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Microencephaly reduces the phosphorylation of the PKC substrate B-50/GAP43 in rat cortex and hippocampus

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The administration of the antimitotic agent methylazoxymethanol (MAM) to rats at day 15 of gestation results in a consistent loss of intrinsic neurons primarily in cortex and hippocampus. These animals when adult, show a cognitive impairment, if tested in specific behavioural tasks. B-50/GAP43 is a neuronal phosphoprotein, specific substrate for protein kinase C (PKC) and involved in the development and plasticity of synaptic connections. Since B-50/GAP43 has been implicated in functional modulation of synapses and in the molecular mechanism underlying cognitive processes, we studied the phosphorylation of B-50 in cortex and hippocampus of control and MAM-treated rats. Here we report that B-50 in MAM-treated rats shows a marked reduction in the phosphate incorporation in the areas affected by the prenatal treatment. In situ hybridization studies demonstrate that the mRNA levels for B-50 are not altered in MAM-treated rats and that the relative amount of the protein, as revealed by Western blot analysis, is also not affected in microencephalic rats. These results suggest that microencephalic animals might represent a useful experimental model to study biochemical correlates of cognitive impairment and synaptic plasticity.

INTRODUCTION

Methylazoxymethanol acetate (MAM) is a strong alkylating agent which kills actively dividing cells. Its administration at day 15 of gestation to pregnant rats produces a dose-dependent microencephaly in the offspring. The most affected regions of the brain are the cerebral cortex and the hippocampal formation, while other telencephalic regions, such as striatum, are less severely affected[6]. Other CNS regions are affected only at doses higher than 25 mg/kg[46].

This specificity is due to the fact that MAM is degraded within 12 h after its administration, hence only neurons actively dividing within this time are killed[22]. In rats, gestational day 15 corresponds to the birth of neuroblasts which will migrate to form the intrinsic neuronal populations in cortex, hippocampus and partly also in striatum[5,21,23].

Since noradrenergic neurons, whose cell bodies are located in the locus coeruleus, are not affected by the treatment, this results in a hyperinnervation of noradrenergic afferents to the cortex and hippocampus. This profound neuronal disorganization results in behavioural changes affecting rather specifically cognitive performances. Learning is impaired in several maze tests and deficits in passive avoidance tests, a paradigm for memory, have been reported[6,19,37].

Much evidence has accumulated recently that phosphorylation processes play a key role in synaptic plasticity. Protein kinase C (PKC) is one of the kinases which has been implicated in the mechanisms underlying long-term changes in plasticity in several models of learning and memory, including classical conditioning of the rabbit nictitating membrane/eyelid response[7,35], long-term potentiation (LTP) in hippocampal slices[2,20,27,29] and passive avoidance training in 1-day-old chicks[3].

The best characterized protein-specific substrate for PKC is a neuron-specific protein described independently by different laboratories and therefore termed in different ways: B-50 (refs. 48,49), GAP43 (ref. 42), F1 (ref. 1) and neuromodulin (ref. 10). The identity between these proteins is based upon biochemical and immunological criteria and by identity of DNA sequences[8,40].

B-50/GAP43 is localized in the inner part of synaptic plasma membranes[17,41,43] and seems to have a role in phospholipid metabolism[11,45], in growth cones during

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development and in axonal regeneration[40] and undergoes a change in its phosphorylation state during long-term potentiation in adult rat hippocampus[12,28]. Recently, it has been unequivocally proven that B-50 is an essential component in neurotransmitter release, at least in those brain regions where its expression is conserved in adulthood[14,15].

All these data indicate that B-50 plays an important role in synaptic function and suggest that its phosphorylation could be causally related to synaptic plasticity associated with learning and memory processes[40].

Therefore microencephalic animals could represent an interesting model for studying biochemical correlates of cognitive deficits and synaptic plasticity. We have studied the PKC-dependent phosphorylation of B-50 in this animal model and we have investigated whether the observed change in the phosphate incorporation in B-50 in MAM-treated rats is due to an alteration of the transcription or translation processes of this protein by means of immunostaining studies and in situ hybridization technique or to a reduction in the PKC activity.

MATERIALS AND METHODS

Animal treatment

Sprague–Dawley pregnant rats were obtained from Charles Rivers (Calco, Italy) and housed in separate cages. On gestational day 15 dams received a single intraperitoneal injection of either 25 mg/kg MAM acetate (Sigma, St. Louis) diluted in sterile saline or of diluent alone. The litters were born on day 22 or 23 of gestation. At 2 months of age rats were killed by decapitation, brains were rapidly removed and cortex and hippocampus dissected according to the method of Glowinski and Iversen[18]. Brain areas from both control and MAM-treated rats were rapidly weighed and a decrease of about 50% in weight of cortex and hippocampus was observed in MAM-rats. All the experiments described were performed comparing exactly the same amount of proteins for the preparations obtained from control and MAM-treated rats.

Phosphorylation assay

Total synaptosomal plasma membranes (SPM) were prepared from cortex of individual control and microencephalic rats according to Kristjansson et al.[25]. Endogenous phosphorylation was performed in conditions known to activate endogenous PKC[25].

Briefly, membranes were resuspended in a cold buffer containing 10 mM Tris–HCl (pH 7.4), 10 mM $MgCl_2$ and 1 mM $CaCl_2$. Protein concentration was determined by the method of Bradford[9] using bovine serum albumine as a standard. Aliquots containing 10 μg of SPM were preincubated 5 min at 30 °C in a final volume of 25 μl and phosphorylated in the presence of [γ-^{32}P]ATP (7.5 μM/2 μCi/tube) (Amersham, 30 Ci/mmol). Incubation was carried out for 15 s; the reaction was terminated in a stopping solution containing: 3% sodium dodecyl-sulfate (SDS), 5% 2-mercaptoethanol, 10% glycerol and 0.002% Bromophenol blue in 0.12 M Tris–HCl, pH 6.8 (final concentrations).

In some experiments PKC (0.12 μg/10 μg SPM proteins, purified according to Kikkawa et al.[24]) was added to the incubation media both of naive SPMs or to heat-inactivated SPMs (100 °C, 2 min).

Separation of proteins by one- and two-dimensional electrophoresis

Samples containing 10 μg of proteins were subjected to one-dimensional SDS–polyacrylamide gel electrophoresis (PAGE) using 11% acrylamide and 0.3% bis-acrylamide in the resolving gel. The resulting gels were stained with Coomassie blue, destained and autoradiographed. In some experiments purified B-50 was utilized as standard. Densitometric analysis was carried out with a Bio-Rad Scanning Densitometer Model 1650 Transmittance/Reflectance (Transmittance mode).

For two-dimensional electrophoretic analysis, the protein phosphorylation reaction was stopped by immersion of the sample in liquid nitrogen. Isoelectrofocussing was done according to the procedure described by O'Farrell[34] with slight modifications (pH gradient 4–9 and 4% acrylamide). The tube gels were then used for conventional SDS–PAGE and proteins were separated according to the molecular mass (resolving gel: 11% acrylamide–0.3% bis-acrylamide). The resultant gels were stained, destained and autoradiographed.

In situ hybridization

Animals were killed by decapitation at 2 months of age. The brains were immediately removed and frozen on dry ice. Brain sections (10 μm) were cut on a cryostat, thaw-mounted on gelatin-coated microscope slides and kept at −80 °C until processed for in situ hybridization histochemistry.

Brains from 3 different control and MAM-treated animals were sectioned, based on their rostro-caudal orientation, at 3 different levels equivalent to the Paxinos and Watson[36] rat coronal sections which are 10.20 mm, 6.20 mm and 3.20 mm from the interaural line, respectively. Two sections for each level and for each animal were collected on subbed slides. One set of sections for each brain level from the different control and MAM-treated animals was processed together in the same in situ hybridization experiment and exposed simultaneously for the same time.

The probe used was a synthetic oligonucleotide designed to be complementary to bases 22–63 (amino acid 8–21) of B-50 sequence[31]. The probe was synthesized and kindly provided by Dr. M. Soria from Farmitalia-Carlo Erba (Milano, Italy). The probe was radiolabeled at the 3′ end with a [^{35}S]dATP as previously described by Lewis et al.[26] to a specific activity of 10^9 cpm/μg using the following reaction mixture (25 μl final volume): 2.5 μl 10 × Cobalt buffer (International Biotechnologies Inc.), 0.4 μM probe, 4 μM [^{35}S]dATP (NEN Du Pont, 1300 Ci/mmol), 10 mM DTT and 20 U terminal deoxynucleotidyl transferase (International Biotechnologies Inc.). Incubation was carried out at 37 °C for 90 min. The labeled probe was separated on NENSORB Nucleic Acid purification cartridge according to the manifacturers' specifications.

Tissue sections were post-fixed for 5 min in freshly prepared 4% paraformaldehyde in 0.1 M phosphate-buffered saline and then transferred in 2 × SSC solution (1 × SSC = 0.15 M sodium chloride, 0.015 M sodium citrate). Each section was overlayered with 300 μl of hybridization buffer which contained 50% formamide, 4 × SSC, 1 × Denhardt's solution, 0.5 mg/ml salmon sperm DNA, 0.25 mg/ml yeast tRNA, 10% dextran sulfate, 10 mM DTT and 1 ng of ^{35}S-labeled probe. The sections were incubated at 37 °C overnight and then washed at room temperature for 2 h in 1 × SSC buffer containing 15 mM 2-mercaptoethanol and 1% sodium thiosulfate, and 2 more h in 0.5 × SSC. Washed tissue sections were dried and exposed to an X-ray film for a period of time ranging from 5 to 10 days. Subsequent densitometric analysis of the X-ray film were performed using a VIPER image analysis system (GESOTEC, F.R.G.) equipped with a Hitachi CCTV camera. The determination of the optical density in the different brain regions was carried out according to the previously established method[12,44]. The Amersham tritium standard was used for calibration.

Western blotting

Affinity-purified anti-B-50 IgG (prepared from serum 8613, dilution 1:4000) prepared according to the method of Oestreicher et al.[33] was used to analyze one-dimensional separation of homogenate proteins by Western blotting, performed according to the method described by Schrama et al.[38].

Fig. 1. Electrophoretic analysis of a SPM preparation obtained from cortex of control and MAM-treated rats. An endogenous phosphorylation assay in conditions known to activate PKC was performed. The autoradiograph relative to the SDS–PAGE electrophoresis shows a major phosphorylated protein band with an apparent molecular weight of 47 kDa. In the two-dimensional electrophoretic analysis the arrow shows a protein with apparent mol. wt. of 47 kDa and isoelectric point 4.5

RESULTS

Endogenous phosphorylation of B-50 in normal and MAM-treated rats

Synaptosomal plasma membranes (SPM) preparations obtained from cortex of control and MAM-treated animals were subjected to endogenous phosphorylation in the presence of [γ-^{32}P]ATP as radiolabeled phosphate donor in conditions known to activate PKC.

Figure 1 shows the autoradiographs of one- and two-dimensional electrophoretic analysis of phosphorylated SPM proteins. The arrows indicate a protein with an apparent mol. wt. of 47 kDa and an isoelectric point of 4.5, in accordance with results previously described for B-50 (ref. 43). Immunoblotting studies (see below) further confirm the identity of the 47 kDa protein as B-50. In vitro addition of H-7, a relatively selective inhibitor of PKC[13], reduces the incorporation of ^{32}P-phosphate into B-50 (data not shown), further demonstrating that this phosphoprotein is a substrate for PKC as previously described[4].

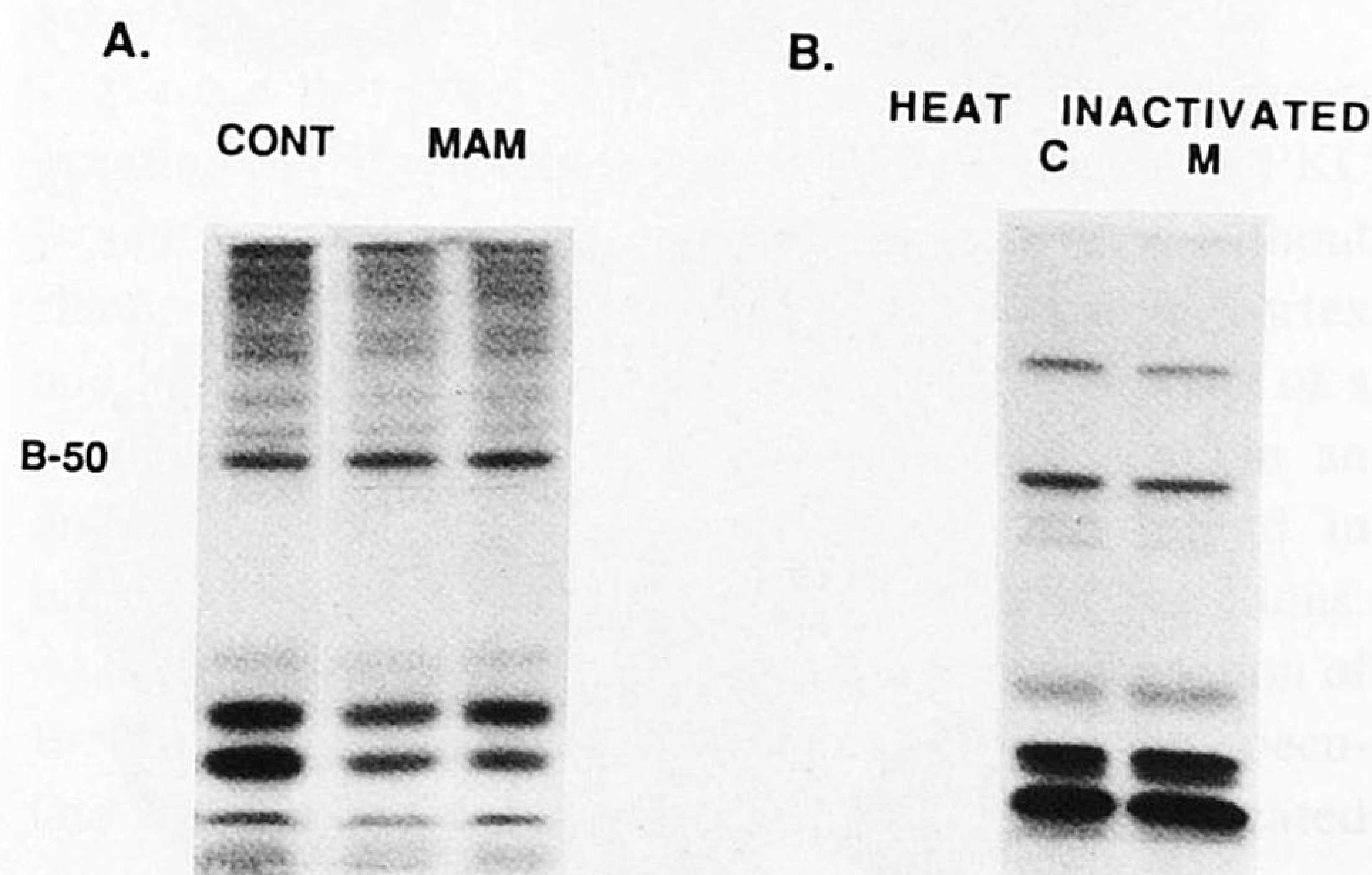


Fig. 2. Autoradiographs of the electrophoretic analysis of a SPM preparation obtained from cortex of control and two MAM-treated rats. Exogenous PKC was added to the incubation media either before (A) or after (B) heat inactivation.

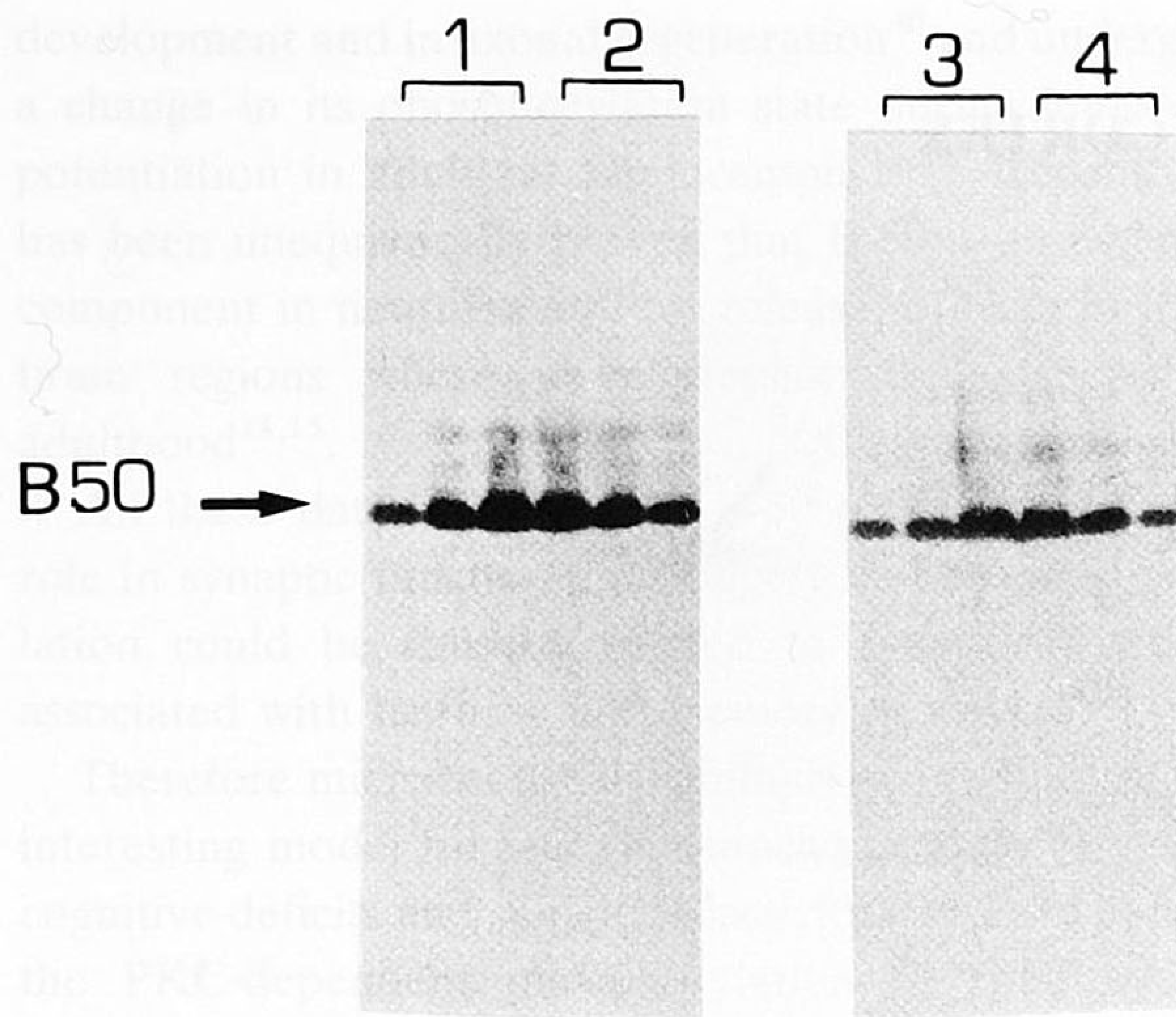


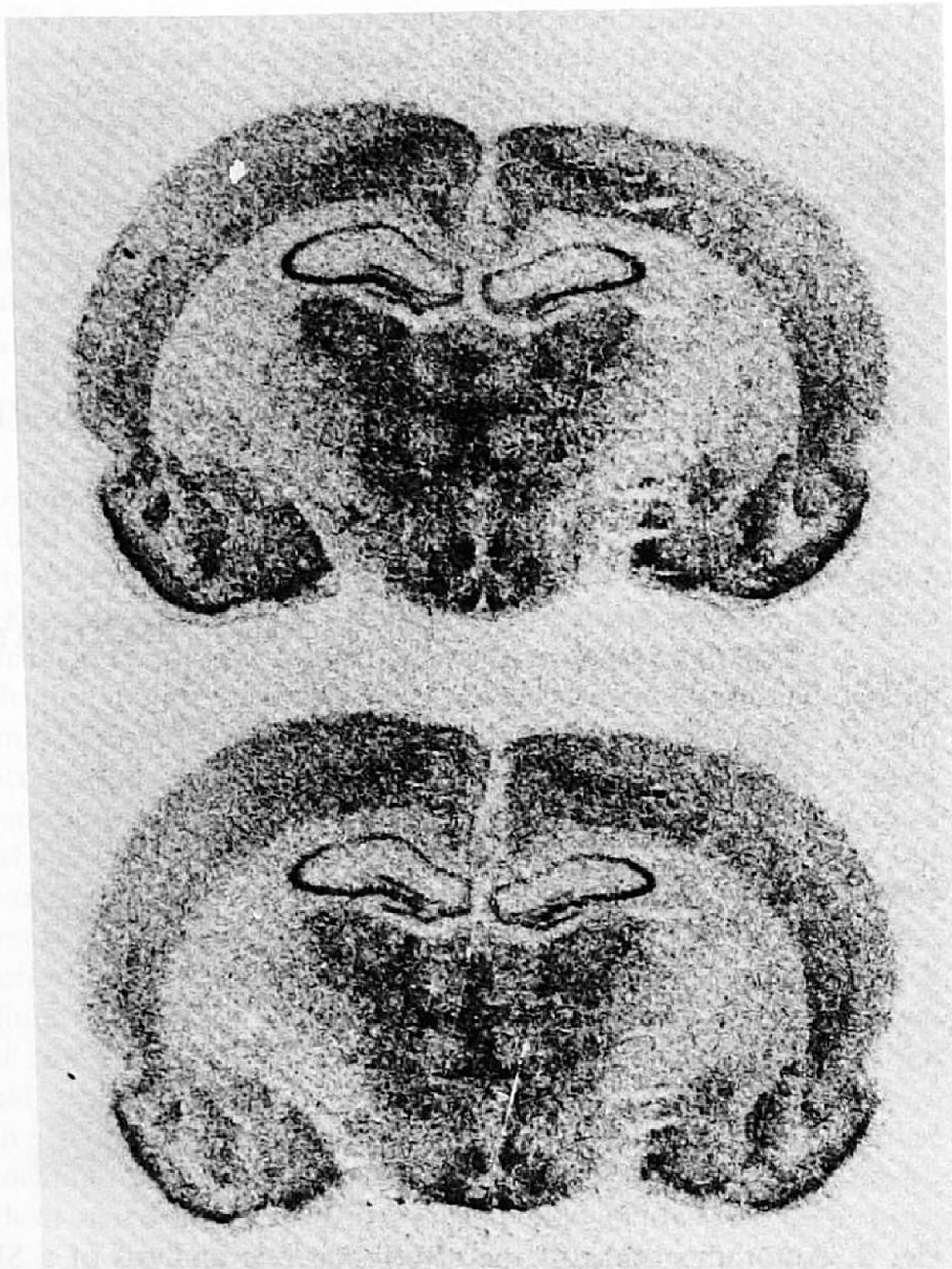
Fig. 3. Western blot of samples obtained from homogenates of cortex and hippocampus of control (lanes 1 and 3: 10, 20, 30 μg of proteins) and MAM-treated rats (lanes 2 and 4; 30, 20, 10 μg of proteins). The amount of B-50 in MAM-treated rats is not altered with respect to controls.

Moreover, Fig. 1 shows that in MAM-treated rats the incorporation of the ^{32}P-phosphate is lower when compared to controls, both in one- and two-dimensional electrophoretic analysis. Total amount of proteins applied to the gels and the Coomassie staining of the gels were the same for both controls and MAM-treated rats. The ^{32}P-phosphate incorporation into B-50 was measured by a densitometric analysis of the corresponding protein band in the one-dimensional electrophoresis of 4 separate experiments and a statistically significant 42% decrease in the phosphate incorporation in this protein in MAM-treated rats was observed (mean $\pm$ S.D. in arbitrary units: controls 865 095 $\pm$ 93 470; MAM 507 199 $\pm$ 95 090; $P < 0.01$).

A similar reduction of ^{32}P-phosphate incorporation was observed also in hippocampus of MAM-treated rats (data not shown).

Effect of exogenous PKC on ^{32}P-phosphate incorporation into B-50

Figure 2 shows the autoradiograph of phosphorylated SPM proteins obtained from cortex of control and microencephalic rats in the presence of in vitro added purified PKC before (A) or after (B) heat inactivation. In both experimental conditions, the ^{32}P-phosphate incor-



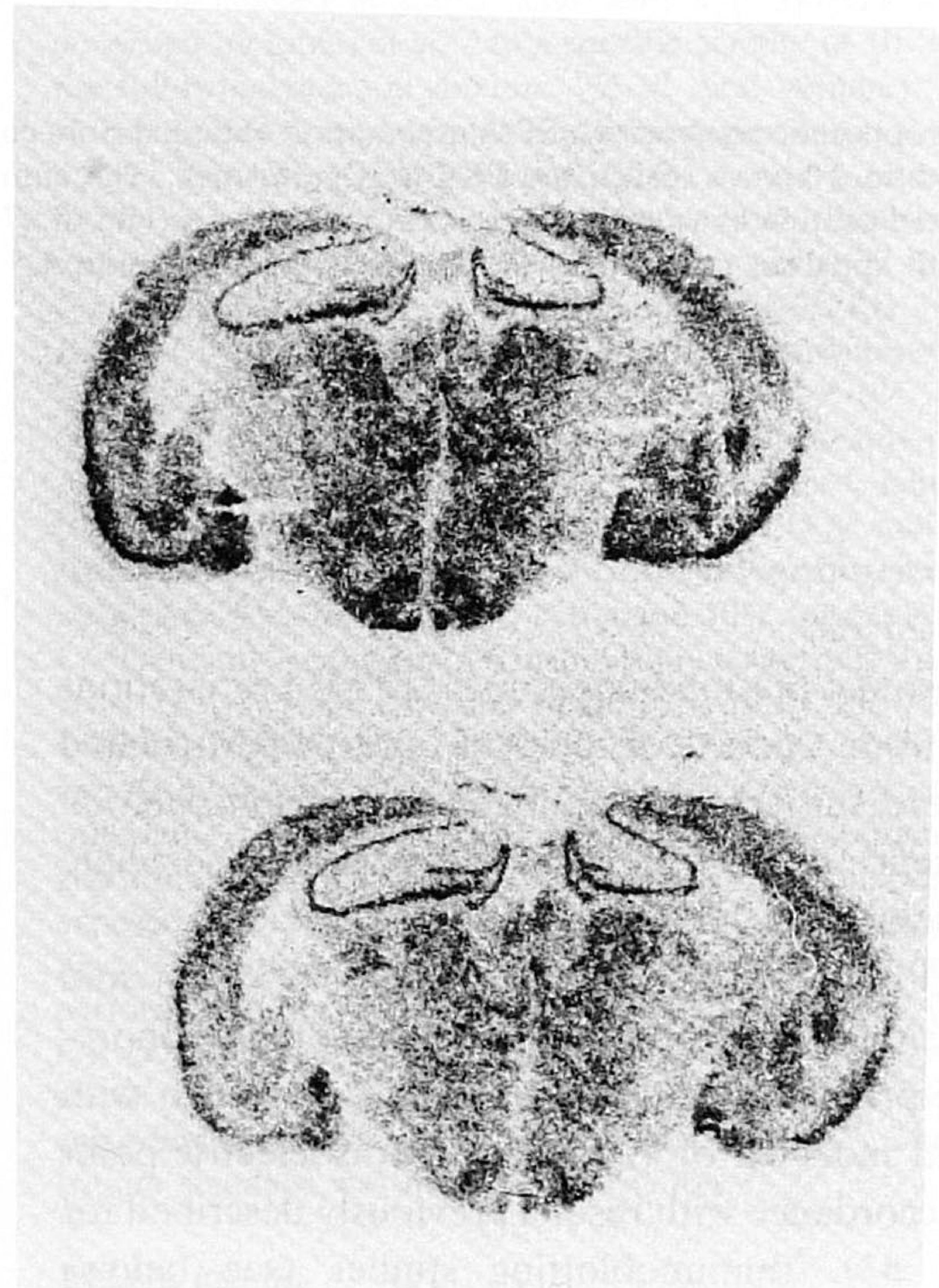


Fig. 4. In situ hybridization studies performed on control and MAM-treated brains utilizing an oligonucleotide probe designed on the amino acid sequence 8–21 of B-50. The synthesis of B-50 is not modified by the prenatal treatment with the antimitotic agent.

TABLE I

Densitometric analysis of in situ hybridization studies

Values are in arbitrary units and are expressed as means ± S.E.M. of 5–12 readings as indicated in parentheses; the Amersham tritium standard was used for the standard curve.

Brain region	*Control*	*MAM-treated*
Cortex	2.063 ± 0.102 (10)	2.222 ± 0.109 (10)
Hippocampus	2.377 ± 0.210 (10)	2.564 ± 0.367 (10)
Striatum	1.497 ± 0.088 (12)	1.616 ± 0.057 (12)
Thalamic area	2.172 ± 0.118 (6)	2.262 ± 0.185 (5)

poration into B-50 is the same in control and MAM-treated rats.

Western blot

To further identify the protein and to investigate whether the total amount of the protein is altered in MAM-treated rats with respect to controls, Western blotting was performed with a specific anti B-50 IgG on samples obtained from homogenates of cortex and hippocampus of control and MAM-treated rats.

Figure 3 shows the Western blot of homogenate proteins separated on a 11% SDS–PAGE; lanes 1 and 3 represent the immunostaining of different amounts of proteins obtained from cortex and hippocampus of control rats (10, 20 and 30 μg) and lanes 2 and 4 represent proteins (30, 20 and 10 μg) obtained from homogenates of cortex and hippocampus of MAM-treated rats, respectively. Judged by this semiquantitative procedure there is no difference between the amount of B-50 in MAM and control animals.

Therefore the immunostaining with the anti-B-50 IgG further confirms the identity of the 47 kDa protein as B-50 in these animals and that its amount, relative to total proteins, is not altered by the treatment.

In situ hybridization studies

To investigate the possible changes of the expression of B-50 in adult MAM-treated rats we performed in situ hybridization studies utilizing an oligonucleotide designed on the amino acid sequence 8–21 of B-50. The autoradiograph of representative sections from control and MAM-treated rats is shown in Fig. 4. The figure clearly shows the dramatic reduction of the cortex and the morphological alteration of the hippocampal region as already reported[47].

The highest hybridization signal was observed in all the CA regions of the hippocampus, the thalamic area and cerebral cortex. Table I shows that no statistically significant differences occur in B-50 expression in MAM-treated rats with respect to controls. This result suggests that in microencephalic animals the synthesis of the protein is not changed and confirms the immunostaining experiments.

DISCUSSION

We report here a statistically significant reduction in the phosphorylation of B-50 in an animal model of microencephaly. In this model, animals are characterized by a relatively selective impairment in tests considered predictive of learning and memory[6,19].

The identity of B-50 in cortex and hippocampus of control and MAM-treated rats is established by (a) one-dimensional, (b) two-dimensional electrophoretic properties and (c) immunoreactivity in Western blot analysis.

The reduction in the phosphorylation of B-50 observed in the brain areas examined, i.e. cortex and hippocampus, in microencephalic rats is statistically highly significant, indicating that in these brain areas the anatomical disorganization induced by the prenatal treatment with MAM is accompanied by biochemical processes known to be of importance in synaptic remodeling. It is interesting to observe that the 42% reduction in the phosphate incorporation is not due to a loss of B-50 in the treated animals. The immunoblots of Fig. 3 clearly show that the amount of B-50 per mg of protein is not different from controls in the MAM-treated rats and this is further confirmed by in situ hybridization studies. Indeed, we demonstrate the typical distribution pattern of mRNA for B-50, with high levels in hippocampus and cortex, as reported by others[13]. This differential distribution is not altered in MAM-treated rats, despite the profound anatomical changes induced by the alkylating agent, and the densitometric analysis does not reveal any difference in the B-50 mRNA signals between control and microencephalic animals both in the high and low density areas.

We can therefore conclude that the decreased incorporation of ^{32}P-phosphate into B-50 by endogenous PKC is significantly reduced in MAM-treated rats, without changes in the relative amounts of the protein in cortex and hippocampus. Moreover, this is the first report of a change in the phosphorylation of B-50 ex vivo in an animal model of a pathological state also found in humans and associated with reduced cognitive functions.

The reasons for this decrease in the phosphorylation of B-50 are at present unclear. However, we might speculate that the enzymatic activity of PKC in MAM-treated rats is reduced with respect to controls. Indeed, exogenous addition of PKC to the SPM preparation obtained from MAM-treated rats seems to restore completely the incorporation of ^{32}P-phosphate into B-50 to values which

are comparable to those of controls (Fig. 2). This finding makes it also unlikely that the effect observed could be due to the action of a soluble PKC inhibitory factor[30,39] which would be present in higher amounts in MAM-treated rats. It must be also noted that a similar incorporation of ^{32}P-phosphate in control and MAM-treated SPM preparations is obtained after heat inactivating enzymatic activities and then adding exogenous PKC, further confirming the specificity for B-50 as substrate for PKC.

Since B-50 contains only one phosphorylation site at serine in position 41, the possibility that different sites are phosphorylated in vitro and in vivo can be excluded[32].

All these data seem to support the hypothesis that the reduction in the phosphorylation of B-50 by endogenous PKC observed in microencephalic animals might be due to a change in enzyme activity. This could be due either to loss of enzyme expression or to reduced translocation of the enzyme from cytosol to plasma membranes or to change in calcium sensitivity. Work is in progress in our laboratory to obtain more direct evidence, both through assay of the enzymatic activity on exogenous substrates and its mRNA expression via the in situ hybridization technique. Moreover, since PKC has been implicated in LTP persistence in hippocampus[17,26] and since its activity is decreased in MAM-treated rats, it would be interesting to evaluate if in these animals LTP can be induced, but not maintained.

In conclusion, we report here that PKC-dependent phosphorylation processes are altered in an animal model characterized by a profound alteration in neuronal circuitries in both cortex and hippocampus and which show deficits in cognitive processes. We propose therefore that this animal model could serve as an in vivo system to study the role of specific PKC substrates, such as B-50, in synaptic remodeling related to learning and memory processes.

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