Neuroprotection After Status Epilepticus by Targeting Protein Interactions With Postsynaptic Density Protein 95

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Abstract

N-methyl-D-aspartate receptors (NMDARs) mediate essential neuronal excitation, but overactivation of NMDARs results in excitotoxic cell death in a variety of pathologic conditions, including status epilepticus (SE). Although NMDAR antagonists attenuate SE-induced brain injury, undesirable side effects have limited their clinical efficacy. Tat-NR2B9c was designed to disrupt protein interactions involving postsynaptic density protein 95 in the NMDAR signaling complex while not interfering with function of the NMDAR ion channel. We examined the ability of Tat-NR2B9c to provide neuroprotection in the hippocampus of rats after 60 minutes of SE induced by the repeated injection of low doses of pilocarpine (10 mg/kg). Tat-NR2B9c was administered 3 hours after the termination of SE, and neuronal densities were assessed 14 days later by stereologic analysis of NeuN-positive cells. After SE, pyramidal cell densities were reduced by 70% in CA1, 34% in CA3, 58% in CA4, and 88% in the piriform cortex. In Tat-NR2B9c-treated rats, neuronal densities in CA1, a subregion of CA3, and CA4 were decreased by only 38%, 4%, and 26%, respectively. Tat-NR2B9c did not reduce cell loss in the posterior piriform cortex. The results indicate that targeted disruption of the NMDAR signaling complex represents a potential therapeutic approach for limiting neuronal cell loss after SE.

Key Words: Epilepsy, Neuroprotection, N-methyl-D-aspartate receptor, Pilocarpine, Status epilepticus.

INTRODUCTION

Status epilepticus (SE), defined as 30 minutes or greater of continuous seizure activity, is a neurologic emergency that results in high mortality and morbidity (1, 2). Status epilepticus is a consequence of the sustained activation of N-methyl-D-aspartate receptors (NMDARs) that mediate excitatory neurotransmission in the central nervous system (3, 4). Overactivation of NMDARs causes widespread neurodegeneration in humans (5) and in animal models of epilepsy, including kindling (6) and the administration of chemoconvulsants such as pilocarpine (7–10) and kainic acid (11, 12) administration models.

Consistent with the critical role for NMDARs in SE-induced neural injury, the systemic administration of NMDAR antagonists provides substantial neuroprotection in rodent models of epilepsy even when they are administered after the onset of SE (13–16). The clinical efficacy of NMDAR antagonists is limited, however, because of their associated side effects that include the induction of psychosis in humans (17–20) and neurotoxicity in rats (21–23). A proposed alternate approach to preventing NMDAR-mediated neurotoxicity is inhibition of excitotoxic NMDAR signaling, for example, resulting from the production of nitric oxide; this may be accomplished by blocking interactions of the NMDAR receptor with downstream signaling molecules (24).

Postsynaptic density protein 95 (PSD-95) is a critical scaffolding protein that links the NMDAR to signaling enzymes within the postsynaptic density; suppression of the expression of PSD-95 selectively attenuated excitotoxicity triggered via NMDARs (25). The synthetic peptide Tat-NR2B9c (that consists of the C-terminal 9 amino acids of the NR2B subunit of NMDARs fused to the membrane transduction domain of the HIV-1-Tat protein) was designed to disrupt neurotoxic signaling from the NMDAR by interfering with protein interactions involving the PDZ1 and PDZ2 domains of PSD-95 (24, 26). Tat-NR2B9c has been shown to provide significant neuroprotection and to preserve cognitive function after transient stroke in rats (24, 27).

Because activation of NMDA receptors is critically involved in the neuropathologic outcomes of SE (3, 4), we investigated the ability of Tat-NR2B9c to provide neuroprotection after SE induced by lithium-pilocarpine. We found that when Tat-NR2B9c was administered 3 hours after the termination of SE, there was a significant reduction of neuronal cell death in the hippocampus of adult rats.

MATERIALS AND METHODS

Induction of Status Epilepticus

All procedures were approved by the University of Toronto Animal Care Committee and were in accordance with the guidelines established by the Canadian Council on Animal Care. Male Wistar rats (Charles River Laboratories, Sherbrooke, Quebec, Canada) weighing between 300 and...
Administration of Peptides

provided in dishes until the rats began to eat hard pellets. for an average of 3 days. Softened rat chow was also and evening of the next day (5 mL, i.p.), as described by (3 mL, i.p.; 2 mL, s.c.); this was repeated in the morning of diazepam, the animals received 5 mL of 0.9% saline occurred in each 5-minute interval after the initiation of SE. 

termined by averaging the highest class of seizure that were continuously monitored after the first injection of pilocarpine; the animals received 5 mL of 0.9% saline before administration. Diazepam was purchased from CDMV Inc. (Saint-Hyacinthe, Quebec, Canada) and used as the commercial solution (5 mg/mL).

The behavioral progression of pilocarpine-induced sei- zures was assessed using a modified Racine scale (29, 30) as follows: Class 1, facial clonus; Class 2, head nodding; Class 3, forelimb clonus; Class 4, forelimb clonus and rearing; Class 5, forelimb clonus, rearing, and 1 fall (loss of postural control); Class 6, forelimb clonus, rearing, and multiple falls; and Class 7, running and jumping. Animals were continuously monitored after the first injection of pilocarpine. The average maximum seizure activity was determined by averaging the highest class of seizure that occurred in each 5-minute interval after the initiation of SE.

After the termination of SE, the animals were kept in a quiet room for 3 days. Immediately after the first injection of diazepam, the animals received 5 mL of 0.9% saline (3 mL, i.p.; 2 mL, s.c.); this was repeated in the morning and evening of the next day (5 mL, i.p.), as described by Glien et al (28). Starting on the second day after SE, animals were tube-fed with softened rat chow mixed with applesauce for an average of 3 days. Softened rat chow was also provided in dishes until the rats began to eat hard pellets.

**NeuN Immunohistochemistry**

At 2 weeks after the termination of SE, animals were anesthetized with a mixture of xylazine (Rompun; 26 mg/kg, i.p.) and ketamine (Ketalar; 174 mg/kg, i.p.; both from CDMV Inc.) and perfused transcardially with 90 mL 0.1 /L phosphate-buffered saline (PBS), pH 7.4, followed by 400 to 500 mL of 4% (w/v) paraformaldehyde in PBS. Brains were removed and left overnight at 4°C in the paraformaldehyde solution. The next day, brains were soaked for cryoprotection in 30% (w/v) sucrose in PBS until they sank at room temperature. The brains were then frozen in 2-methylbutane at −35°C and stored at −80°C. Forty-μm-thick coronal sections were prepared with a freezing microtome. Sections were placed in 24-well culture plates containing anti-freeze solution (0.05 mol/L of sodium phosphate buffer, pH 7.4, 30% (w/v) ethylene glycol, 15% (w/v) glucose), and stored at −20°C.

For each animal, 3 coronal brain sections containing the dorsal hippocampus were selected from Bregma −3.2 to −3.72 mm, with the first section presenting all hippocampal subfields, exhibiting only the dorsal portion of the lateral ventricle, and containing the most ventral portions of the capsular division (CeC) and lateral division (CeL) of the central amygdala. The subsequent sections were selected ventrally at 240-μm intervals and exhibited the lateral ventricle extending to the ventral portion of the brain, replacing the structures CeC and CeL detected in the initial section. Section were rinsed in PBS (3 × 5-minute washes), reacted overnight at 4°C with NeuN antibody (1:1000; Chemicon, Billerica, MA) in 0.2% (v/v) goat serum, 0.3% (v/v) Triton X-100 in PBS, followed by 2 hours at room temperature with Cy3 conjugated secondary antibody (1:200; Chemicon) in 0.2% (v/v) goat serum, 0.3% (v/v) Triton X-100 in PBS.
Neurons were imaged using a Zeiss LSM510 Laser Scanning Confocal microscope equipped with a 40×/1.3 oil-immersion objective lens; neuronal densities were determined using the unbiased optical dissector technique as described by West and Gundersen (32). Briefly, 3 counting frames (120 × 60 μM) were positioned in the CA1, CA3, and posterior piriform cortex (PPC); 2 counting frames were positioned in the CA4 region, and 1 counting frame was placed in CA2 of the left brain hemisphere, as shown in Figures 1A and C. Right brain structures were visually inspected and found to be comparable to the left side. Neurons were identified as NeuN-positive cells that contained a relatively large (>8 μm) soma (33). Chromophilic somas contained within each counting frame or those touching the inclusion borders of the frame (upper and right borders) were counted (32). The dissector height equivalent to the known tissue height before staining (40 μm) was used in all calculations (34), and upper or lower exclusion borders in the z-plane were excluded because previous studies demonstrated that no significant variation in neuronal density occur whether such borders are used or not (35–37). Unless otherwise specified, cell densities for individual animals represent the average densities of all counting frames in a particular region for 3 brain sections. All results are expressed as neurons per cubic millimeter.

Statistical Analysis
Statistical analysis was performed using Statistica 6.0 software. Significant differences were determined using 1-way analysis of variance. The Newman-Keuls post hoc test was used to determine difference between treatment groups. χ² analysis was performed to analyze mortality rates. Significance was set at a p value of 0.05 or less.

RESULTS

Induction of Status Epilepticus
Of 63 animals that received pilocarpine, 16 (25%) did not develop SE after 6 injections of pilocarpine and were not used for this study. The other animals developed SE after 1 (n = 2), 2 (n = 23), 3 (n = 16), or 4 (n = 6) pilocarpine injections; SE developed within 22 to 120 minutes (mean, 68 ± 22 min) after the initial pilocarpine injection. The overall fatality rate for animals that entered SE was 30% and did not differ among experimental groups (Table 1).

All animals that developed SE displayed Stage 4/5 convulsive seizures within the first 10 minutes. During the 60 minutes of SE, the animals experienced seizures varying between 2 and 4/5 on the Racine scale (29) and did not regain consciousness. There was no significant difference between groups with respect to the average maximum seizure activity recorded during SE, which approximated to 3.0 for all groups (Table 1). All groups experienced similar weight gain during the recovery period (Table 1), and by 14 days, there was no difference in mean body weight between groups (Table 1).

SE Induced by Repeated Low Doses of Pilocarpine Results in Neurodegeneration in the Hippocampus and Piriform Cortex
The hippocampus and piriform cortex play critical roles in the development and maintenance of limbic seizures and are the most susceptible brain regions to SE-induced damage (9, 38–40). Therefore, we focused our analyses on these regions. Preliminary experiments demonstrated that neurodegeneration in both the hippocampus and the piriform cortex occurred rapidly within the first few days after the termination of SE and was complete within 2 weeks (Dykstra and Gurd, unpublished observations). Therefore, the effect of SE on neuronal cell death was assessed 14 days after the termination of seizures.

We initially determined the effect of SE on neuronal cell density in animals that experienced SE but that did not receive any additional treatment. Preliminary comparisons of pyramidal cell counts in each of the 3 coronal brain sections
showed that the extent of SE-induced cell death did not vary between sections. For example, the average cell densities in the CA1 region before and after SE were 110,796 ± 23,841 and 33,654 ± 24,097 (mean ± standard deviation [SD]; n = 7), 106,271 ± 24,551 and 33,234 ± 24,207, 104,692 ± 18,128, and 35,879 ± 21,056 for the 3 sections from the most proximal to the most distal from Bregma. Similar results were obtained for the other hippocampal subfields. We also compared the effect of SE on cell density in individual counting frames to determine whether SE-induced pyramidal cell loss was uniform within discrete structures. The data in Table 2 show that the extent of cell loss in individual counting frames was similar regardless of position within the specific hippocampal subfield or in the PPC. This indicates that the effect of SE on neurodegeneration was independent of cell location within the region. Therefore, cell densities were determined by averaging the densities for all counting frames in all 3 sections for a particular region. Status epilepticus resulted in a marked loss of pyramidal neurons in the CA1, CA3, and CA4 regions of the dorsal hippocampus and in Layer II of the PPC (Fig. 1B, D). Neuronal densities (cells per cubic millimeter ± SD) before and after SE were 104,993 ± 18,982 and 18,982 ± 22,093 (70% cell loss) in the CA1 region, 64,705 ± 3,575 and 37,616 ± 12,412 (34% cell loss) in the CA3 region, 64,704 ± 9,697 and 31,084 ± 20,344 (58% cell loss) in the CA4 region, and 48,417 ± 12,123 and 5,851 ± 5,103 (88% cell loss) in the PPC. Status epilepticus did not result in a significant change in cell density in the CA2 region (81,018 ± 10,195 cells/mm³ in naive animals, 68,297 ± 12,678 cells/mm³ in SE rats; Fig. 1E). The effect of SE on the dentate granule cell layer and the hilus was also assessed. Visual inspection indicated that in most animals (6/7), there was no, or only minor, cell loss in the dentate granule cell layer. In the single animal in which cell death was clearly apparent, the loss consisted of the entire upper blade. Neuronal degeneration in the hilus was more variable than in CA1, CA3, or CA4, with an average decrease in cell density of 38% ± 30% (mean ± SD; n = 4). For these reasons, the effects of Tat-NR2B9c on cell densities in CA2, the hilus, or the dentate granule cell layer were not assessed. Overall, the decrease in hippocampal and piriform cortex pyramidal cells after SE induced using the repeated low-dose pilocarpine procedure was similar to neuronal loss reported in previous studies using variations of the pilocarpine model (9, 41–43).

**Tat-NR2B9c Reduces SE-Induced Neurodegeneration in the Hippocampus**

We next determined the effect of Tat-NR2B9c on SE-induced neuronal cell loss. For these experiments, animals received Tat-NR2B9c, its inactive homolog Tat-NR2B9AA, or saline 3 hours after the termination of SE; cell densities were determined 2 weeks later. Results for individual animals are plotted in Figure 2. Mean cell densities for each brain region were as follows. CA1: shams (107,253 ± 4,339 cells/mm³; mean ± SEM; n = 11), saline (36,699 ± 7,002 cells/mm³; n = 8), Tat-NR2B9c (66,049 ± 11,993 cells/mm³; n = 10), Tat-NR2B9AA (30,767 ± 7,606 cells/mm³; n = 8); CA3: shams (62,812 ± 1,354 cells/mm³), saline (44,618 ± 5,702 cells/mm³), Tat-NR2B9c (52,916 ± 4,124 cells/mm³), Tat-NR2B9AA (41,354 ± 6,401 cells/mm³); CA4: shams (67,847 ± 2,923 cells/mm³), saline (27,391 ± 7,657 cells/mm³), Tat-NR2B9c (50,170 ± 8,238 cells/mm³), Tat-NR2B9AA (32,118 ± 7,012 cells/mm³); PPC: shams

### TABLE 1. Comparison of the Effect of Treatment on Mortality, Seizure Severity, and Weight Gain After Status Epilepticus (SE)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total Animals</th>
<th>Survived*</th>
<th>Died*</th>
<th>SE Severity†</th>
<th>Initial Weight, g</th>
<th>Final Weight, g</th>
</tr>
</thead>
<tbody>
<tr>
<td>SE + saline (administered after SE)</td>
<td>11</td>
<td>8</td>
<td>3 (27.3%)</td>
<td>3.06 ± 0.12</td>
<td>402 ± 15</td>
<td>468 ± 18 (16.6%)</td>
</tr>
<tr>
<td>SE + Tat-NR2B9c (3 nmol/g, administered after SE)</td>
<td>14</td>
<td>10</td>
<td>4 (28.6%)</td>
<td>2.98 ± 0.10</td>
<td>395 ± 12</td>
<td>431 ± 22 (9.2%)</td>
</tr>
<tr>
<td>SE + Tat-NR2B-A (3 nmol/g, administered following SE)</td>
<td>11</td>
<td>8</td>
<td>3 (27.3%)</td>
<td>2.90 ± 0.12</td>
<td>405 ± 15</td>
<td>462 ± 12 (14.2%)</td>
</tr>
<tr>
<td>SE + Tat-NR2B9c (3 nmol/g, administered during SE)</td>
<td>7</td>
<td>4</td>
<td>3 (42.9%)</td>
<td>3.13 ± 0.14</td>
<td>393 ± 9</td>
<td>441 ± 15 (12.3%)</td>
</tr>
<tr>
<td>SE + Tat-NR2B9c (9 nmol/g, administered during SE)</td>
<td>4</td>
<td>3</td>
<td>1 (25.0%)</td>
<td>3.09 ± 0.18</td>
<td>387 ± 10</td>
<td>430 ± 27 (11.2%)</td>
</tr>
<tr>
<td>Sham (no SE)</td>
<td>11</td>
<td>11</td>
<td>0</td>
<td>No SE</td>
<td>379 ± 7</td>
<td>433 ± 11 (14.5%)</td>
</tr>
</tbody>
</table>

*The number of animals that survived or died within 14 days after SE. Numbers in parentheses represent the percentage of animals that died.
†Average maximum seizure activity for all animals determined as in Materials and Methods section (mean ± standard deviation [SD]).
‡Initial and final group weights evaluated immediately before and at 2 weeks after SE induction (mean ± SD). Values in parentheses indicate percent weight gain. No significant differences in mortality rates (χ² analysis), seizure severity (repeated ANOVA), or percent weight gain (1-way ANOVA) were detected among treatment groups.

ANOVA, analysis of variance.

### TABLE 2. Comparison of Status Epilepticus-Induced Pyramidal Cell Loss in Individual Counting Frames

<table>
<thead>
<tr>
<th>Brain Region</th>
<th>Counting Frame a</th>
<th>Counting Frame b</th>
<th>Counting Frame c</th>
</tr>
</thead>
<tbody>
<tr>
<td>CA1</td>
<td>71.9 ± 19.9</td>
<td>76.9 ± 17.9</td>
<td>71.6 ± 12.2</td>
</tr>
<tr>
<td>CA3</td>
<td>37.4 ± 25.7</td>
<td>30.2 ± 23.3</td>
<td>29.9 ± 22.4</td>
</tr>
<tr>
<td>CA4</td>
<td>64.3 ± 36.4</td>
<td>54.2 ± 25.6</td>
<td>—</td>
</tr>
<tr>
<td>PPC</td>
<td>85.3 ± 16.4</td>
<td>89.6 ± 16.7</td>
<td>91.2 ± 20.2</td>
</tr>
</tbody>
</table>

Individual counting frames were positioned in hippocampal subfields and the posterior piriform cortex as depicted in Figure 1. Numbers represent percent decrease of pyramidal cells ± standard deviation (n = 5). Pyramidal cell loss within individual regions was similar regardless of the placement of the counting frame (1-way ANOVA).

ANOVA, analysis of variance.
The administration of Tat-NR2B9c resulted in a statistically significant overall reduction in pyramidal cell loss in hippocampal subfields CA1 and CA4 relative to saline-treated animals, although there was considerable animal-to-animal variation in the efficacy of the peptide. In CA1, the decrease in pyramidal cell density in animals that received Tat-NR2B9c was 38% ± 35% (mean ± SD) versus a decrease of 66% ± 18% in saline-treated animals (p < 0.05). Similarly, in CA4, administration of Tat-NR2B9c reduced SE-induced cell loss from 60% ± 28% in saline controls to 26% ± 39% in animals that received the peptide (p < 0.05). In neither case did Tat-NR2B9AA, the inactive homolog of Tat-NR2B9c, exhibit any neuroprotective effect; this is consistent with the active peptide having mediated its effects via its interaction with PDZ domains. In contrast to the results for CA1 and CA4, Tat-NR2B9c did not reduce overall cell loss in either CA3 or the PPC compared with saline-treated animals (Fig. 2).

**FIGURE 2.** Tat-NR2B9c reduces pyramidal cell loss in the dorsal hippocampus when administered 3 hours after status epilepticus (SE). Animals received saline, Tat-NR2B9c, or Tat-NR2B9AA 3 hours after the termination of SE and were killed 14 days later. Coronal sections were stained with NeuN and cell densities determined. (A) Representative NeuN stained images of the pyramidal cell layer of indicated regions of the hippocampus and of Layer II of the posterior piriform cortex (PPC). Bar = 20 μm. Magnification: 400×. (B) Administration of Tat-NR2B9c reduced SE-induced cell loss in CA1 and CA4, but not in CA3 or the PPC. Cell densities for individual animals are plotted as open circles. Dash indicates mean cell density for the group. * Indicates a significant difference from sham group (p < 0.05); # indicates a significant difference from saline and Tat-NR2B9AA groups (p < 0.05); † indicates a significant difference from saline group (p < 0.05) and approaching significance with Tat-NR2B9AA group (p = 0.08).
neurodegeneration was similar in each of the counting frames placed within CA1, CA3, and CA4 and in the PPC in animals that received either saline or the inactive peptide, Tat-NR2B9AA (Fig. 3; gray and oblique stripped bars). In contrast to the uniform effect of SE on cell death, however, comparison of cell densities within individual counting frames identified differences in the neuroprotective actions of Tat-NR2B9c within CA1 and CA3. The neuroprotective effect of Tat-NR2B9c varied in the medial to lateral direction across CA1, increasing with proximity to the subicular border (Fig. 3A; black bars). Thus, in the presence of the peptide, 81% ± 13% (86,226 ± 14,027 cells/mm³) in animals that received Tat-NR2B9c vs 106,691 ± 5,051 in control animals; p < 0.05) of pyramidal neurons remained in counting frame 1a, adjacent to the subicular border, after SE, whereas only 44% ± 9% (47,685 ± 9,707 cells/mm³) in animals that received Tat-NR2B9c vs 109,638 ± 4,542 in control animals) remained in counting frame 1c, adjacent to CA2 (Fig. 3A; black bars). Similarly, although Tat-NR2B9c had no overall protective effect in CA3 (Fig. 2), comparison of individual counting frames showed that, whereas there was no protective effect in the areas represented by counting frames 3a and 3b, there was nearly complete protection in the area represented by counting frame 3c, the area proximal to the transitional border with CA4 (Fig. 3B; black bars). Differential neuroprotective effects of Tat-NR2B9c were not observed in either CA4 or PPC.

### FIGURE 3. Tat-NR2B9c exhibits differential neuroprotection within different regions of the CA1 and CA3 subfields of the hippocampus. Neuronal densities in individual counting frames in hippocampal subfields were compared between treatment groups. Results are expressed as mean ± standard error of mean. Open bars indicate shams (n = 11); gray bars, saline (n = 8); black bars, Tat-NR2B9c (n = 10); oblique stripped bars, Tat-NR2B9AA (n = 8). * Indicates a significant difference from sham group (p < 0.05); # indicates a significant difference from saline and Tat-NR2B9AA groups (p < 0.05); † indicates significant difference from saline group (p < 0.05) and approaching significance relative to Tat-NR2B9AA group (p = 0.067); ** significant difference between counting frames a and c.

**Tat-NR2B9c Did Not Provide Neuroprotection in CA1 When Administered During SE**

We next determined whether Tat-NR2B9c provided protection if it was administered during SE. For these experiments, animals received Tat-NR2B9c after 10 minutes of continuous seizure activity; SE was allowed to continue for an additional 50 minutes. Administration of the peptide at this time had no affect on seizure severity or survival rates (Table 1), and Tat-NR2B9c administration resulted in no neuroprotection in CA1 (Fig. 4). Mean cell densities included shams (107,253 ± 4,339 cells/mm³; mean ± SEM), saline (36,996 ± 7,002 cells/mm³), Tat-NR2B9c at 3 nmol/g (26,427 ± 5,847 cells/mm³), and Tat-NR2B9c at 9 nmol/g (28,340 ± 10,290 cells/mm³). Increasing the dosage of peptide from 3 to 9 nmol/g did not increase the neuroprotective properties of the peptide under these conditions.

### DISCUSSION

We determined the ability of Tat-NR2B9c, a synthetic peptide designed to selectively disrupt the NMDAR signaling complex, to reduce SE-induced neurodegeneration. Tat-NR2B9c has been shown to reduce brain damage after an ischemic challenge, whereas the function of the NMDAR ion channel was maintained (24, 25, 27). Cui et al (26) analyzed the interaction of Tat-NR2B9c with all known potential

![Figure 4](http://jnen.oxfordjournals.org/)
Human binding partners and found that it was highly specific for the PDZ2 domain of PSD-95, suggesting that its neuroprotective action is due to the disruption of protein interactions involving PSD-95. Although NMDAR antagonists are highly neuroprotective in rodent epilepsy models, even when they are administered after the onset of SE, clinical trials with these compounds have failed because of poor tolerance and efficacy (44–48). Tat-NR2B9c offers an alternative approach to disrupting NMDAR-mediated pro-death signaling while sparing signaling pathways linked to survival and plasticity (24, 49, 50).

The primary finding of this study is that Tat-NR2B9c significantly attenuated hippocampal damage induced by an episode of SE. Importantly, substitution of the C-terminal ser and val of Tat-NR2B9c by alanine residues to prevent interaction with the PDZ domains of PSD-95 (24, 26, 31) completely eliminated the neuroprotective effect of the peptide; this supports the hypothesis that neuroprotection is due to disruption of protein interactions involving the PDZ domains of PSD-95. We initially reported that Tat-NR2B9c preferentially reduced the coimmunoprecipitation of NR2B and PSD-95 from rat brain extracts (24). More detailed binding analysis subsequently demonstrated that Tat-NR2B9c has a high affinity for the PDZ2 domain of PSD-95 and inhibits the binding of NR2 subunits, as well as neuronal nitric oxide synthase (nNOS), to this domain with IC50 values in the low micromolar range (26). Nitric NOS is a component of the NMDAR complex, and it is activated by the influx of Ca2+ ions via the NMDAR ion channel (51). Nitric oxide production is enhanced after SE (52), and inhibition of nNOS is neuroprotective (53, 54) and anti-epileptogenic (55, 56). Taken together, these findings support a model in which the neuroprotective actions of Tat-NR2B9c result from disruption of the NMDAR signaling complex. In particular, the dissociation of nNOS from PSD-95 by competing for PDZ domain binding sites on PSD-95 is consistent with known mechanisms of SE-induced cell death (50, 57).

An unexpected observation was the regional specificity of neuroprotection by Tat-NR2B9c within CA1 and CA3, the greatest protection of pyramidal neurons within these regions occurring proximal to the CA1/subicular border and CA3/CA4 border, respectively. The molecular basis for the differential neuroprotective effects of Tat-NR2B9c remains unclear, but the finding suggests the presence of previously unidentified differences in the properties of these neurons. The severity of SE-induced neurodegeneration was similar across all counting frames within CA1 or CA3, indicating that the differential effects of Tat-NR2B9c cannot be explained by variable sensitivities to the initial insult. The projection patterns of pyramidal cells change gradually as one moves across CA3 from the border with CA2 to that with CA4 (58, 59), and it is tempting to speculate that the increase in neuroprotection from CA3a (essentially no protection) to CA3c (nearly complete protection) may be related to the varying pattern of connectivity of CA3 pyramidal neurons. Alternatively, subunit composition is an important determinant of NMDAR function (60, 61) and may affect association of the receptor with members of the PSD-95 family of membrane-associated guanylate kinases (62, 63), as well as with downstream signaling molecules such as nNOS (64, 65). The results therefore suggest the occurrence of differences between protected and nonprotected neurons in subunit composition of the NMDAR and/or in protein interactions involving PSD-95 within the NMDAR signaling complex. Although differences in the subunit composition of NMDARs between hippocampal subfields and between dorsal and ventral CA1 have been reported (66–68), we are not aware of any studies examining either NMDAR subunit composition or the NMDAR signaling complex within different regions of the same hippocampal subfield; this is an important subject for future investigation.

Although the administration of Tat-NR2B9c significantly reduced neuronal cell loss when the peptide was given after the termination of SE, it was ineffective when it was given during SE. Previous studies have shown that 40 to 60 minutes of continuous seizure activity is required before neuron loss occurs (7, 43), suggesting that molecular mechanisms that occur in the earlier stages of SE are distinct from the prodeath signaling that occurs after prolonged SE. In general accord with this suggestion, SE results in numerous changes in the composition and properties of the postsynaptic density, including the recruitment and activation of several signaling molecules (69, 70), enhanced phosphorylation of the NMDA receptor and other proteins (69, 71) and changes in protein interactions or molecular organization (72). These seizure-induced changes to the composition and structure of the postsynaptic signaling apparatus may alter the way in which Tat-NR2B9c interacts with its binding partners and render the neuron more susceptible to the neuroprotective actions of the peptide. Alternatively, although peptides fused to the Tat protein transduction domain readily penetrate the blood-brain barrier (73–75) and enter neurons (24), enhanced efficacy of Tat-NR2B9c at later times may also result from increased accessibility to neurons as a result of seizure-related damage to the blood-brain barrier (76). In contrast to the present findings, NMDA receptor antagonists were reported to provide protection to several brain regions, including the hippocampus and piriform cortex when they were administered only 15 minutes into SE induced with pilocarpine (15, 16). The difference between these results and those described here with Tat-NR2B9c presumably relate to the different mechanisms of action of the drugs, that is, antagonists blocking channel function and Tat-NR2B9c modulating signaling downstream of the NMDA receptor.

In summary, the present study demonstrates that the use of Tat-NR2B9c for the targeted disruption of the NMDAR signaling complex represents a viable approach for limiting neuron loss after SE. Because preservation of neurons within the hippocampus reduces the behavioral consequences of SE (77), it now becomes important to determine whether Tat-NR2B9c also offers long-term protection against SE-induced neurological deficits.

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