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Functional Peculiarities of MAP2 in DBA/2J Inbred Mice as a Component of Genetic Predisposition to Seizures

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We compared the content of high molecular microtubule-associated protein-2 and its phosphorylation by cAMP- and Ca^{2+}/calmodulin-dependent protein kinases in the brain of DBA/2J and C57Bl/6 inbred mice. The revealed differences in protein content and phosphorylation can be attributed to the mechanisms mediating audiogenic seizures in DBA/2J mice and to differential sensitivity of these inbred lines to seizure-inducing factors.

Key words: *MAP2; phosphorylation; seizures; hereditary epilepsy*

Many studies showed significant differences in the sensitivity to seizure-inducing agents (sound, electric stimulation, convulsants) between C57Bl/6 and DBA/2J inbred mice [6,9]. Thus, in DBA/2J mice, generalized audiogenic seizures are most pronounced on postnatal day 21 and disappear completely on day 60, whereas C57Bl/6 mice are resistant to all seizure-inducing agents [1,2,12]. It was assumed that lower seizure threshold in DBA/2J mice compared to C57Bl/6 mice is genetically determined. It was shown that changes in the content of high molecular isoforms of microtubule-associated protein-2 (MAP2) in neuronal soma and dendrites can be regarded as a marker of pathological plasticity [3,5]. It was also shown that MAP2 phosphorylation by cAMP- and Ca^{2+}/calmodulin-dependent protein kinases regulates the dynamics of cytoskeleton tubulin microtubules and contributes to long-term plastic changes in neurons [11]. Little is known about MAP2 regulation in animals with genetically determined seizures. Here we compared MAP2 content and phosphorylation in the brain of DBA/2J and C57Bl/6 mice.

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MATERIALS AND METHODS

The study was carried out on 21- and 60-day-old male DBA/2J and C57Bl/6 mice (Biocenter, Pushchino). The animals were decapitated under light ether anesthesia. The cortex and hippocampus were rapidly removed in 0.32 M sucrose and homogenized in a Teflon-glass homogenizer at 4°C in a buffer containing 10 mM Hepes-NaOH, pH 7.2, 1 mM EGTA, 0.5 mM EDTA, 1.5 mM $Na_4P_2O_7$ and protease inhibitors: 10 mM benzamidine, 100 ng/ml aprotinin, and 100 ng/ml leupeptin. Protein content in homogenates was determined according to Bradford [4]. Aliquots of homogenates were frozen and stored in liquid nitrogen.

Reagents from Sigma and Isotop (γ-^{32}P-ATP) were used in the study.

Homogenate aliquots containing 50 μg protein were preincubated at 30°C for 1 min. cAMP-dependent phosphorylation was initiated by adding ATP, cAMP, and $MgCl_2$ to a medium containing 50 mM Hepes-NaOH, pH 7.0, 1 mM EGTA, 0.5 mM EDTA, 1 mM dithiothreitol, 1.5 mM $Na_4P_2O_7$, 0.5 mM 3-isobutyl-1-methylxanthine, 10 mM $MgCl_2$, 20 μM ATP (γ-^{32}P-ATP, 3 μCi/sample), 100 μM cAMP, 5 μM okadaic acid, 2.5 mM NaF, 10 mM β-glycerophosphate, and protease inhibitors (final reaction volume 50 μl). One minute after initiation the reaction was stopped

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by adding 10 μl concentrated solubilizing buffer for polyacrylamide gel (PAAG) electrophoresis in the presence of sodium dodecyl sulfate followed by 3-min boiling.

For evaluation of Ca^{2+}/calmodulin-dependent phosphorylation the reaction was initiated with ATP, $CaCl_2$, calmodulin, and $MgCl_2$. The reaction was carried out in a medium (final volume 50 μl) containing 50 mM Hepes-NaOH, pH 7.4, 1 mM EGTA, 0.5 mM EDTA, 1 mM dithiothreitol, 1.5 mM $Na_4P_2O_7$, 0.1 mM phenylmethylsulfonyl fluoride, 10 mM $MgCl_2$, 50 μM ATP (γ-^{32}P-ATP, 3 μCi/sample), 2.5 mM $CaCl_2$, 5 mg/ml calmodulin, 5 μM 6-22 amid fragment of protein kinase A protein inhibitor, 2 μM 19-36 fragment of Ca^{2+}/phospholipid-dependent protein kinase inhibitory domain, 5 μM okadaic acid, 2.5 mM NaF, 10 mM β-glycerophosphate, 10 mM benzamidine, 100 ng/ml aprotinin, and 100 ng/ml leupeptin. The reaction was stopped 2 min after initiation as described above.

Phosphoproteins were separated in 10% sodium dodecyl sulfate PAAG. Autoradiographs of dry gels were obtained on Kodak X-OMAT/AR5 films. ^{32}P incorporation into MAP2 was evaluated by densitometry.

For immunodot analysis isolated cortex and hippocampus were homogenized in a buffer (1 and 0.5 ml, respectively) containing 10 mM Hepes-NaOH, pH 7.2, 1 mM EGTA, 0.5 mM EDTA, 0.3 mM phenylmethylsulfonyl fluoride, 50 mM benzamidine, 300 ng/ml aprotinin, and 300 ng/ml leupeptin. The homogenate was incubated at 4°C for 30 min and centrifuged 60 min at 100,000 g (4°C). Supernatant (cell cytosol) was collected and protein concentration was measured; the aliquots were stored at -20°C. Protein samples (2 μl, 3.6 μg protein) were applied to nitrocellulose membrane (22-μ pore diameter). MAP2 content was determined using monoclonal antibodies (mouse IgG, HM2 clone) against high molecular MAP2 isoforms (MAP2A and MAP2B). Preliminary immunoblot analysis showed that these antibodies react with protein band corresponding to MAP2. MAP2 content was evaluated by the intensity of peroxidase staining using a calibration.

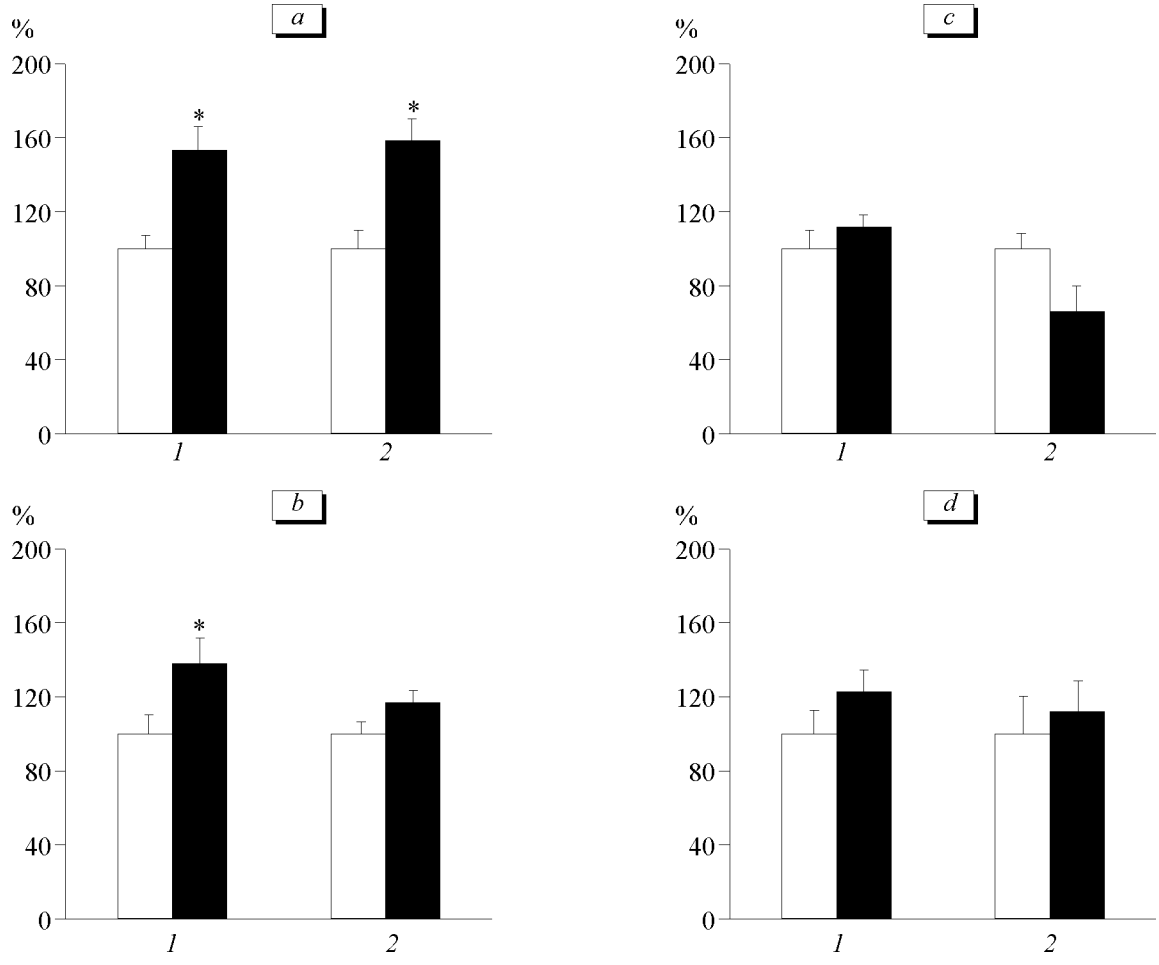

Fig. 1. Comparison of MAP2 post hoc phosphopylation in the cortex (*1*) and hippocampus (*2*) of C57Bl/6 (open bars) and DBA/2J (dark bars) mice on postnatal days 21 (*a, c*) and 60 (*b, d*). *a, b*) cAMP-dependent phosphorylation, *c, d*) Ca^{2+}/calmodulin-dependent phosphorylation. Here and in Fig. 2: *p<0.05 compared to C57Bl/6 mice.

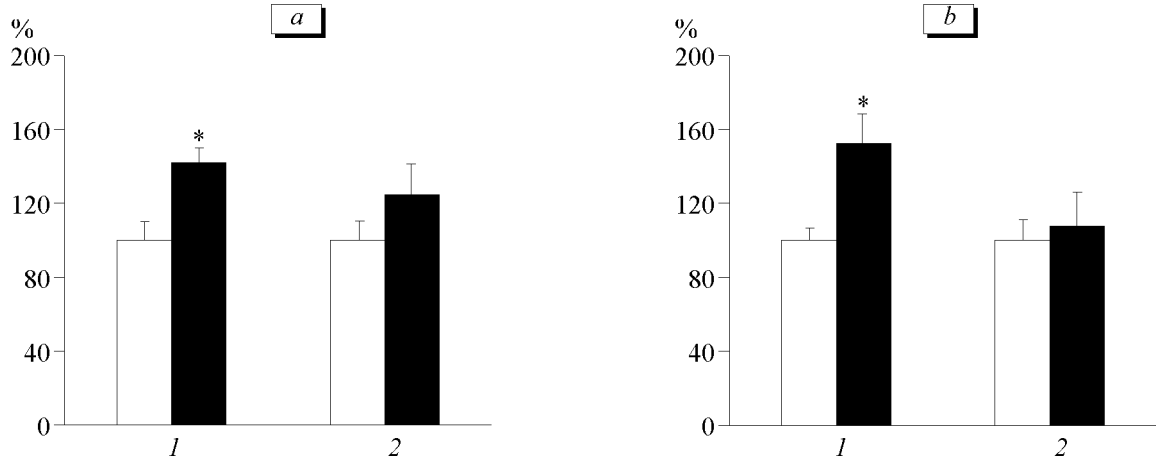

Fig. 2. MAP2 content in the cortex (*1*) and hippocampus (*2*) of C57Bl/6 (open bars) and DBA/2J (dark bars) mice on postnatal days 21 (*a*) and 60 (*b*).

The significance of differences was evaluated by Student *t* test. Differences were considered significant at $p<0.05$.

All data are presented as means±standard errors.

RESULTS

In vitro cAMP-dependent post hoc phosphorylation revealed a marked increase in ^{32}P incorporation into MAP2 in the cortex and hippocampus (153.26±12.56 and 158.29±12.14%, respectively, $p<0.05$) in DBA/2J mice compared to C57Bl/6 on postnatal day 21. In the cortex, ^{2}P incorporation into MAP2 remained increased on postnatal day 60 (137.90±13.65, $p<0.05$, Fig. 1, *a, b*). The observed increase in ^{32}P incorporation into MAP2 revealed by *in vitro* post hoc phosphorylation reflects increased number of protein kinase-accessible (nonphosphorylated) acceptor sites on MAP2 molecule *in vivo* [10].

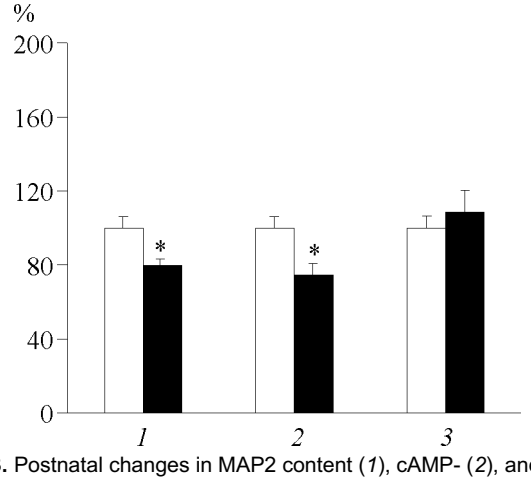

Fig. 3. Postnatal changes in MAP2 content (*1*), cAMP- (*2*), and Ca^{2+}/calmodulin-dependent (*3*) post hoc MAP2 phosphorylation in the cortex of DBA/2J mice on postnatal days 21 (open bars) and 60 (dark bars). *$p<0.05$ compared to postnatal day 21.

It cannot be excluded that the revealed changes in cAMP-dependent protein kinase MAP2 phosphorylation in DBA/2J mice are determined by higher MAP2 content in the cortex and hippocampus. Immunodot analysis revealed a higher content of MAP2 in the cortex (141.90±7.75%, $p<0.05$) and hippocampus (124.56±16.49%) of DBA/2J mice on day 21 (Fig. 2, *a*). Thus, on postnatal day 21 (maximum audiogenic seizures), DBA/2J mice demonstrated increased MAP2 content in the cortex and hippocampus associated with *in vivo* increase in the number of phosphorylation-accessible sites compared to those in C57Bl/6 mice. It appears that in 21-day-old DBA/2J mouse brain the level of MAP2 phosphorylation in cAMP-dependent protein kinase-specific sites is lower than in corresponding brain structures of C57Bl/6 mice. cAMP-dependent protein kinase MAP2 phosphorylation is known to protect this phosphoprotein against calpain proteolysis [8,11] suggesting higher vulnerability of MAP2 to proteolytic enzymes in DBA/2J mouse brain. Thus, increased MAP2 content in DBA/2J mice can be regarded as a compensatory mechanism decelerating its proteolysis. The observed peculiarities of cAMP-dependent protein kinase MAP2 phosphorylation in the cortex and hippocampus of 21-day-old DBA/2J mice suggest modified regulation of the dynamics of tubulin microtubules and their binding to other elements of the cytoskeleton [7, 13]. It is interesting that the revealed differences between the examined mouse lines in cAMP-dependent MAP2 phosphorylation and content persist up to postnatal day 60 in the cortex, but not in the hippocampus, which points to juvenile features of MAP2 functioning in the cortex of adult DBA/2J mice.

Ca^{2+}/calmodulin-dependent post hoc phosphorylation revealed no significant interlinear differences in ^{32}P incorporation into MAP2 in the cortex and hippocampus on postnatal days 21 and 60 (Fig. 1, *c, d*).

However, increased MAP2 content in the brain of DBA/2J mice of both age groups (Fig. 2, *a, b*) points to decreased *in vivo* MAP2 phosphorylation level in Ca^{2+}/calmodulin-dependent protein kinase-specific sites, which enhances MAP2 affinity to microtubule primers and promotes their further elongation [11].

When comparing 21- and 60-day-old DBA/2J mice we revealed a decreased MAP2 content in the cortex (79.56±4.04%, $p<0.05$) and ^{32}P incorporation after cAMP-dependent post hoc phosphorylation (74.46± 6.47%, $p<0.05$) on postnatal day 60 (Fig. 3, *a, b*) and unchanged phosphate incorporation into MAP2 by Ca^{2+}/calmodulin-dependent protein kinase (Fig. 3, *c*). No significant differences in these parameters between age DBA/2J mouse groups were revealed (data not shown). It cannot be excluded that changes observed in DBA/2J mouse brain during postnatal development (Fig. 3) correlate with the disappearance of audiogenic seizures on postnatal day 60.

The revealed differences in MAP2 content and phosphorylation between DBA/2J and C57Bl/6 mice can be associated with mechanisms mediating audiogenic seizures in DBA/2J mice and with differential sensitivity of these lines to seizure-inducing agents in the adulthood.

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