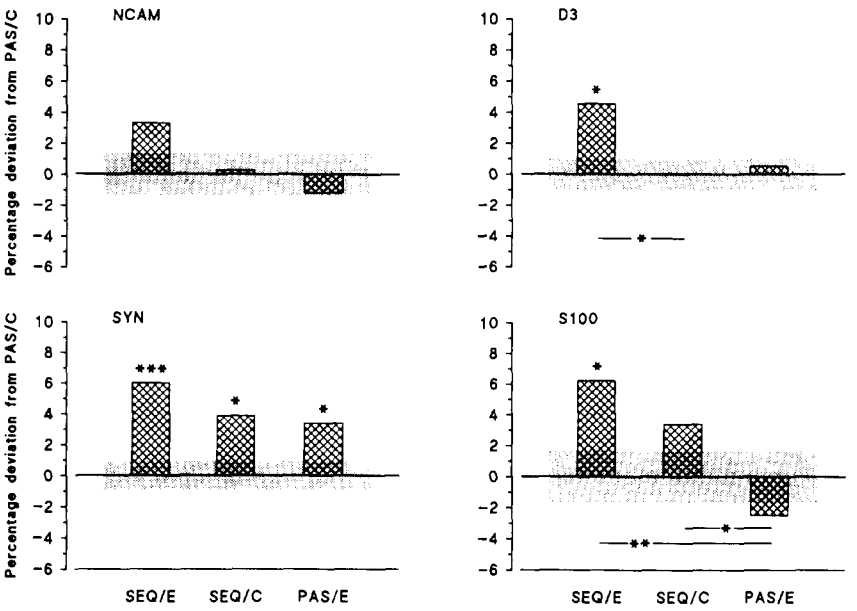

FIG. 5. Relative specific concentrations of the four marker proteins in the "total forebrain" of the SEQ/E, SEQ/C, and PAS/E groups as percentage deviations from the relative specific concentrations of the same marker proteins in the PAS/C group. The Studentized range standard error of means was calculated from the one-way ANOVA, corrected for significant plate-to-plate variation in immunoelectrophoresis, and is indicated by shading. Significant differences between the PAS/C group and other groups are indicated above the bars, while other significant group differences are indicated below (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

TABLE 2

SUMMARY OF THE OUTCOME OF TWO-WAY ANOVA AND THE CORRESPONDING STUDENTIZED RANGE STANDARD ERROR OF MEANS (SEM) FOR THE FOUR MARKER PROTEINS IN FOUR "STRUCTURES"

	ECS	SEQ	ECS/SEQ Interaction	SEM
Prefrontal cortex				
NCAM	$p < 0.001$	NS	$p < 0.01$	1.4
D3	NS	NS	NS	1.2
SYN	$p < 0.05$	NS	$p < 0.01$	1.3
S100	NS	NS	NS	3.6
Occipital cortex				
NCAM	$p < 0.01$	NS	NS	1.6
D3	NS	NS	NS	1.6
SYN	$p < 0.01$	NS	NS	1.8
S100	NS	NS	$p < 0.05$	2.2
Hippocampus				
NCAM	NS	NS	NS	1.6
D3	NS	NS	NS	1.3
SYN	NS	NS	NS	1.7
S100	NS	NS	NS	1.5
Total forebrain				
NCAM	NS	NS	NS	1.4
D3	$p < 0.01$	NS	NS	1.1
SYN	$p < 0.01$	$p < 0.001$	NS	1.0
S100	NS	$p < 0.01$	NS	1.8

tal group) was never exposed to any behavioural training or testing. Consequently, the observed synaptic modifications may not only reflect the actual process of task acquisition, but could alternatively be related to the level of (test-associated) activity or handling. Future experiments (including additional control groups) are needed to clarify the exact nature of the behaviour-associated synaptic changes.

In a study of the synaptic modifications accompanying the acquisition of a "spatial delayed alternation" task (35), it was found that in the four brain regions studied (the prefrontal and occipital cortex and two subdivisions of the neostriatum) no quantitative, synaptic modification could be seen in animals that for a number of sessions had been performing the delayed alternation procedure at a high level of proficiency. In the same study, animals subjected to an unsolvable spatial task were found to have a significantly reduced concentration of synaptic marker proteins (including the presently studied NCAM, D3, and synaptophysin proteins) in the prefrontal cortex. It was hypothesized that while no quantitative, synaptic modifications remained in the studied structures in the final training phases, a reduction of the synaptic mass of the prefrontal cortex accompanied the behavioural attempts at finding a solution to a "spatial" task. The term "hypothesis frustration" was suggested for the repetitive (and unsuccessful) attempts identifying the spatial "hypothesis" (28) that would yield a consistent success in task performance.

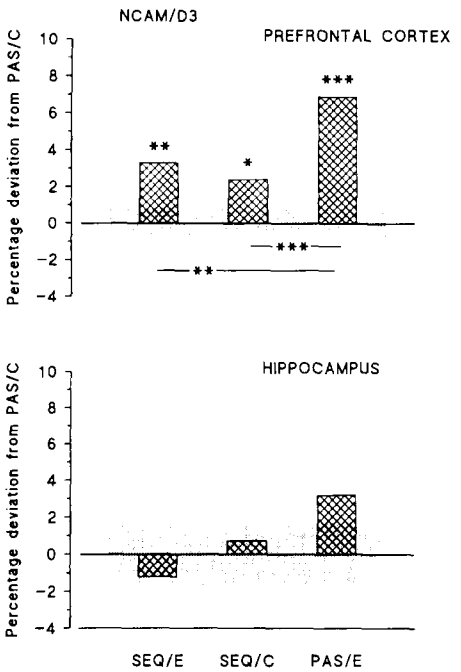

FIG. 6. The ratio between relative specific concentrations of NCAM and D3 in the prefrontal cortices and hippocampi of the SEQ/E, SEQ/C, and PAS/E groups as percentage deviations from the ratios of the relative specific concentrations of the same marker proteins in the PAS/C group. The Studentized range standard error of means was calculated from the one-way ANOVA, corrected for significant plate-to-plate variation in immunoelectrophoresis, and is indicated by shading. Significant differences between the PAS/C group and other groups are indicated above the bars, while other significant group differences are indicated below ($*p < 0.05$; $**p < 0.01$; $***p < 0.001$).

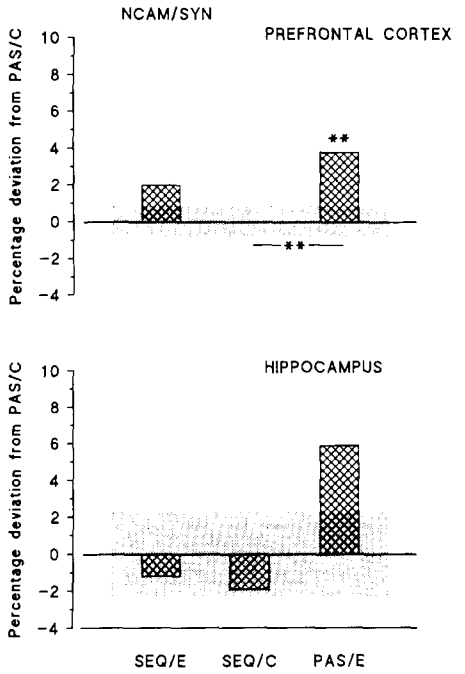

FIG. 7. The ratio between relative specific concentrations of NCAM and synaptophysin in the prefrontal cortices and hippocampi of the SEQ/E, SEQ/C, and PAS/E groups as percentage deviations from the ratios of the relative specific concentrations of the same marker proteins in the PAS/C group. The Studentized range standard error of means was calculated from the one-way ANOVA, corrected for significant plate-to-plate variation in immunoelectrophoresis, and is indicated by shading. Significant differences between the PAS/C group and other groups are indicated above the bars, while other significant group differences are indicated below ($*p < 0.05$; $**p < 0.01$; $***p < 0.001$).

The second study addressing the correlations between problem solving behaviour and synaptic modifications (34) focused on NCAM, D3, a mitochondrial marker protein (MM), and a cytoplasmatic marker protein (CM) in the prefrontal and "inferotemporal" cortex of rats. The "learning" group of that experiment was subjected to training in a visual, pattern-discrimination task administered in a "Y-maze." These animals were sacrificed at the point in time when their error score indicated that they switched from application of inadequate to adequate "hypotheses." The second experimental group was exposed to an unsolvable task administered in an apparatus identical to the one in which the "learning" group was trained—an experimental setup allowing the animals to apply visual as well as spatial "hypotheses." In both the studied cortical areas of both experimental groups significantly increased concentrations of the D3 and MM proteins were found. However, the animals subjected to an unsolvable task exhibited significantly greater concentration increases than the "learning" group. The results seem to indicate that in both cortical regions of both groups the synaptic mass increased by growth of mature synapses (as reflected by increased concentration of D3 without an accompanying increase in NCAM). Additionally, the increased concentration of the MM protein indicated an increased energy demand in the studied cortical regions. It was speculated that while the changes in the "inferotemporal" cortex might reflect the neural counterparts of

selection and/or evaluation of visual "hypotheses," the effects on the prefrontal cortex reflected a similar process related to spatial "hypotheses."

In spite of the already mentioned restrictions on the conclusions that can be drawn from the present experiment, comparisons to previous studies may at least partly clarify the presently demonstrated phenomenon. The four experimental groups of the first two studies of this research line (34,35) were subjected to extensive handling, "behavioural activation," and motivational processes rather similar to the "nonspecific factors" affecting the "learning" group of the present experiment. In all four experimental groups of previous experiments analyses included studies of the NCAM and D3 proteins in the prefrontal cortex. Still, in no instance did we find a pattern of change indicating increased synaptic remodelling in this cortical area. In the two experimental groups in which the analysis included measurement of the prefrontal cortical concentration of synaptophysin the outcome was that the synaptophysin concentration either remained unchanged or was significantly reduced as part of a general reduction in synaptic mass. From these results it may tentatively be concluded that the present finding of an increased number of small synaptic vesicles and increased synaptic remodulation in the prefrontal cortex is unlikely to be a reflection of such "nonspecific" task factors as motivation, activity, or handling. Rather, the prefrontal synaptic modifications (and potentially even the increased concentration of small synaptic vesicles in other forebrain structures) may be more specifically related to the acquisition of the presently applied sequential, operant task.

It should be noted that among the presently studied structures task-related increases in synaptic remodulation were only found in the prefrontal cortex, and increased concentration of small synaptic vesicles only in the prefrontal cortex and total forebrain. As already mentioned, lesions of the rat's prefrontal cortex significantly impair the retention of the presently applied sequential, operant task [(33); J. Mogensen and S. Holm, in preparation].

Our first three studies of synaptic changes associated with learning and problem solving behaviour [(34,35) and the present study) have focused on three different behavioural tasks and on different points in the learning process. Consequently, it is presently not possible to conclude whether the differences in the synaptic modifications demonstrated in the three studies reflect task-specific synaptic processes or different aspects of a unitary process subserving problem solving behaviour and information storage in the brain. Ongoing studies focus on these problems by performing a longitudinal analysis of synaptic modifications during early as well as later phases of the acquisition of a single behavioural task.

While most models of the neural processes subserving information storage in the brain emphasize modification of existing synapses [e.g., (7,13,37)], other models [e.g., (8–10)] include the formation of new synapses—thereby being more in agreement with the present results. Most of the cellular methods (including electronmicroscopy) utilized in studies of the neural substrate of memory encounter substantial difficulties when faced with the task of establishing whether or not behaviour-correlated changes in neural structures include the formation of new synapses. Consequently, most studies supporting the notion of learning-associated synaptogenesis include only rather indirect demonstrations of sprouting [for reviews see (8–10)].

Modifications in the concentration of synaptic vesicles may participate in the mediation of the learning process both as a component in more generalized changes in the synaptic mass [e.g., (35)] or as a more specific process [e.g., (3) and present data].

In the present study both ECS and the process of acquisition and/or performance of the operant task were in the prefrontal cortex accompanied by increases in synaptic remodelling and the concentration of small synaptic vesicles (the latter even occurring in other forebrain structures). Since the synaptic modifications accompanying both stimulation and task acquisition—within the categories offered by the present study—appear to be similar and appearing within the same structures, it is tempting to speculate that at least one of the ways ECS reflects itself in behavioural impairment is via modifications of synaptic processes essential to the establishment and/or "consolidation" of the engram.

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