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A single amino acid determines the subunit-specific spider toxin block of α-amino-3-hydroxy-5-methylisoxazole-4-propionate/kainate receptor channels

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ABSTRACT **Joro spider toxin (JSTX) is one of the most potent antagonists of glutamatergic AMPA/KA (α-amino-3-hydroxy-5-methylisoxazole-4-propionate/kainate) receptor channels in invertebrates and vertebrates. A differential blocking effect on certain types of glutamatergic synapses—e.g., parallel and climbing fiber synaptic inputs to rat cerebellar Purkinje neurons—has been shown by using a synthetic analog of the spider toxin. By investigating the molecular basis of the JSTX action on the recombinant AMPA/KA receptors GluR1–GluR4 and GluR6 expressed in *Xenopus* oocytes, we found that submicromolar concentrations of JSTX exert a subunit-specific block. Thus, receptor subunits forming a receptor channel with a linear current–voltage (*I–V*) relationship (GluR1/2, GluR2/3, and GluR6) were not affected, while receptor subunits with rectifying *I–V* relationships (GluR1, GluR3, GluR4, and GluR1/3) were reversibly blocked by JSTX. By using receptor-subunit mutants obtained by site-directed mutagenesis, we have identified a single amino acid position (glutamine in the proposed second transmembrane domain) that is critical for the JSTX block. Since this site has previously been shown to control the *I–V* relationship of the AMPA/KA receptor channel and to participate in the regulation of the channel's permeability for calcium ions, our findings suggest that JSTX binds close to the central pore region of the channel.**

Joro spider toxin (JSTX) (Fig. 1*a*), the toxin of the spider *Nephila clavata*, and the closely related arthropod toxins *Nephila* toxin (NSTX) and argiotoxin are potent antagonists of excitatory synaptic transmission and glutamate-mediated currents in various types of neurons (1–8). The common structural motif of these toxins is a polyamine chain with an aromatic side chain and a positively charged terminal amino group. Both the aromatic side chain and the positively charged amino group seem to be essential for the binding of the toxins to glutamate receptors (7–9). To investigate the molecular basis of toxin binding to glutamate receptors, we studied the blocking effect of JSTX on recombinant AMPA/KA receptors GluR1–GluR4 and GluR6 expressed in *Xenopus* oocytes. Several functional properties of recombinant AMPA/KA receptors have been reported (10–21). Thus, the subunit GluR1 or GluR3 combined with the subunit GluR2 has a linear current–voltage (*I–V*) relationship and a low permeability for calcium, while combinations lacking GluR2 have an inwardly rectifying *I–V* relationship and a high calcium permeability (16–19). In contrast, the receptor subunit GluR6 is characterized by a linear *I–V* relationship and a high calcium permeability (14, 20). Here we report the block by JSTX of specific AMPA/KA receptor subunits and the identification of a single amino acid within the predicted membrane-spanning region M2 which is critically involved in the JSTX block. Interestingly, the same amino acid which is responsible for the blocking of JSTX also determines the shape of the *I–V* curve and the calcium permeability of some receptors (16, 17, 20).

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MATERIALS AND METHODS

Preparation of *Xenopus* Oocytes and mRNA. The procedures for preparing and maintaining the oocytes followed the methods of Methfessel *et al.* (22). Briefly, after a 3-hr incubation with collagenase (Worthington; 2.8 mg/ml), the residual follicle cell layer of the oocytes was skinned. The electrophysiological measurements from oocytes were done 3–4 days after the injections with mRNA (2 ng per oocyte). The mRNA was prepared as described by Hollmann *et al.* (10). The coexpression of the receptor subunit GluR2 with GluR1 or GluR3 was achieved by the injection of a 10-fold higher concentration of GluR2. All subunits were "flop-splice" (32) variants of the receptor clones. The glutamate receptor clones and all recombinant products were maintained in the plasmid pBluescript SK(−) (Stratagene). The mutant clones were made by using the oligonucleotide site-directed mutagenesis system 2 of Amersham. Chemicals were bought from Sigma, except for JSTX-3, which was bought from Research Biochemicals (Natick, MA), and 1-naphthylspermine, which was a generous gift from Nobufumi Kawai, Tochigi-ken, Japan.

Electrophysiology and Data Analysis. Current recordings were performed by using the two-electrode voltage-clamp system Turbo Tec 01C ("npi electronic," Tamm, Germany) as described (19, 21, 22). During measurements the recording chamber was continuously perfused with saline solutions kept at room temperature (21–24°C). Normal saline (frog Ringer solution) was 115 mM NaCl/2.5 mM KCl/1.8 mM $CaCl_2$/10 mM Hepes, pH 7.2 with NaOH. Perfusion solutions also contained 150 μM niflumic acid (Sigma) to block the transient component of intrinsic calcium-activated chloride currents in *Xenopus* oocytes. Recording electrodes were made of a fine glass pipette (1–2 MΩ) filled with 2 M KCl. Signals were filtered at 0.1 kHz with an eight-pole Bessel filter (Frequency Devices, Haverhill, MA) and stored on video tapes by a PCM/VCR recording device (Instrutech, Mineola, NY). The software used for analysis was obtained either from Instrutech or Luigs and Neumann (Ratingen, Germany).

RESULTS

High-Affinity, Reversible Blocking Action of JSTX. Fig. 1*b* shows the blocking effect of JSTX on an oocyte expressing

Abbreviations: JSTX, Joro spider toxin; AMPA, α-amino-3-hydroxy-5-methylisoxazole-4-propionate; KA, kainate.

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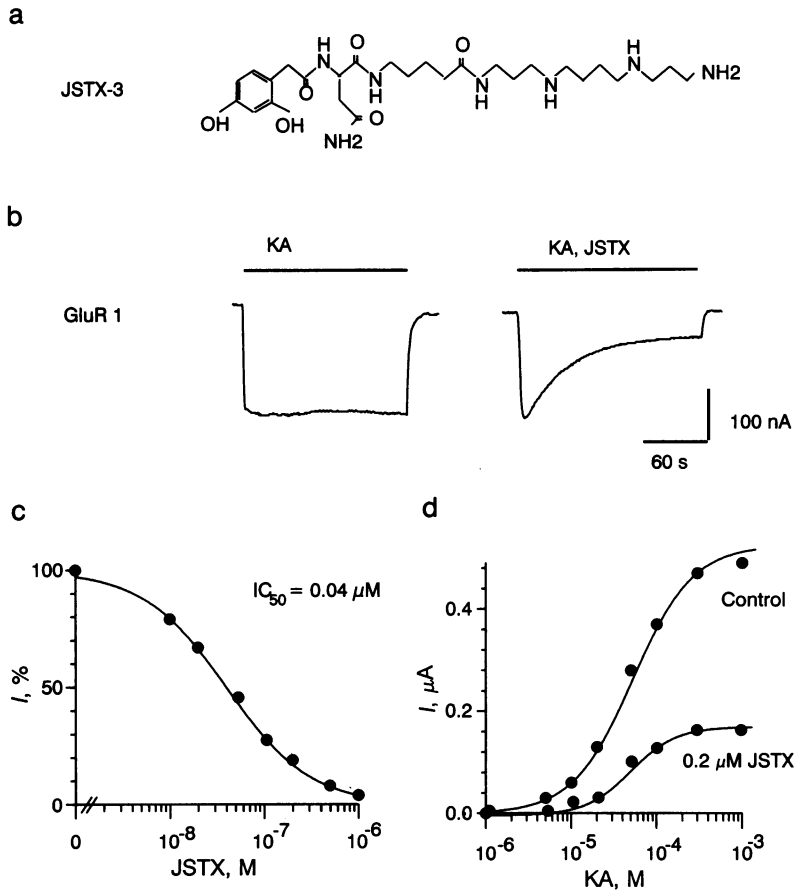

FIG. 1. Block of kainate (KA)-mediated currents through α-amino-3-amino-5-methylisoxazole-4-propionate (AMPA)/KA receptor channel GluR1 by JSTX. (*a*) Chemical structure of JSTX, which was utilized in its synthetic form (Research Biochemicals, Natick, MA) for the experiments described in this report. (*b*) KA-induced currents were measured in the two-electrode voltage-clamp configuration during application of 300 μM KA (*Left*) and 300 μM KA plus 0.2 μM JSTX (*Right*). Drug applications were performed by fast bath perfusion, which yielded a complete change of the bath solution within 3 s. Membrane potential was held at −100 mV during this experiment. (*c*) Dose–response curve for the receptor GluR1. For this experiment, JSTX was applied in increasing concentrations from 10 nM to 1 μM. The blocking effect was determined 3 min after the beginning of the JSTX application. The resulting dose–response curve was fitted with a least-squares algorithm, yielding a half-maximum inhibition, IC_{50}, of 40 nM and a Hill coefficient of 0.98 for this experiment. (*d*) Dose–response curves for 10 μM–1 mM KA with GluR1 in the absence (control) or presence of 0.2 μM JSTX. The maximum KA-induced current was reduced in the presence of the toxin, but the EC_{50} values were virtually unaffected (control, EC_{50} = 29 μM; 0.2 μM JSTX, EC_{50} = 20 μM).

the AMPA/KA receptor GluR1. The control trace (left) shows the characteristic, nondesensitizing current response produced by the application of KA to an oocyte clamped at a voltage of −100 mV. Addition of JSTX to the KA-containing perfusion medium caused a block (right trace of Fig. 1*b*) which developed over a period of several minutes. In oocytes held at negative voltages the JSTX block was only partially reversible (<60% recovery after 5 min of continuous perfusion with control solution). A more effective recovery (80–95% of the control value) was obtained when the oocyte was clamped to 0 mV for 60 s in the presence of kainate, suggesting a voltage dependence of the blocking action of JSTX (7, 23). Fig. 1*c* demonstrates the high affinity of the toxin block. The concentration that inhibited 50% of the KA-mediated current response (IC_{50}) through GluR1 was about 40 nM JSTX. Fig. 1*d* shows dose–response relations for KA-mediated currents in the absence (control) and the presence of 0.2 μM JSTX. The EC_{50} values (the concentration of KA producing a half-maximal response) remained virtually unaffected by the presence of JSTX (control, 29 μM; 0.2 μM JSTX, 20 μM; oocyte expressing subunit GluR1). This suggests that JSTX is a noncompetitive inhibitor of AMPA/KA receptor channels and that the JSTX binding site is different from the agonist binding site.

Subunit-Specific Block of JSTX. Fig. 2 and Table 1 demonstrate the subunit specificity of the JSTX block. Receptors composed of subunits GluR1, GluR3, and GluR4 and the subunit combination GluR1/3 were blocked by the toxin with an affinity in the nanomolar concentration range. In contrast, the combinations GluR1/2 (mRNA ratio 1:10), GluR3/2 (1:10), and GluR6 were not affected by concentrations up to 1 μM JSTX (Table 1). Fig. 2 compares the effect of 0.5 μM JSTX on KA-evoked currents for receptors GluR3 and GluR1/2. The dose–response curve of the JSTX block for GluR3 (Fig. 2*a Lower*) shows the IC_{50} value for JSTX to be about 30 nM. There was a clear correlation between JSTX-sensitive and -insensitive AMPA/KA receptor combinations and the shape of their *I–V* curves (Table 1). All subunit combinations with an inwardly rectifying *I–V* relationship were blocked by nanomolar concentrations of JSTX, whereas receptor combinations with a linear *I–V* relationship were not affected by such low concentrations of the toxin. Similar subunit-specific effects could be obtained by using compounds structurally related to JSTX, such as its synthetic analog 1-naphthylspermine or the wasp toxin argiotoxin 636 (7, 8, 23).

A Single Amino Acid Determines the JSTX Blocking Effect. Fig. 3 compares the amino acid sequences of glutamate receptor subtypes GluR1–GluR4 and GluR6 in the predicted

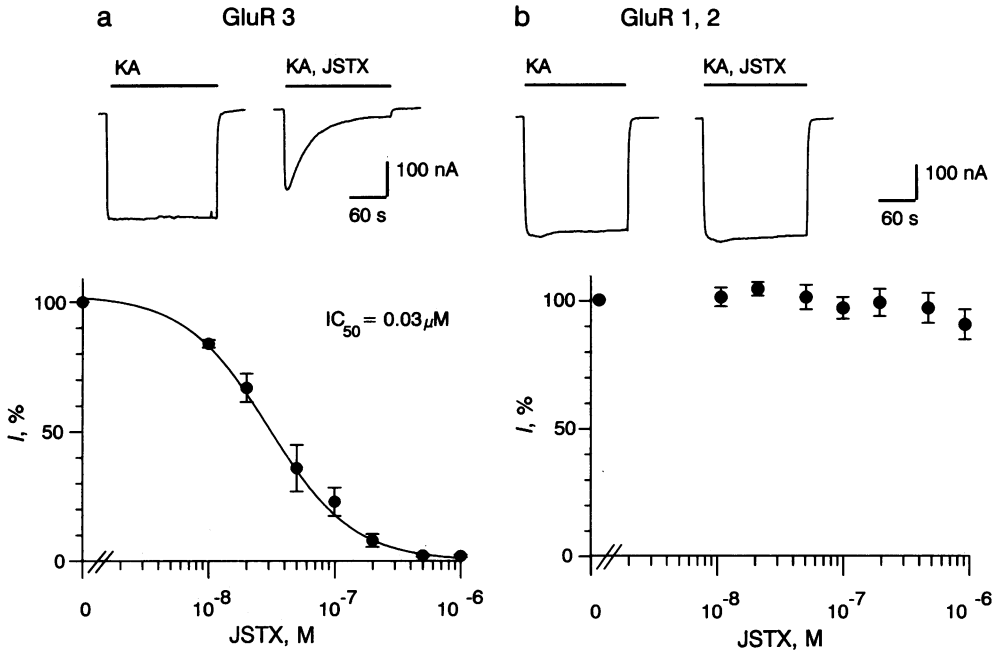

FIG. 2. Subunit-specific block of JSTX of AMPA/KA receptors. (*a*) (*Upper*) Control traces (300 μM KA) and traces obtained after application of 300 μM KA plus 0.5 μM JSTX for receptor GluR3. Currents were blocked by 90% after application of 0.5 μM toxin. (*Lower*) Averaged dose–response curve for receptor GluR3. Each data point represents the average of four experiments; current values were normalized to the control response in the absence of JSTX. (*b*) (*Upper*) KA-induced currents before and after application of 0.5 μM JSTX to an oocyte expressing the receptor subunit combination GluR1/2. To suppress the formation of GluR1 homooligomers, the mRNAs were injected with a GluR1/GluR2 ratio of 1:10. (*Lower*) Averaged current responses obtained from the subunit combination GluR1/2 ($n = 6$) after JSTX application. The small decrease in current responses may be due to "run down" of the current response, which was observed sometimes also in the absence of the toxin.

membrane-spanning region M2 (10–13). The amino acid sequence in this region is highly conserved in the receptor subunits GluR1–GluR4, which belong to one glutamate receptor subfamily. The sequence of GluR6 is less conserved, since GluR6 belongs to another receptor subfamily (14, 15). By using site-directed mutagenesis it has been shown that the change of a single amino acid in the membrane-spanning region M2 is responsible for the shape of the *I–V* curve, and in some, but not all glutamate receptors, it regulates the calcium permeability of the glutamate receptor channel (16, 17, 20). GluR2, the subunit which, in coexpression with GluR1 or GluR3, produces a receptor with a linear *I–V* relationship, contains the positively charged amino acid arginine at position 586. Replacing this amino acid with the neutral amino acid glutamine results in a receptor combination, GluR1/2(R586Q), with a rectifying instead of a linear *I–V* curve. Correspondingly, the change of arginine to glutamine at the analogous position in the receptor subunit GluR3(Q590R) results in a mutant receptor with a linear instead of a rectifying *I–V* relation (Fig. 4 *Lower*). We tested

Table 1. Blocking effect of JSTX on various receptor subunit combinations

Receptor combination	% block by 0.5 μM JSTX	*I–V* curve	No. of cells	Calcium permeability
Wild type				
GluR1	88 ± 9	Rectifying	13	High
GluR3	96 ± 3	Rectifying	7	High
GluR1/3	87 ± 22	Rectifying	9	High
GluR1/2	No block	Linear	5	Low
GluR2/3	No block	Linear	5	Low
GluR4	82 ± 12	Rectifying	5	High
GluR6(R591)	No block	Linear	5	High
Mutants				
GluR2(R586Q)	72 ± 14	Rectifying	5	High
GluR1/2(R586Q)	63 ± 15	Rectifying	5	High
GluR1/3(Q590R)	No block	Linear	7	Low
GluR3/3(Q590R)	No block	Linear	6	Low
GluR6(R591Q)	81 ± 10	Rectifying	6	High

The various receptor combinations were expressed in *Xenopus* oocytes. In the case of the receptor combinations GluR1/2 and GluR3/2 the mRNAs were injected in a ratio of 1:10, with a 10-fold higher concentration of GluR2 to suppress the formation of either GluR1 or GluR3 homooligomeric receptors. The ratio of injected mRNAs of the mutant subunit combination GluR1/3(Q590R) was 1:2; for GluR3/3(Q590R) the ratio was 1:4 to obtain a linear *I–V* relationship. In case of the receptor type GluR6(R591) and GluR6(R591Q), the oocytes were in general preincubated with concanavalin A (10 μM) prior to the electrophysiological recordings, to obtain an increase of the KA-induced current responses (14).

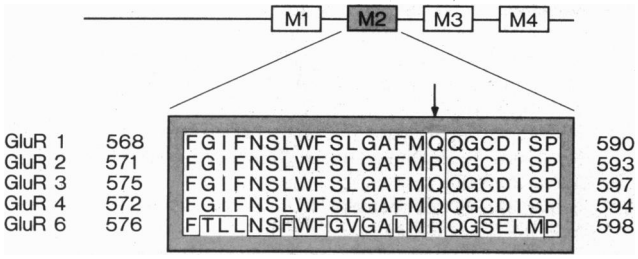

FIG. 3. Comparison of amino acid sequence in the putative transmembrane region M2. The amino acid sequence of the putative transmembrane region M2 is shown for GluR1–GluR4 and GluR6. Identical amino acids are framed, and the amino acid position critical for JSTX block is marked with an arrow. Amino acid positions can be deduced from the number of the first and last amino acid of each sequence, respectively. A, alanine; C, cysteine; D, aspartate; E, glutamate; F, phenylalanine; G, glycine; I, isoleucine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan.

whether this amino acid position was also responsible for the JSTX block. The wild-type receptor combination GluR1/3 was blocked by the toxin, but the mutant receptor combination GluR1/3(Q590R) was JSTX-insensitive (Fig. 4). This observation is consistent with the hypothesis that JSTX interacts with a site on AMPA/KA receptor channels which is directly coupled to the channel and determines the ion permeation properties.

DISCUSSION

Our results demonstrate that JSTX functions as a noncompetitive, subunit-specific antagonist of AMPA/KA glutamate receptor channels. As a general rule, receptor combinations with a rectifying *I–V* relationship and glutamine at the analogous amino acid position (e.g., GluR1, GluR3, GluR1/3, and GluR4) were blocked by JSTX with an apparent affinity in the nanomolar range (Table 1). In contrast, receptor combinations characterized by a linear *I–V* relation and containing an arginine residue in the transmembrane region M2 (e.g., GluR1/2, GluR2/3, and GluR6) were not blocked by the toxin. Except for the subunit GluR6, which is calcium-permeable but has a linear *I–V* relation (20), the toxin block correlated also with a high calcium permeability of the AMPA/KA receptors. We did not address in the present study the important problem of how JSTX blocks glutamate-mediated synaptic transmission in neurons (1–6). JSTX has been shown to block excitatory postsynaptic currents with linear *I–V* relationships in hippocampal pyramidal cells (4, 5). Further, a synthetic analog of JSTX, 1-naphthylspermine, blocked postsynaptic responses of parallel but not climbing fibers in cerebellar Purkinje neurons, both of them being characterized by linear *I–V* relations (6). An explanation for these findings, which are inconsistent with the results that JSTX blocks inwardly rectifying but not linear recombinant receptors, could be the different toxin preparations that were used in the two experiments. While the subunit-specific block on recombinant AMPA/KA receptors was observed in the nanomolar concentration range with synthesized toxin, the blocking effects in hippocampal neurons were observed by using a purified toxin preparation (4, 5). The synthetic analog 1-naphthylspermine, which was used to demonstrate the differential blocking of parallel and climbing fibers in cerebellar neurons, was applied at 250 μM (6). This concentration also blocked recombinant AMPA/KA receptors with linear and rectifying *I–V* relationships in the heterologous expression system (M.B. and B.U.K., unpublished observations). The specific molecular mechanisms underlying the blocking action on GluR channels in neurons (4–6) compared with recombinant GluR channels will be an interesting area for future investigations.

By using site-directed mutagenesis we have identified the amino acid position (Fig. 3, Table 1) which is critical for the blocking action of the toxin in the subunits GluR2, GluR3, and GluR6. This amino acid is located in the predicted second membrane-spanning region, M2 (e.g., at position 590 for GluR3) (Fig. 3). Replacement of the uncharged amino acid glutamine in the wild-type GluR3 by the positively charged amino acid arginine results in a mutant which is no longer sensitive to JSTX (Fig. 4). By contrast, the mutation of the corresponding homologous site in GluR2 (position 586, see Fig. 3 and Table 1) converts the JSTX-insensitive wild-type

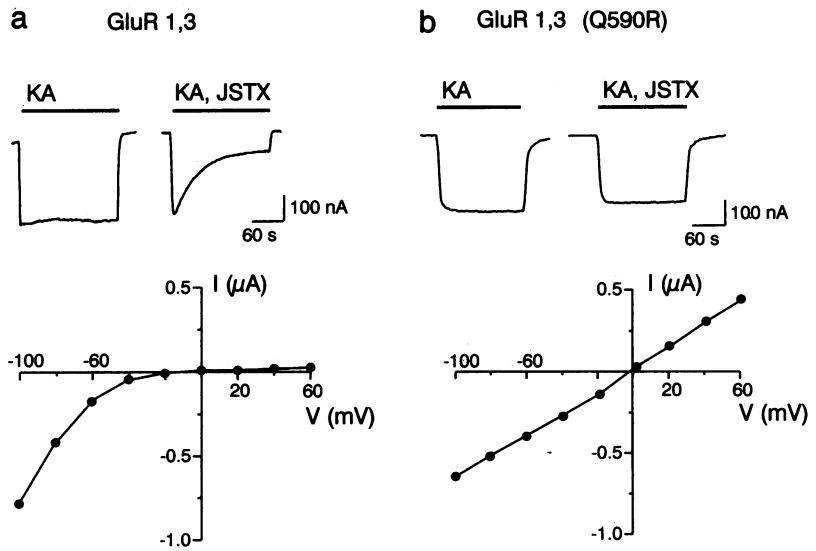

FIG. 4. One amino acid residue determines the subunit-specific JSTX block. (*a*) (*Upper*) Current responses induced by 300 μM KA (*Left*) and after application of 300 μM KA plus 0.5 μM JSTX (*Right*) for the receptor combination GluR1/3. For this oocyte, mRNA was injected at a GluR1/GluR3 ratio of 1:1. (*Lower*) *I–V* relationship of the subunit combination GluR1/3 for membrane potentials between −100 mV and +60 mV. (*b*) (*Upper*) Currents obtained from oocytes expressing the mutant subunit combination GluR1/3(Q590R) (expressed at an mRNA ratio of 1:2) after application of 300 μM KA (*Left*) and 300 μM KA plus 0.5 μM JSTX (*Right*). (*Lower*) *I–V* curve for the mutant subunit combination GluR1/3(Q590R) in the absence of the toxin. All data points in the *I–V* curves were corrected for the oocyte's leak currents, which were always <100 nA.

receptor to a JSTX-sensitive mutant. For all subunits tested, it has been shown previously that this amino acid position also determines conductance rectification (refs. 16 and 17; see also Fig. 4) and, further, that it regulates the ion selectivity of the channel (16, 17, 24, 25). Taken together, these findings strongly suggest that the JSTX binding site is located close to the central pore region of the receptor.

One of the potentially important practical implications of the differential JSTX block may be the development of clinically useful drugs able to reduce excitatory activity in selected areas of the central nervous system. Indeed, a spider toxin structurally related to JSTX (argiotoxin 636) has already been shown to protect against audigenic seizures in mice (26). The possibility to inhibit distinct glutamate receptor combinations in specific neurons or glial cells (25, 27) carries a promising potential for the treatment of a number of degenerative brain diseases, such as Parkinson and Alzheimer diseases, and the prevention of ischemic damage after epileptic seizures, which might be linked to a dysfunction of excitatory synapses (7, 28–31).

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