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Short Communication

MPTP-induced central dopamine depletion exacerbates experimental autoimmune encephalomyelitis (EAE) in C57BL mice

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Abstract. It is obvious that the central nervous system plays a role in the regulation of an immune response. However, the mechanisms of this regulation are poorly understood. The goal of the present study was to examine the role of one of the neurotransmitters – dopamine, in this process. We used experimental autoimmune encephalomyelitis (EAE), an autoimmune disease with its effector phase in the CNS, as a model to study the effect of central dopamine depletion on the development of an immune response. Dopamine depletion was achieved by treatment with 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropiridine (MPTP; 40 mg/kg), whereas EAE was elicited by immunization with MOG 35-55 (150 μg) in complete Freund's adjuvant (CFA), supplemented with *Mycobacterium tuberculosis*. As determined by HPLC, striatal dopamine contents in mice treated with MPTP were significantly lower compared to vehicle-treated controls. Remarkably, striatal depletion of dopamine prior to EAE induction resulted in an earlier onset of the disease and an augmentation of its clinical signs. Moreover, the striatal dopamine-depleted mice demonstrated an increased concentration of IL-1β and decreased concentration of TGFβ in the spinal cord, compared to EAE mice. Since MPTP itself does not have any direct effect on immune cells, it strongly suggests that the observed changes in EAE induction and progression after MPTP administration depended on lower dopamine level. Further studies are required to find out the cellular mechanism of the dopamine action.

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Key words: Experimental autoimmune encephalomyelitis – MPTP – IL-1 β – TGF β – Dopamine – Dopaminergic system

Introduction

Experimental autoimmune encephalomyelitis (EAE) is an inflammatory demyelinating disease of the central nervous system (CNS) that serves as a model for multiple sclerosis (MS) [1]. For a vast majority of mouse strains, EAE is a chronic and monophasic disease with sparse inflammatory lesions in the CNS [2]. C57BL mice are genetically susceptible to EAE, which can be induced in these animals by a single injection of the myelin oligodendrocyte glycoprotein (MOG) emulsified in complete Freund's adjuvant (CFA) [3]. Compared to a significant body of work elucidating the role of the sympathetic nervous system in the regulation of the immune system, much less attention has been devoted to the role of the central dopaminergic system.

MPTP is a neurotoxin which selectively injures the central dopaminergic system [4]. Studies conducted by Renoux and colleagues, show that MPTP intoxication leads to immune system depression, assayed as a decreased T lymphocyte activation [5]. Bieganowska and colleagues reported that mice treated with MPTP show a depressed proliferation of mitogen-activated lymphocytes in the spleen, and a decreased number of antibody-producing spleen cells [6]. There is evidence that pargyline treatment, which prevents the dopaminergic neuron injury after MPTP intoxication, blocks the described suppression of the immune system [7]. This information, together with the fact that MPTP does not affect the immune cell function *in vitro,* suggest that cen-

tral dopaminergic system is required for a normal functioning of the immune system. In support of this hypothesis, a dopaminergic system injury has also been shown to cause an enhanced tumor growth [8]. Moreover, 6-hydroxydopamine (6-OHDA) treatment, which reduces both dopamine and norepinephrine concentrations in the CNS, impairs humoral immune responsiveness and activation of spleen T suppressor lymphocytes [9]. Nistico and colleagues have shown that the D_1 receptor stimulation in various dopaminergic areas of the brain causes a modulation of NK cell activity [10].

An important role in the regulation of the immune response during EAE seems to be played by the autonomic nervous system. For example, Chelmicka-Schorr and coworkers have shown that sympathectomy evoked by 6-OHDA treatment causes augmentation of EAE [11]. Conversely, Karpus and colleagues demonstrated that CNS depletion of catecholamines prior to the induction of EAE suppresses EAE symptoms without affecting the number of mononuclear cells infiltrating the spinal cord [12].

The present study was undertaken to examine the role of central dopamine depletion in regulation of autoimmune reaction, using EAE as a model. We used a specific neurotoxin MPTP to selectively reduce dopamine content in the CNS, and evaluated the effects of the dopamine depletion on the subsequent course of the autoimmune response in EAE.

Methods

Animals

C57BL mice (males; 8–12 weeks, 20–25 g) were housed under standard conditions with a free access to food and water. Supplementary food and water were provided on the cage floor for disabled animals.
The animals were divided into four groups:

- Control group, which received four intraperitoneal (i. p.) injections of 0,9 % NaCl in one-hour intervals, and one subcutaneous (s. c.) injection of distilled water in both flanks and nape area. The injection volumes were identical with those used in the corresponding MPTP and MOG administration protocols;
- MPTP group, which received MPTP-HCl in four i. p. injections, in one-hour intervals, at the dose of 10 mg MPTP/kg body weight/dose, resulting in the total dose of 40 mg/kg;
- in both flanks (50 µl) and nape (100 µl) areas, and one iv PT injection in the dose of 200 ng, administered on days 0 and 2 after immunization;
- MPTP+MOG group, which first received MPTP, and was immunized with MOG 35-55 seven days later, according to the schedule described for the MPTP and MOG groups, respectively.

MPTP administration

MPTP-HCl (2 mg/ml; Sigma, Poland) was dissolved in 0,9 % NaCl and delivered in four i. p. injections of 10 mg/kg b. w. each, at 1-hr intervals, to reach the total dose of 40 mg/kg. The control group received 4 i. p. injections of 0,9 % NaCl according to the same regimen as MPTP.

Induction of the experimental autoimmune encephalomyelitis (EAE)

EAE was induced by s.c. nape and both flanks injections of 150 µg of MOG 35-55 peptide (Neocomps, France) in complete Freund's adjuvant (CFA;Difco, Detroit, USA), enriched with *Mycobacterium Tuberculosis,* on day 7 after MPTP administration, supplemented by i. v. injections of 200 ng of PT (Sigma, Germany).

Preparation of Pertussis Toxin (PT) solution:

PT solution (LIST Biological Laboratories, USA) was prepared in distilled water at the concentration of 1 µg/µl.

Clinical assessment of animals

A clinical score of EAE was assigned to each animal daily for 43 days. The clinical score was graded on the scale of 0 to 5, as follows: 0, no clinical signs; 1, flaccid tail; 2, hind limb weakness and abnormal gait; 3, complete hind limb paralysis; 4, complete hind limb paralysis with forelimb weakness or paralysis; 5, moribund or deceased. Intermediate scores (–0.5) were assigned if the neurological signs exhibited a lower severity than typically observed. Several parameters of disease, as previously described [13], were examined for evaluation of the severity of EAE and the influence of MPTP treatment. The following parameters were applied:

Mean clinical score – an average of clinical scores for all mice within a group on a particular day.

Incidence – the number of mice within a group that developed a clinical score of 1 or greater, in comparison to the starting number of mice in that group.

Mean day of the onset – an average number of days from the start of the experiment to the time when animals from a given group begin to show the clinical signs of the disease.

Mean duration of course – an average of days with a clinical score of 1 or greater for all mice within a group.

Mortality – the number of mice within a group that died as a result of a very severe EAE.

Histopathological evaluation of inflammation:

For a histological evidence of EAE, four to six mice from MOG, MPTP+MOG and control groups were sacrificed on the day when clinical signs first appeared (at least 1 point of disease severity). Animals were deeply anaesthetised with chloral hydrate (400 mg/kg i. p.), and transcardially perfused with heparinized 0.9 % NaCl solution followed by 2 % paraformaldehyde-lysine-periodate fixative (PLP). The spinal cords were removed, post-fixed in PLP for 4–6 h, then immersed in 20 % sucrose solution overnight at 4 °C and rapidly frozen. Transverse sections through the lumbar segments of the spinal cord (20 µm thick), were cut with a cryostat, picked up on gelatinised slides, and stored at –20 °C until hematoxylin and eosin staining.

Every tenth section was stained, localization, number of inflammatory infiltrations and size of infiltration were determined. From every spinal cord 10 sections were chosen, mean number of inflammatory cells in the infiltration for an each animal were counted.

The presence of infiltrating cells was determined by an investigator blinded to the identity of the experimental groups.

HPLC analysis of dopamine (DA) levels in the striatum

HPLC evaluation was performed on the 7^{th} , 14^{th} , 28^{th} and 50^{th} day following MPTP intoxication.

Striata were rapidly dissected from brain tissue, weighed, homogenized in 1000 µl of ice cold 0,1 N HClO4, and centrifuged at 13,000 × *g* for 15 min. The supernatant was collected, filtered (0,2 µm pore size; Whatman, USA) and examined for its DA content.

Dopamine (standard from Research Biochemicals International, RBI), dopamine metabolite DOPAC (3,4 dihydroxyphenylacetic acid;

RBI), HVA (homovanilic acid; Sigma), 5-HT (5-hydroxytryptamine; Sigma), 5-HIAA (5-hydroxyindolacetic acid; Sigma) were measured using high-performance liquid chromatography (HPLC) with electrochemical detection and glassy carbon electrode. The electrochemical potential was set at 0, 8 V with respect to the Ag/AgCl reference electrode.

The chromatograph system consists of an autosampler automatic injector (Knauer Basic Marathon), pump (Mini-Star K-500; Knauer), an electrochemical detector (L-3500A; Merck).

The mobile phase comprised 32 mM sodium phosphate (Sigma), 39 mM citric acid (Sigma), 1 mM octan sulfonic acid (Aldrich).

54 µM ethylenediaminetetraacetic acid (EDTA, Sigma) in deionized, 18,3 mΩ polished water containing 0,15 % acetonitrile (Merck) and 6,5 % methanol (Merck).

Separation of monoamines was achieved with a C-18 column (250 mm × 4 mm reverse phase, Nucleosil, 5 µm particle size; Macherey-Nagel, Germany) and mobile phase flow rate maintained at 0,8 ml/min.

Samples were quantified by comparison with standard solutions of known concentrations using HPLC software, and area under the peaks was quantified.

Data were collected and analyzed by Eurochrom 2000 for Windows (Knauer).

Cytokine evaluation

For RT-PCR (Reverse trancriptase-polymerase chain reaction) analysis four to six mice from MOG, MPTP+MOG and control groups were sacrificed on the day when clinical signs first appeared (7th day for MPTP+MOG group, 21st day for MOG group). Total RNA was isolated from spinal cord tissue using TRI reagent (Sigma), in accordance with the manufacturer's instructions. The RNA product was resuspended in 20 µl diethyl pyrocarbonate (DEPC)-treated water. The quality of RNA samples was confirmed by the electrophoresis of RNA through the 1,5 % agarose gel containing ethidium bromide and visualization by UV illumination. The RNA was stored at –70 °C until use. Total RNA was reverse transcribed at 42 °C for 1 h with moloney murine leukemia virus (MMLV) reverse transcriptase according to the instruction of the manufacturer of the reagent (Sigma). Following the RT reaction the cDNA products were stored at –20 °C until use. The cDNA was amplified using adequate primers.

IFNγ -5'-AGC GGC TGA CTG AAC TCA GAT TGT AG-3'
5'-GGTC ACA GTT TTC AGC TGT ATA GGG-3'
-size (bp) 243

TGF β -5'-CGG GGC GAC CTG GGC ACC ATC CAT GAC-3'
5'-CTG CTC CAC CTT GGG CTT GCG CAA CAC-3'
- size (bp) 406

IL-1β -5'-TCA TGG GAT GAT GAT GAT AAC CTG CT-3'
5'-CCC ATA CTT TAG GAA GAC ACG GAT-3'
- size (bp) 502

GAPDH -5'-TGA AGG TCG GAG TCA ACG GAT TTG GT-3'
5'-CAT GTG GGC CAT GAG GTC CAC CAC-3'
- size (bp) 493

Negative control reaction without template or MMLV reverse transriptase was included in PCR amplification with primer set in parallel. As a control to eliminate variations for sample-to-sample differences in RNA extraction and convertion to cDNA, we amplified the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The thermal cycling parameters were as follows:

IFN-γ, IL1-β, –95 °C, 2 min; 94 °C, 50 s; 60 °C, 50 s, 35 cycles; and 72 °C, 5 min; TGF-β_1,: 94 °C, 1 min; 60 °C, 30 s; 72 °C, 30 s, 30 cycles; and 72 °C, 7 min. GAPDH: 94 °C, 5 min; 94 °C, 30 s; 57,5 °C, 45 s; 72 °C, 1 min; 30 cycles; and 72 °C, 10 min. PCR products were separated on 1,5 % agarose gels stained with ethidium bromide and recorded under UV light with camera linked to an image analyzer (One-descan, Scanalitics. Inc.). The result was evaluated as a relative unit determined by normalization of the optical density (OD) of cytokine band to that of the GAPDH band. Two or three cytokine PCR assays per sample were performed.

Statistical analysis

Data were analysed by one-way analysis of variance (ANOVA) using Statistica for Windows software. For comparison of data between two groups, Mann-Whitney U-test was used. The differences were considered significant at the level of $p < 0.05$.

Results

1. EAE course

Clinical signs of EAE were observed in all the animals which received MOG 35-55.

In mice from the MOG group, EAE had a monophasic form. Specifically, until day 18, the mean clinical score did not exceed 0,6 points. The mean clinical score reached its maximum (2,7 points) on day 20. From day 21, the mean clinical score gradually decreased, reaching the level of 0,5 points on day 23 (Fig. 1). The mean day of the onset was 18,8 ± 4,02 and the mean duration time of the course was 3,17 ± 0,75 days (Table 1).

In mice with injured dopaminergic system (MPTP+MOG group), EAE showed a biphasic form (Fig. 1). The first EAE relapse was observed 6 days after the immunization. The maximal clinical score reached 2,4 points, and it lasted 6 days. Mean clinical scores, during first five days, were thus significantly higher as compared to MOG group ($p < 0,01$). The second relapse started on day 16th, when it reached 2,75 points, and lasted for 12 days. Mean clinical scores were similar as in MOG group, and was higher only on day 18 ($p < 0,05$). From day 24th, the mean clinical score was 0,5 points. The complete remission of symptoms was observed from day 38th (Fig. 1). The mean day of the onset was earlier as comparing to MOG group ($p < 0.05$), and the mean duration of a course was longer than in MOG group ($p < 0.05$) (Table 1).

2. Evaluation of the inflammatory infiltrates in the spinal cord

In the lumbar spinal cord, the inflammatory infiltrates containing lymphocytes and macrophages were visible on almost every section. Infiltrates located usually around the vessels forming typical cuffs but some cells spread into tissue.

Table 1. Parameters of EAE course in MPTP+MOG and MOG groups.

Group	MPTP+MOG	MOG
Incidence	100 % (34/34)	100 % (19/19)
Mean day of onset	7,33 ± 4,37*	18,8 ± 4,02
Mean duration of course	9,87 ± 2,13*	3, 17 ± 0,75
Mortality	11,7 %	37 %

* significant difference between groups $p < 0,05$

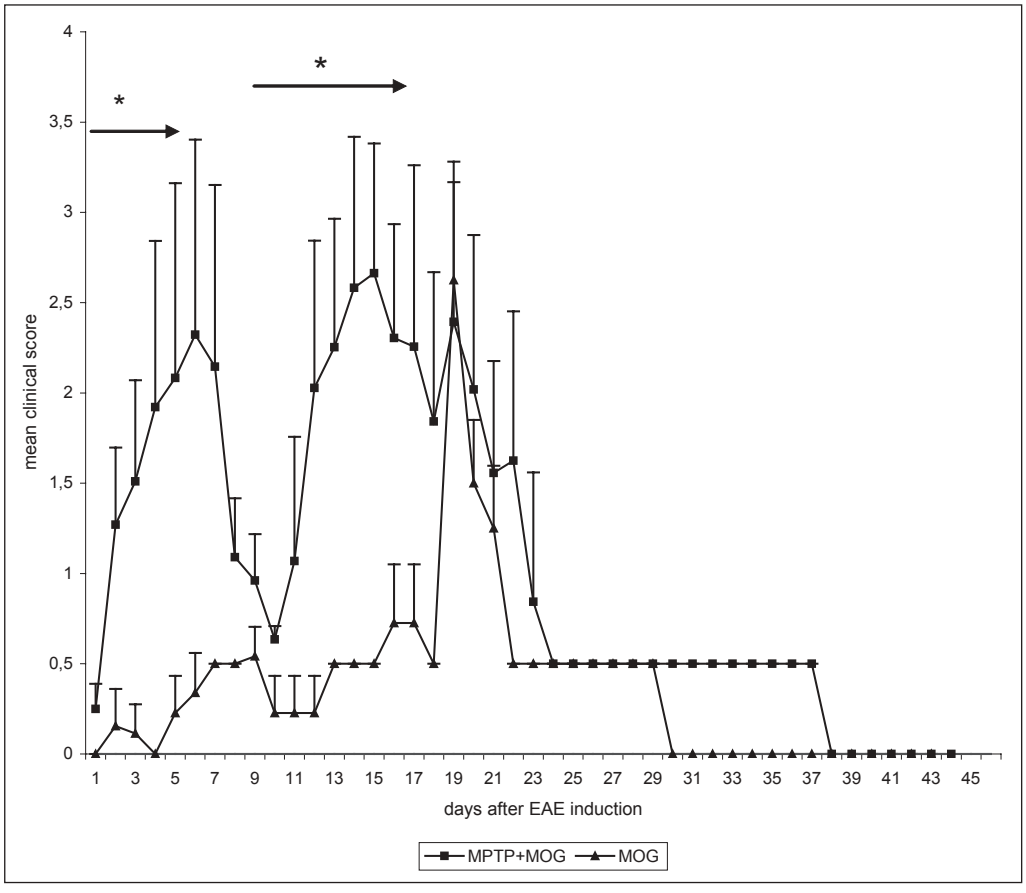

Fig. 1. Mean clinical scores of EAE in mice from MPTP+MOG and MOG groups (cumulative data from 3 observations; MPTP+MOG -34 animals and MOG -19 animals). Stars above arrows denote level of significance comparing MPTP+MOG mice with MOG mice for the particular days (Mann Whitney U test, p<0, 05).

Table 2. Evaluation of inflammatory infiltrates in the spinal cord. Stars show the mean value with ±SEM

Group of mice	Mean number of inflammatory cells in the infiltration	Mean number of infiltrations/section
MPTP+MOG	39,1* ± 7,26	0,35 ± 0,06
MOG	14,1 ± 3,82	1 ± 0,43

* Significant difference comparing to MOG group p < 0,05

The mean number of the inflammatory cells in the infiltration was significantly higher in mice from the MPTP+MOG group as compared to the MOG group (p < 0,05) (Table 2).

3. Evaluation of dopamine content in striata

Dopamine concentrations were measured on 7th, 14th, 28th, 50th day after MPTP intoxication. Mice which had not been treated with MPTP did not show any decreases in striatal dopamine content assessed at the beginning and at the end of the observation (Fig. 2). Mice from the MPTP+MOG group exhibited a significant 37 % decrease in the striatal dopamine concentration on the EAE induction day, i. e. day 7, as compared to vehicle-treated animals. On day 14, i. e. 7 days after the EAE induction, striatal dopamine content constituted only 25 % of the control value (control 7) (p < 0.05). Although dopamine levels began to climb in subsequent weeks (41 % of control on day 28, and 66 % on day 50), all these values were significantly lower compared to control (p < 0.05).

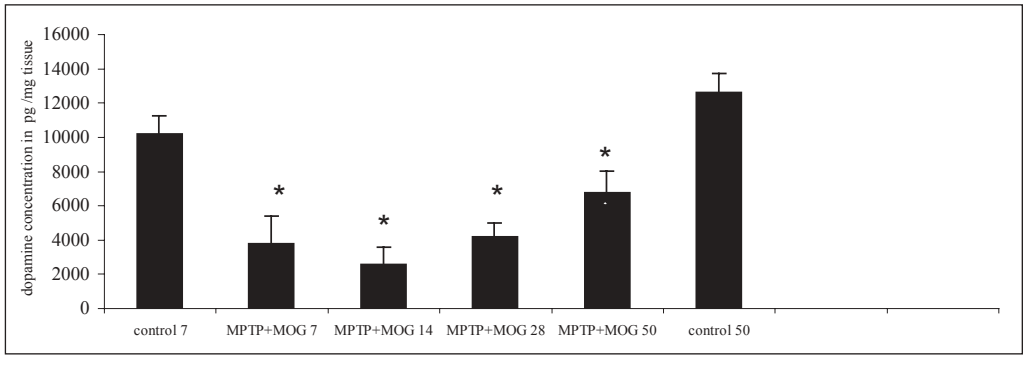

Fig. 2. Dopamine concentrations in striata of control and MPTP+MOG animals during observation (7, 14, 28 and 50 days after MPTP administration). Bars show the mean value with±SEM of 6 animals per group per day

* significant difference comparing to control group p<0,05

4. Cytokines mRNA content in the spinal cord

Expression of mRNA of three cytokines (IL-1β, IFN-γ and TGFβ) were assessed in the spinal cord of control, MPTP, MOG and MPTP+MOG animals groups on the day when clinical signs firstly appeared .

In MPTP group no changes in cytokines mRNA expression in the spinal cord were observed. Expression of IL-1β mRNA was significantly increased only in MPTP+MOG group as compared to control ($p < 0,02$) and was significantly higher than in MOG group ($p < 0,02$).

In MOG group the expression of IL-1β was similar as in the control.

The expression of IFN-γ did not change during the relapse in both groups of immunized animals.

TGF-β concentration was significantly decreased both in MOG group ($p < 0,05$) and in MPTP+MOG group ($p < 0,02$), comparing to the control. However, the decrease in MOG group was greater than in MPTP+MOG group ($p < 0,05$).

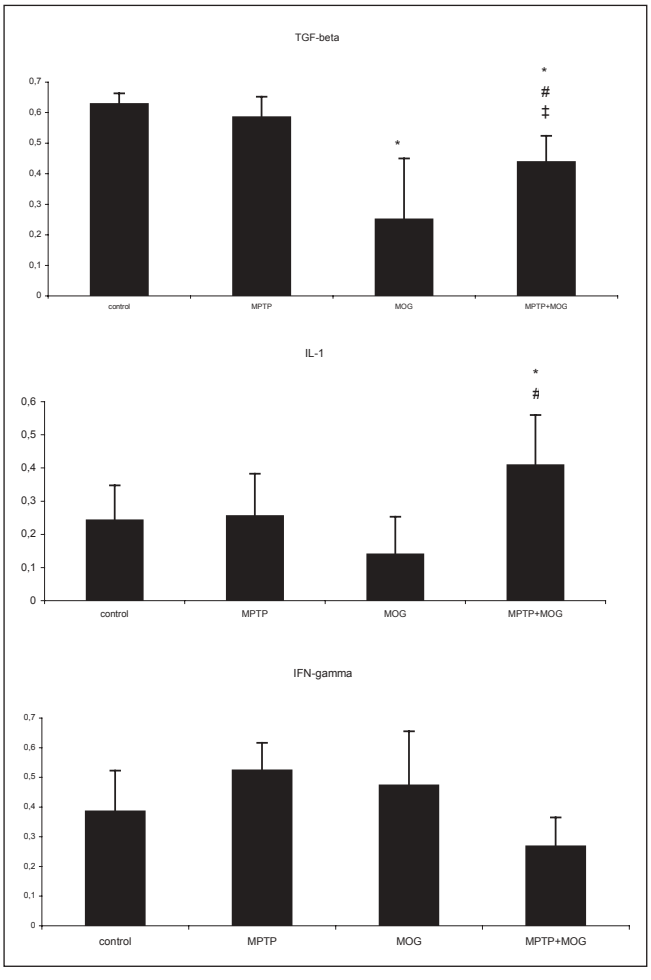

Fig. 3. Cytokines mRNA expression in the spinal cord in control and in experimental groups on the day of first clinical signs. Bars indicate mean + SD of 6 animals/ group.
significant difference comparing to MOG $p< 0,05$, * significant difference comparing to control $p<0,05$, ‡ significant difference comparing to MPTP $p<0,05$

Discussion

The novel finding of our study is that the injury of the dopaminergic system evoked by MPTP administration modulates clinical course and inflammatory reaction during EAE. We observed earlier onset of the disease in mice with injured dopaminergic system as well as changes in clinical course of the disease. Similarly, an inflammatory reaction in the spinal cord was more pronounced in MPTP-treated mice.

Besides that dopamine itself was shown to modulate immune cells, central dopamine could affect local immune responses, leading to changes in the autoimmune response. Several lines of evidence for the central regulation of the immune system by the nigrostriatal system were obtained from studies on patients suffering from Parkinson's disease. The total number of lymphocytes decreases and changes in CD4+ and CD8+ lymphocyte subpopulations are noted [14]. An increased concentration of adenosine deaminase activity – a proof for the lymphocyte activation, have been correlated with Parkinson's disease [15]. It has been previously shown that MPTP administration affects immune responses: decreases mitogen-induced splenic lymphocyte proliferation and a number of antibody-forming splenic cell population. All these data show decrease of inflammatory response after central dopamine depletion i. e.MPTP administration. Our results demonstrated, however, more intensive autoimmune response in MPTP treated mice: earlier beginning of the disease, two phases vs one and with higher clinical score, as well as higher cumulative disease index. The mortality rate in mice with injured dopaminergic system was lower than in only MOG 35-55-treated mice.

Neurotransmitters concentrations alterations during autoimmune reaction in various CNS regions have been shown by various authors. White et al. [16], showed, that both NA and DA are depleted in the spinal cord of mice suffering from EAE. Similarly, Krenger et al. [17], showed that during entire course of EAE levels of 5-HT and NA are reduced in the spinal cord. In the craniothoracical region 5-HT concentration was unchanged; NA was reduced during the relapse, returning to normal level during the first recovery. In these studies alteration in catecholamines might be a consequence of local inflammation, or of the injury to the brain stem catecholaminergic neurons. Recently, Hofstetter et al. showed, reduced immune cell infiltration and a reduced number of MOG p 35-55 specific IFN-gamma producing cells in female knockout mice lacking the 5-HT transporter [18].

The exacerbation of EAE symptoms has been shown by Chelmicka-Schorr et al. [19] after 6-OHDA -mediated sympathetic nervous system injury. An increase of the humoral response, as well as a decreased number of T suppressor lymphocytes in spleens of animals treated with 6-OHDA, have been also shown by Miles et al. [20]. It has been postulated that exacerbation of EAE signs can be caused by inflammatory cell activation, as well as by an increase in proinflammatory cytokine production. [3] In our study, we also observed significantly increased expression of IL-1β mRNA in MPTP+MOG group that correlated with clinical progression of the disease.

IL-1β is involved in the pathogenesis of experimental autoimmune encephalomyelitis (EAE) and multiple sclero-

sis (MS) [21]. It is suggested to act as a mediator in neuronal apoptosis in vitro and in vivo, IL-1β mRNA has been found in MS plaques and in CSF of the EAE mice [22]. IL-1β also acts as an important regulatory factor in inflammation by modulation of various endothelial cell functions. It stimulates astrioglosis and expression of a number of adhesion molecules [23]. Interleukin-1 receptor antagonist (IL-1Ra) moderates the induction of experimental autoimmune encephalomyelitis (EAE) [24]. In our study we observed significant increase in IL-1β mRNA expression in the spinal cord only in MOG+MPTP as compared to control. We suspect that this increase could be responsible for observed changes in clinical course of the disease. Proinflammatory cytokine IL-1β could stimulate T cell proliferation and B cell activation, thus causing more severe EAE course.

Increased IL-1β mRNA expression could not be a direct result of MPTP intoxication, because in MPTP group we did not observe any changes in IL-1 level. Increased IL-1 level may be than possibly caused by disturbed central dopamine content. We observed also decrease in TGFβ concentration in both MOG and MPTP+MOG groups as compared to control. Changes of TGFβ expression in the spinal cord has been shown in a wide array of diseases, including MS and EAE [25]. Systemic injection of small amounts of TGFβ has a profound effect in protecting mice from the inflammatory demyelinating lesions of EAE [26]. TGF β production by the antigen induced regulatory T cells has been shown to be the mechanism responsible for prevention of CNS autoimmunity after epicutaneous application of myelin basic protein (MBP) to the skin prior to the induction of EAE [27].

TGFβ concentration was shown to be elevated during remission phases of EAE [28].

Higher IL-1β mRNA and low TGFβ mRNA expressions in MPTP + MOG group, as well as more severe inflammation in the spinal cord are going well together with more severe clinical course of EAE in these mice. These changes were not just caused by MPTP as in MPTP group no alteration in cytokines mRNA expressions in the spinal cord were observed. They might be caused by some central stimuli connected with dopamine depletion in the nigrostriatal system.

In summary, the observed changes of EAE course, in mice treated with MPTP were associated with an enhanced inflammatory reaction in the spinal cord, as well as with disturbances of the balance among some cytokines. Dopaminergic neuron injury leads to the decrease in dopamine concentration in striatum, which probably produced an alteration of the autoimmune response and progression of the inflammation in the spinal cord. Thus, our study showed that autoimmune reaction was regulated by dopamine system in the brain. Implementation of this novel and promising model may prove extremely beneficial in exploring the role of the dopaminergic system in the pathogenesis of EAE.

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