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Selective alteration in B-50/GAP-43 phosphorylation in brain areas of animals characterized by cognitive impairment

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When methylazoxymethanol acetate is administered to pregnant rats at gestational day 19, the offspring are greatly impaired in the learning of a two-way active avoidance task and these behavioral changes are paralleled by a change in the phosphorylation of the protein B-50/GAP-43 in hippocampus but not in cortex. The expression of the protein is not altered, indicating that the phosphorylation of B-50 is a sensitive marker of alterations in synaptic plasticity associated with impairments of learning abilities in rats.

The administration of the potent alkylating agent methylazoxymethanol acetate (MAM) to pregnant rats induces a mitotic arrest of cells actively dividing in the fetus^{7,10}. Since the cell-killing effect of MAM is confined to a period extending from 2 to 24 h after its administration, only neurons actively dividing within this narrow time window are affected. This makes MAM highly suitable to induce specific changes in different brain functions and areas, depending upon the time of its administration¹¹. The time dependency of the MAM effect is particularly interesting because, by changing the time of its injection during gestational life, different populations of neurons in the central nervous system are selectively affected. We have recently studied molecular correlates of alterations in synaptic plasticity associated with cognitive deficits in animals exposed to MAM at gestational day (GD) 15, a treatment inducing a marked hypoplasia of telencephalic structures, such as cortex and hippocampus. Indeed we have demonstrated that the phosphorylation of the nervous tissue specific protein B-50/GAP-43³ — a protein selectively localized on the inner leaflet of the presynaptic plasma membrane¹⁶ and implicated in

neurite outgrowth¹⁵, in transmembrane signal transduction and transmitter release⁵ and in synaptic plasticity as evidenced by LTP in hippocampus⁹ — is markedly altered in cortex and hippocampus of cognitively impaired animals⁸. MAM exposure at GD15 induces a quite dramatic reduction in these telencephalic structures. To further investigate the relationship between MAM induced brain damage, behavioral abnormalities and changes at the molecular level, we have administered MAM at a later gestational day, GD19, when the major part of neuroblasts have already undergone their final mitosis and only a few selected groups of neurons, such as granule cells of hippocampal dentate gyrus, are in active dividing phase². Because their proliferation extends up to the first week of postnatal life, a single MAM administration at GD19 will affect only a limited number of these neurons^{11,14}.

On GD19, Sprague–Dawley pregnant rats (Charles River, Calco, Italy) received a single intraperitoneal injection of either 25 mg/kg of MAM (Sigma, St. Louis) in sterile saline or of vehicle alone. At two months of age offspring were randomly selected from

each litter and assigned either to the behavioral testing or to the biochemical assays. Learning performance was assessed by an active avoidance test in a two-way shuttle box, as previously described¹. Endogenous phosphorylation of B-50 was evaluated in synaptosomal plasma membrane (SPM) preparations obtained from cortex and hippocampus ($n = 9$) of control and GD19 MAM-treated rats and phosphorylated in conditions known to activate membrane bound PKC.

[γ -³²P]ATP was used as in vitro phosphate donor and the reaction was carried out in buffer containing (final concentrations; mM): Tris 10, MgCl₂ 10, CaCl₂ 0.1, pH7.4 at 30 °C. Phosphorylated proteins were separated on 11% SDS-PAGE, stained and subjected to autoradiography. The expression of B-50 mRNA was evaluated by means of in situ hybridization utilizing a probe recognizing amino acid residues 8–21 in the B-50 sequence. The probe was radiolabeled at the 3'-end with ³⁵S to a specific activity of 10⁹ cpm/ μ g and purified on a NEN-SORB cartridge⁸. The labeled oligonucleotide was hybridized to brain coronal sections (10 μ m) obtained at two different levels (6.20 and 3.20 mm from the interaural line, according to the Paxinos and Watson atlas¹³) of control and GD19 MAM-treated rats⁸.

Fig. 1 shows results obtained in the active avoidance test. Control rats learned to avoid the electric shock through the grid floor at the presentation of the conditioned stimuli already after the first block of 10 trials and reached maximal avoidance at the fourth block (mean % of avoidance: 70–80); MAM-treated rats remained at the basal level even after 9 blocks of 10 trials, indicating a total inability to learn, at least in this experimental condition. Therefore, despite the absence of gross morphological changes, these animals are markedly cognitively impaired, suggesting that GD19 MAM-treatment might affect a small group of dentate

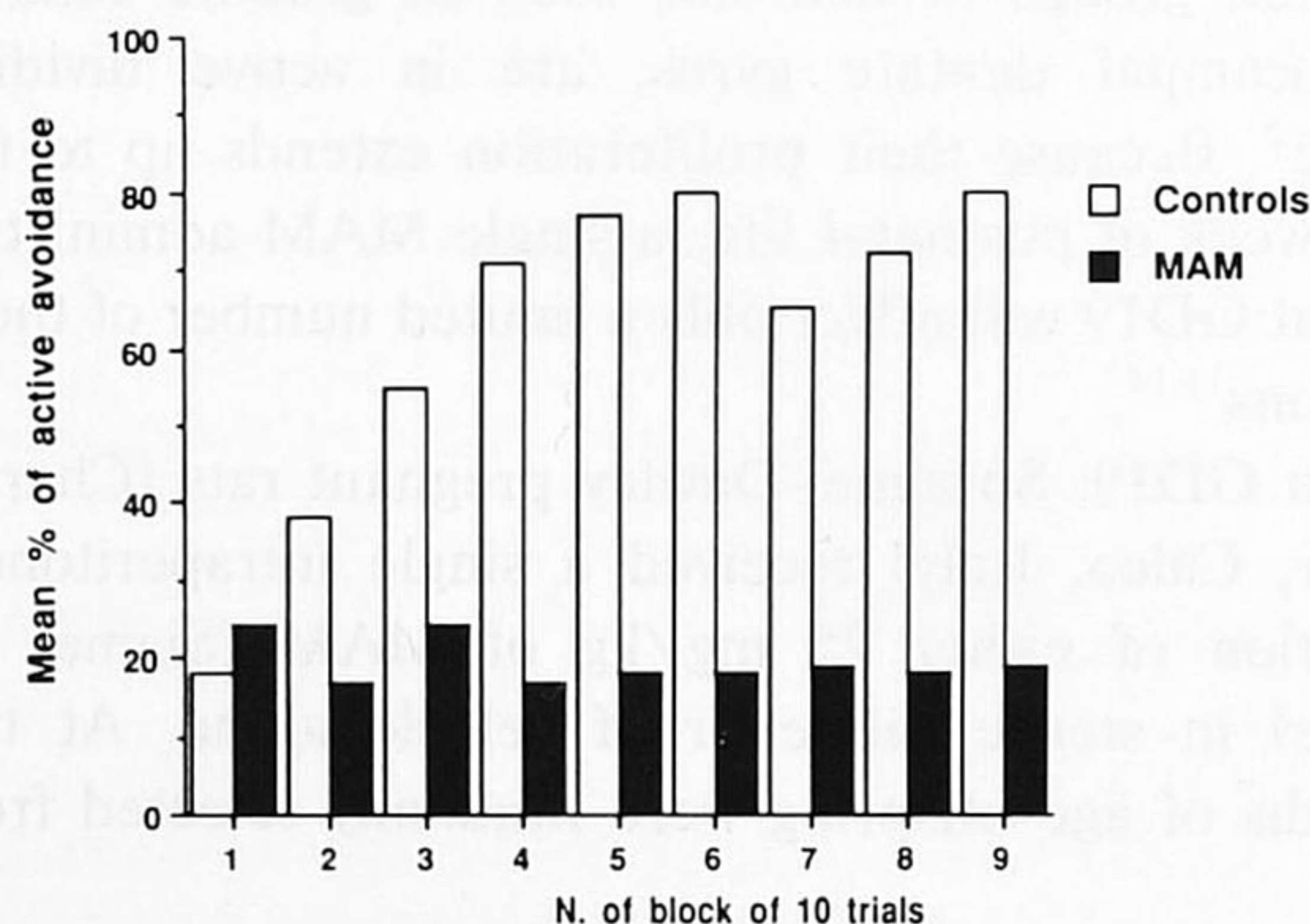


Fig. 1. Mean percent avoidance per block of 10 avoidance trials in control (open bars) and GD 19 MAM-treated offspring (black bars). Repeated measures ANOVA ($F_{1,10} = 19.93$, $P < 0.005$, GD 19 group vs controls).

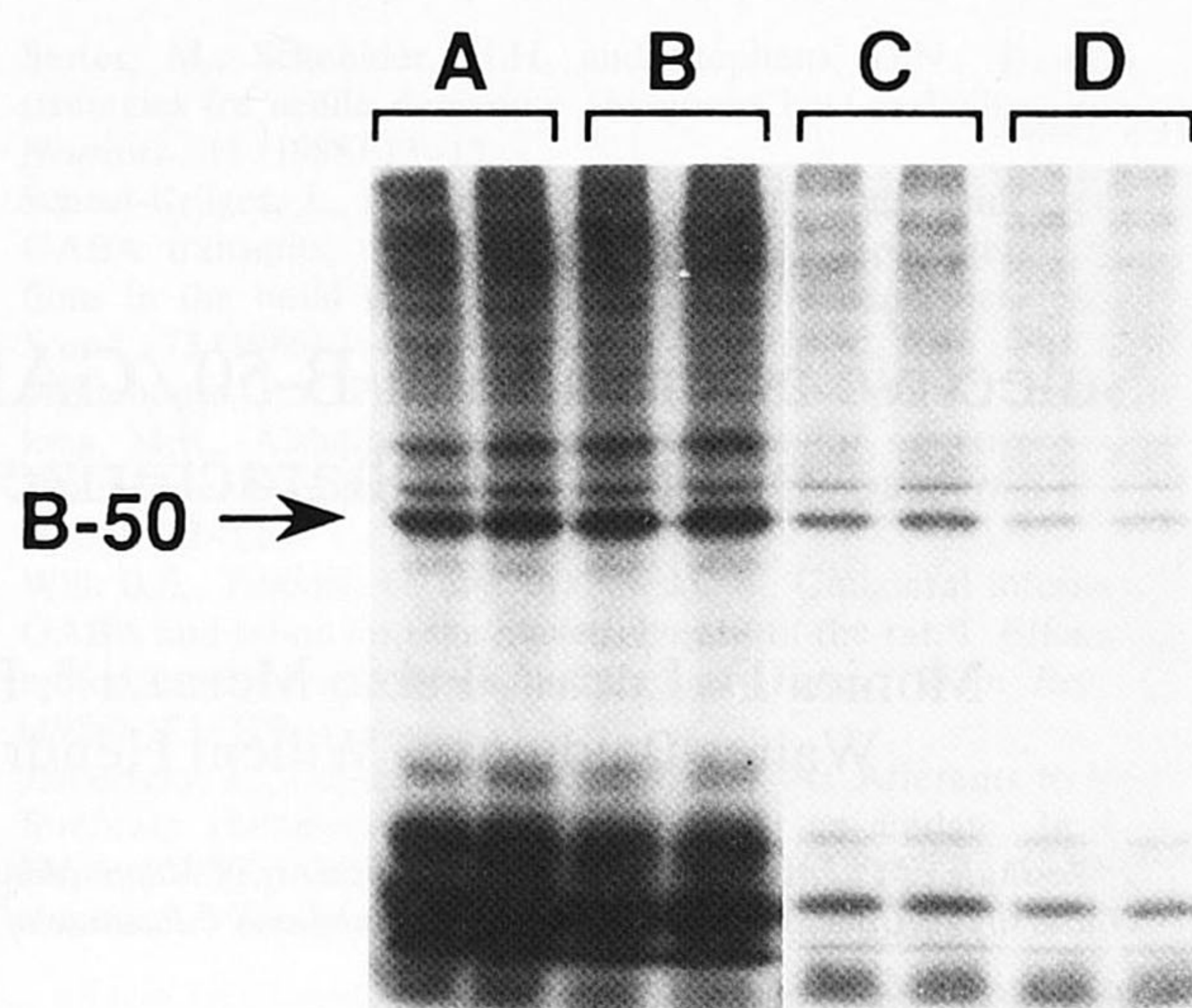


Fig. 2. Autoradiograms of phosphorylated SPM obtained from cortex of control (A) and treated (B) rat brains and hippocampus of control (C) and treated (D) animals. B-50 (arrow) phosphorylation was analyzed by densitometry and showed a statistically significant reduction of 44% in SPM of treated rats as compared to controls (Mean of 3 independent experiments, 3 animals each).

granule cells which are evidently important for the expression of this learning behavior.

Post-hoc phosphorylation experiments have been performed to assess B-50 phosphorylation in different brain areas of GD19 MAM-treated rats. A representative autoradiogram after one dimensional separation of phosphorylated SPM proteins is shown in Fig. 2. Lanes A and B refer to the endogenous post-hoc phosphorylation assay in cortex: ³²P incorporation into B-50 is not altered in treated rats as compared to control animals. In hippocampus, phosphorylation of B-50 is lower in MAM-treated animals (lane D) than in controls (Lane C). The ³²P incorporation into B-50 in hippocampus, quantified densitometrically (3 independent experiments, each of 3 animals) is significantly decreased (44%) in MAM-treated rats as compared to controls (control = 100 ± 9.2 ; treated = 56.5 ± 3). These data suggest that indeed a cell loss might have occurred in hippocampus and that the observed modifications in the PKC-dependent phosphorylation processes are most likely due to a compensatory response to the prenatally induced hippocampal lesion. In the cortex no statistically significant difference in B-50 phosphorylation was found, reflecting the lack of effect of MAM on cortical neuroblasts according to the time table of origin of neurons intrinsic to this brain area².

To ascertain that the change in B-50 phosphorylation was not due to a change in the expression of the protein as the result of the MAM treatment, we performed in situ hybridization studies. High levels of B-50 mRNA were found in the cortex, hippocampal

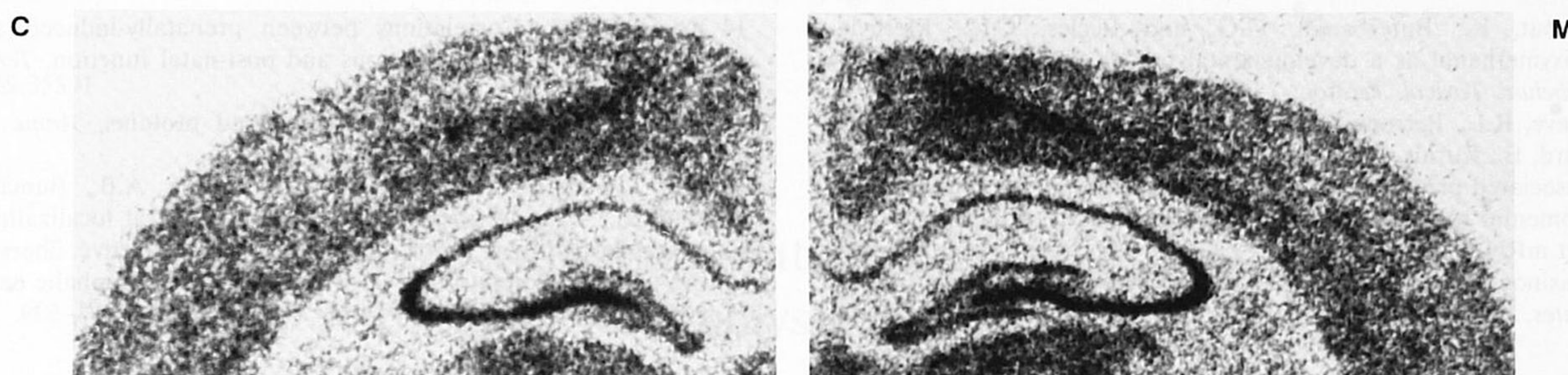


Fig. 3. Representative autoradiograms of in situ hybridization studies using an oligonucleotide probe designed to recognize amino acid sequence 8–21 of B-50. C, control; M, MAM-treated.

formation and thalamic areas, as previously reported^{6,12}. In the hippocampus the hybridization signal was not uniformly distributed in the different subfields: in the CA₃ region the signal was significantly higher than that observed in the other regions, in line with data on the immunolocalization of the protein⁴. Indeed the CA₃ region, which shows the highest labeling with the oligonucleotide probe for B-50, contains the cell bodies whose collaterals project to the CA₁ region, where the highest immunolabeling was described⁴. This uneven pattern was observable also in GD19 MAM-treated rats, as revealed by the computer assisted image analysis (NIH Image, Bethesda, USA) reported in Table I. No significant differences in the intensity of the hybridization signal could be detected between MAM-treated and control animals in any of the regions analyzed.

Therefore, the change in the phosphorylation of B-50 is not associated with changes in its expression as a consequence of the treatment. This suggests that alterations in the endogenous phosphorylation of B-50 could reflect changes in the functional activity and not simply in the disorganization of the neuronal circuitry in the areas affected by MAM.

In conclusion, our data show that changes in the phosphorylation of B-50, a specific substrate for PKC, occur only in brain areas affected by the treatment

with the neurotoxin, as already shown previously for GD15 MAM-treated rats. The selective reduction in B-50 phosphorylation observed in the hippocampus in the GD19 group is of particular importance, because it shows that this occurs in the absence of major neuronal losses or neuronal disorganization and this change could correlate with the selective impairment in the acquisition in the two-way active avoidance task, indicating the phosphorylation of B-50, rather than the expression of the protein, as a sensitive marker of alterations in synaptic plasticity in the mature nervous system.

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TABLE I

Densitometric analysis of in situ hybridization studies

Values are expressed as optical density (arbitrary units). ^a Means \pm S.D. of 16 readings (4 animals and from each animal two slices at each of the 2 levels, see text). ^b Means \pm S.D. of 20 readings (5 animals and from each animal two slices at each of the two levels, see text).

Brain region	Control ^a	MAM-treated ^b
Cortex	38 \pm 3	37 \pm 2
Dentate gyrus	40 \pm 3	39 \pm 3
CA ₁	39 \pm 4	38 \pm 3
CA ₃	51 \pm 6 *	50 \pm 6 *

* $P < 0.01$ vs respective CA₁ and dentate gyrus subfields.

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