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LIPOPOLYSACCHARIDE CHANGES SIALYLATION PATTERN IN THE MOUSE CENTRAL NERVOUS SYSTEM

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Sialylated glycoconjugates seem to play crucial role in the mechanisms that control the most important functions of the body. Sialylation is an important mechanism for the regulation of intercellular interactions that underlie neuronal plasticity as well as immune defense in the central nervous system (CNS). In this study, we analyzed the effect of lipopolysaccharide (LPS) on sialylation pattern in several regions of CNS. Additionally, we tested the effects of inflammatory stimulation on Siglec-F expression in microglial cells. Using lectin blotting with *Maackia amurensis* and *Sambucus nigra* agglutinins and immunostaining with antibody directed against PSA-NCAM we demonstrated altered expression of sialylated glycoconjugates differentially due to LPS-induced inflammation. We found that LPS caused significant increase of α2,3- and α2,6-linked sialic acids in the hippocampus and spinal cord. In the prefrontal cortex, the level of α2,3-linked sialic acids in selected glycoconjugates tended to be increased ($p>0.05$), while α2,6-linked sialic acids were reduced ($p<0.05$), while the expression of PSA-NCAM in all analyzed structures were significantly higher in comparison to the control group. The expression of Siglec-F in microglial cells stimulated with LPS remained unchanged. Given the significance of glycans in the brain biology we can conclude that sialic acids and their receptors Siglec may be crucial regulators of immune response in the CNS.

Key words: *brain, microglia, inflammation, lipopolysaccharide, sialic acid, Siglec-F, inducible nitric oxide synthase, interleukin -1beta*

INTRODUCTION

Neurodegeneration in the central nervous system (CNS) is strongly associated with innate immunity. Immune response in the brain is mediated by intracellular interactions of microglial cells with components of microenvironment which regulate signalling pathways responsible for activation or inhibition of immune cells (1-3). Sialic acid, monosaccharide covalently attached to the end of oligosaccharide chains in glycoproteins and glycolipids, is one of the most important glycans expressed at the cell surface (4, 5). In contrast to their role as inhibitors of adhesion, sialic acids act as ligands for several carbohydrate binding proteins which regulate recognition processes in innate immunity (6-8). Sialic acids are the ligands for the Siglec receptor family (Sialic acid binding immunoglobulin-like lectin) that play important role in immune defense during the initial contact of cells with pathogens may be crucial in degenerative and regenerative processes. Changes in the level of sialoglucoconjugated macromolecules were detected in several diseases such as cancer, diabetes, alcoholism and after exposure to degenerative factors such as stress, heavy metals and narcotic substances (9-15). Elevated concentrations of sialylated glycoproteins in serum are known as a marker of the acute phase response to inflammatory stimuli (16). Similarly, high amount of sialolyglycoconjugates were detected in synovial tissues of acute rheumathoid arthritis cases (17, 18). Recent studies showed that intracerebral injection of amyloid-β induces proinflammatory cytokine production which may correspond with alteration of sialylation pattern in the hippocampus (19, 20). These alterations of the glycocalyx can be recognized by immune cells *via* sialic acid binding receptors and induce classical inflammatory or regenerative processes. There is no information on the sialylation state in the CNS after exposure to acute inflammatory stimuli. Interestingly, several studies have demonstrated that sialic acids and their receptors such as human Siglec-11 and mouse Siglec-F may be involved in neuroprotection in neuron-microglia co-cultures (21, 22). In this study we address the question whether inflammatory stimuli may influence sialylation state of glycoproteins in the mouse CNS. We analyzed the effects of acute LPS administration on the expression of α2,3-, α2,6- and α2,8-linked sialic acids as well as their receptor Siglec-F in the CNS tissues.

MATERIAL AND METHODS

Animals

Female BL6/C57 mice, weighing 19–20 g were housed in groups of five per cage, under constant temperature (22±2°C) and lighting (12 h light/dark cycle) conditions. The experimental procedures were conducted between 8:00 and 13:00. The animals had free access to food and water. Mice were injected daily (i.p.) for 4 days with LPS (1 mg/kg, Sigma Aldrich) or appropriate volume of 0.9% NaCl. Four hours after final

injection mice were decapitated, CNS tissues were removed on ice and the hippocampus and prefrontal cortex were dissected out from the brain (3). Tissues were homogenized in extraction buffer (6 mM Tris-buffer, 10 mM EDTA and 2% SDS, pH 7.0) as described Braza-Boils *et al*. (23) and stored at –80°C until assay. All described procedures were approved by the local Ethics Commission for Animal Experimentation.

Isolation of microglia from adult mouse brain

Brain tissues prepared from control and LPS treated mice were homogenized in 5 ml cold 10% fetal serum in PBS (4°C) and filtered through a 40 µm nylon mesh under constant agitation. Percoll gradient was prepared by underlaying 4 ml of 30% Percoll with 37% Percoll (Sigma Aldrich). Filtered tissue homogenates were diluted with PBS and centrifuged at 1500 rpm at room temperature for 5 min. Pellets were resuspended in 70% Percoll and added to the bottom of the tube by long infusion needle underlaying 37% Percoll. Samples were centrifuged at 2000 rpm at RT for 30 min without braking. Myelin collected in 30% Percoll was removed using water pump and the interface between 37% and 70% Percoll was collected, washed with PBS and centrifuged at 1500 rpm 4°C for 5 min. Cells were collected, homogenized in extracting buffer and stored at –80°C until assay.

Microglial cell cultures

Embryonic stem cells derived microglia (ESdM) were a gift from Prof. Harald Neumann (Life&Brain Center, University of Bonn). Cells were cultured in DMEM/F-12 supplemented with 1% N2 supplement (Invitrogen) and 5% fetal calf serum (Sigma Aldrich). Cells were stimulated with LPS (500 ng/ml) for 48 h. After experimental procedure cells were collected, homogenized in extracting buffer and stored at –80°C until assay.

Western blot

Protein (20 g) from brain or cellular homogenates were loaded into 10% SDS-polyacrylamide gel, electrophoresed and transferred to PVDF membrane. Blots were incubated with primary monoclonal antibodies directed against Siglec-F (R&D System, 1:1000), PSA-NCAM (Milipore&Chemicon, 1:1000), Iba1 (Wako, 1:1000), IL-1β (Abcam, 1:1000), iNOS (Abcam, 1:1000) and β-actin (Sigma Aldrich, 1:3000) at 4°C overnight and alkaline phosphatase-conjugated secondary antibody (1:10,000, Sigma Aldrich) for 1 h at room temperature. Immunoreactive proteins were visualized with BCIP/NBT Liquid Substrate System (Sigma Aldrich) for alkaline phosphatase. Membranes were scanned and analyzed densitometrically using Quantity One software (Biorad Laboratories, Inc.). The densitometric values were expressed as a percentage of control (100%). Protein concentration in each sample was estimated by the method of Bradford using bovine serum albumin as a standard.

Determination of α2,3- and α2,6-linked sialic acids

The α2,3- and α2,6-linked sialic acids in the mouse brain homogenates were analyzed using the DIG Glycan Differentiation Kit (Roche, Germany) following the manufacturer's instructions. Terminal sugar structures were recognized by *Maackia amurensis* agglutinin (MAA) and *Sambucus nigra* agglutinin (SNA), that bind α2,3- and α2,6-linked sialic acids, respectively.

Statistical analysis

The data are expressed as means ±S.D. For statistical analysis an unpaired two-tailed t-test (InStat, GraphPad Software, San Diego, USA) was used. Significant differences were deemed at $p<0.05$.

RESULTS

Effect of lipopolysaccharide on Siglec-F, Iba1, IL-1β and iNOS expression in microglial cells

To study the effects of LPS on microglial cells, we stimulated cultured ESdM with LPS (500 ng/ml) for 48 h. To confirm microglial immune response *in vivo*, BL6/C57 mice were treated with LPS (1 mg/kg) for 4 days and then microglial cells were isolated in Percoll gradient. Western blot analysis was performed to analyse the protein expression of Siglec-F, Iba1, IL-1β and iNOS in cell lysates. The results are presented in the *Figs. 1A, 1B*. Unpaired t-test of IL-1β level in cultured ESdM, expressed as percent of control, evidenced statistically significant differences between control and LPS treated group ($t=11.159$, $df=18$, $p<0.05$). Lipopolysaccharide significantly increased IL-1β by 76.36% as compared to the control group. Similarly, systemic administration of LPS significantly increased IL-1β expression by 7% in mouse microglial cells ($t=2.185$, $df=18$, $p<0.05$). Unpaired t-test of iNOS expression in ESdM culture showed statistical differences between control and LPS-treated group ($p<0.05$, $t=13.469$, $df=18$). LPS significantly (29%) increased expression of iNOS in cultured microglial cells. This tendency (4.29%) in LPS-treated animals was insignificant ($p>0.05$, $t=1.096$, $df=18$). Expression of Iba1 in cultured ESdM cells exposed to LPS was significantly increased (by 12%) as compared to the control group ($p<0.05$, $t=7.630$, $df=18$) whereas this tendency in microglial cells derived from LPS-treated animals tended to be increased (by 9%) but was not significant ($p>0.05$, $t=1.472$, $df=18$). Siglec-F was detected in microglial cell lysates as protein of about 65 kDa. LPS decreased Siglec-F expression in ESdM culture and microglia isolated from adult brain tissue by 2.37% and 2.07%, respectively, as compared to the not stimulated cells. These differences were not significant ($p>0.05$, $t=0.3$, $df=18$).

Lipopolysaccharide increases PSA-NCAM expression in the mouse central nervous system

Analysis of PSA-NCAM immunolabelled by monoclonal antibody showed that systemic inflammation had statistically significant effect on its expression in selected structures of the central nervous system. In detail, repeated administration of LPS for 4 days increased PSA-NCAM expression in mouse hippocampus, prefrontal cortex and spinal cord by 75% ($p<0.05$, $t=17.768$, $df=18$), 69% ($p<0.05$, $t=12.771$, $df=18$) and 52% ($p<0.05$, $t=15.573$, $df=18$), respectively, as compared to the control group (*Fig. 2*).

Lipopolysaccharide- induced changes in α2,3- and α2,6-linked sialic acids in the mouse central nervous system

Representative Western blots and corresponding density histograms presented in the *Fig. 3* illustrate how LPS modulated the amount of glycoproteins containing α2,3- and α2,6-linked sialic acids in several regions of the mouse central nervous system. Detailed statistical analysis of selected peaks showing highest differences between both groups is presented in *Table 1*. For example, a densitometry analysis of hippocampal glycoproteins visualized as bands with molecular weight of about 100 kDa (bands B2) showed LPS-induced significant increase in α2,3- and α2,6-linked sialic acids by 41% ($p<0.05$, $t=7.523$, $df=8$) and 38% ($p<0.05$, $t=2.851$, $df=8$), respectively,

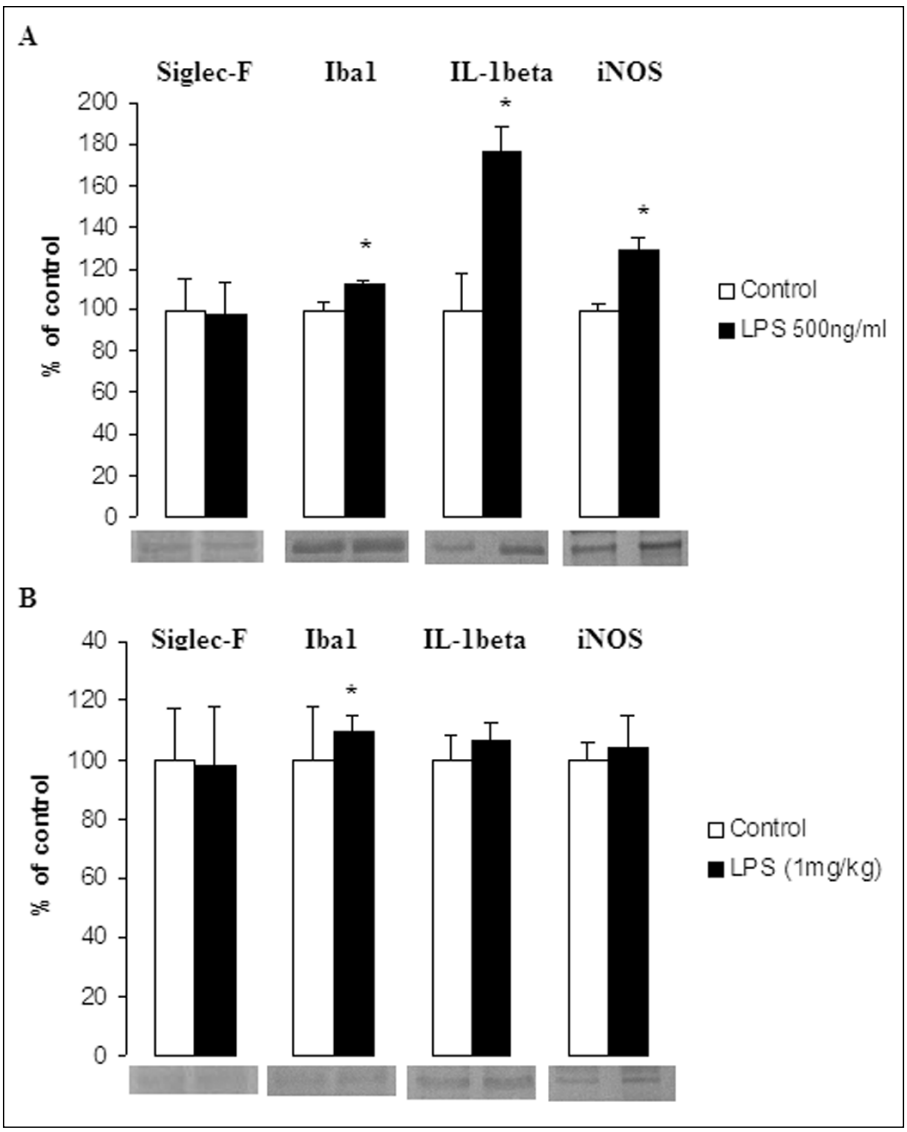

Fig. 1. Effect of lipopolysaccharide on Siglec-F, Iba1, IL-1β and iNOS expression in cultured microglia (A) and microglial cells isolated from CNS tissues (B). Representative Western-blots and corresponding quantification bars (mean expression ±S.D. of the values obtained from n=10 mice) are shown. Data are presented as a percentage of control group; *p<0.05 *vs*. control.

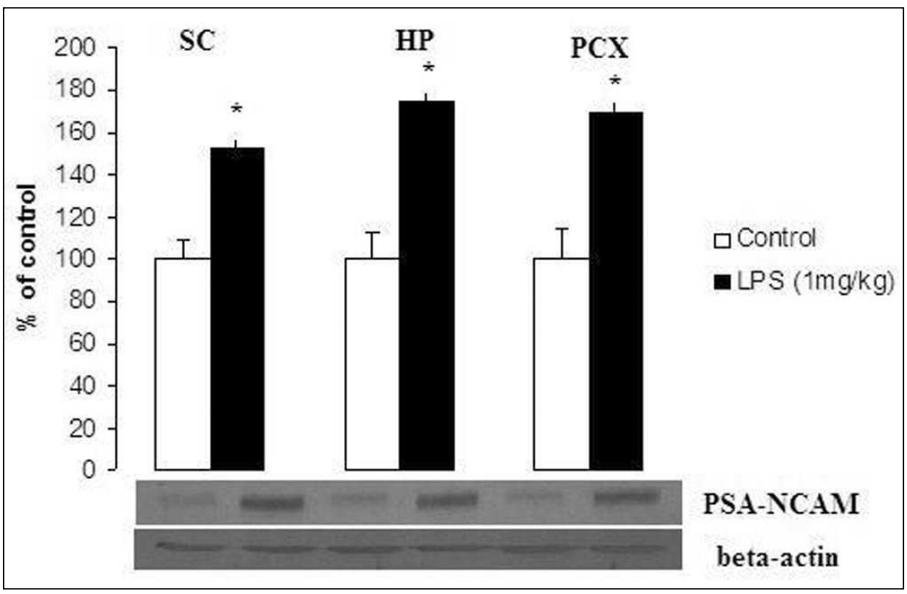

Fig. 2. Representative Western-blots PSA-NCAM in spinal cord, hippocampus and prefrontal cortex of control mice and mice treated with lipopolysaccharide (LPS, 1 mg/kg, i.p.). Corresponding quantification bars (mean expression ±S.D. of the values obtained from n=10 mice) are shown. Data are presented as a percentage of control group. *p<0.05 *vs*. control.

as compared to the control group. In contrast, glycoproteins in prefrontal cortex visualized as bands B2 with molecular weight of about 100 kDa showed significant decrease in α2,3- and α2,6-linked sialic acids by 22% ($p<0.05$, $t=3.953$, $df=8$) and 40% ($p<0.05$, $t=3.414$, $df=8$), respectively, as compared to the control group.

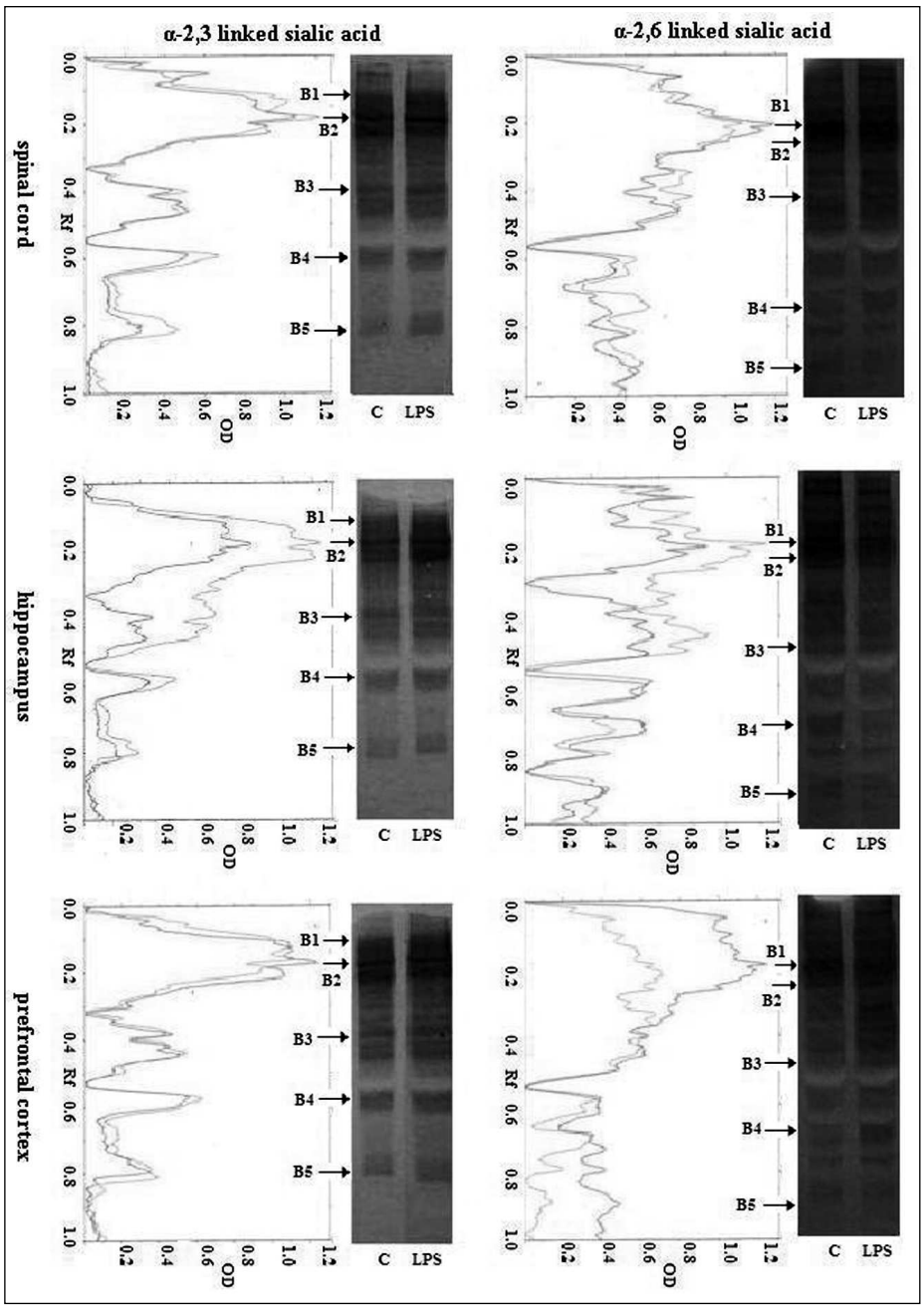

Fig. 3. Representative Western-blots of α2,3- and α2,6-linked sialic acids in spinal cord, hippocampus and prefrontal cortex of control (C) and lipopolysaccharide treated mice (LPS, 1 mg/kg, i.p.). Corresponding density histograms are also shown (brown histograms - control; green histograms - group treated with LPS).

DISCUSSION

Given the importance of cell surface glycosylation in the brain, changes in the composition of sugar chains of glycoproteins and glycolipids can be crucial for the processes of repair and regeneration of CNS after injury and exposure to degenerative factors. In the present study we addressed the question whether inflammatory stimuli modulate degree of sialylation of proteins expressed in the mouse CNS. To investigate the influence of inflammatory factors on sialylation state in the mouse CNS we used the model of systemic inflammation which was based on the intraperitoneal administration of LPS for 4 days as described Cardona *et al*. (3). Lipopolysaccharides from many bacterial species initiates acute inflammatory response in mammals that is typical of the host reaction to tissue injury or infection (24, 25). Therefore that inflammation is a critical component of neurodegenerative diseases, LPS is widely used as a modulator of neuropathology in animal models (26, 27). The mechanism whereby LPS induces CNS injury is not fully understood but seems to be linked with toll-like receptor 4 (TLR4) activation and initiation of inflammatory response resulting in systemic hypoglycemia, perturbation of coagulation, cerebral hypoperfusion, and activation of inflammatory cells in the CNS (28). Numerous experimental data showed that LPS induces cerebral inflammatory response leading to white matter and myelin damage as well as degeneration and reduction in dopaminergic neurons (28-30). Nolan *et al*. demonstrated that systemic injection of LPS blocks the expression of long-term potentiation (LTP) in the hippocampus which is coupled with increased IL-1β concentration and c-Jun NH(2)-terminal kinase activity, as well as an increase in the number of cells displaying apoptotic features in the hippocampus (31). Structural and functional changes in the brain induced by CNS remain in close relationship with subsequent behavioral disturbances such as social inhibition, depressive-like syndrome and learning deficits

Table 1. Densitometric quantification of selected glycoproteins (*Fig. 3A-E*; peaks B1-B5) expressing α2,3- or α2,6-linked sialic acids separated by SDS-PAGE and labeled with MAA or SNA lectins. Data (mean expression ±S.D. of the values obtained from n=5 mice) are presented as a percentage of control group; *p<0.05 *vs*. control.

	α2,3-linked sialic acid	***α2,6-linked sialic acid***
Spinal cord		
B1	118 ± 13% *	87 ± 13%
B2	110 ± 10%	99 ± 6%
B3	118 ± 14% *	127 ± 14% *
B4	117 ± 5% *	177 ± 32% *
B5	166 ± 15% *	103 ± 12%
Hippocampus		
B1	148 ± 23% *	132 ± 24% *
B2	141 ± 10% *	138 ± 31% *
B3	629 ± 46% *	143 ± 12% *
B4	140 ± 31% *	74 ± 28%
B5	144 ± 24% *	104 ± 17%
Prefrontal cortex		
B1	100 ± 6%	54 ± 18% *
B2	78 ± 5% *	60 ± 20% *
B3	270 ± 27% *	101 ± 16%
B4	117 ± 45%	80 ± 18%
B5	112 ± 17%	48 ± 13% *

measured by different memory tests (32-35). Although the influence of LPS on brain functions is widely known, some effects such as modulation of sialylation degree and its importance in neurodegeneration and/or regeneration remains unclear. From lectin blotting with MAA (specific for α2,3 sialic acids) and SNA (specific for α2,6 sialic acids) and immunostaining with monoclonal antibody 2-2B directed against PSA-NCAM (specific for α2,8 sialic acids), we demonstrated the expression of brain membrane sialylated glycoproteins changed by LPS-induced inflammation. As expected, in all analyzed regions of mouse CNS we have found expression of PSA-NCAM, which was strongly increased, particularly in the hippocampus, after LPS administration. These observation are not surprising because expression of PSA-NCAM has been found to be sensitive indicator of many degenerative processes in the brain. The level of PSA-NCAM in the CNS is normally low except the hippocampus where its high level is associated with intense neuronal plasticity observed during the formation of memory traces. Previous studies have demonstrated that almost any degenerative process in the brain appears to be accompanied by changed PSA-NCAM expression. Fostel *et al*. (36) have demonstrated that PSA-NCAM immunoreactivity increases in the mouse spinal cord following peripheral inflammation induced by bilateral, intraplantar injection of 6% carrageenan. Authors suggest that these changes may be indicative of spinal synaptic rearrangement associated with chronic pain. Furthermore, the analyzed brain tissue of patients with Alzheimer's disease and Parkinson's disease showed increased neurogenesis in several brain regions manifested in upregulation of PSA-NCAM expression (37, 38). Similar observation was noted in mice model of amyotrophic lateral sclerosis (39). It may present compensatory mechanism directed toward the replacement of functional neurons in damaged brain areas. As it is widely known, this mechanism does not counterbalance for further loss of these cells which can be caused by the toxic effects of surrounding environment but it may suggest that promoting of polysialylation during some neurodegenerative disorders may be helpful in repair processes. On the other hand, observations of patients with multiple sclerosis do not confirm these conclusions. Myelination in the CNS depends on communication between damaged axons and myelin-forming cells but increased expression of PSA-NCAM in demyelinated area inhibits repair processes whereas enzymatic removal of PSA with EndoN intitiates myelination and enhances efficiency of this process (40). In addition to changed expression of PSA-NCAM, our study revealed that proinflammatory stimulation also increases the expression of α2,3- and α2,6-linked sialic acid residues in the CNS. Both, hippocampus and spinal cord, presented higher profile of sialylated glycoconjugates while in the prefrontal cortex these changes were not so significant. Jou *et al*. suggest that sialic acids, predominantly contained in brain glycolipids, can trigger inflammatory responses *via* TLR4 in brain glia (41). It was shown that gangliosides can be released by damaged neurons into the extracellular space resulting in production of various inflammatory mediators such as cytokines and inducible nitric oxide synthase (42, 43). Findings from *in vitro* and *in vivo* studies suggest that several cytokines secreted in response to inflammatory stimulation can increase excitability of the brain leading to neuronal changes in brain areas associated with behavior (44-46). Altered sialic acid expression on the neuronal surface can impact neuron polarization which is in line to altered neuronal excitability observed after injury (47-51). In addition, sialic acids seem to be important modulators of immune response during brain pathology. In this study we analyzed additionally the effects of LPS on Siglec-F expression in microglial cells. Both cultured ESdM and primary microglia isolated from tissue did not present significant differences in Siglec-F expression after inflammatory stimulation. Interestingly, the altered level of IL-1β and iNOS was accompanied by increased expression of microglial marker Iba1 suggesting a positive response to the inflammatory stimulation, and this effect was significantly stronger *in vitro*. Yang and Wang (52) suggest that active or inhibitory immune responses may depend on different phase of inflammation. It was shown that during acute phase Siglecs containing immunoreceptor tyrosine-based activation motif (ITAM) may promote activity of immune cells by binding with sialylated glycoconjugates on the cellular surface. During the chronic phase the surface of immune cells is dominated by Siglecs containing immunoreceptor tyrosine-based inhibitory motif (ITIM) that reduces cytotoxicity of these cells (52). The *in vivo* study with Siglecs precisely involved in particular phases of CNS inflammation may be interesting observation in the future. Finally, it is difficult to conclude

whether changes in the sialylation state during inflammation is associated with altered expression of individual glycoproteins or is linked with severity of the mechanisms responsible for transferring of sialic acids to the protein cores. Our previous experiments showed that intraperitoneal injection of corticosterone, potent anti-inflammatory agent, reduces PSA-NCAM expression and sialidase activity in the rat hippocampal synaptosomes (53). Coughlan *et al*. (54) demonstrated that corticosterone and other adrenal gland hormones influence the sialic acid metabolizing enzymes in the brain. Analysis of mRNAs for sialyltrasferases involved in the synthesis of sialylated glycoconjugates revealed the up-regulated expression of β-galactoside α2,3-sialyltransferases (ST3Gal I and ST3Gal III), β-N-acetylgalactosaminide α2,6-sialyltransferase (ST6GalNAc) and β-galactoside α2,6-sialyltransferase (ST6Gal I) during liver and brain inflammation or injury in mouse (16, 28). Incubation of macroscopically healthy fragments of human bronchial mucosa with proinflammatory interleukin-6 (IL-6) or interleukin-8 (IL-8) significantly increases expression of α2,3- and α2,6-sialyltransferases suggesting that inflammation may affect glycosylation of various glycoproteins (55).

In this study we showed that inflammation in the CNS results in increased amounts of sialic acids which are key determinants of degenerative processes in the brain. Depending on the pathogenesis of neurodegeneration, both promoting and inhibition of sialylation can be useful. The current state of knowledge about therapeutic ways in neurodegeneration suggests recruitment of stem cells into damaged regions of brain (56). According to recent studies sialic acids are crucial factors for chemotactic migration of transplanted embryonic stem cell-derived precursors and interaction with the host brain environment (57-59). Recognition of sialylated molecules by several Siglec receptors can limit damage by immune cells during brain inflammation. Better understanding of precise mechanisms underlying sialylation and its importance in the CNS can open new therapeutic way in neurodegenerative disorders.

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