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Glutamate Regulates the Activity of Topoisomerase I in Mouse Cerebellum

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Abstract Topoisomerase I (topo I) is a nuclear enzyme which participates in most DNA transactions. It was shown to be inhibited in depolarized neurons by poly adenosine diphosphate (ADP)-ribosylation of the enzyme protein. We demonstrated previously an age and sex dependent topo I activity and enzyme protein level in the various regions of mouse brain. A specific distribution pattern of topo I was observed and the inhibitory neurons exhibited the highest enzyme activity and protein level in both the nucleus and the cytoplasm. Here, we show that neurotransmitters (glutamate and gamma-aminobutyric acid (GABA)) regulate the activity of topo I in mouse cerebellum sections. Glutamate exhibited a significant time-dependent inhibition of topo I activity but no effect of the enzyme protein level. GABA in contrary only slightly and transiently inhibited topo I activity. The inhibitory effect of glutamate was mediated by Ca^{+2} and by ADP-ribosylation of topo I protein and the glutamate ionotropic receptors were involved. Glutamate also diminished the inhibitory effect of topotecan on topo I. These results point to distinct and highly specific effects of the major neurotransmitters on topo I activity in the cerebellum suggesting that topo I possesses a specific role in the brain which differs from its known biological functions.

Keywords Topoisomerase I · Brain · GABA · Glutamate · Calcium

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Introduction

DNA topoisomerases (topo) are essential nuclear enzymes which participate in and are responsible for the determination of the topological state of the DNA. The eukaryotic type I topoisomerase (topo IB) plays an important role in various DNA transactions and in the maintenance of genomic stability [1, 2]. Its inactivation leads to embryonic death at the four–16 cell stage gestation [3]. topo IB can relax both positive and negative supercoiled DNA by the formation of a transient single-strand DNA break in which the active site tyrosine becomes attached to the 3′ phosphate end of the cleaved strand followed by rotation of the DNA and religation process [2, 4, 5]. In addition to this nicking–closing activity, topo I plays critical nonclassic roles in genomic stability and gene-specific transcription [6]. topo I may also regulate transcription by acting as a specific protein kinase for SR proteins, therefore contributing to RNA splicing [7]. The activity of topo IB is regulated by posttranslational modifications of the enzyme protein. In vitro phosphorylation of mammalian topo I, predominantly at serine, by casein kinase II and protein kinase C is necessary for its DNA relaxation activity while dephosphorylation decreased its activity [8]. Phosphorylation of topo I by the c-Abl tyrosine kinase enhanced its activity [9].

The topo I protein is subjected to poly adenosine diphosphate (ADP)-ribosylation by poly ADP-ribose polymerase (PARP) which down regulates its DNA relaxation activity [8, 10]. PARP-1 inhibits the formation of topo I-linked DNA and exhibited the inhibitory effects on topoisomerase I cleavage reactions in a dose-dependent manner [11]. It was suggested that the mechanism responsible for the inhibition might be due to the increase in the net negative charge of ADP-ribosylated topo I, which may cause a repulsion between topo I and the DNA. In addition, it was shown that activated poly ADP-ribosylated PARP-1 [12] and also PARP-1 itself [11] promoted the religation activity of topo I in the presence of camptothecin (CPT). This PARP-1 effect on topo I was shown to be modulated by ATP [13]. Recently, we also showed that topo I is modified by O-GlcNacylation which regulates its activity [14]. Mammalian DNA topoisomerases are the targets of several anticancer drugs in current clinical use [15, 16]. Only a few of these cytotoxic agents cross the blood-brain barrier (BBB) and among them is the anti-topo I inhibitor, topotecan (TPT), which is a water soluble derivative of CPT [17]. Several studies have shown that CPT induce apoptosis in neurons [18, 19]. The drug-induced apoptosis has been directly correlated with the level of topo I activity and the formation of DNA-enzyme cleavable complexes [20]. In a previous work, it was shown that significant topo I activity was present in rat brain cortical neurons prepared as primary neuronal cultures from 18–19-day-old embryos [21]. Following depolarization of the neurons, topo I had undergone ADP-ribosylation by a Ca^{+2}-dependent activation of PARP, suggesting a possible role for topo I in this process [21]. We also demonstrated the activity of topo I in the various brain regions of the mouse and found that in the visual cortex, cerebellum, and striatum, the activity of topo I was three–fourfold higher compared to that found in the hippocampus and hypothalamus. In addition, the distribution patterns of topo I protein revealed high levels of topo I in the cytoplasm and in the nucleus of inhibitory neurons. Most interesting, the activity and the level of the enzyme were age and gender dependent. It increased from birth to maturity and decreased, more significantly in males, with senescence [22]. These data point to a possible, yet unknown, involvement of topo I activity and regulation in various brain functions. Here, we examined the regulatory effects of neurotransmitters (glutamate and GABA) on topo I activity, level, and modification in mouse cerebellum slices. Our results show a striking but distinct effect in which glutamate treatment was followed by a prolonged decrease in topo I activity while GABA treatment exhibited a short transient decreased followed by an increased enzyme activity. In addition, the effect of glutamate was caused by ADP-ribosylation of topo I protein mediated by Ca^{+2}-activated PARP. Finally, our results suggest that the glutamate effects are mediated via the ionotropic (*N*-methyl-D-aspartate NMDA and alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)) receptors.

Materials and Methods

Preparation of Coronary Sections from Mice Cerebellum

All animal procedures used were approved by the animal experimentation ethics committee at Ben-Gurion University. CD-1 male mice (10–12 weeks) were anesthetized with a ketamin (50 mg/kg)/Rompun (10 mg/kg) mix and decapitated. Brain slices were prepared as previously described [23]. Briefly, brains were quickly removed from the skull, washed, and placed in an oxidized ringer solution at 4°C. The cerebellum was immersed with oxidized ringer solution at 4°C and coronary sections of 400 μm were prepared. The sections were given a 1-h recovery period before any further treatments. The slices were treated with various concentrations and for different intervals of neurotransmitters: GABA or glutamate (Sigma, Rehovot, Israel). topo I activity and protein level were measured as well as posttranslational modifications of the enzyme protein. Immunohistochemistry and immunofluorescent methods with anti-topo I antibody were used.

Preparation of Nuclear and Cytoplasmic Extracts

Coronary cerebellum sections of 400 μm were isolated and immersed in phosphate-buffered saline (PBS). The samples were homogenized using a manual homogenizer (pestle B). The homogenates were centrifuged at 1,700 rpm at 4°C for 7 min and the pellets were subjected to nuclear and cytoplasmic extractions as previously described [22, 24]. A mixture of protease inhibitors (final concentrations—2 μg/ml aprotinin, 2 μg/ml leupeptin, 1 μg/ml pepstatin A, 2 μg/ml antipain, and 100 μg/ml phenylmethylsulphonyl fluoride) were added to the extraction buffers. Total protein concentration was determined using the Bio-Rad (Hercules, CA, USA) protein assay kit.

topo I Assay

topo I assay was performed as previously described [22]. Equal concentrations of nuclear or cytoplasmic proteins derived from the various treatments were added to a topo I reaction mixture containing, at a final volume of 25 μl, 20 mM Tris-HCl (pH 8.1), 1 mM dithiothreitol, 20 mM KCl, 10 mM $MgCl_2$, 1 mM ethylenediaminetetraacetic acid (EDTA), 30 μg/ml bovine serum albumin, and 300 ng pUC19 supercoiled DNA plasmid (MBI, Fermentas, Han-

over, MD, USA). Following incubation at 37°C for 30 min, the reaction was terminated by adding 5 μl of stopping buffer (final concentration—1% sodium dodecyl sulfate, 15% glycerol, 0.5% bromophenol blue, and 50 mM EDTA (pH 8)). The reaction products were analyzed by electrophoresis on a 1% agarose gel using a TBE buffer (89 mM Tris-HCl, 89 mM boric acid, and 62 mM EDTA) at 1 V/cm, stained by ethidium bromide (1 μg/ml), and photographed using a short-wavelength UV lamp (ChemImager 5500, Alpha Inotech, CA, USA). Densitometric analysis of the results were performed using the EZQuant-Gel image processing and analysis software (EZQuant, Rehovot, Israel), and the percentage of topo I activity was calculated using the following equation: [1−(sample/control)]×100.

Determination of the Level of topo I Protein

Antibodies Anti-topo I antibody [22, 24] was purchase from Santa Cruz Biotechnology (Santa Cruz, CA, USA; lot no. D101) and anti-β-actin antibody [24] from ICN (Irvine, CA, USA; lot no.8739F). Equal amounts (40 μg) of nuclear and cytoplasmic proteins derived from the cerebellum sections were analyzed by Western blot analysis as previously described [25, 26] using either an anti-topo I antibody (1:2,000) or an anti-β-actin antibody (1:1,000). The immunocomplexes were detected by enhanced chemiluminescence (ECL; Santa Cruz Biotechnology). Densitometric analysis was performed as described above. The level of topo I protein was calculated using the equation: [topo I/β-actin]×100.

Immunohistochemistry and Immunofluorescence Analysis

Section Preparation Cerebellar sections from the various treatments were prepared as aforementioned described. The sections (400 μm) were then immersed in paraformaldehyde fixative solution for 3 h and dehydrated in ethanol 70% overnight. Following the next day, the sections were dehydrated in ethanol, 80% for 15 min, 90% for 15 min ×2, 100% for 15 min ×2 to xylene (for 30 min ×2), and embedded in paraffin (1:1 xylene paraffin mix for 20 min at 62°C and then with paraffin for 60 min). A rotary microtome was used to produce 5-μm sections, which were mounted on Superfrost plus slides. The sections were dried on a metal plate heated to 42°C overnight and stored in a cool and dry place until subjected to immunoassays.

Immunohistochemistry Paraffin-embedded sections were deparaffinized in xylene and rehydrated in decreasing concentrations of ethanol. All sections were initially treated with an anti-endogenous peroxidase solution containing 80 ml methanol, 10 ml H_2O_2 (30%), and 10 ml dH_2O for 15 min. The slide-mounted sections were then rinsed with dH_2O and heated in a microwave for 15 min in 0.01 M citric acid buffer, pH 6.0. The tissue was rinsed with PBS and then incubated for 30 min with a normal rabbit serum (Vector Burlingame, CA, USA) diluted in PBS. The sections were then incubated for 1 h in goat anti-topo I polyclonal IgG antibody (Santa Cruz, CA, USA; lot no. D1403) at a dilution of 1:100 at room temperature. After rinses in PBS, the sections were again incubated for 1 h in biotinylated antigoat IgG (1:100) followed by incubation in Vectastain ABC reagent diluted 1:100 for 1 h. The HRP was developed in 0.04% diaminobenzidine tetrahydrochloride prepared in PBS, with 0.003% H_2O_2. The specificity of the anti-topo I polyclonal antibody used in these experiments was previously demonstrated [22] and also examined here by pre-incubation of the antibody (dilution 1:100) with 200 units of purified topo I peptide for 3 h at 4°C prior to the aforementioned procedure.

Immunofluorescence

Antibodies Rabbit anti-GAD 65/67 was purchase from Sigma-Aldrich (St. Louis, MO, USA; lot no. G5163) and goat anti-topo I polyclonal IgG antibody from Santa Cruz, CA, USA (lot no. D1403). The endogenous peroxidase step was eliminated. Sections were incubated with the goat anti-topo I antibody (1:100) and with rabbit anti-GAD65/67 (1:1,000) for 1 h at room temperature. After rinsing in PBS, the sections were incubated for 1 h in affinity-purified cy2- and cy3-conjugated goat/rabbit IgG (1:50/1:100; Jackson ImmunoResearch, West Grove, PA, USA). After rinsing with PBS, the tissue was mounted with antifade mounting medium (Kierkegaard, Gaithersburg, MD, USA) and examined with a Nikon BX-50 Optiphot microscope equipped for fluorescence imaging using appropriate excitation and barrier filters. For nuclear staining, sections were incubated with 0.1 μg/ml 4′,6-diamidino-2-phenylindole for 20 min, at room temperature, rinsed with PBS, and mounted with antifade mounting medium (Kierkegaard). The photographs were prepared using Adobe PhotoShop 7.

Results

topo I Activity in Nuclear and Cytoplasmic Extracts Derived from Consecutive Cerebellum Sections After 1 h Recovery

To examine the effect of neurotransmitters on topo I activity in cerebellum slices, we first established an experimental system in which, prior to the neurotransmitter treatments,

no differences in topo I activity and topo I protein level and distribution between the samples are observed. Three consecutive cerebellum sections were recovered from three male mice (CD-1, 10–12 weeks old) in such a way that each specimen contained three cerebellum sections from three different mice and from three different planes (illustrate in Fig. 1a). Nuclear and cytoplasmic extracts were prepared and equivalent nuclear (50 ng) or cytoplasmic (200 ng) protein concentrations were added to a topo I reaction mixture. Although topo I is a nuclear protein, we also examined its activity in the cytoplasm since we have previously demonstrated the existence of topo I activity and protein in the cytoplasm of inhibitory neurons [22]. Indeed, no significant differences in the level of topo I DNA relaxation activity between the three nuclear or cytoplasmic specimens were observed (Fig. 1b compare lanes 2–4 and lanes 5–7, c). Examination of topo I protein in the nuclear aforementioned samples was performed by Western blot analysis with specific anti-topo I antibody. The results revealed a similar topo I protein level in the examined samples (Fig. 1d). The effect of exposure of the cerebellum slices for 1 h in oxygen bubbled ringer solution on the integrity of the tissue was examined by hematoxylin staining of paraffin-embedded coronary cerebellum sections. The granular and molecular layers as well as the Purkinje cells were apparently intact (Fig. 1e). In addition, the distribution of topo I and glutamic acid decarboxylase (GAD) proteins was examined in these cerebellum slices by immunofluorescence procedure using the indicated antibodies. No apparent changes on topo I protein distribution was observed by the treatment. Compatibly with our previous results [22], topo I protein was present in the nuclei of neurons and in the nucleus and cytoplasm of Purkinje cells (Fig. 1f). These results suggest that this experimental protocol is suitable for further examination of the effect of neurotransmitters on topo I.

Decrease in topo I Activity in Cerebellum Sections Treated with Glutamate or GABA

To determine the effect of the neurotransmitters, cerebellar sections were treated with GABA (50 μM) or glutamate (30 μM) for 1 or 5 min. Sections were then quickly washed in O_2 perfuse ringer solution, and nuclear proteins were extracted and analyzed for topo I activity. Equivalent amounts of nuclear (50 ng) proteins derived from the various treatments were added to a topo I reaction mixture and the reaction products were analyzed. A significant decrease in topo I activity (43±3.4%) was observed in cerebellum sections treated with glutamate for 1 min (Fig. 2a compare lane 4 to 2, c). Treatment of the slices with GABA for 1 min was also followed by a significant decrease in topo I activity (35±2.8%; Fig. 2a compare lane 3 to 2, c).

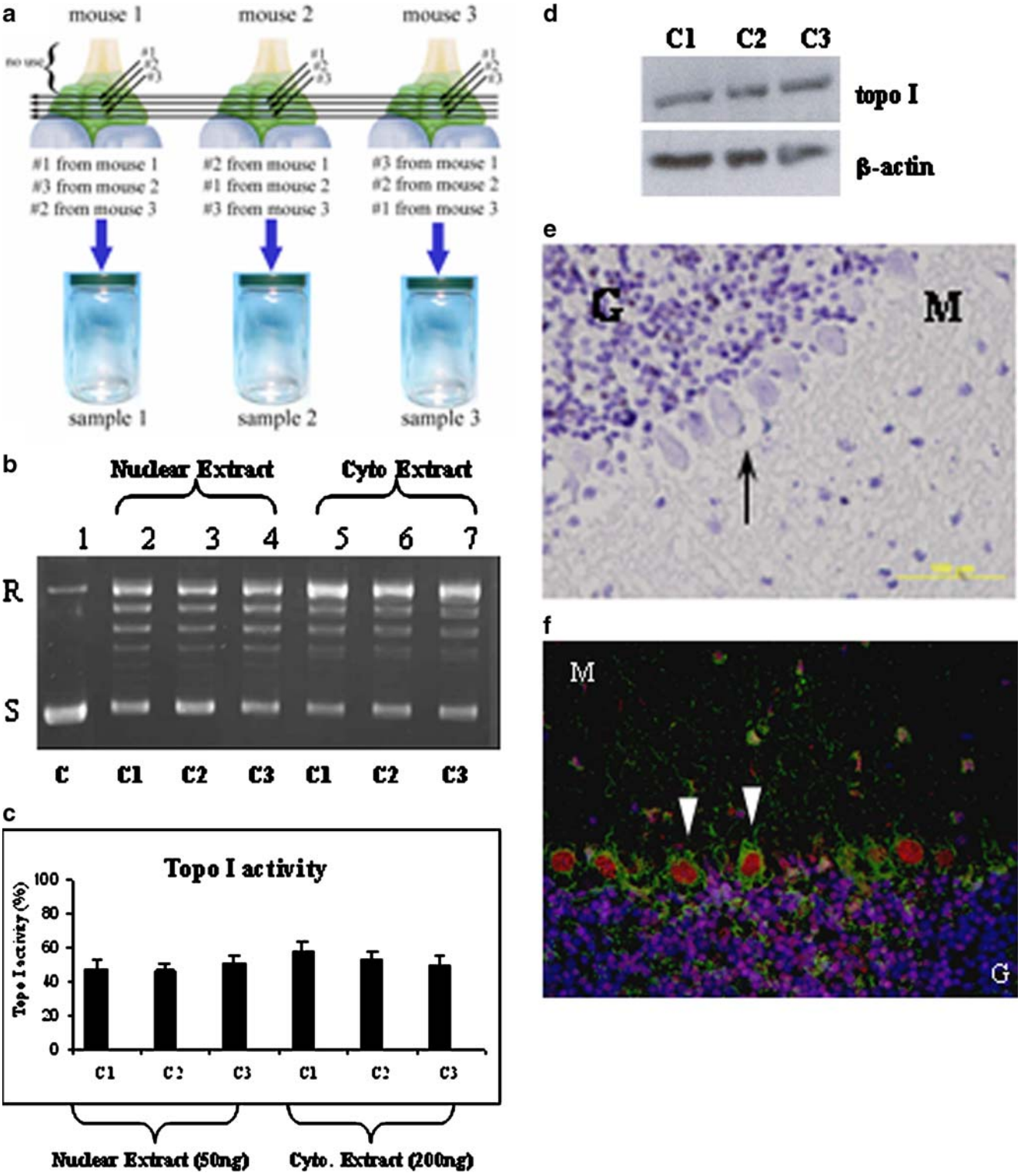
Longer treatment (5 min) with glutamate further enhanced the decrease in topo I activity to 75±6% (Fig. 2b compare lane 4 to 2, c). In contrast, the 5-min treatment with GABA did not inhibit the nuclear topo I activity and even an increase in the activity was observed (Fig. 2b compare lane 3 to 2, c). No effect on the level of topo I protein was observed in glutamate or GABA-treated sections as determined by Western blot analysis with specific anti-topo I antibody (Fig. 2d). To examine a possible direct effect of glutamate or GABA on topo I activity, these neurotransmitters were added to a topo I reaction mixture containing nuclear extract derived from untreated cerebellum sections. No effect on topo I activity was observed (Fig. 2e). Altogether, the results of this part indicate a significant relatively long inhibitory effect of glutamate but not GABA on topo I activity in the cerebellum.

Recovery of topo I Activity in Glutamate-Treated Cerebellum Slices

To examine the recovery of topo I activity after glutamate treatment, the cerebellum sections were treated with glutamate (30 μM) for 1 min, washed twice with oxygenated ringer solution, and incubated for additional 15 or 30 min without glutamate; the untreated control samples were subjected to the same procedure. Nuclear proteins were then extracted and analyzed for topo I activity. The results depicted in Fig. 3 demonstrate a significant recovery in topo I activity in sections that were incubated for 15 or 30 min in ringer solution only following the pretreatment with glutamate (Fig. 3 compare lanes 4 and 5 to lane 3 and 2, b).

Glutamate Inhibits topo I Activity by a Ca^{+2}-Dependent Pathway

Glutamate triggers a major rise in the intracellular level of Ca^{+2} [27]. Therefore, to characterize the mechanism by which glutamate treatment inhibits topo I activity, we further determined the effect of Ca^{+2} depletion on topo I activity in glutamate-treated cerebellum slices using 1,2-bis (*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid acetoxymethyl ester (BAPTA/AM), an intracellular Ca^{+2} chelator. Cerebellar sections were pre-incubated in ringer buffer containing 25 mM of BAPTA/AM for 20 min followed by the addition of glutamate (30 μM) for 1 min. The results depicted in Fig. 4a show that while treatment with glutamate inhibited the enzyme activity (Fig. 4a lane 3, b), pre-incubation of the cerebellum sections with BAPTA/AM (25 mM) prior to glutamate treatment abolished this inhibition and even slightly increased topo I activity (compare lane 5 to lanes 3 and 2 and b), suggesting that the inhibitory effect of glutamate on topo I is calcium



dependent. To substantiate this finding, the cerebellar slices were immersed in a ringer solution in the presence or absence of Ca^{+2} (2 mM) prior to glutamate treatment. The results depicted in Fig. 4c revealed that glutamate treatment decreased topo I activity only in the presence of Ca^{+2} (compare lane 3 to 5). Moreover, in the absence of Ca^{+2}, the addition of glutamate did not affect topo I activity (compare lane 2 to lane 4). This result suggests that glutamate affect topo I activity mainly by the ionotropic pathway. To determine the specific role of the ionotropic receptors AMPA and NMDA, the cerebellar slices were pre-incubated with competitive antagonists of AMPA/kinate glutamate receptor—6-

◀ **Fig. 1** topo I activity, enzyme protein level distribution in nuclear, and cytoplasmic protein extract derived from a mixture of three consecutive cerebellum slices. **a** An illustration of the preparation of the three consecutive cerebellum slices. **b** Equivalent amounts of nuclear (50 ng) or cytoplasmic proteins (200 ng) were added to a specific topo I reaction mixture. The reaction products were analyzed on agarose gel electrophoresis. The gel was stained with ethidium bromide and photographed using short UV lamp. *C* pUC 19 supercoiled plasmid only, *R* relaxed form of the pUC19 DNA, *S* supercoiled form of the pUC19 DNA. **c** Densitometric analysis of the results for 50 ng nuclear protein and for 200 ng cytoplasmic proteins were performed. The percentage of topo I activity was calculated as described in the "Materials and Methods" section. The results are means±SD of three different experiments. **d** Of nuclear protein extract derived from a mixture of three consecutive cerebellum slices, 40 μg was analyzed by Western blot assay using anti-topo I antibody (*upper panel*) or anti-β-actin antibody (*lower panel*). The immunocomplexes were identified by ECL. **e** Paraffin-embedded cerebellar sections were stained with hematoxylin to reveal the different layers in the cerebellum. *Arrow* Purkinje cells, *g* granular cell layer, *m* molecular cell layer. Magnification ×40. **f** Immunofluorescence labeling of the cerebellum slices by anti-topo I antibody (*red*), anti GAD (*green*), and nuclei (*blue*). Magnification ×40. *M* molecular layer, *G* granular layer, *arrow heads* Purkinje cells

cyano-7-nitroquinoxaline-2,3-dione (CNQX)—or with the specific NMDA receptor antagonist—2-amino-5-phosphonopentanoate (AP5). Each of these inhibitors was added separately at a concentration of 20 μM to the ringer solution and the cerebellar slices were immersed in this solution for 10 min prior to the glutamate application. Both the NMDA and AMPA inhibitors prevented the glutamate-induced decrease of topo I activity (Fig. 4d compare lanes 3 and 6 to lane 2). The exposure of the brain slices to NMDA or AMPA inhibitors without glutamate failed to inhibit and even increased topo I activity (Fig. 4d compare lanes 4 and 5 to lane 1) suggesting that both ionotropic receptors participate in the Ca^{+2}-dependent glutamate-induced decrease of topo I activity.

PARP Inhibitors Prevented the Glutamate, but not GABA, Induced Decrease in topo I Activity

Previous work showed that in depolarized neurons, topo I had underwent significant posttranslational modification (ADP-ribosylation) by a fast activation of PARP mediated via IP3 induced Ca^{+2} release [21, 28]. To determine whether glutamate inhibited topo I activity by PARP activation through elevated Ca^{+2} concentrations, cerebellar sections were produced as described in Fig. 1 and treated with glutamate (30 μM) with or without pre-incubation with PARP inhibitor (3AB 0.1 mM). While application of glutamate decreased as expected topo I activity (Fig. 5a lane 3, b) pre-incubation of the cerebellar sections for 20 min with PARP inhibitor (3AB) prior to glutamate treatment diminished by 80% the decrease in topo I activity (Fig. 5a compare lane 4 to 3 and 2, b). Treatment of the brain slices with 3AB for 20 min did not significantly affect topo I activity (Fig. 5c lane 4, d). Pretreatment of the brain slices with 3AB prior to GABA treatment did not significantly affect the inhibitory effect of GABA treatment on topo I activity (Fig. 5c compare lane 5 to 3, d). The results of this part indicate that the inhibitory effect of glutamate on topo I activity are mediated at least in part by PARP.

The Activity of topo I is Inhibited by TPT

To further characterize the effect of glutamate on topo I in the brain, we examined the influence of glutamate on the inhibition of topo I by TPT. topo I is the only known target of CPTs, chemotherapeutic drugs, and one of them, TPT, is known to cross the BBB and therefore may be an effective in vivo inhibitor of the activity of topo I in the brain [17]. CPTs inhibit the religation reaction of topo I by selectively inducing a stabilization of covalent reversible adduct between a tyrosine residue of the enzyme and the 3′ end of the single-strand DNA molecule and thus introduce a single DNA strand break. In replicating cells, an irreversible double DNA strand breaks occurs when a replication fork meets a cleavable complex. These breaks may lead to cell cycle arrest, activation of apoptosis pathways, and to cell death [29]. We initially established the exposure time of cerebellar sections to TPT required for the inhibition of topo I activity. Live cerebellar sections were immersed in ringer solution containing TPT (60 μM) for different intervals (15, 30, and 60 min) and topo I activity was determined. The results depicted in Fig. 6a, b show that 60 min of exposure to TPT are required for a significant inhibition of topo I activity (60%) in the cerebellar sections (compare lanes 3–5 to lane 2 and b).

To examine the combined effect of TPT and glutamate treatment on the activity of topo I, cerebellar sections were first exposed to TPT (60 μM) for 60 min prior to glutamate treatment (30 μM for 1 min). Equivalent nuclear protein concentrations (50 ng) were added to a topo I reaction mixture and analyzed as described. As expected, administering of TPT or glutamate alone reduced topo I activity (Fig. 6c lanes 3 and 4, d); interestingly, however, pretreatment with TPT followed by the addition of glutamate slightly but significantly reduced (from 65% to 50%, $p=0.0082$) the inhibitory effect of TPT (Fig. 6c lane 5, d).

Discussion

Previous work from our laboratory revealed that although neuron are fully differentiated nonmitotic cells, they show a significant level of topo I activity which differed between the various brain regions, and one of the regions presenting a significant level of topo I activity was the cerebellum

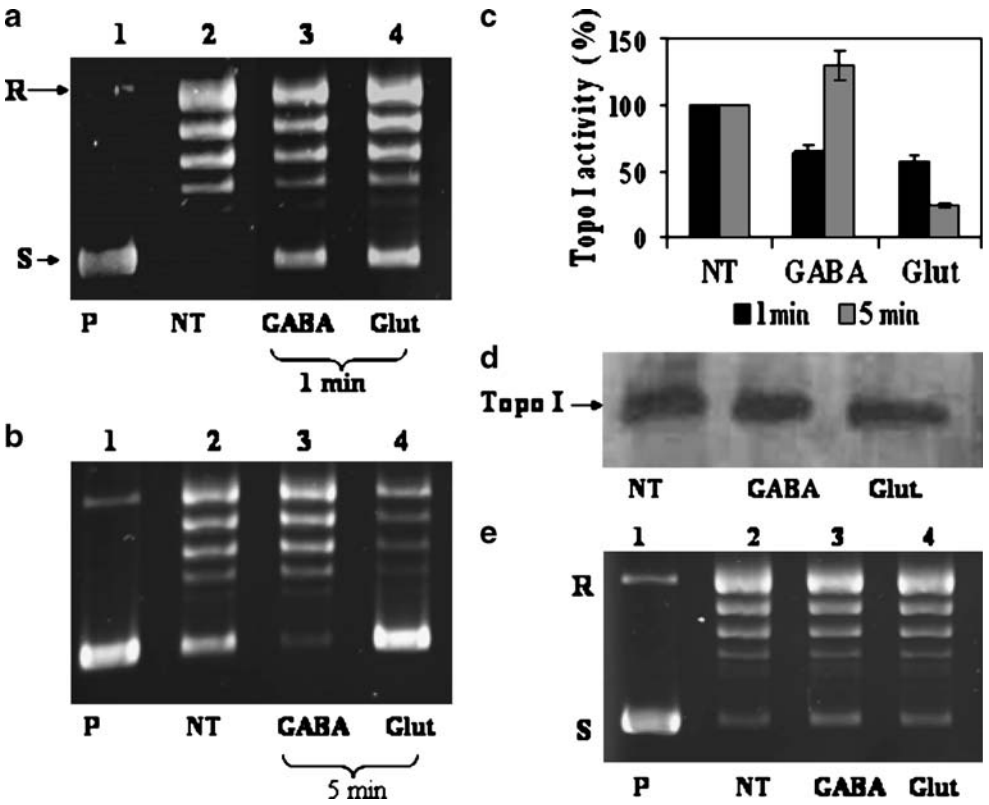


Fig. 2 The effect of neurotransmitters on topo I activity and expression in cerebellum sections. Cerebellar sections were treated with GABA (50 μM) or glutamate (30 μM) for 1 (**a**) or 5 min (**b**). Nuclear protein extracts were added to topo I reaction mixture. topo I reaction products were analyzed on agarose gel electrophoresis (**a** and **b** are representative pictures). Densitometric analysis of the results followed by calculation of topo I activity are depicted in **c**. The results are means±SD of three different experiments. *P* pUC 19 supercoiled plasmid only, *NT* no treatment, *Glut* glutamate, *R* relaxed form of the pUC19 DNA, *S* supercoiled form of the pUC19 DNA. **d** The level of topo I protein in the aforementioned treated cerebellar sections. Of nuclear proteins derived from the treated cerebellar sections, 40 μg were analyzed by Western blot assay using anti-topo I antibody. The immunocomplexes were identified by ECL. **e** The addition of GABA or glutamate to the reaction mixture did not influence topo I activity derived from cerebellar nuclear extracts. Nuclear protein extracts (50 ng) from untreated cerebellar sections were added to topo I reaction mixture containing GABA (*lane 3*), glutamate (*lane 4*), or none (*lane 2*) Reaction products were analyzed as described. *C* pUC 19 supercoiled plasmid only, *R* relaxed form of the pUC19 DNA, *S* supercoiled form of the pUC19 DNA

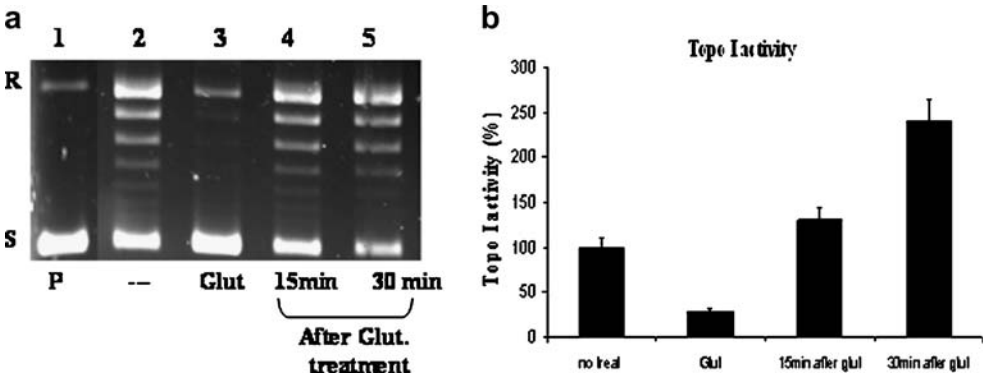


Fig. 3 Time dependence of glutamate application on topo I activity. **a** A representative picture. Nuclear protein extracts (50 ng) from cerebellar sections that were treated with glutamate (30 μM) for 1 min, washed twice with O_2 perfuse ringer solution, and incubated for 15 or 30 min were added to topo I reaction mixture. Reaction products were analyzed for topo I activity as described. *C* pUC 19 supercoiled plasmid only, *R* relaxed form of the pUC19 DNA, *S* supercoiled form of the pUC19 DNA. **b** Calculation of topo I activity. The results are means±SD of three different experiments

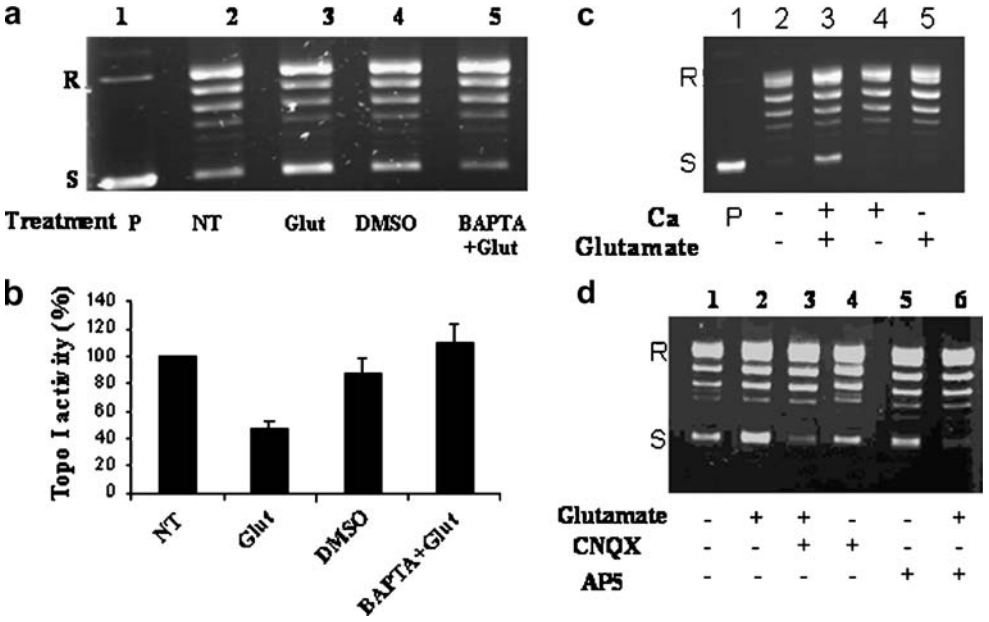


Fig. 4 Calcium dependent of glutamate-induced inhibition of topo I activity in cerebellar sections. topo I activity was determined in nuclear protein extracts (50 ng) from cerebellar sections that were treated with **a** and **b** glutamate with or without pre-incubation of BAPTA/AM (25 mM); **c** glutamate (30 μM) for 1 min in the presence or absence of Ca^{+2}; **d** glutamate (30 μM) for 1 min after pre incubation with 20 μM of NMDA inhibitor (*AP5*) or AMPA inhibitor (*CNQX*) for 10 min.. **b** topo I activity calculation—the results are means±SD of three different experiments. *C* pUC 19 supercoiled plasmid only, *R* relaxed form of the pUC19 DNA, *S* supercoiled form of the pUC19 DNA

[22]. In addition, a fast modification of topo I activity and topo I protein following depolarization of neurons was reported [21]. These data led us to investigate the effects of neurotransmitters on the activity of topo I in mice cerebellum.

First, it was important to establish a semi-in vivo system in which (1) the cerebellar sections will remain viable under the experimental conditions and (2) no differences in topo I activity and protein level and distribution, prior to the neurotransmitters treatment, will be observed between the various samples. Indeed, the cerebellar slices immersed in the oxygenated ringer solution for 1 h demonstrated an intact tissue containing the granular and molecular layers as well as Purkinje cells. Of interest is that the nuclei of the cells in the granular layer are small, compact, and with condensed chromatin but the nuclei of the Purkinje cells are much larger and with looser chromatin density suggesting very active gene expression processes in Purkinje cells.

The cerebellar sections were sustained under physiological conditions and subjected to various treatments. From

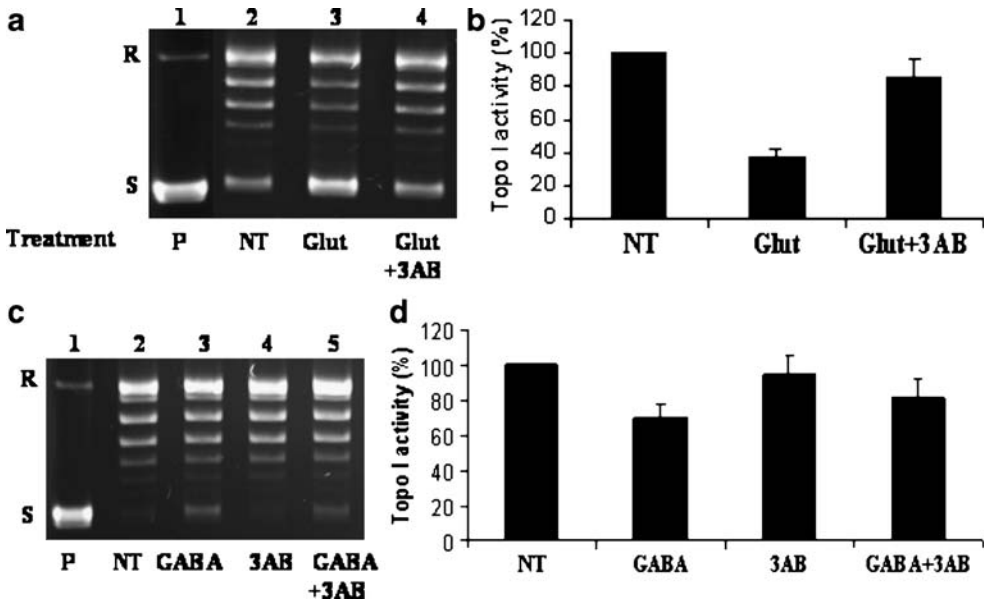


Fig. 5 PARP inhibitors prevented the glutamate-induced decrease in topo I. Equivalent nuclear protein extracts(50 ng) from cerebellar sections that were treated with glutamate (**a**, **b**) or with GABA (**c**, **d**) with or without pre-incubation of 3AB (0.1 mM) were added to topo I reaction mixture. Reaction products were analyzed for topo I activity as described; the results (in **b** and **d**) are means± SD of three different experiments. *C* pUC 19 supercoiled plasmid only, *R* relaxed form of the pUC19 DNA, *S* supercoiled form of the pUC19 DNA

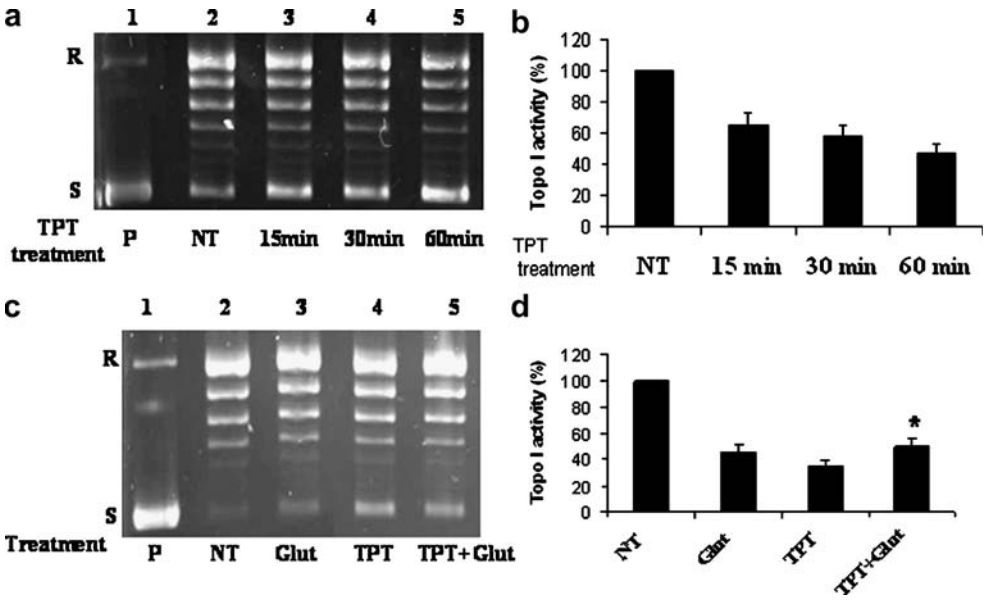


Fig. 6 Glutamate suppress the inhibition of topo I activity in cerebellar sections by TPT. Equivalent nuclear protein extracts (50 ng) from cerebellar sections that were treated with TPT (30 μM) for different intervals (15, 30, and 60 min) were added to a topo I reaction mixture (**a** a representative pictures). Reaction products were analyzed for topo I activity as described; the results (in **b**) are means± SD of three different experiments. *C* pUC 19 supercoiled plasmid only, *R* relaxed form of the pUC19 DNA, *S* supercoiled form of the pUC19 DNA. **c**, **d** The combined effect of TPT with glutamate on topo I activity. Equivalent nuclear protein extracts (50 ng) derived from cerebellar sections that were pretreated with TPT (30 μM) followed by glutamate treatments were added to topo I reaction mixture. Reaction products were analyzed for topo I activity as described (**c**) and topo I activity was calculated. The results (**d**) are means±SD of three different experiments. *p=0.0082 compared to TPT alone. *C* pUC 19 supercoiled plasmid only, *R* relaxed form of the pUC19 DNA, *S* supercoiled form of the pUC19 DNA

three male CD-1 mice (age 10–12 weeks), the cerebellum was removed and three sections of 400 μM were produced from each cerebellum; thus, each sample contained three cerebellum sections from three different mice from three distinct positions. The activity and level of topo I in these samples were demonstrated to be equal. Moreover, we showed by immunofluorescence staining with anti-topo I antibody that the experimental conditions did not change the distribution pattern of topo I in the mouse cerebellum. The results are compatible with previous data [22] and demonstrate a highly immunopositive staining for topo I in the cerebellum. The cells that expressed the highest staining for the enzyme were identified as Purkinje cells and were colocalized with GABAergic markers.

In this study, we focused on the influences of GABA and glutamate the major inhibitory and excitatory neurotransmitters, respectively. A time- and dose-dependent decline on the activity of topo I was observed when cerebellum sections were treated with glutamate and this decline was consistent at various glutamate concentrations. Although treatment with GABA for 1 min showed a slight inhibitory effect on topo I activity, this effect was not consistent and diminished in the 5-min treated cerebellar slices. In addition, we demonstrated that the influence of glutamate on the activity of topo I is reversible since a time-dependent recovery (and even an increase) in the activity of the enzyme occurs after the inhibition by glutamate. Moreover, no effect on topo I activity was detected when GABA or glutamate were directly added to the reaction mixture containing nuclear proteins derived from untreated cerebellar sections. The decrease in topo I activity following glutamate treatment might be due to a reduction in the level of topo I protein; however, no significant effect on the level of topo I protein was observed in the various treatments. Altogether, these data suggest that glutamate or GABA probably affect topo I activity by posttranslational modifications of the enzyme protein.

Posttranslational modifications of topo I that inhibit its activity include dephosphorylation of phosphoserine residues [30] or ADP-ribosylation of the enzyme proteins [10, 11]. In previous work, it was shown that depolarized primary cell cultured neurons exhibited a Ca^{+2}-dependent fast activation of PARP induced through IP3. This PARP activation was shown to result in poly ADP-ribosylation of topo I, which as a consequence downregulates its activity [21, 28]. This led us to assume that in the CNS of mice, topo I activity might be regulated by Ca^{+2}-dependent PARP activation following glutamate treatment. Glutamate triggers a robust increase in the Ca^{+2} concentrations in the cell; therefore, we examined the possibility that the effect of glutamate on topo I is Ca^{+2} dependent. Brain sections were exposed to glutamate in the presence and absence of BAPTA/AM, a Ca^{+2} chelator, or in the presence or absence of Ca^{+2} ions in the ringer solution. Indeed, decreasing the

Ca^{+2} concentrations by BAPTA or omitting the Ca^{+2} from the ringer solution revealed that the inhibitory effect of glutamate on topo I activity was diminished. This indicates that the decrease in topo I activity by glutamate treatment is Ca^{+2} dependent. To determine the glutamate ionotropic receptors that participate in the effect on topo I activity, common inhibitors of AMPA and NMDA receptors (AP5 and CNOX, respectively) were used. NMDA and AMPA inhibitors diminished the glutamate-induced decrease of topo I activity. Moreover, the exposure of the brain slices to NMDA or AMPA inhibitors alone, without glutamate treatment, increased topo I activity. This suggests that both ionotropic receptors probably participate in the Ca^{+2}-dependent glutamate-induced decrease of topo I activity. The increase in topo I activity in slices treated with the ionotropic receptor inhibitors (compared to that found in the untreated brain slices) is probably due to the reduction in the inhibition of topo I by the endogenous glutamate.

Native AMPA receptor channels are impermeable to calcium ions, a function controlled by the GluR2 subunit. Sustained activation of AMPA by glutamate depolarizes the postsynaptic cells releasing the NMDA channel inhibition, and thus, NMDA receptor is activated. NMDA receptors are permeable to calcium ions; therefore, activation of NMDA will lead to a calcium influx into the postsynaptic cells [31]. The sequential AMPA/NADM receptors activation and the finding that inhibition of each ionotrophic receptor alone was enough to diminish the glutamate inhibitory effect of topo I, suggesting that the influx of calcium ions as a consequence of the sequential AMPA/NADM activation is necessary for the glutamate-induced topo I inhibition.

To demonstrate the involvement of PARP in the glutamate-induced topo I inhibition, we pretreated the brain slices with 3AB a known PARP inhibitor [32]. Indeed, inhibition of PARP diminished the inhibitory effect of glutamate on topo I activity. Since PARP-1 demonstrated a different effect on topo I in the presence of topo I inhibitor, CPTs [11–13, 33], we examined the effect of glutamate on topo I activity in the presence of TPT. CPT (and its analogs including TPT) inhibits the religation reaction of topo I by selectively inducing a stabilization of the cleavable complexes. Indeed, a decline in the activity of topo I in the cerebellar sections subjected to TPT was observed suggesting that the topo I enzyme in the cerebellum is sensitive to TPT. When the sections were pre-incubated with TPT and then exposed to glutamate for 1 min, a relative increase in topo I activity was observed, which suggest that glutamate treatment diminished the inhibitory effect of TPT on topo I. This contradictory effect of glutamate on topo I can be explained by the findings that PARP-1 could facilitate the religation activity of topo I when the enzyme is linked to the DNA in the presence of CPT [11–13, 33]. Therefore, activation of PARP by glutamate in the presence of TPT will indeed result in the diminishing of the glutamate inhibitory effect on topo I.

A recent study suggests that poly(ADP-ribose) binds to the splicing factor ASF/SF2 and regulates its phosphorylation by topo I [33]. Therefore, one may postulates that glutamate, by activation of PARP, may affect also the kinase activity of topo I and thus influences the alternative splicing in the brain cells. This assumption should be investigated.

Altogether, our findings suggest that the pathway in which glutamate influences the activity of topo I is through the sequential activations of AMPA/NAMDA receptors leading to increasing of the intracellular Ca^{+2} concentrations, resulting in a fast activation of PARP which ADP-ribosylated topo I protein that downregulated topo I activity. In the presence of topo I inhibitor, TPT, the activation of PARP-1 by glutamate will facilitated the religation process of topo I and diminished the inhibitory effect of glutamate on topo I.

The glutamate ionotropic receptors are important for normal brain function and are considered as primary candidates for the molecular basis of learning and memory [34]. It was shown that long-term memory in *Aplysia californica* requires poly ADP-ribosylation [35], and it was suggested that PARP-1 might be involved in memory of injury [36]. Therefore, the regulation of topo I activity in the brain by ionotropic receptors through activation of PARP suggests that topo I may play a role in learning and memory.

The fast modification of topo I activity following exposure to major neurotransmitters suggests that neuronal activity is a critical factor in the regulation of this enzyme in the brain, a process that may lead to brain specific regulation of gene expression which is mediated by this cross talk between topo I and neuronal activity. Our results further suggest that this cross talk between neuronal activity and topo I may also have important clinical implication in the brain considering the use of anti topo I inhibitor, TPT, for the treatment of brain tumor metastases.

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