

IL-10 Gene Knockout Reduces the Expression of mGlu Receptor 1a/b and Decreases the Glutamate-Dependent Production of Nitric Oxide

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Received 6 August 2014; revised 23 September 2014; accepted 12 October 2014

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Abstract

IL-10 provides trophic and survival effects directly on neurons, promotes axonal outgrowth, and stimulates neuroregeneration. In this study, we analyzed the activities of arginase and nitric oxide synthase (NOS) in synaptoneurosomes derived from brain cortex of C57BL/6 IL-10 gene-knockout (KO) and wild-type (Wt) mice and determined that the synaptoneurosomes derived from KO mice present lower arginase II activity and lower spermine content than those derived from Wt mice, whereas the basal NOS activity in the KO synaptoneurosomes was higher than that observed in the control synaptoneurosomes. Moreover, our results indicate that the plasma membranes isolated from the KO mice brain exhibit significantly lower spermine-induced enhancement of [³H] MK-801 binding than the plasma membranes from the brain of Wt mice. Glutamate increases the production of nitric oxide (NO) in Wt synaptoneurosomes in a dose-dependent manner, whereas in the KO synaptoneurosomes, this amino acid does not affect the synthesis of NO. The glutamate-dependent acceleration of NO synthesis in Wt synaptoneurosomes was abrogated by LY367385, an antagonist of mGluR1a/b. The western blot analysis of the synaptoneurosomal proteins demonstrates that the expression of the subunits of NMDAR (NMDAR2A and NMDAR2B), the level of NMDAR-bound nNOS and the expression of iNOS are not changed in KO mice and that only the level of mGluR1a/b is markedly reduced in the synaptoneurosomes of KO mice. We conclude that a neuroprotective and neuroregenerative property of IL-10, in addition to its effects on polyamine metabolism and the spermine-dependent modulation of NMDAR, may involve the regulation of mGluR1a/b expression.

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How to cite this paper: Koriauli, S., Barbakadze, T., Natsvlshvili, N., Dabrundashvili, N., Kvaratskhelia, E. and Mikeladze, D. (2014) IL-10 Gene Knockout Reduces the Expression of mGlu Receptor 1a/b and Decreases the Glutamate-Dependent Production of Nitric Oxide. *J. Biomedical Science and Engineering*, 7, 1019-1029. <http://dx.doi.org/10.4236/jbise.2014.713099>

Keywords

IL-10, Polyamine Biosynthesis, Nitric Oxide, Metabotropic Glutamate Receptor

1. Introduction

It has been demonstrated that interleukin-10 (IL-10), an anti-inflammatory cytokine, provides trophic and survival effects directly to neurons, modulates neurite plasticity and outgrowth, and has a pivotal importance in neuroregeneration [1]-[4]. These effects of IL-10 are mediated by direct interactions with the neuronal IL-10 receptor and are distinct from the known anti-inflammatory actions of this cytokine on immune cells [5]. One of the targets of IL-10 is the arginase gene, the expression of which is strongly induced in a STAT-3 dependent manner [6]. Arginase, an enzyme of the urea cycle, has two isozymes: types I and II. Arginase I is a cytoplasmic protein that is primarily expressed in the liver, whereas arginase II is a mitochondrial protein with a widespread tissue distribution [7]. The function of arginase is to convert L-arginine to L-ornithine, a precursor in the production of polyamines. Polyamines play a fundamental role in the developing nervous system: the inhibition of polyamine biosynthesis results in defects in neuronal morphogenesis, whereas exogenous polyamines stimulate axonal regeneration [8]. Polyamines play important roles in the maintenance of normal neuronal function by regulating the neurotransmitter receptor and modulating learning and memory. Polyamines also influence neurotransmission by interacting with ionotropic (e.g., NMDAR-N-methyl-D-aspartate type of glutamate receptor) glutamate receptors [9]-[11].

The increased expression of arginase accompanied by the resulting degradation of the NO precursor arginine would decrease NO generation by NOS (nitric oxide synthase) and thus potentially protect neurons and axons [12] [13]. In the surviving motor neuron subpopulations, NO production may be impaired by the low levels of arginine caused by the high basal activity of arginase, which suggests that the ability of nitric oxide to reach a toxic level depends on the balance between neuronal NOS and arginase activity [12] [14] [15]. It is well established that NO synthesis in neurons is regulated predominantly by glutamate receptors. Neuronal nitric oxide synthase (nNOS), through its interaction with PSD-95 with NMDAR-NR2B, can be efficiently activated by NMDAR-mediated Ca^{2+} influx [16]. Ca^{2+} -dependent nNOS activation leads to nitric oxide (NO) production, which may be toxic when found in excess and combined with other reactive oxygen species (ROS). In addition, the activation of mGluRs (metabotropic glutamate receptors) in neurons may result in the release of high levels of Ca^{2+} from intracellular compartments, which can also contribute to Ca^{2+} -dependent regulatory processes [17]. Many studies indicate that mGluRs activation leads to neuroprotection or excitotoxicity [18]-[20], which suggests that these receptors may constitute a promising multipotential therapeutic approach [21].

We hypothesize that the beneficial effects of IL-10 on neurons depend on the upregulation of arginase genes, which may be potentially neuroprotective by enhancing polyamine biosynthesis and reducing the concentration of NO. Moreover, we hypothesize that the action of IL-10 may involve the modulation of glutamate-dependent NO generation. To investigate the action of glutamate on the production of NO in C57BL/6 IL-10 gene knock-out (KO) and wild-type (Wt) mice, we used synaptoneurosome as a model system. Synaptoneurosome preparations contain a population of presynaptic and postsynaptic structures [22] that retain the ability to display many of the molecular effects that characterize the intact brain, including neurotransmitter release, receptor-mediated signal transduction, and protein synthesis [23]. Synaptoneurosome preparations provide an excellent model for the study of glutamate-dependent biochemical responses in nervous tissue [24]-[26].

In this study, we found that the synaptoneurosome derived from the brain cortex of KO mice present decreased glutamate-dependent NO synthesis compared with the synaptoneurosome from Wt animals, and this reduction in NO synthesis is due to the weak expression of mGluR1a/b in KO mice. Furthermore, we observe that the KO synaptoneurosome exhibit lower mitochondrial activity of arginase II and a lower content of spermine in the synaptoplasm compared with the corresponding Wt fractions, whereas the basal NOS activity in the KO synaptoneurosome is higher than that found in the Wt synaptoneurosome. In addition, our results indicate that the levels of the NMDAR subunits (NR2A and NR2B) in plasma membranes isolated from the brain cortex of KO mice is not changed compared with those from Wt mice; however, the plasma membranes from KO mice present a significantly lower spermine enhancement of [^3H] MK-801 binding than the plasma membranes from

the brain cortex of Wt mice. These data suggest that IL-10, through both mGluR1-dependent pathways and polyamine biosynthesis, can regulate NO metabolism in neurons and that this effect may signify a neuroprotective and neuroregenerative action of IL-10.

2. Materials and Methods

2.1. Materials

All of the reagents were purchased from Sigma-Aldrich (Sigma-Aldrich Inc., USA) unless otherwise specified. Monoclonal antibodies against NMDAR-2A, NMDAR-2B, mGluR1a/b, iNOS, and nNOS were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.2. Animals and Social Conditions

Male C57BL/6J wild-type and *IL-10*-deficient C57BL/6J mice (mice homozygous for the $IL10^{tm1Cgn}$ targeted mutation, 8 - 12 weeks of age) were purchased from Jackson Laboratories (Bar Harbor, ME, USA) and maintained by the Institute of Medical Biotechnology of Tbilisi State Medical University (Tbilisi, Georgia). These *IL-10* mutant mice are viable and fertile under specific pathogen free conditions. *IL-10*-deficient phenotype is associated with altered lymphocyte and myeloid profiles, elevated serum amyloid A levels, altered responses to inflammatory or autoimmune stimuli, increased depressive-like behavior [27] and decreased neuroprotection [28].

All of the experimental procedures were approved by the Institute of Medical Biotechnology of Tbilisi State Medical University Animal Care and Use Committee.

2.3. Preparation of Synaptoneurosomes, Synaptoplasma, Plasma Membranes, and Mitochondria

The synaptoneurosomes was prepared according to the method described by Kim *et al.* [26] with some modifications. Briefly, the mice ($N = 6$) were decapitated, and their brains were removed and dissected. The cortices were homogenized in homogenizing buffer containing 50 mM HEPES, pH 7.5, 125 mM NaCl, 2 mM KCl, 100 mM sucrose, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 U/mL aprotinin, and 5 mg/mL pepstatin A (buffer A) using a glass-Teflon homogenizer. The homogenate was filtered through three layers of 100- μ m nylon mesh filters and eight layers of 100- μ m nylon mesh filters. The final filtrate was centrifuged briefly ($4000 \times g$, 1 min), and the supernatant was centrifuged ($7000 \times g$, 15 min) to pellet the synaptoneurosomes, which were then diluted in buffer A.

To obtain the plasma membrane, synaptoplasma, and mitochondria preparations, the synaptoneurosomes were lysed with 200 μ L of 0.1% Triton X-100 at 4°C for 30 min. The lysate was homogenized with an insulin syringe, and 100 mL of 25 mM Tris-HCl was added. The homogenate was centrifuged at $1000 \times g$ for 10 min, the pellet was removed, and the supernatant was centrifuged at $9500 \times g$ for 30 min to obtain the mitochondrial pellet. The post-mitochondrial supernatant was centrifuged at $20,000 \times g$ for 20 min, and the pellet was resuspended in 20 mM Tris-HCl, pH 7.4 buffer containing 0.1 mM PMSF, 2 mM EDTA, 5 mg/mL pepstatin, and 5 U/mL aprotinin (buffer B) to yield a suspension with a concentration of 5 mg protein/mL and was frozen at -40°C until use (plasma membrane preparation). The supernatant obtained after the last centrifugation was used as the “synaptoplasma” preparation.

The plasma membrane proteins were solubilized with 1% sodium deoxycholate at a detergent/protein ratio of 4/1 (mg/mg) in buffer B for 1 h at 4°C and then centrifuged at $20,000 \times g$ for 2 h. The supernatants were used as the solubilized plasma membrane preparations.

2.4. Determination of NOS Activity and NO

The NOS activity was determined in medium containing 50 mM K_2PO_4 buffer, pH 7.2, 400 μ M L-arginine, 500 μ M NADPH, 0.1 μ M calmodulin, 2 mM $CaCl_2$, 2 μ M FAD, 2 μ M FMN, 10 μ M tetrahydrobiopterin, 0.01 U/mL nitrate reductase, and 100 μ g of synaptoneurosomal protein. To study the dose-dependent effect of glutamate, the synaptoneurosomes were incubated for 30 min at 37°C with various concentrations of glutamate (10 - 100 μ M). After incubation, the synaptoneurosomes were centrifuged for 20 min at $16,000 \times g$, and the supernatants

were treated with 200 μM 2,3-diaminonaphthalene (DAN) and incubated for 10 min at room temperature in the dark before sodium hydroxide (NaOH) was added. The fluorescence was measured using a spectrofluorometer (Jenway Fluorimeter 6200) with maximum excitation and emission wavelengths of 350 nm and 425 nm, respectively.

The total amount of nitric oxide in the synaptoplasma was determined after mixing 500 μL of the clear soluble preparation of synaptoplasma with 100 μL of Griess reagent (1% sulfanilamide and 0.1% naphthalene-ethylenediamine dihydrochloride in 5% H_3PO_4). After 15 min, the product formation was colorimetrically (550 nm) determined. As a standard, NaNO_2 was reacted with Griess reagent.

2.5. Arginase Activity

The arginase activity was determined in the synaptoplasmic and mitochondrial fractions according to Munder *et al.* [7] with slight modifications. First, 10 mM MnCl_2 was added to the protein fractions, and the enzyme was activated by heating for 10 min at 56°C. Arginine hydrolysis was conducted by incubating the lysate with 100 μL of 0.5 M L-arginine (pH 9.7) at 37°C for 15 - 120 min. The reaction was stopped by the addition of 900 μL of a H_2SO_4 (96%)/ H_3PO_4 (85%)/ H_2O (1/3/7, v/v/v) mixture. The urea concentration was measured at 540 nm after the addition of 40 mL of α -isonitrosopropiophenone (dissolved in 100% ethanol) followed by heating at 95°C for 30 min. One unit of enzyme activity was defined as the amount of enzyme that catalyzes the formation of 1 micromole of urea per min.

2.6. MK-801 Binding Assay

The binding assay was performed in duplicate at room temperature in a volume of 250 μL . Each tube contained 50 μL of the plasma membrane suspension (final protein concentration ~ 0.4 mg/mL), 2 nM [^3H] MK-801, and a range of concentrations of spermine (1 - 10,000 nM) or sodium nitroprusside (SNP; 50 - 50,000 nM) in 50 mM Tris-HCl (pH 7.4) buffer. Non-specific binding was defined by unlabelled MK-801 at 500 nM. The assays were terminated after 45 min via rapid filtration through Whatman GF/B glass fiber filters. The assay tubes and filters were rapidly washed three times with ice-cold assay buffer. After drying, the filters were transferred to vials and soaked overnight in 5 mL of toluene scintillant. The radioactivity bound to the filters was determined using an LKB Rack-PTM counter with onboard quench correction.

2.7. Measurement of Polyamines by HPLC Analysis

The content of polyamines in the synaptoplasma was measured according to Sethi *et al.* [29] with slight modifications. First, 350 - 350 μL of the synaptoplasma was deproteinized with an equal amount of 0.6 N HClO_4 , and the mixture was centrifuged at $15,000 \times g$ for 20 min. The supernatants were then neutralized with 2N NaOH. After the addition of benzoyl chloride and incubation for 25 min at room temperature, the benzoyl-polyamines were extracted twice in diethyl ether. The extracted layers were dried in a stream of nitrogen, and the remaining residue was dissolved in acetonitrile (100 μL). Then, 20 μL of the samples was analyzed by HPLC at wavelengths of 198, 229, and 254 nm.

The HPLC analysis was conducted using an Agilent 1260 infinity quaternary LC instrument (quaternary pump 1260, autosampler 1290, and UV detector 1260). The samples were injected and separated using a C-18 (Nova-Pac C-18, 4 micron, 3.9×150 mm, Waters) column and initially eluted using a gradient elution profile. The elution profile was the following: 20% solvent A (acetonitrile) to 100% A over a 10-min period, 100% A for 3 min, and from 100% A to 20% A over a 2-min period. The flow rate was 0.5 mL/min, and the total run time was 17 min.

2.8. Immunoblotting

For the analysis of iNOS, nNOS, NMDAR-2A, NMDAR-2B, and mGluR1a/b, 50 μg of proteins from the synaptoneuroosomes was denatured at 90°C for 5 min, separated by SDS-PAGE on 15% gels, and transferred to nitrocellulose membranes. After blocking with 5% bovine serum albumin and 0.05% Tween 20 in Tris-HCl buffered saline, the membranes were incubated with the corresponding primary antibodies in the blocking solution. The immunolabeled bands were visualized using enhanced chemiluminescence (Amersham Biosciences) and analyzed by densitometric scanning. The intensities of the bands were within the linear range of the amount of

protein loaded.

The protein concentration was determined through a dye-binding method (Bio-Rad).

2.9. Statistical Analysis

The statistical analyses were performed using either an unpaired t test or one-way ANOVA and Scheffe's post hoc analysis when appropriate. The results were considered significant when $p < 0.05$. The results are expressed as the means \pm SEM from at least three independent experiments.

3. Results

3.1. Arginase Activity, Polyamine and NO Levels in KO and Wt Synaptoneuroosomes

The AI and AII isozymes of arginase were detected in the neurons, and their expression was particularly high in the cerebral cortex [30]. The arginase II isozyme is predominantly found in the mitochondria, and its primary function is thought to be the production of ornithine, which is a precursor in the synthesis of polyamines. Because the expression levels of both types of isozymes are controlled by IL-10 [6], we first examined the activity of arginase in the mitochondrial and soluble (synaptoplasmic) fractions of the synaptoneuroosomes (**Figure 1(a)**). We found that the activity of the mitochondrial enzyme (AII isozyme) is lower in KO mice compared with Wt mice, whereas the activity of the synaptoplasmic enzyme (AI isozyme) was similar in both mice (data not shown).

Polyamines are the major products of ornithine metabolism. To assess how an alteration in the activity of arginase II is reflected in the metabolism of polyamines, we determined the content of spermine, the end product of polyamine metabolism, in the synaptoplasmic fractions. The HPLC results show that the content of spermine in the synaptoplasm of Wt mice is higher than that found in the synaptoplasm of KO mice (**Figure 1(b)**). These data suggest that IL-10 deficiency, in addition to reducing arginase activity, down-regulates the synthesis of spermine. Because arginase converts arginine to ornithine, thereby competitively depriving L-arginine from NOS production, we then determined the content of NO in the synaptoplasmic fractions and found that the content of NO is higher in the KO synaptoplasm than in the Wt synaptoplasm (**Figure 1(c)**). These data indicate that the reduction of arginase in KO mice may lead to an accelerated utilization of arginine for NO synthesis.

3.2. Role of mGluR1 in Glutamate-Dependent Nitric Oxide Production

Because fluctuations in the NO content in the nervous tissue depend on the intensity of glutamatergic neurotransmission, we then determined the activity of NOS in the presence of various concentrations of glutamate. Our results show that glutamate increases the synthesis of NO in a concentration-dependent manner only in Wt synaptoneuroosomes, whereas in the KO synaptoneuroosomes, the production of nitric oxide does not change significantly (**Figure 2**). However, the basal activity of NOS was higher in the KO than in Wt synaptoneuroosomes. These data show that, despite the increased basal activity of NOS in KO synaptoneuroosomes, the production of NO is less sensitive to glutamate in IL-10-deficient mice.

It is well established that overstimulation of NMDA receptors can increase the local Ca^{2+} concentration, which activates neuronal nitric-oxide synthase (nNOS) [31]. Moreover, accumulating evidence suggests that group I metabotropic glutamate receptors trigger Ca^{2+} release from intracellular calcium stores by inositol triphosphate (IP3) [17] and may increase the activity of NOS [32]. Therefore, the impairments observed in the glutamate-dependent NOS activity may be due to a lower expression of NMDA-type or metabotropic glutamate receptors in KO mice. Thus, we then determined the contents of the NMDAR2A and NMDAR2B subunits of NMDAR, mGluR type1a/b, and nNOS in Wt and KO synaptoneuroosomes. The level of nNOS, as well as the levels of the NMDAR subunits (NR2A and NR2B), were not changed significantly in the KO mice compared with the Wt mice. However, the level of mGluR1a/b was markedly decreased in the plasma membrane of the synaptoneuroosomes from KO mice (**Figure 3**).

To confirm the hypothesis that the activity of mGluR type1a/b may alter the glutamate-dependent NO synthesis in synaptoneuroosomes, we determined the content of NO in the presence of an antagonist of mGluR1a/b, *i.e.*, LY367385. The data show that the addition of LY367385 to the synaptoneuroosomes abrogates the stimulatory effect of glutamate on NO synthesis only in the Wt synaptoneuroosomes, whereas in the KO synaptoneuroosomes, this antagonist does not significantly change the production of NO (**Figure 4**). These data confirm the

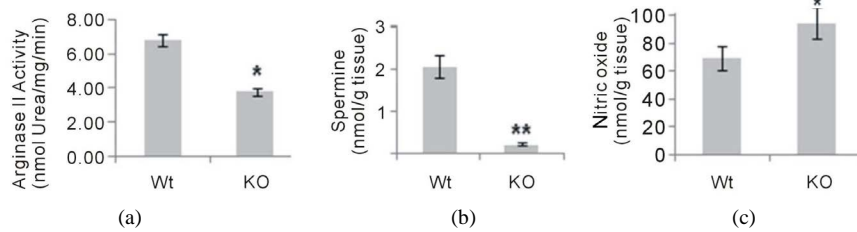


Figure 1. Changes in the activity of arginase II and the contents of spermine and NO in KO and Wt mice. (a) The arginase II activity was analyzed in the mitochondrial fractions obtained from the synaptoneurosomes of Wt and KO mice brains as described in Materials and Methods; (b) The synaptoplasmic fractions from KO and Wt mice were subjected to HPLC analysis to determine the spermine level and analyzed as described in Materials and Methods; (c) The content of NO in the KO and Wt synaptoneurosomes were determined by the Griess reaction as described in Materials and Methods. The values are expressed as the means \pm S.E.M. * $P < 0.05$ and ** $P < 0.01$ compared with the corresponding Wt fraction, as determined through *t* tests.

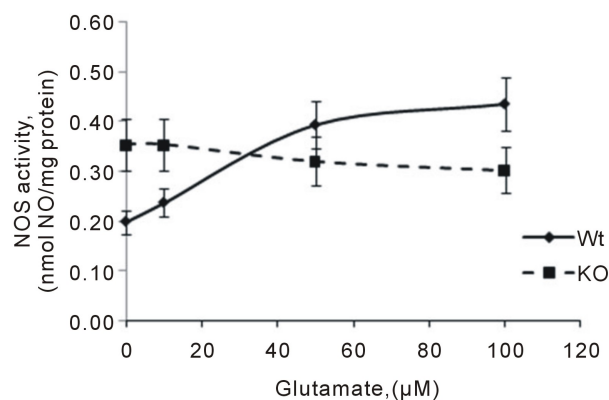


Figure 2. Glutamate-dependent production of nitric oxide in KO and Wt synaptoneurosomes. The NOS activity in the neurosynaptosomes derived from KO and Wt mice brains was determined. The incubation was performed with various concentrations of glutamate for 30 min at 37°C, and the production of nitric oxide was analyzed as described in Materials and Methods. The values are expressed as the means \pm S.E.M.



Figure 3. Western blot analysis of nNOS, iNOS, NR2A, NR2B, and mGluR1 in synaptoneurosomes of KO and Wt mice brains. nNOS, iNOS, NR2A, NR2B, and mGluR1, were analyzed directly in the synaptoneurosomes. For the immunoblots, 50 μ g of total proteins from each fraction were loaded per lane, resolved by SDS-PAGE, transferred to nitrocellulose membranes, and probed with anti-nNOS, anti-iNOS, anti-NR2A, anti-NR2B, and anti-mGluR1a/b. β -Actin was also visualized by Western blotting to confirm equal loading of the fractions. The blot is representative of four similar experiments.

hypothesis that the glutamate-dependent synthesis of NO in the Wt synaptoneurosomes may be increased by the activation of mGluR and that the production of NO is impaired by a deficiency in mGluR1a/b.

3.3. Spermine and Sodium Nitroprusside Enhancement of [³H] MK-801 Binding

There is some evidence that IL-10 blocks the inhibitory effect of IL-1 β on glutamate release and long-term potentiation (LTP) [33]. Because NMDAR is involved in LTP and taking into account that the level of NMDAR in KO mice is not changed, we then analyzed the modulatory sites of NMDAR. Among the many sites of NMDAR that may be modulated by the action of IL-10, there are sites that are sensitive to both polyamines and NO [34].

Thus, we examined the effects of spermine on the binding of MK-801 to the plasma membranes isolated from the brain of KO and Wt mice. The profiles associated with the spermine-induced enhancement of [^3H] MK-801 binding were analyzed, and the two main components of the curves (an enhancement phase and an inhibitory phase at higher concentrations) were found in both types of mice. The curve analysis showed that the brain plasma membrane from Wt mice present a significantly higher mean maximal enhancement than the plasma membrane from KO, and no other differences were statistically significant (Figure 5). These data suggest that the NMDAR in KO mice has lost its sensitivity to spermine.

Both the NR2A and NR2B subunits of NMDAR undergo *S*-nitrosylation on cysteine thiol groups by endogenous NO and exogenously applied *S*-nitrosothiols [35]. Therefore, we then attempted to evaluate the effects of sodium nitroprusside (SNP), which is often used as a NO donor, on [^3H] MK-801 binding to NMDAR in synaptoneurosomes and found that the IC₅₀ values of Wt and KO synaptoneurosomes for SNP do not differ significantly. The IC₅₀ for SNP of the Wt synaptoneurosomes was $283.6 \pm 20.9 \mu\text{M}$, whereas that of KO synaptoneurosomes was $254.9 \pm 32.3 \mu\text{M}$. These data suggest that the IL-10 gene knockout does not change the ability of NO to modulate NMDAR in mice.

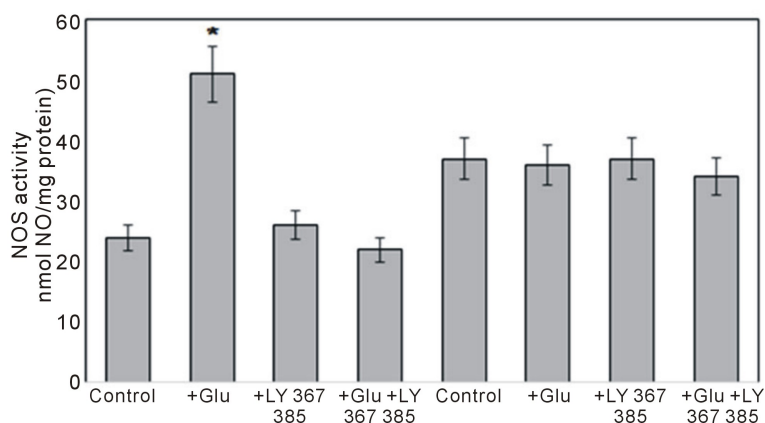


Figure 4. Antagonist of mGluR1-LY367385 blocks glutamate-dependent production of nitric oxide in KO synaptoneurosomes. The synaptoneurosomes derived from KO and Wt mice brains were incubated for 30 min at 37°C with and without 100 μM glutamate and 10 μM LY367385. The activity of NOS was determined as described in Materials and Methods. The values are expressed as the means \pm S.E.M. * $P < 0.05$ compared with the corresponding control, as determined through *t* tests.

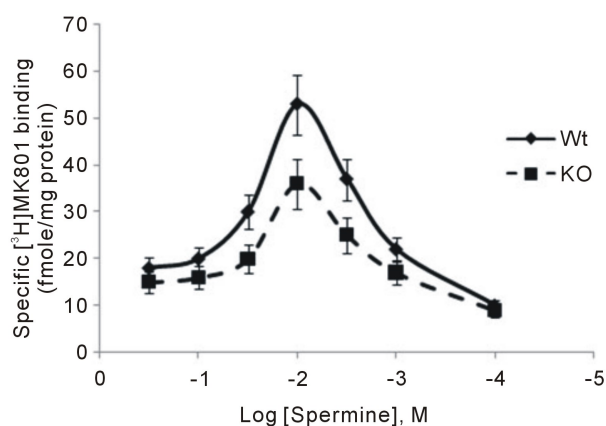


Figure 5. Effects of spermine on the specific [^3H] MK-801 binding in plasma membranes derived from KO and Wt synaptoneurosomes. The assays were performed as described in Materials and Methods. The plasma membranes derived from KO and Wt synaptoneurosomes were incubated with 2 nM [^3H] MK-801 and a range of concentrations of spermine (1 - 10,000 nM) for 45 min at room temperature as described in Materials and Methods. Non-specific binding was defined by unlabelled (500 nM) MK-801. The enhancement of specific [^3H] MK-801 binding with each spermine concentration was calculated as fmol bound per mg of protein. The values are expressed as the means \pm S.E.M.

4. Discussion

The cytokine IL-10 has been shown to improve neurologic outcome after central nervous system injury and render neurons less vulnerable to detrimental effects [1] [2] [36]-[38]. IL-10 acts as a growth factor [39], regulates neurogenesis in the normal adult brain, promotes axonal outgrowth, stimulates neuroregeneration [4], and provides trophic and survival effects against glutamate-dependent excitotoxicity [5]. IL-10 can change long-term potentiation, which suggests that a target of the cytokine is the system participating in glutamatergic neurotransmission [33].

Nitric oxide (NO) is an important signaling molecule that is widely used in the nervous system. Accumulating evidence suggests that nitrergic signaling modulates neuronal homeostasis and intrinsic excitability under physiological conditions [40]. Metabotropic and ionotropic glutamate receptors are considered to regulate NO production via Ca^{2+} -induced nNOS activation [41] [42]. Our data indicate that the synaptoneurosomes of IL-10-deficient mice present lower glutamate-dependent NOS activity compared with the control synaptoneurosomes. Moreover, we find that the glutamate-dependent acceleration of NO-synthesis is inhibited by an antagonist of mGluR1, namely LY367385, only in the Wt synaptoneurosomes, whereas this sensitivity of NOS is completely lost in the KO mice. Furthermore, our experiments show that the expression of mGluR1 is significantly decreased in KO mice, whereas the expression of the NMDAR subunits NR2A and NR2B is unchanged. These data suggest that the NOS activity in the Wt synaptoneurosomes depends on the mGluR receptor to a certain extent and that the reduced expression of mGluR in IL-10-deficient mice leads to the diminution of glutamate-dependent NO synthesis.

In this study, we have analyzed the effect of spermine on [^3H] MK-801 binding to NMDA receptor. Polyamines are complex allosteric modulators of the NMDA receptor [43]. At low concentration polyamines (3 - 100 M) enhanced the binding of [^3H] MK-801, whereas at high concentrations of spermine (>100 μM), [^3H] MK-801 binding was inhibited [44], suggesting that polyamines modulate the NMDA receptor by at least two distinct actions. We have found that NMDA receptor from KO mice cortex exhibited lower affinity to spermine compared with NMDA receptor from Wt mice in [^3H] MK-801 saturation analysis. Because only NR1A/NR2B subtype of NMDA receptor contain a binding site for the both, stimulatory and inhibitory components of polyamine actions [45], our data suggest that the cortex of IL-10 deficient mice comprising a splicing isoforms of NMDA receptor with low affinity to spermine. e.g. NR1A subunit with deleted region in N-terminal domain cassette [34].

The present data also indicate that a deficiency of IL-10 reduces the expression of the mitochondrial arginase II isoform, whereas the induction of the arginase I isoform is not changed. There is some evidence suggesting that the induction of arginase I and II genes is regulated differentially by IL-10. Arginase II has been shown to be induced directly by IL-10, whereas this cytokine increases arginase I expression only when synergized with IL-4 [6]. Both IL-10 and IL-4 operate through the STAT3 and STAT6 transcription factors [46] [47], the expression of which is coordinately regulated by various transcription elements, such as CCAAT/enhancer binding protein b (C/EBP) [48] [49]. CCAAT/enhancer binding protein b is a member of the C/EBP family of transcription factors, which is regulated at multiple levels during several physiological and pathophysiological conditions through the action of various hormones and cytokines [50]. C/EBP promotes the transcription of a wide range of genes, including the mGluR1 gene [51] [52]. In addition, gene C/EBP is itself specifically upregulated by IL-10 [6]. Thus, a deficiency in IL-10 may downregulated the expression of C/EBP-protein and C/EBP-related genes, including STAT3, and thereby the mGluR1 genes [51] [52]. Our observation that a deficiency in IL-10 causes the downregulation of mGluR1a/b expression in mice confirms the hypothesis that IL-10 may act through C/EBP. Taking into account that the activity of arginase II and the content of mGluR are decreased in the KO mice, it is possible to conclude that IL-10 deficiency primarily affects genes that are activated by CCAAT/enhancer binding protein. However, additional experiments are needed to confirm this hypothesis.

Taken together, these results suggest that the knockout of the IL-10 gene in mice decreases the expression of mGluR1 and the expression of arginase II. These perturbations initiate a cascade of biochemical events that leads to the reduction in polyamine synthesis and to the downregulation of mGluR-dependent NO-production. The reduced level of polyamines decreases the NMDA-receptor responses to spermine, whereas the downregulation of mGluR reduces the modulatory effects of NO. Taking into account the facts that mGluR is involved in synaptogenesis, neuronal differentiation, synaptic transmission, and plasticity [53] and that polyamines are important for the development and regeneration of neurons [8], these results indicate that IL-10 may have a positive effect on the regenerative and cognitive functions of the brain. These studies are consistent with the hypo-

thesis that IL-10, in addition to having an immunomodulatory action in immune cells, provides neuroprotection that may have direct beneficial effects on the neurological outcome after central nervous system injury.

Acknowledgements

The authors thank Dr. M.Gongadze for providing the C57BL/6J mice. This work was supported, in part, by N5048 research grant from the Science and Technology Center in Ukraine (STCU) and Shota Rustaveli National Science Foundation (SRNSF). Partial support for these studies was provided by a grant-in-aid from the Ilia State University.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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