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Behavioral and Regional Neurochemical Sequelae of Hippocampal Destruction in the Rat[1]

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BÄR, P. R., W. H. GISPEN AND R. L. ISAACSON. *Behavioral and regional neurochemical sequelae of hippocampal destruction in the rat.* PHARMAC. BIOCHEM. BEHAV. **14**(3) 305–312, 1981.—In an attempt to determine the nature of changes found in selected remaining brain regions following hippocampal damage, endogenous *in vitro* protein phosphorylation was measured in membranes obtained from n. accumbens, n. caudatus, septal area, hypothalamus and frontal neocortex in rats with bilateral hippocampal destruction, animals with damage limited to postero-lateral neocortex, and in sham operated subjects. Measurements were made in separate groups of animals 8 and 28 days after surgery. The membrane protein profiles measured by SDS polyacrylamide slab gel electrophoresis from tissues obtained from all groups were remarkably similar as were the phosphoprotein profiles. The most prominent bands for phosphoprotein activity had estimated molecular weights of 43K, 48K, 50K, and 82K dalton. Specific changes were found after the two forms of brain damage and at the two postoperative times at which the animals were sacrificed. Eight days after surgery an enhanced endogenous phosphorylation was found in the caudate and accumbens samples in the hippocampectomized group, whereas no differences were seen in these areas between cortically lesioned and sham operated rats. Twenty eight days after surgery, both brain lesioned groups displayed an enhanced endogenous phosphorylation of membrane proteins compared to sham operates in these regions. In addition, changes were also found in the neocortical samples. These results indicate that there are neurochemical changes occurring over time after surgery. The changes in membrane phosphoproteins may be related to altered synaptic efficacy. Novelty-induced and water immersion-induced excessive grooming were also studied in these animals. Animals with hippocampal destruction tended to groom less than other animals at both postoperative periods but were not different from other animals when tested for excessive grooming after water immersion.

Membrane phosphorylation Hippocampus Neocortex Caudate N. Accumbens
Changes after brain damage Excessive grooming

IT is likely that the behavioral effects found after brain damage can be best understood in terms of the progression of secondary changes in remaining tissues initiated by the primary lesion [1, 23, 38]. The hippocampus is a useful structure for experimental studies of changes after lesions because the effects of its destruction have been so intensively studied (see [21,33]). Recently, the progressive behavioral changes occurring after hippocampal damage have been investigated [24, 25, 27, 28, 29]. However, determinations of physiological alterations related to these behavioral changes have been relatively ignored experimentally. In such studies it is desirable to determine the alterations in neuronal activity that are occurring in various regions throughout the brain, ones that may be several synapses removed from the site of damage. One such study undertaken in this laboratory evaluated changes in ^{3}H 2-deoxy-D-glucose uptake in selected brain regions after hippocampal damage [35]. While some interesting results were obtained, this method is limited to

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evaluating regional changes occurring in the period between the administration of the labeled glucose analog and sacrifice, a period in which the behavioral alterations may not be manifest. Furthermore, if unusual behaviors are being exhibited during this period, presumably ones induced by the lesion, then it would be impossible to distinguish uptake differences induced by the lesion from those secondary to the unusual behaviors. Therefore, we undertook to determine the effect of hippocampal and neocortical destruction on the endogenous phosphorylation of membrane proteins. This process does not depend on the detection of perturbations in neuronal activity occurring at the time at which the biochemical measurements are made.

Similar determinations have been applied to various brain regions after the administration of ACTH, cyclic nucleotides, putative neurotransmitters, and opiates [18, 44, 46]. The technique involves the dissection of selected brain regions, the preparation of membrane fractions, and the exposure of the resulting preparations to $|\gamma^{-32}P|$ATP. The presence of protein kinase(s), the substrate proteins, and phosphoprotein phosphatases [36] results in the complex endogenous phosphorylation of membrane proteins. Membrane phosphoproteins have been associated with the efficacy of synaptic transmission [18] and the stimulation of well-defined neural pathways leads to changes in synaptic protein phosphorylation in *post hoc, in vitro* assays [1,5].

In the studies to be reported selected samples of brains were dissected from the brains of animals with bilateral destruction of the hippocampus, bilateral destruction of the neocortex overlying the hippocampus, or sham operated rats. The surgery was performed as previously described [22]. The regions selected were based on regions anatomically associated with the hippocampus and ones implicated in changes after hippocampal damage by pharmacologic tests [23]. These included the nucleus accumbens, caudate nucleus, the hypothalamus, the septal area, and dorsal frontal neocortex. Because of the known behavioral changes that occur at different times after hippocampal damage, independent tissue samples were processed for the biochemical evaluation of protein phosphorylation capabilities at 8 and 28 days after surgery.

Incidental to the major thrust represented by the biochemical studies, we also tested animals for the occurrence of novelty-induced and water immersion-induced excessive grooming. While these experiments were conducted as adjuncts to the other work, we felt they could provide further information about the behavioral effects of hippocampal lesions. Excessive grooming, whether induced by intracerebroventricular administration of ACTH fragments [6] or by novel circumstances [32,34] has been shown to be reduced by bilateral hippocampal lesions. We felt that it might be possible to relate the regional pattern of membrane protein phosphorylation changes found after hippocampal damage to those found after the administration of behaviorally effective ACTH fragments.

METHOD

Animals and Surgery

Sixty male Wistar rats, weighing approximately 150 g at the time of surgery, obtained from TNO, Zeist, The Netherlands, were divided into three experimental groups. One group (HPX) received bilateral aspiration-induced lesions of the hippocampus and overlying neocortex. A second group (C) had only the neocortex overlying the hippocampus aspirated. The surgical procedures have been described [22]. For the hippocampal lesion group, the lesions remove the dorsal and middle regions of the hippocampus virtually completely and most of the ventral portions of the structure, as well. About 85% of the hippocampus is removed. In both the hippocampal and neocortical lesion groups, the neocortex overlying the middle and dorsal portions of the hippocampus is destroyed. A third group (S) of animals received sham operations in which the scalp was opened and bilateral burr holes were made in the skull in the area used to create the initial bone defect in the other groups. The burr holes did not interrupt the underlying dura. After this procedure, the animals had the scalp incisions sutured. No antibiotics were given to the animals. All animals were housed in individual cages in a colony room with a 12:12 light-dark cycle (lights on at 8:00 a.m.).

Experimental Procedure

The first experiment used data from 10 HPX, 10 C, and 10 S rats that were tested in the excessive grooming test 7 days after surgery and were sacrificed the following day. The second experiment also involved 10 HPX, 10 C, and 10 S rats. Seven and 23 days after surgery they were tested in the excessive grooming test. On the 24th day they were immersed in water and immediately thereafter were observed for water immersion-induced grooming. On the 28th day after surgery they were sacrificed. After sacrifice, the endogenous phosphorylation of brain membrane proteins were measured.

Brain Dissection and Tissue Fractionation

The rats were sacrificed by decapitation and their brains were quickly removed and placed on ice. Dissection of the various brain regions was performed according to Gispen *et al.* [13]. The nucleus accumbens was dissected from the brains of all subjects. In addition, from half of the subjects of each group the nucleus caudatus and hypothalamus were dissected, whereas from the other half the septum and frontal cortex were taken. The order in which the rats were sacrificed and what brain parts were selected for study from a particular brain were determined randomly. The tissues were weighed and individually homogenized in ice cold 0.32 M sucrose (1:10 w/v) using small homogenization devices according to Potter and Elvehjem (clearance 0.15 mm, 8 up and down strokes, 700 rpm). All further treatment was performed at 0–4°C. The homogenates were spun for 10 min at 1,000 g to remove cell debris and nuclei. The supernates were saved and spun for 20 min at 10,000 g in a cooled centrifuge. The supernates were aspirated and the crude mitochondrial pellets, containing synaptosomes and cell membranes, as well, were resuspended in buffer (50 nM Na-acetate, 10 nM Mg-acetate, pH 6.5). After standing for 5 min, the osmotically shocked membranes were collected by spinning for an additional period of 20 min at 10,000 g and resuspended in the same buffer (±1.5 mg/ml). Duplicate aliquots of 5 μl were then taken for protein determination according to the Lowry method [30]. The samples were further diluted with the buffer to a final protein concentration of 1 mg/ml.

Endogenous Phosphorylation Assay

The procedure was in essence that described by Zwiers *et al.* [45]. The assay was carried out in small plastic vials in a final volume of 25 μl. The incubation mixture consisted of

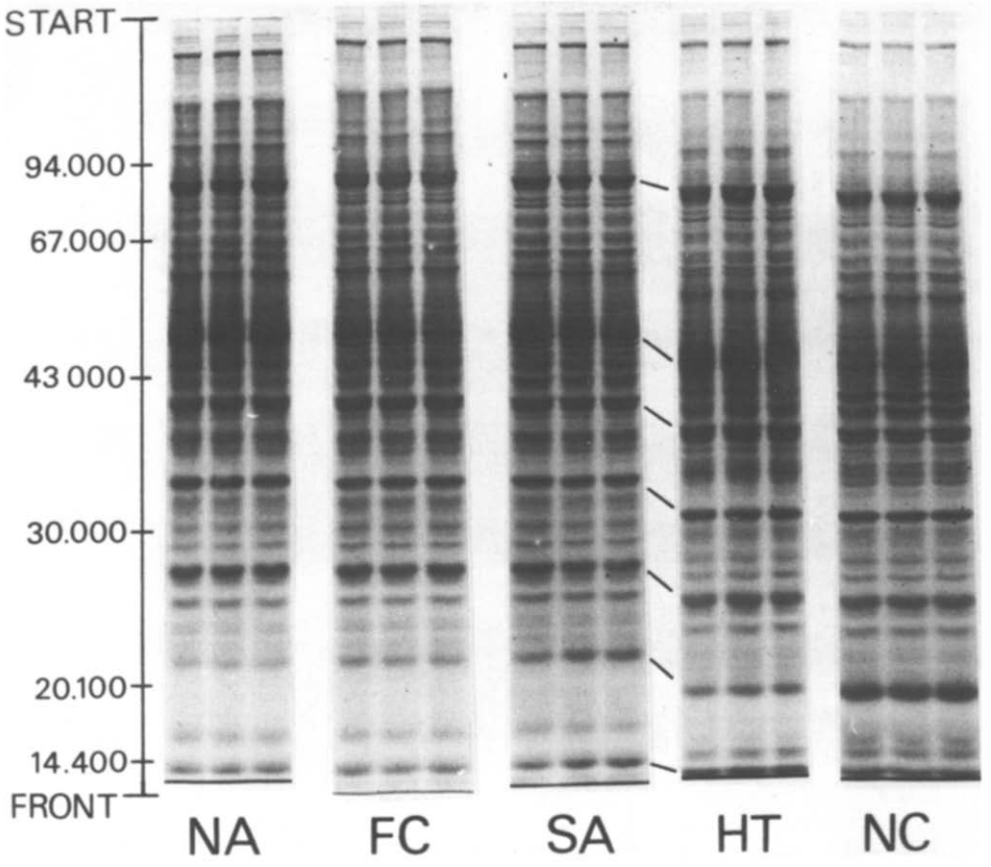

FIG. 1. Protein pattern of different brain regions after polyacrylamide gel electrophoresis. Protein mixture is applied at the top ("START"), the position of the tracking dye is indicated with "FRONT." Positions of marker proteins are also indicated. Abbreviations: NA: Nucleus Accumbens; NC: Nucleus Caudatus; SA: Septal Area; HT: Hypothalamus; FC: Frontal Cortex.

7.5 μM ATP; 2–4 μCi $[\gamma\text{-}^{32}P]$ATP; 15 μg protein; 50 nM Na-acetate; 10 nM Mg-acetate (pH 6.5). From each membrane sample, triplicates were tested. After preincubation of the protein fraction for 5 min at 30°C, the phosphorylation reaction was started by adding the ATP. Endogenous phosphorylation of proteins under these conditions reaches a maximum value within seconds [43]. After 15 sec the reaction was stopped by adding a "stop mix" resulting in final concentrations of 62.5 nM Tris-HCl, pH 6.5; 2% SDS; 10% glycerol; 0.001% Bromophenol Blue, and 5% 2-mercaptoethanol. Aliquots of 30 μl were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) on slab gels, as previously described [45]. The gels were stained for proteins using Fast Green, destained and dried, and subjected to autoradiography using Kodak Royal X-Omat X-ray film as described previously [43,45]. The radioactivity of individual bands was measured by photometric scanning of the autoradiographs using a Zeiss PMQ11 spectrophotometer and a linear gel scanner (slit width 0.02 mm, wave length 50 mm). The correlation between absorbence and incorporated ^{32}P-phosphate was checked by cutting 1 mm slices of the gel and counting the ^{32}P in these slices. Exposure times were chosen to allow a linear relationship between radioactivity and grain density per band [46], and phosphorylated standard protein samples were run to account for inter-gel differences. After scanning the autoradiographs, peak heights above background were computed as relative measure of radioactivity in the protein bands [40]. Differences across groups per protein band were established using a one-way Analysis of Variance followed by Student's t-test. The molecular weights of the various protein bands were established using a set of standard proteins obtained from Pharmacia, Uppsala [1].

Grooming Test

Rats were placed individually in glass observation cages (24×12.5×14 cm) in a dimly lit soundproof observation room after transport from the colony room. Observation was carried out through a one-way mirror. Eight to ten rats were observed simultaneously and the observations were carried out between 9:00–12:00 a.m.

Preliminary experiments had established an inter-observer correlation >0.90. The placement of the rats in their observation cages marked the beginning of the behavioral session. Grooming behavior was recorded starting 5 min after the animals were placed in the observation boxes using a 15th sec time sampling procedure previously described [14]. In short, every 15th sec the observers recorded whether or not the animal displayed any element of grooming behavior.

For the study of excessive grooming after water immersion, the rats were forced to swim in a large container with water (22°C) for three min. They then were taken out, briefly

wrapped in a towel to remove excess water, and placed in the observation cages for observation as described above.

RESULTS

Regional Endogenous Membrane Protein Phosphorylation

The protein patterns obtained after SDS-polyacrylamide gel electrophoretic separation of membrane proteins and staining with Fast Green are shown in Fig. 1. The samples, also used in the endogenous phosphorylation assay, were obtained from the following brain regions: nucleus accumbens (NA), nucleus caudatus (NC), septal area (SA), hypothalamus (HT), and dorsal frontal neocortex (FC). No obvious differences exist between the protein patterns of these areas (the apparent differences seen in the low MW protein region are due to gel batch differences; as always protein markers and intergel reference protein were present, and corrections could be made for these differences).

Figure 2 shows three typical autoradiographs and their respective scans. The pattern in 2A is representative for frontal neocortex, nucleus accumbens, nucleus caudatus, and the septal area. The most prominent phosphorylated bands are indicated with arrows; their estimated molecular weights were found to be 43K, 48K, 50K, and 82K. The MW 50K band was not, or only slightly, phosphorylated in the hypothalamus, irrespective of treatment or time after operation (Fig. 2B). In the nucleus caudatus the 48K band was not or very little phosphorylated four weeks after the operation (Fig. 2C). Apart from these differences, the pattern of phosphorylated proteins showed a great similarity across the regions studied. Among the four bands that were most constantly present, the MW 48K band has been studied by Zwiers *et al.* [46,47], the band (or bands) at 82K probably is identical to Protein 1 from Ueda *et al.* [41]. The 50K band has been found to respond to electrical stimulation of rat hippocampal slices [2] and also is sensitive to treatment of hippocampal slices with methionine-enkephalin [1].

Effects of Hippocampal Destruction on Endogenous Membrane Protein Phosphorylation

At the time of sacrifice the animals and their brains were evaluated for general health, appropriateness of the lesion, and lack of infection. Tissue was only subjected to analysis if it came from healthy subjects without signs of infection and with appropriate lesions. The final *n* of each group is shown in the figures.

Eight days after surgery. No significant changes were seen in the frontal cortex and hypothalamus when endogenous phosphorylation was studied eight days after the operation. However, in the membrane fractions obtained from n. accumbens and the caudate marked stimulations of endogenous phosphorylation were found: (1) the 50K band incorporated significantly more ^{32}P in both areas, and (2) the 48K band incorporated more ^{32}P in the caudate (Fig. 3A/B, left panels). In all regions there were no differences between sham-operated animals and cortex lesioned animals. In an independent pilot study we could not detect differences in regional endogenous phosphorylation or membrane proteins between brain regions obtained from animals one and four weeks after sham surgery (data not shown).

Four weeks after surgery. In tissue obtained from animals sacrificed four weeks after surgery several significant effects in both endogenous phosphorylation were found in the dorso-frontal neocortex: Both the 48K and 82K bands showed an increase in phosphate incorporation in the HPX

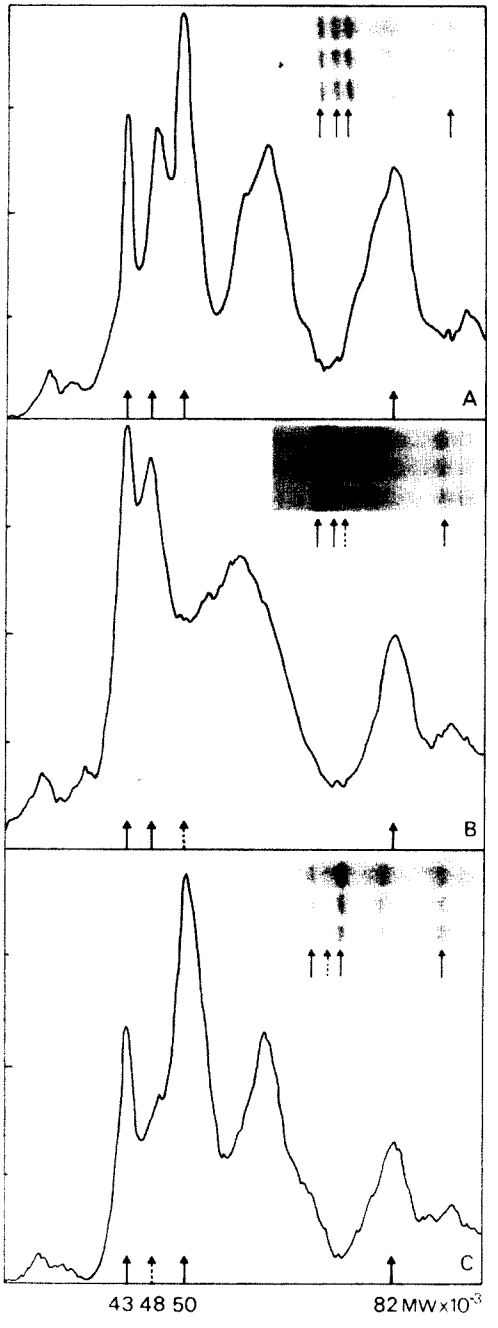

FIG. 2. After protein separation and autoradiography scans are made to obtain a phosphoprotein profile and to quantitate the differences in ^{32}P-phosphate incorporation into different protein bands. The inserts show the parts of the autoradiograph from which the scans were made; front and start are not shown. On the X-axis the molecular weights of the most prominent bands are indicated, the four arrows corresponding with the four arrows in the inserts. Dotted arrows indicate bands into which no or only very little label has been incorporated. On the Y-axis the absorbence at 500 nm (slit width 0.02 mm) is given in arbitrary units. (A) Phosphoprotein profile of most areas studied: n. accumbens, n. caudatus, septal area and frontal cortex. (B) Phosphoprotein profile of the hypothalamus: no label was incorporated into the 50K band (dotted arrow) both after 8 and 28 days. (C) Phosphoprotein profile of the n. caudatus after 28 days: only very little incorporation into the 48K (dotted arrows) band was measured.

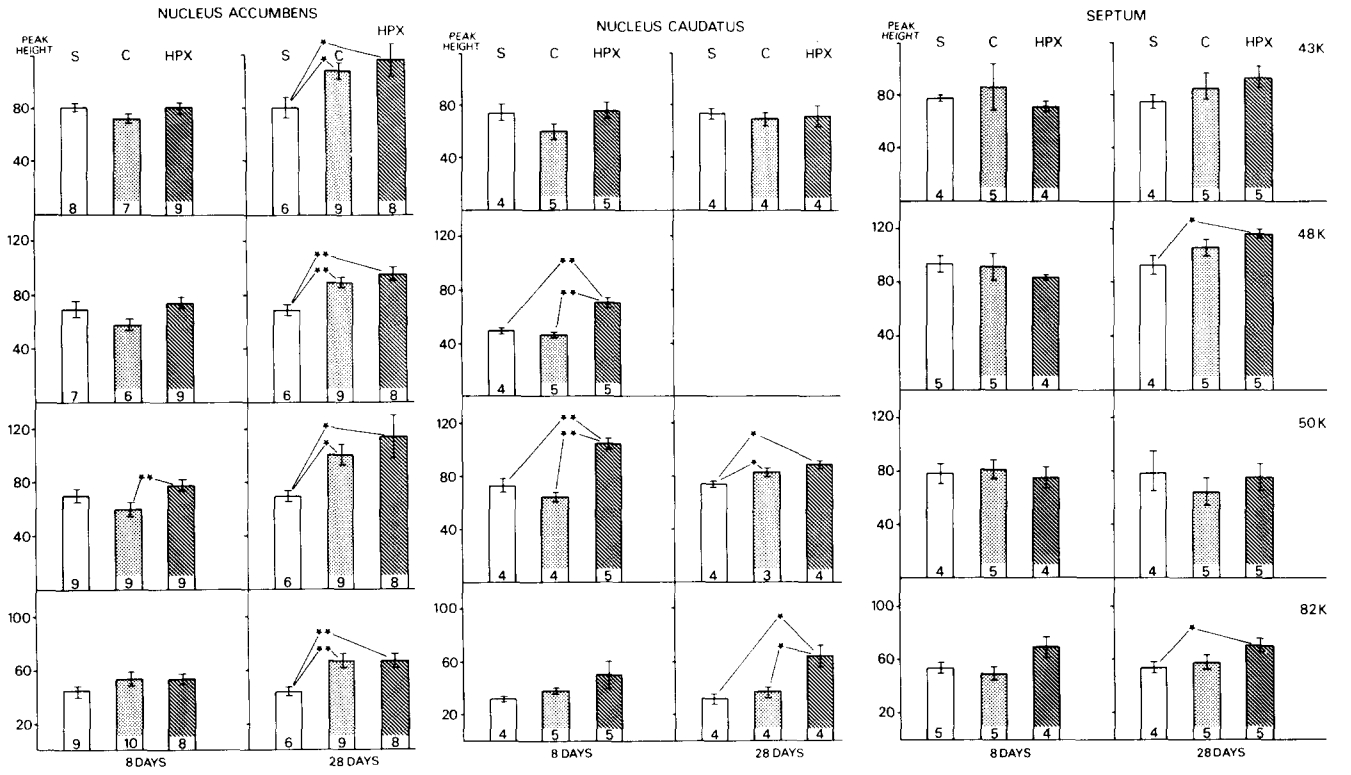

FIG. 3. Effects of hippocampal aspiration (HPX), cortex aspiration (C), or sham operation (S) on endogenous membrane protein phosphorylation. Incorporation of ^{32}P-phosphate into protein bands is given as peak height above background, obtained through scans from autoradiograms as described in section "Endogenous phosphorylation assay." The X-axis gives the peak height in mm above background. Blank columns: sham controls (S), dotted columns: cortex lesioned groups (C) and striped columns: hippocampal lesioned animals (H). In each column is given the number of animals of which data were obtained, the SEM is given also in each column by means of a bar. Significant differences between groups are indicated by means of one asterisk ($p<0.05$, Student t-test, two-tailed, see text), or two asterisks ($p<0.01$). Left panels present data obtained 8 days after the operation, right panels 28 days after the operation.

group; no differences were found between sham operated (S) and cortex lesioned (C) animals (Fig. 4).

All four protein bands in n. accumbens indicated enhanced ^{32}P incorporation in both the brain lesioned groups (Fig. 3A, right panels). In the caudate the 82K band evidenced an enhanced ^{32}P-phosphate incorporation after hippocampal destruction. The 50K band showed small but significant changes in the C and HPX groups while, as mentioned earlier, changes in the phosphorylation of the 48K band were below detection level (Fig. 3B, right panels). In the septal area the endogenous phosphorylation of the 82K band of tissue from HPX animals was greater than that of S and C groups. The 48K band in this area incorporated more phosphate in the tissue from the HPX animals (Fig. 3C, right panels). No selective ^{32}P-phosphate changes were found in the hypothalamus. Importantly, protein patterns as obtained by Fast Green staining of gels did not show any group differences at both 8 or 28 days after surgery in any tissue samples.

Novelty-Induced Grooming

The means and standard errors of the means (SEM) of the animals tested 7 days after surgery are presented in Fig. 5A. In order to eliminate error due to possible differences in score distributions, the data were analyzed with non-parametric procedures. The grooming scores have been divided into two parts: the first 10 min and the next 50 min. Kruskal-Wallis Analysis of Variance indicated that there were significant lesion effects for both grooming intervals (for 1st 10 min, $p<0.01$: for next 50 min, $p<0.05$). For the first 10 min period subsequent Mann-Whitney U tests indicated that all groups differed from each other. For the last 50 min period, the same trend can be seen in the data but the difference between the hippocampally lesioned and neocortically damaged groups was not significant.

The means and standard errors of the means of the grooming scores for the three groups of animals tested 23 days after surgery are given in Fig. 5B. Again, the grooming scores have been divided into two portions, the first 10 min and the subsequent 50 min. Kruskall-Wallis Analysis of Variance indicated a significant group effect only for scores in the first 10 min period ($p<0.01$). The animals with hippocampal lesions groomed less in this interval than did either those with neocortical damage ($p<0.01$) or sham operated controls ($p<0.01$), as evaluated by Mann-Whitney U tests. There was no difference between the cortically lesioned and sham operated animals.

On the following day when the animals were tested after water immersion, there were no differences in grooming among the groups, although there was an increase in grooming scores for all groups (Fig. 5C). Significant increases were

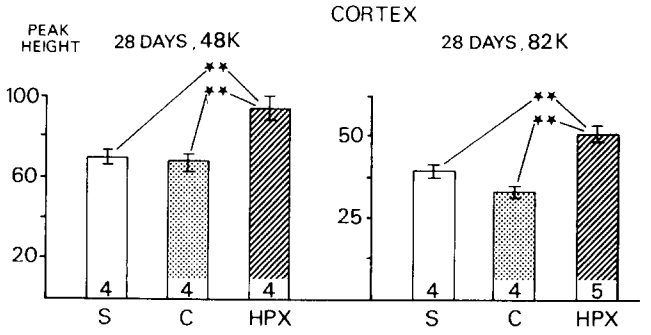

FIG. 4. Effect of treatment on endogenous membrane protein phosphorylation in frontal cortex membrane preparations 28 days after operation. Treatment consisted of hippocampal aspiration (HPX), cortical aspiration (C), or sham operation (S) as described in section *Animals and Surgery*.

shown for each group of animals in both grooming scores ($p<0.01$).

Evaluation of Brain Damage

Because of the fact that brains of the lesioned animals were used for the biochemical assays, it was not possible to process them for histological evaluation. Nevertheless, it appears that the lesions were comparable to those used in other studies of the effects of hippocampal destruction on behavior. All the surgery was performed by one of the investigators (RLI) who has considerable experience with such aspiration-induced lesions. At the time of sacrifice the general destruction of the hippocampus and neocortex was evaluated by all of the investigators. The tissue from animals with lesions that encroached on the thalamus or were in any way inappropriate was not used.

DISCUSSION

The *post hoc* endogenous membrane protein phosphorylation experiments carried out in this study are not subject to easy interpretation. Differences in the endogenous phosphorylation of a given protein band, between brain regions, or across treatment groups for a particular region, may be the result of differential effects of the activity of either membrane-bound protein kinases or protein phosphatases, or both. Furthermore, the availabilities of substrate protein and the phosphate donor (ATP) will contribute to the overall resultant activity seen in the *post hoc* assay [36]. Since there were no differences in protein profiles across treatment groups for a given region, it is unlikely that the effects we found after hippocampal destruction are due to differences in the amount of phosphorylatable substrate protein. The short incubation time and the rapid exhaustion of the donor ATP under the presently applied conditions [43] rule out an effect produced by differences in the availability of ATP. With the methods used, the activity of the protein kinases present is preferentially measured [9, 36, 43, 46]. However, it is impossible to distinguish between an enhanced activity of the protein kinase as measured in the *post hoc* assay or an enhanced activity *in vivo* of the corresponding phosphoprotein phosphatase. The latter would result in a relatively dephosphorylated state of the substrate protein *in vivo*. This would provide the protein kinase with more phosphorylatable sites on the protein under optimal kinase conditions *in vitro*. The

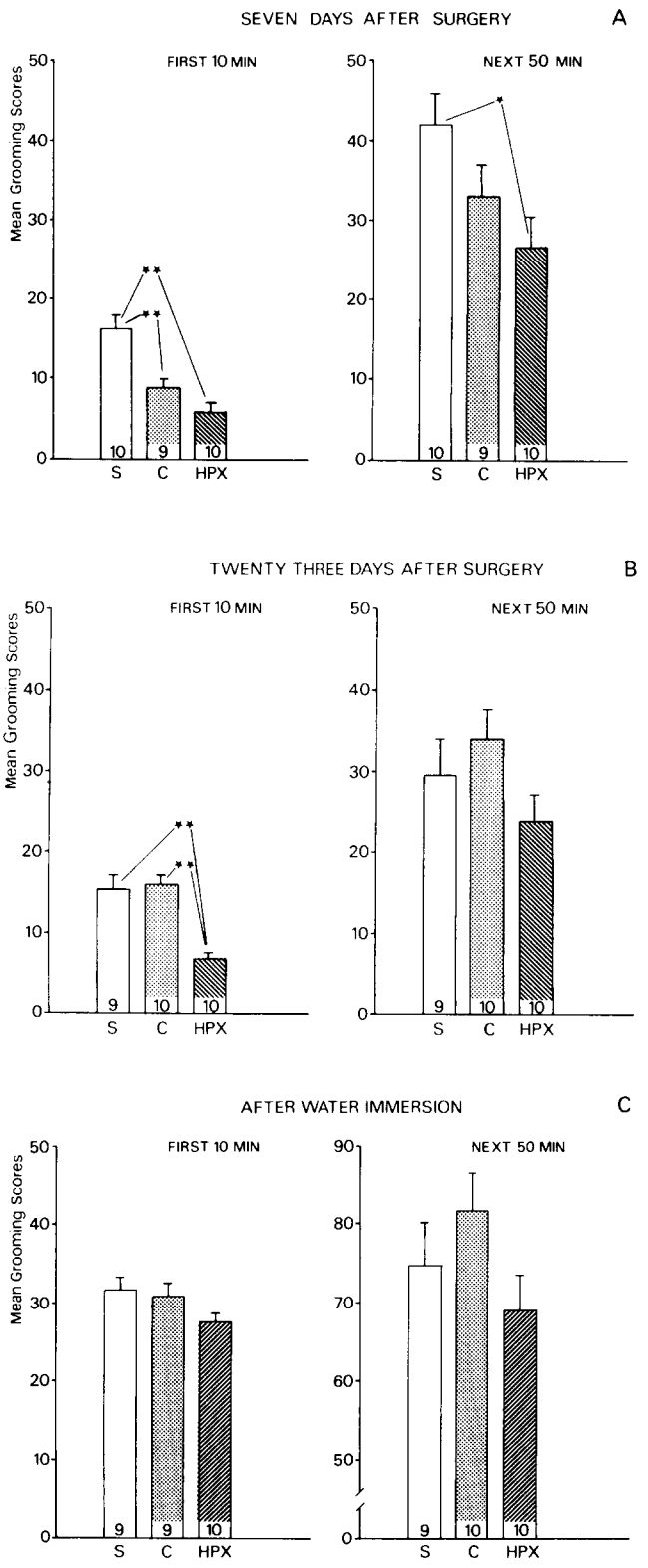

FIG. 5. Total grooming scores for first 10 min (left) and subsequent 50 min (right) of rats at 7 (panel A) and 23 (panel B) days after surgery and after water immersion (panel C). HPX: hippocampectomized, C: cortical lesions, S: sham operation. Bars represent mean ± SEM (number of rats per group indicated in bars). *$p<0.05$, **$p<0.01$ (Student t-test, two tailed).

significance of this latter interpretation is underscored by recent experiments [12] in which the phosphorylation of protein I (in this report 82K) in brain slices in relation to experimental manipulation of the slice was studied. The studies of Berman *et al.* [3,4] on the differences between brain membrane phosphoprotein profiles obtained under *in vivo* and *in vitro* conditions warrant caution in this respect. Because we can only speculate about the mechanisms underlying the presently reported changes in endogenous phosphorylation of membrane proteins as a result of brain lesions, we only refer to changes in phosphorylation.

Using a similar approach, other groups of investigators have reported changes in brain membrane protein phosphorylation related to brain function. Various behavioral experiences of mice and rats have been reported to affect *post hoc* endogenous protein phosphorylation [11, 20, 37]. Furthermore, electrical stimulation of intact synaptic pathways in rat hippocampal slices induced specific changes in *post hoc* membrane protein phosphorylation dependent on the presence of calcium and the nature of the electric stimulation [2,5]. In the latter study [5] the phosphorylation of the 50K band was specifically affected, and in a related study on the effect of enkephalins and opiates on hippocampal membrane protein phosphorylation, specific effects on the 50K band were reported [1].

From the studies on the cAMP-mediated effects on nerve cell excitability and membrane protein phosphorylation it was inferred that the degree of phosphorylation of certain synaptic proteins would underlie short-term changes in synaptic connectivity in certain neuronal pathways [17,18]. In addition, the requirement for calcium for transmitter exocytosis [10,26] and phosphorylation of non-cAMP dependent synaptic proteins [8, 19, 31, 39] underscore the potential importance of synaptic phosphoproteins to neurotransmission.

If the membrane phosphorylation changes we have found are related to the efficacy of synaptic transmission, then we have evidence that the effects of hippocampal damage result in selectively altered transmission in the caudate 8 days after damage. This effect would not be anticipated on the basis of known, direct synaptic connections. By 28 days after surgery, this caudate effect remains but a similar phosphorylation change is now found in animals with only neocortical damage. At this longer postoperative time both forms of brain damage produce presumed alterations in synaptic transmission responsiveness in the accumbens, a large effect found in all measured protein bands. However, specific effects of the hippocampal damage are found in the dorsal frontal neocortex and in the 82K band of the caudate tissue samples.

These results indicate that the membrane phosphorylation changes are not limited to regions connected to the hippocampal formation on a monosynaptic manner and, on the other hand, that monosynaptic anatomical connections do not insure the production of the membrane alterations. The results lead to new ideas concerning the nature of interactions among neuronal systems and the course of changes occurring after damage.

The failure of the 48K protein band in the caudate to show detectable amounts of phosphorylation 28 days after surgery cannot be explained. Possibly a longer exposure time to the radioactivity might have produced a visible reaction, but all of the autoradiograms had the same exposure times and reactions were found in the other samples. Therefore, we must conclude that for unknown reasons this band was less phosphorylated in the caudate relative to the other preparations. Further research with caudate samples from lesioned or sham operated animals at different postoperative times will help determine the generality of this result.

The mechanisms by which hippocampal destruction reduces novelty-induced excessive grooming are not patently obvious from the present data. This type of lesion is one of the few central nervous system lesions that affect either novelty- or ACTH-induced excessive grooming (see [15]). Previously it had been shown that local pharmacologic systems related to dopaminergic transmission in either n. accumbens or n. cuadatus could interfere with excessive grooming [7, 16, 42], but the large phosphorylation changes in all studied bands we obtained in n. accumbens do not appear related to the decrease in excessive grooming since it was found in both types of lesioned animals. However, Cools *et al.* [7] have proposed that the expression of excessive grooming depends on the balance of activity in dopaminergic activities in systems primarily related to the caudate and accumbens nuclei rather than to specific levels of activity in either. It is conceivable that 28 days after surgery the animals with only neocortical lesions have established a new "balance" in membrane responsiveness (changes in both accumbens and caudate) and the balance is not restored in animals with hippocampal lesions possibly associated with the change found in the 82K band. If so, the decrease in excessive grooming found one week after the lesion may be due to different types of changes in the caudate. This speculation must be specifically examined in the future, although in support of this idea Reinstein [34] found that the intra-accumbens injection of a dopamine agonist restored excessive grooming in hippocampally lesioned animals differentially at various postoperative periods.

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