Neuroprotection by Tosyl-Polyamine Derivatives through the Inhibition of Ionotropic Glutamate Receptors

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ABSTRACT

Tosyl-polyamine derivatives such as N-[(4-[(guanidinobutylamino)butyl]4-methylbenzenesulfonyl)amido-3-hydroxy-5-methyl-4-isoxazolepropionic acid trihydrochloride (TsHSPMG) have been found to strongly inhibit macroscopic currents through heteromeric N-methyl-D-aspartate (NMDA) receptors (NR1/NR2A, NR1/NR2B) and Ca$^{2+}$-permeable $\alpha$-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors expressed in Xenopus laevis oocytes on voltage-clamp recording. In the present study, it was found that the inhibition of NMDA receptor activity induced by tosyl-polyamine derivatives was voltage-dependent. Some mutations located in the intracellular region of the channel pore, such as NR1 E621Q and NR2B W607L, reduced the inhibition by tosyl-polyamine derivatives, suggesting that tosyl-polyamine derivatives penetrate deeply into the channel pore of NMDA receptors. The neuroprotective effects of tosyl-polyamine derivatives against cell injury caused by NMDA were investigated in cultured rat hippocampal neurons. Addition of 1 mM TsHSPMG to medium ablated the neurotoxicity induced by NMDA, and a similar effect was observed with 30 $\mu$M memantine. The neuroprotective effects of tosyl-polyamine derivatives on NMDA-induced seizures in mice were also assayed. Intracerebroventricular or intravenous injection of TsHSPMG (0.1 or 0.5 mg/kg) decreased the seizures induced by intraperitoneal injection of NMDA in mice. These findings indicate that tosyl-polyamine derivatives exhibit neuroprotective effects not only in primary cultured neurons but also in mice.

The ionotropic glutamate receptors are ligand-gated ion channels that mediate excitatory neurotransmission in the brain. Three pharmacologically defined classes of ionotropic glutamate receptors were originally named based on their agonist selectivity: N-methyl-D-aspartate (NMDA), $\alpha$-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), and kainate (Dingledine et al., 1999). In the central nervous system, NMDA receptors play critical roles in a variety of neurophysiological phenomena, including neurodevelopment, synaptic plasticity, and excitotoxicity because of their high permeability to Ca$^{2+}$. The neurodegeneration associated with a variety of acute and chronic disorders (e.g., ischemic stroke, Parkinson’s disease, Alzheimer’s disease, and dementia) is due in part to overactivation of NMDA receptors (Sattler and Tymianski, 2001). Inhibitors of NMDA receptors have thus been developed as anticonvulsants and neuroprotective agents.

The NMDA receptor consists of at least two types of subunits, NR1 and NR2 (Dingledine et al., 1999). One subunit includes three transmembrane domains (M1, M3, and M4)
plus a cytoplasm-facing re-entrant membrane loop (M2). The M2 loop region in NR1 and NR2 subunits is a critical determinant of divalent cation permeability and Mg$^{2+}$ blockade. NR1 is a single-gene product expressed as eight alternatively spliced mRNAs, and NR2A, NR2B, NR2C, and NR2D are distinct gene products. NMDA receptors probably consist of tetrameric and heteromeric subunit assemblies that differ in physiological and pharmacological properties depending on differences in NR2 subunits (Danyes and Parsons, 1998). NMDA receptor channels are blocked by a variety of structurally dissimilar blockers, including Mg$^{2+}$, ketamine, MK-801, memantine, various spider toxins, and trienylspermidine (Huettner and Bean, 1988; Igarashi et al., 1997; Dingledine et al., 1999; Kashiwagi et al., 2002).

Polyamines (putrescine, spermidine, and spermine) are ubiquitously present in prokaryotic and eukaryotic cells. In the central nervous system, specific interactions of polyamines with several structurally and functionally distinct types of cation channels have been reported previously (Williams, 1997). Among these, the most striking are the blockade of some types of K$^+$ channels and modulation of NMDA receptors. Spermine has complex effects on NMDA receptors, including two types of stimulation and one type of voltage-dependent blockade (Williams et al., 1995; Williams, 1997; Masuko et al., 1999a, 2008; Jin et al., 2008). One of the effects of spermine is "glycine-independent" stimulation, observed in the presence of saturating concentrations of glutamate and glycine. With recombinant NMDA receptors, this type of stimulation is observed only at receptors containing splice variants of NR1 that lack the exon-5 insert, expressed together with the NR2B subunit (Durand et al., 1993; Williams, 1994). Inhibition by extracellular spermine is strongly voltage-dependent, being more pronounced at hyperpolarized than at depolarized membrane potential. Single-channel patch-clamp recording has been used to study the mechanism of this effect. Spermine (10–1000 µM) was found to decrease single-channel conductance and decreased average channel open-time (Rock and MacDonald, 1992).

We recently reported the inhibitory effects of cyclic polyamines such as CP2323 on NMDA receptor currents (Masuko et al., 2008). In addition, we found that CP2323 has potent neuroprotective effects against excitotoxic neurotoxicity mediated via inhibition of NMDA receptors. The inhibitory effects on NMDA receptors of some linear polyamine derivatives such as dansyl-spermine (DnSPM), N-[3-(4-(3-amino propylamino)butylamino)propyl]-5-(dimethylamino)naphthalenene-1-sulfonamide trihydrochloride, anthraquinone polyamines, and anthraceneaminopropyl)-5-(dimethylamino)naphthalenene-1-sulfonamide dansyl-spermine (DnSPM, NMDA, AMPA Clones, and Numbering of Residues. The NR1 clone used in this study is the NR1A variant (Moriyoshi et al., 1991), which lacks the 21-amino acid insert encoded by exon-5. This clone was a kind gift from Dr. S. Nakashima (Osaka Bioscience Institute, Osaka, Japan). The rat and mouse NR2A and NR2B clones (Kutsuwada et al., 1992; Monyer et al., 1992) were kindly supplied by Dr. M. Mishina (Graduate School of Medicine, University of Tokyo, Tokyo, Japan) and Dr. P. H. Seeburg (Center for Molecular Biology, University of Heidelberg, Heidelberg, Germany). The mouse NR2C and NR2D clones (Ikeda et al., 1992; Kutsuwada et al., 1992) and the mouse GluR1 and GluR2 clones (Sakimura et al., 1990) were also kind gifts from Dr. M. Mishina. The preparation of NR1 and NR2B mutants has been described previously (Williams et al., 1998; Kashiwagi et al., 2002, 2004; Jin et al., 2007). Amino acids are numbered from the initiator methionine in each subunit. This differs from the numbering system used in some other laboratories, in which residues are numbered from the start of the mature peptide (Kuner et al., 1996; Beck et al., 1999).

Expression in Oocytes and Voltage-Clamp Recording. Adult female Xenopus laevis (Saitama Experimental Animals Supply Co., Ltd., Saitama, Japan) were chilled on ice, and the preparation and maintenance of oocytes were carried out as described previously (Masuko et al., 1999a, 2007a). Capped cRNAs were prepared from linearized cDNA templates using mMessage mMACHINE kits (Ambion, Austin, TX). Oocytes were injected with NR1A and NR2 cRNAs at a ratio of 1:5 (0.2–4 ng of NR1A plus 1–20 ng of NR2). Where indicated, oocytes were injected with cRNAs of GluR1 (50 ng) or GluR1/GluR2 (10 ng of GluR1 plus 20 ng of GluR2) instead of NR1 and NR2 cRNAs. Macroscopic currents were recorded with a two-electrode voltage-clamp method using the CEZ-1250 dual-electrode voltage-clamp amplifier (Nihon Koden, Tokyo, Japan). Electrodes were filled with 3 M KCl. Oocytes were continuously superfused (approximately 5 ml/min) with a Mg$^{2+}$-free saline solution (96 mM NaCl, 2 mM KCl, 1.8 mM BaCl$_2$, and 10 mM HEPES, pH 7.5). This solution contained BaCl$_2$ rather than CaCl$_2$, and, in most experiments, oocytes were injected with K$^+$/Ba$^2+$-containing solutions (100 mM BaCl$_2$; 40 mM; pH 7.5) on the day of recording to eliminate Ca$^{2+}$-activated Cl$^-$ currents (Leonard and Kelso, 1990; Masuko et al., 2004).

Alamar Blue and Trypan Blue Assay in Neuroblastoma SH-SY5Y Cell Line. The SH-SY5Y neuroblastoma cell line was purchased from American Type Culture Collection (Manassas, VA). Cells were grown in Dulbecco's modified eagle medium (DMEM) (Invitrogen, Carlsbad, CA), supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml), and 10% (v/v) heat-inactivated fetal bovine serum (FBS) (Invitrogen). Cells were maintained at 37°C in a CO$_2$ incubator in a saturated humidity atmosphere containing 95% air and 5% CO$_2$. Cell viability was measured by alamar blue staining, which indicates mitochondrial activity (O'Brien et al., 2000), and by trypan blue staining of dead cells. When cells became 50% confluent, they were treated with polyamine derivatives or memantine for 24 h without FBS. Then, alamar blue stock solution was added to prepare 10% alamar blue. After 6 h, the reduced form of alamar blue was measured at a wavelength of 570 nm. Percentage of cell viability in the presence of test compound was expressed as a percentage of that in the vehicle control. For trypan blue testing, SH-SY5Y cells were cultured in the presence of polyamine derivatives or memantine for 24 h, and viable cell number was counted in the presence of 0.2% trypan blue.

Primary Hippocampal Cell Culture. Hippocampi were dissected from the brains of 19-day Wistar rat fetuses and placed in Brooks-Logan solution (137 mM NaCl, 2.7 mM KCl, 6 mM Na$_2$HPO$_4$, 1.7 mM KH$_2$PO$_4$, 44 mM sucrose, 25 mM glucose, and 10 mM HEPES, pH 7.4) (Dawson et al., 1991). The hippocampal tissues were then dissociated in Brooks-Logan solution containing 0.2% trypsin.
solution at 37°C for 20 min. Subsequently, tissues were triturated by repeated passage through a constricted Pasteur pipette. Dispersed tissues were allowed to settle for 3 min, and the supernatant was transferred to a fresh tube and centrifuged at 1000 rpm for 1 min. The pellet was resuspended in a modified DMEM containing 21 mM KCl, 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin.

Viable cells were counted, and cells were then plated onto 24-well plates coated with polyethylenimine (Sigma-Aldrich) at 1 × 10⁵ per well. Cell cultures were kept in a humidified atmosphere of 95% air and 5% CO₂ at 37°C. After 2 days, the medium was replaced with fresh medium containing 10% horse serum (Invitrogen) instead of FBS and 3 mM cytosine arabinoside (Nacalai Tesque, Kyoto, Japan).

After every 2 days of culture, half of the medium was replaced with fresh medium containing 100 ng/ml nerve growth factor.

**NMDA-Induced Toxicity in Rat Hippocampal Neurons.** After 7-day culture, hippocampal neuronal cells were washed with a modified HEPES-buffered saline (146 mM NaCl, 21 mM KCl, 2 mM CaCl₂, 10 mM d-glucose, and 10 mM HEPES, pH 7.4) and exposed to 1 mM NMDA with 10 μM glycine at 37°C for 1 h in the presence or absence of polyamine derivatives or NMDA receptor antagonists in modified HEPES-buffered saline. Cells were cultured in FBS-free modified DMEM. After 24 h, to quantify cell death, lactate dehydrogenase (LDH) released into the cell culture medium from injured cells was estimated by measuring the production of diformazane at 570 nm (Decker and Lohmann-Matthes, 1988). Values were normalized to the activity of LDH released from vehicle-treated cells (100%) and are expressed as percentages of the control.

**Neuroprotective Effects of Polyamine Derivatives against NMDA-Induced Seizures in Mice.** Male ddY mice (25–30 g; 5 weeks old) were allowed to acclimate with free access to food and water and were maintained on a 12:12-h light/dark cycle. Polyamine derivatives (0.1 mg/kg i.c.v.; 0.5 mg/kg i.v.) or memantine (2 mg/kg i.v.) were administered intraperitoneally 30 min before NMDA injection. The drug effects were compared with those of vehicle

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**Fig. 1.** Effects of polyamine derivatives on glutamate receptors. A, structure of polyamine derivatives (TsSPM, DnSPM, TsSPMG, TsHSPM, and TsHSPMG). B, effects of 1 μM polyamine derivatives were determined in oocytes expressing NMDA (NR1/NR2A, NR1/NR2B, NR1/NR2C, and NR1/NR2D), Ca²⁺-permeable AMPA (GluR1), and Ca²⁺-nonpermeable AMPA (GluR1/GluR2) receptors and voltage-clamped at −70 mV. GluR1 and GluR1/GluR2 receptor currents were evoked by 100 μM kainate. Macromolecular currents in the presence of polyamine derivatives were expressed as a percentage of the control response at NMDA or AMPA receptors. Values are presented as mean ± S.E.M. from four to six oocytes. C, voltage-dependent inhibition by polyamine derivatives of NMDA receptor currents was measured. Effects of 0.3 μM TsSPM, DnSPM, and TsSPMG; 0.1 μM TsHSPM; and 0.03 μM TsHSWMPG on NR1/NR2A and NR1/NR2B receptors, and 3 μM TsSPM, DnSPM, TsSPMG, and TsHSPM and 1 μM TsHSPMG on NR1/NR2C and NR1/NR2D receptors was measured at −20 and −100 mV. Values are presented as mean ± S.E.M. from four to six oocytes for each subunit combination.

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i.c.v.; 10 mg/kg i.v.) was administered by intracerebroventricular or intravenous injections at 30 min before intraperitoneal injection of NMDA (115 mg/kg). NMDA-injected mice exhibited intense seizure behavior characterized by explosive running and jumping, followed by posturing, opisthotonos, clonic movements of the limbs, and tonic hindlimb extension. The duration of seizure behavior of mice after the first such observed behavior was measured for 60 min. Analysis of variance with the Bonferroni test for post hoc comparison was used for statistical analysis. All procedures used in this study were performed in accordance with the Guidelines established by the College of Pharmacy at Nihon University for the Care and Use of Laboratory Animals.

Results

Effects of the Polyamine Derivatives TsSPM, TsSPMG, TsHSPM, TsHSPMG, and DnSPM on Glutamate Receptors Expressed in X. laevis Oocytes. Spermine potentiates NMDA receptor currents at −20 mV, a depolarized membrane potential, in the presence of saturating concentrations of glycine (glycine-independent stimulation), an effect that involves an increase in the frequency of channel opening and reduction of desensitization of NMDA receptors (Williams, 1997). However, none of the polyamine derivatives we tested (TsSPM, TsSPMG, TsHSPM, TsHSPMG, and DnSPM shown in Fig. 1A) potentiated NMDA receptor currents (Fig. 1C). The inhibitory effects of 1 μM polyamine derivatives on NMDA receptors were then examined at −70 mV (Fig. 1B). All polyamine derivatives exhibited inhibitory effects on NR1/NR2 receptor currents induced by 10 μM glutamate with 10 μM glycine. The inhibition of NR1/NR2A and NR1/NR2B receptors by polyamine derivatives was more pronounced than that of NR1/NR2C receptors; 14, 13, 2.9, and 33 μM for NR1/NR2C receptors; and 14, 13, 15, 3.1, and 30 μM for NR1/NR2D receptors, respectively (Fig. 2, A–D; Table 1). Sigmoidal inhibition curves (solid lines) were drawn to fit the data with a Hill coefficient (0.7–1.3) using the Prism 4 software program (GraphPad Software Inc., San Diego, CA). TsHSPMG was approximately 100-fold more potent at NR1/NR2A and NR1/NR2B receptors than at NR1/NR2C and NR1/NR2D receptors.

Identification of Amino Acid Residues Involved in the Blockade by Polyamine Derivatives. To identify the amino acid residues involved in the channel blockade of NMDA receptors by polyamine derivatives, the effects of mutations were examined on the residues composing the channel pore, where voltage-dependent channel blockers are generally bound (Fig. 3A). Amino acid residues for which mutations reduced the inhibition by tosyl-polyamine derivatives were more than 20% are highlighted by open circles. The inhibitions at −100 mV were more prominent than those at −20 mV, suggesting that the inhibition by polyamine derivatives is voltage-dependent. The IC50 values of TsSPM, TsSPMG, TsHSPM, TsHSPMG, and DnSPM were 0.37, 0.36, 0.093, 0.011, and 0.31 μM for NR1/NR2A receptors; 0.45, 0.36, 0.16, 0.029, and 0.33 μM for NR1/NR2B receptors; 43, 21, 13, 2.9, and 33 μM for NR1/NR2C receptors; and 14, 13, 15, 3.1, and 30 μM for NR1/NR2D receptors, respectively (Fig. 2, A–D; Table 1). Sigmoidal inhibition curves (solid lines) were drawn to fit the data with a Hill coefficient (0.7–1.3) using the Prism 4 software program (GraphPad Software Inc., San Diego, CA). TsHSPMG was approximately 100-fold more potent at NR1/NR2A and NR1/NR2B receptors than at NR1/NR2C and NR1/NR2D receptors.

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tors. The narrowest region, composed of M2 loops of NR1 and NR2 subunits, is a critical determinant of divalent cation permeability and channel blockade by Mg\(^{2+}\) and other organic channel blockers (Kashiwagi et al., 2002). The inhibitions by all polyamine derivatives except DnSPM were significantly reduced in NR1/NR2B receptors containing NR1 (N616Q), NR2B (N615Q), and NR2B (N616Q), located in the narrowest region of the channel pore (Fig. 3, B and C). The inhibition by DnSPM was stronger in mutant NMDA receptors containing NR1 (N616Q) or NR2B (N615Q) than in wild-type receptors. Furthermore, the inhibition by all polyamine derivatives tested was significantly reduced in NMDA receptor mutants located in the vestibule of the channel and extracellular region, such as NR1 (Y647L), NR1 (N650A), NR1 (L655A), NR1 (D669N), NR1 (T807S), NR2B (W559L), and NR2B (Y646L) (Fig. 3, B and C). It has been suggested that triazenlepyspermidine mainly interacts with the narrowest region of the channel pore together with the vestibule of the channel and extracellular region of NMDA receptors (Igarashi et al., 1997; Kashiwagi et al., 2002) and that anthraquinone polyamines interact with the intracellular region of the channel in addition to the regions in which tribenzylspermidine interacts (Kashiwagi et al., 2004; Jin et al., 2007). Thus, the binding site of TsSPM, TsSPMG, TsHSPM, and TsHSPMG on NMDA receptor may be similar to that of anthraquinone polyamines.

### Cell Toxicity of Polyamine Derivatives in Neuroblastoma SH-SY5Y Cells.

To examine cell toxicity, neuroblastoma SH-SY5Y cells were exposed to polyamine derivatives or memantine for 24 h, and cell viability was assessed by alamar blue or trypan blue. On the alamar blue assay, the IC\(_{50}\) (50% inhibition of mitochondrial enzyme activities) value of memantine was 146 \(\mu\)M on the alamar blue assay, and the LC\(_{50}\) (50% lethal concentration) value of memantine was 141 \(\mu\)M on the trypan blue assay.

### NMDA Channel Block

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<tr>
<th>NMDA Channel Block</th>
<th>Cell Toxicity</th>
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<td>NR1/NR2A</td>
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<td>IC(_{50})</td>
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<td>TsSPM</td>
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<td>TsSPMG</td>
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<tr>
<td>DnSPM</td>
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<td>Memantine</td>
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Comparison between polyamine derivatives and memantine in NMDA channel block and cell toxicity experiments were performed as described under Materials and Methods. Cell toxicity data for memantine are from Masuko et al. (2007b). The IC\(_{50}\) (50% inhibition of mitochondrial enzyme activities) value of memantine was 146 \(\mu\)M on the alamar blue assay, and the LC\(_{50}\) (50% lethal concentration) value of memantine was 141 \(\mu\)M on the trypan blue assay.
Fig. 3. Effects of polyamine derivatives on wild-type and mutant NR1/NR2B receptors. A, schematic illustration of the NR1 and NR2B subunits, which contain three transmembrane segments (M1, M3, and M4), a re-entrant loop (M2), an extracellular N-terminal domain, an extracellular loop between M3 and M4, an intracellular loop between M1 and M2, and an intracellular C-terminal domain. The inhibitory effects of polyamine derivatives on the mutants were examined at the indicated amino acid residues in the figure. B, representative traces showing the effects of 0.3 μM TsHSPM and 0.1 μM TsHSPMG on wild-type and mutant NR1/NR2B receptors. NMDA receptor currents were evoked by 10 μM glutamate with 10 μM glycine and voltage-clamped at −70 mV. C, effects of 1 μM TsSPM, DnSPM, and TsSPMG; 0.3 μM TsHSPM; and 0.1 μM TsHSPMG were determined in oocytes expressing wild-type and mutant NR1/NR2B receptors, voltage-clamped at −70 mV. Values are presented as mean ± S.E.M. from four to six oocytes.
NMDA receptor antagonists 10 to 100-fold from 2 to 0 mM, 3 to 30 mM, 25 to 300 μM 5,7-dichlorokynurenic acid (5,7-DCKA); 3–30 μM ifenprodil; and 3 to 30 μM memantine on NMDA-induced neuronal cell death. LDH release from cultured hippocampal neurons, as index of neuronal cell death, was measured at vehicle-treated cells (100%) and are expressed as percentage of the control. Data were normalized to the activity of LDH release from the regulatory domain (N-terminal domain), to which ifenprodil and Zn2+ bind (Masuko et al., 1999a; Paoletti et al., 2000). In this study, we found that tosyl-polyamine derivatives (TsSPM, TsSPMG, TsHSPM, and TsHSPMG) directly inhibit macroscopic currents at heteromeric NMDA receptors through penetration into the channel pores of NMDA receptors.

The cell toxicity of DnSPM, which has a dansyl group, was stronger than that of TsSPM, which has a tosyl group, although the degrees of inhibition of NMDA receptor activity by the two were almost the same. We previously synthesized cleft-type cyclophane compounds, such as TsDCn and DnCn, which have tosyl and dansyl groups, respectively. TsDCn and DnCn exhibited neuroprotective effects against excitatory neurotoxicity through inhibition of NMDA receptors. The degrees of inhibition of TsDCn and DnCn were almost the same, although the cell toxicity of DnCn was much stronger than that of TsDCn (Masuko et al., 2007b). Therefore, we synthesized TsSPM and TsSPMG, which have guanidyl groups, and TsHSPM and TsHSPMG, which have homospermine instead of spermine moieties, because the guanidyl group and homospermine moiety may not be sensitive to polyamine oxidases (spermine oxidase and acetylpolyamine oxidase), which degrade spermine to produce acrolein, a toxic unsaturated aldehyde (Sharmin et al., 2001). TsSPM, TsHSPM, and TsHSPMG (at concentrations up to 300 μM) did not exhibit cell toxicity in the presence of FBS (data not shown).

There are a few NMDA receptor antagonists available for clinical use, including ketamine and, most importantly, memantine, both of which act as channel blockers. The IC50 values for ketamine and memantine were 1.4 and 1.0 μM, respectively, for NMDA receptors expressed in X. laevis oocytes in voltage-clamp recording (Ogata et al., 2006). The inhibitory potencies of TsHSPM (IC50 = 0.093 μM) and TsHSPMG (IC50 = 0.011 μM) for NR1/NR2A receptors were greater than that of memantine, a therapeutic drug for Alzheimer’s disease, under the experimental conditions. However, the inhibition by TsHSPM and TsHSPMG for NMDA receptor was reversible, because NMDA receptor current recovered immediately in X. laevis oocyte (Fig. 3B) and hippocampal neuron (data not shown) after removal of polyamine derivatives.

It was shown that polyamine derivatives worked better at
the hyperpolarized potential than at the depolarized potential (Fig. 1C). However, polyamine derivatives decreased the duration of seizure behavior induced by NMDA (Fig. 5). In addition, dansyl-spermine exhibited neuroprotective effect through inhibition of NMDA receptors in focal cerebral ischemia model in mice using middle cerebral artery occlusion method (Li et al., 2004). These data suggest that polyamine derivatives inhibit the activity of NMDA receptors under the pathological depolarized conditions.

The potency of many open-channel blockers, including MK-801, polyamine derivatives such as tribenzylspermidine and anthraquinone polyamines, and memantine, was greatly reduced in the mutant NR1 (N616Q), NR2B (N615Q), and NR2B (N616Q) receptors (Kashiwagi et al., 2002, 2004; Jin et al., 2007). Similarly, inhibition by polyamine derivatives, except for that by DnSPM, were significantly affected in NR1/NR2B receptor mutants in the narrowest region containing NR1 (N616Q), NR2B (N615Q), and NR2B (N616Q) receptors (Kashiwagi et al., 2002, 2004; Jin et al., 2007). The narrowest constriction in wild-type NR1/NR2B receptor mutants was estimated to be ∼0.55 nm, and this was increased to 0.67 nm in NR1/NR2A (N615Q) receptors, 0.75 nm in NR1 (N616G)/NR2A receptors, and 0.87 nm in NR1 (N616G)/NR2A (N615G) receptors, with a diameter of the naphthalene ring of DnSPM of 0.8 to 0.85 nm (Chao et al., 1997). It seemed that extension of the narrowest region in mutant NMDA receptors increased the inhibition by DnSPM. In addition, mutant NMDA receptors containing NR1 (Y647L), NR1 (N650A), NR1 (L655A), NR1 (D669N), NR1 (T807S), NR2B (W559L), and NR2B (Y646L), located in the vestibule of the channel and extracellular region, significantly reduced inhibition by all of the polyamine derivatives tested. The inhibitory potencies of anthraquinone polyamines and anthracene polyamines were also reduced in the mutants of these positions (Jin et al., 2007). Our results suggest that tosyl-polyamine derivatives interact with amino acid residues of the extracellular region, vestibule of the channel, narrowest region of the channel, and intracellular region of the NMDA receptor, at almost the same site of that of interaction with anthraquinone polyamines and anthracene polyamines.

Comparison was made between tosyl-polyamine derivatives and memantine for inhibitory effects on NMDA receptors, toxicity in neuroblastoma SH-SY5Y cells, neuroprotection of cultured hippocampal neurons, and antiseizure effects in mice (Figs. 4 and 5; Table 1). TsHSPM and TsHSPMG exhibited strong inhibition of NMDA receptors expressed in X. laevis oocytes, weak toxicity in SH-SY5Y cells, and strong neuroprotective effects against NMDA toxicity in hippocampal neurons. Moreover, TsHSPM and TsHSPMG significantly decreased the duration of seizure behavior induced by NMDA in mice. These findings suggest that TsHSPM and TsHSPMG may be useful as a lead compound of the development of therapeutic drugs for Alzheimer's disease. The tosyl-polyamine derivatives seemed to pass through the blood-brain barrier, because intravenous administrations of TsHSPM and TsHSPMG prevented NMDA-induced seizures in mice to a significant extent (Fig. 5B).
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