Modulation of Protein Tyrosine Phosphatase Activity Alters the Subunit Assembly in Native N-Methyl-D-aspartate Receptor Complex

Karima Ferrani-Kile and Steven W. Leslie

Division of Pharmacology and Toxicology, College of Pharmacy, and the Waggoner Center for Alcohol and Addiction Research, The University of Texas at Austin, Austin, Texas

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ABSTRACT

The N-methyl-D-aspartate (NMDA) receptor is crucial for development and neuroplasticity as well as excitotoxicity. The biochemical basis of the disassembly and reassembly of NMDA receptor has never been reported. Using coimmunoprecipitation, Western blotting, and mass spectrometry, we show that inhibition of tyrosine phosphatases triggers disassembly of NR1, NR2A, and NR2B in cortical NMDA receptor complexes. Furthermore, the disassembly of the NMDA receptor subunits is immediate, dose-dependent, and reversible and seems to occur through mechanisms linked to Src kinases. Together, these results define a novel role for tyrosine phosphatases in the complex mechanism of NMDA receptor regulation.

The N-methyl-D-aspartate (NMDA) receptor is a ligand-gated and voltage-dependent ion channel permeable to calcium, which, in neuronal cells, triggers diverse functions and pathologies ranging from synaptic plasticity, ischemic damage, pain, inflammation, and alcohol sensitivity (Woodward, 2000; Cull-Candy et al., 2001; Vajda, 2002). NMDA receptors are heteromeric assemblies composed of multiple subunits; these include NR1 subunits, a family of four distinct NR2 subunits (A, B, C, and D) and two NR3 subunits (A and B) (Luo et al., 1997; Dingledine and Conn, 2000; Chatterton et al., 2002). The function of these receptors is regulated by a series of phosphorylation and dephosphorylation processes mediated by protein kinases and protein phosphatases, respectively (Wang and Salter, 1994; Smart, 1997). Numerous studies have demonstrated the important role that protein tyrosine kinases (PTKs), such as Src kinase family, play in the regulation of NMDA receptors (Ali and Salter, 2001). These PTKs have been shown to phosphorylate NR2 subunits at their tyrosine residues (Moon et al., 1994; Lau and Huganir, 1995) and may potentiate NMDA receptor function both by modulation of the channel gating and by an increase in the number of NMDA receptors at the cell surface (Yu et al., 1997; Grosshans et al., 2002).

In contrast to PTKs, the purpose of protein tyrosine phosphatases (PTPs) in the regulation of NMDA receptors remains elusive. PTPs play a critical role in regulating intracellular signal transduction pathways responsible for controlling cell growth, differentiation, motility, and metabolism (for review, see Tonks and Neel, 1996). In addition, numerous studies showed that modulation of the enzymatic activity of PTPs might constitute a therapeutic approach for the treatment of cancer, diabetes, and certain immunological disorders (van Huijsduijnen et al., 2002). A recent study has shown that PTP inhibition prevents death of injured substantia nigra neurons in adult rats (Lu et al., 2002) and rescues cells from delayed neuronal death after transient forebrain ischemia in gerbil hippocampus (Kawano et al., 2001).

The mechanism by which PTP inhibition exerts its neuroprotective effects has not been investigated. Since NMDA receptors play a key role in mediating excitotoxic damage occurring after brain injury (Beal, 1992) and are involved in several neurodegenerative diseases (Vajda, 2002), we hypothesized that PTP inhibition could affect these receptors. In support of this hypothesis, a study reported that PTP inhibitors such as sodium orthovanadate (SOV) and phenyl-
arsine oxide (PAO) down-regulate NMDA-mediated synaptic potentials (Coussens et al., 2000).

To further investigate the mechanism of PTP action on the NMDA receptor complex, experiments involving coimmunoprecipitation under nondenaturing conditions, Western blotting and mass spectrometry were performed and revealed that inhibition of PTPs causes a reversible disassembly of NR1, NR2A, and NR2B subunits of the NMDA receptor complex via a pathway involving Src family kinases.

Materials and Methods

Antibodies and Chemicals. For immunoprecipitation, a cocktail of NR1 antibodies was used. The cocktail contained a polyclonal NMDA1 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA) and a monoclonal antibody anti-NR1 (Upstate Biotechnology, Lake Placid, NY). For the probing, anti-phosphotyrosine (anti-pTyr) (4G10 clone and anti-PSD-95 (Upstate Biotechnology); anti-NR1 (BD Biosciences PharMingen, San Diego, CA); and anti-NR2A, anti-NR2B, and anti-NR2A/B (recognizing both NR2A and NR2B subunits) antibodies (Chemicon International, Temecula, CA) were used. Phenyldisulfide and sodium orthovanadate were purchased from Calbiochem-Novabiochem (San Diego, CA). Other chemicals used were from Sigma-Aldrich (St. Louis, MO) or from Calbiochem-Novabiochem.

Cortical Slice Preparation and Drug Treatments. Experiments were performed on cortical slices obtained from 3-week-old Sprague-Dawley rats. All procedures were conducted in strict adherence with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Brains were rapidly removed from the skulls and put in an ice-cold oxygenated artificial cerebrospinal fluid (ACSF) consisting of 120 mM NaCl, 25 mM NaHCO3, 3.3 mM KCl, 1.8 mM CaCl2, 2.4 mM MgSO4, 10 mM dextrose. Cortex was then dissected out, and several 400-μm slices were cut using a tissue chopper and transferred to a holding chamber containing ACSF. The ACSF in the chambers was bubbled with 95% O2 and 5% CO2 for 10 min at 4°C, 2% SDS, and 0.1% bromphenol blue, and 4% 2-mercaptoethanol. All samples were boiled for 5 min, centrifuged for 1 min, and 30 μl of proteins were loaded in a 7.5% SDS-polyacrylamide gel. The loaded gel was run through electrophoresis and transferred to a nitrocellulose membrane from Bio-Rad. The nitrocellulose membrane was blocked, washed, and incubated overnight with 1:1000 dilution of one of the following antibodies: anti-pTyr (4G10), anti-NR2A/B, anti-NR1, anti-PSD-95, or anti-β-actin. After washing, the membranes were then incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies and developed by chemiluminescence reagent from NEN Renaissance (PerkinElmer Life and Analytical Sciences, Boston, MA) using a Kodak Digital Science 4400CF imager. Between incubations with antibodies that recognize the same bands, the membranes were stripped in 62.5 mM Tris, pH 6.8, 2% SDS, and 0.7% β-mercaptoethanol. The membranes were not stripped more than once.

Mass Spectrometry. Proteins that coimmunoprecipitated with NR1 subunits were separated by 10% SDS-PAGE and visualized by Coomassie staining. Protein bands from around 160 to 190 kDa were excised and digested with trypsin, according to the method of Shevchenko et al. (1996). The resulting peptide mixture was analyzed using liquid chromatography (LC)-electrospray ionization-tandem mass spectrometry (MS/MS). Experiments were performed using a microcapillary LC system (Agilent 1100 series) on-line with an Esquire ion trap mass spectrometer (Bruker, Newark, DE). Peptides were separated using a C18 column (Higgins Analytical, Southborough, MA) and a short linear gradient; 2 to 65% buffer A: 0.1% formic acid and 0.01% trifluoroacetic acid in 100% high-performance liquid chromatography grade water in 5 min at a flow rate of 1.2 μl/min. All peptides were eluted from the column within 35 min using buffer B: 0.1% formic acid, 0.01% trifluoroacetic acid, 12% isopropyl alcohol, and 68% acetonitrile in 10% high-performance liquid chromatography grade water. In the standard operating mode, the positive ions, generated by charge droplet evaporation, entered the analyzer through the orthogonal spraying interface of the ion trap. Settings of the mass analyzer were optimized according to the operation manual (Bruker and Esquire LC operation manual). For optimal results, up to 12 precursor ions were automatically selected for fragmentation within each fragmentation cycle. A list of the masses containing precursor ion fragmentation data was generated and used for searches. Protein identifications were performed using the search engine Mascot.

Cerebrocortical Cell Culture. Cerebral cortex was dissected from gestational day 21 Sprague-Dawley rat pups. Cells were dispersed by gentle mechanical agitation and brief exposure to 0.25% trypsin and 0.01 mg/ml DNase (Sigma-Aldrich) at 37°C. Isolated cells were washed, counted, filtered, and plated at the concentration of 2.2 × 10^6 cells/cm^2 on collagen and poly-l-lysine-treated flasks and grown in a 37°C and 5% CO2 incubator. Media consisted of equal amounts of Dulbecco’s modified Eagle’s medium modified with 25 mM HEPES and 4500 mg/l glucose, and minimum Eagle’s medium modified with 25 mM HEPES. Final medium contained 10% horse serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 250 mg/ml amphotericin B. On the third day of culture, 1-β-arabinofuranosylcytosine (Sigma-Aldrich) was added to stop the proliferation of glia cells. On the fifth day of culture, medium was changed and replaced with Neurobasal (Invitrogen, Carlsbad, CA) supplemented with B27.
(Invitrogen) and antibiotics. The medium was then replenished as a 50% replacement twice/week until the harvest day at days in vitro (DIV) 21.

**MTT Cytotoxicity Assay.** Cytotoxicity potential of SOV was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) reduction assay kit (Sigma-Aldrich), according to the manufacturer’s instructions. The cytotoxicity assay was performed in 24-well plates. Cortical cells DIV 21 were incubated for 1 h with different concentrations of SOV, NMDA (100 μM in the presence of 10 μM glycine), or vehicle alone. The cells were then rinsed and incubated with complete media without phenol red for 24 h at 37°C. MTT was added and cell viability assessed by measuring the absorbance (A) of each well at 595 nm. Results were expressed as percent viability = ([A 595 (treated cells) − background]/[A 595 (untreated cells) − background]) × 100.

**Data Analyses and Statistics.** The results were expressed as a ratio where phosphorylated NR2A/B (pTyr) subunits were divided by the expression of total NR2A/B subunits, where NR2A/B subunits ratio where phosphorylated NR2A/B (pTyr) subunits were divided by the expression of NR1 subunits and where NMDA receptor subunits were divided by PSD-95 or by β-actin. One-way repeated measures and two-ways analyses of variance were computed using SPSS software (SPSS Inc., Chicago, IL). For all analyses, p < 0.05 was considered statistically significant.

**Results**

**PTP Inhibition Enhances Tyrosine Phosphorylation and Simultaneously Decreases the Amount of NR2A/B Subunits in the NMDA Receptor Complex.** Because both SOV and PAO, two structurally distinct PTP inhibitors, were reported to down-regulate NMDA-mediated synaptic potentials (Coussens et al., 2000), we tested both of these PTP inhibitors on the tyrosine phosphorylation and subunit levels in the NMDA receptor complex (Fig. 1). Cortical slices were incubated in the presence of SOV (3 mM; 1 h) (Fig. 1, a and b) or PAO (10 μM; 1 h) (Fig. 1, c and d) and subjected to immunoprecipitation under nondenaturing conditions followed by Western blots. The analysis of the Western blots revealed that after treatment with SOV, tyrosine phosphorylation of NR2A and NR2B, referred to in the present report as NR2A/B subunits, increased significantly to 192 ± 11% (Fig. 1a), and this effect was accompanied by a decrease to 59 ± 14% of total NR2A/B subunits (Fig. 1b). Similar results were observed for PAO with an increase in tyrosine phosphorylation to 323 ± 53% (Fig. 1c) and a decrease in NR2A/B subunits to 40 ± 9% (Fig. 1d). In the present report, SOV, which has been previously assessed in vivo (Watkins et al., 1993; Hulley et al., 2002), was chosen to study the effects of PTP inhibition on NMDA receptor.

**Sodium Orthovanadate Is Not Toxic for the Cortical Neurons.** To investigate the cytotoxic potential of SOV, we performed a viability assay on cortical neurons in culture for DIV 21. Cells in wells were treated with vehicle alone, 100 μM NMDA as a positive control, or 3, 5, 10, and 30 mM SOV for 1 h (Table 1). The viability of the cells was assessed 24 h after the treatment, using an MTT assay. The results show that SOV did not affect the viability of the cells, even at 30 mM, whereas NMDA induced 38 ± 5% cell death. Taking these results into consideration, we decided to test the effect of higher concentrations of SOV on the phosphorylation and subunit levels of NMDA receptor.

**PTP Inhibition Affects Tyrosine Phosphorylation and NR2A/B Subunit Amount in a Dose-Dependent Manner.** Cortical slices were treated with 1, 5, 10, and 30 mM SOV and subjected to immunoprecipitation as described above. The analysis of the Western blots revealed that increases in SOV concentration resulted in augmentation in tyrosine phosphorylation of NR2A/B subunits to 336 ± 63% for 5 mM, 776 ± 104% for 10 mM, and completely disappeared at 30 mM SOV (Fig. 2a). Similar to the results mentioned above, SOV effect was associated with a simultaneous decrease of total NR2A/B subunits to 58 ± 5% in the presence of 5 mM and to 22 ± 8% in the presence of 10 mM, whereas at 30 mM SOV, NR2A/B subunits were not detectable in the NR1 immunoprecipitates (Fig. 2b). The increase in tyrosine phosphorylation and the reduction of NR2A/B subunit levels in NMDA receptor were both dose-dependent and displayed a negative correlation with a correlation coefficient of −0.97.

**Mass Spectrometry Analysis of NR2A/B Subunits before and after PTP Inhibition.** To confirm the disappearance of NR2A and NR2B subunits from the NMDA receptor.

### Table 1

<table>
<thead>
<tr>
<th>Percentage of viable cells</th>
<th>Control</th>
<th>Sodium Orthovanadate</th>
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<tr>
<td></td>
<td>100</td>
<td>62 ± 8*</td>
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<tr>
<td></td>
<td>3</td>
<td>119 ± 2</td>
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<td>5</td>
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<td></td>
<td>30</td>
<td>91 ± 9</td>
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*p < 0.05, NMDA versus control.*
Complex, we used a proteomic approach to detect NR2A and NR2B subunits in control and treated slices with 30 mM SOV. Knowing that NR2A and NR2B subunits are detected around 170 to 180 kDa, protein bands ranging from 160 to 190 kDa were manually excised from a Coomassie Blue-stained SDS-polyacrylamide gel, washed, and in-gel digested. As an example, the chromatogram of a control sample (slices treated with vehicle alone) is shown in Fig. 3a. Mass spectrum of peptides contained in the peak at retention time 32.4 min is shown in Fig. 3b. The peptide with a mass to charge ratio of 515.8, \( m/z = 2 \) was automatically selected for fragmentation by collision-induced dissociation. The fragmentation pattern of this peptide is shown in Fig. 3c. A list of masses was generated from the precursor ion information and fragmentation pattern. A data search performed with the search engine Mascot, using this list of masses, allowed for identification of the peptide and the protein. In this instance, the MS/MS fragmentation pattern matched the sequence GVDDALLSLK from the NR2B subunit (Fig. 3c).

Table 2 shows the summarized mass spectrometry results for the identification of the peptide and the protein. In this list of masses, allowed for the identification of the peptide and the protein. In this instance, the MS/MS fragmentation pattern matched the sequence GVDDALLSLK from the NR2B subunit (Fig. 3c). Table 2 shows the summarized mass spectrometry results for the identification of the peptide and the protein. In this list of masses, allowed for the identification of the peptide and the protein. In this instance, the MS/MS fragmentation pattern matched the sequence GVDDALLSLK from the NR2B subunit (Fig. 3c).

The solvent method with buffer A containing DOC and Triton X-100 used in this study has been shown to be the most effective in solubilizing NMDA receptor complexes (Luo et al., 1997). However, to ensure that the apparent loss of NR2A/B subunits after PTP inhibition could also be observed with a different method, NMDA receptor complexes were immunoprecipitated with buffer B, a non-denaturing and Triton X-100-free buffer that contains DOC, Nonidet P-40, SDS, and NaCl (Fig. 5, a and b). Similar to the results obtained with buffer A, immunoprecipitated NMDA receptor complex using buffer B from pretreated cortical slices displayed both an enhanced tyrosine phosphorylation (25% ± 17%) (Fig. 5a) and a reduction of NR2A/B subunits (47% ± 9%) (Fig. 5b). Thus, the effect of PTP inhibition is confirmed in a Triton X-100-free solubilization buffer.

Because PDZ proteins such as PSD-95, play an important role in anchoring and regulating NMDA receptors, as well as in the early event of assembly, processing, and delivery of receptor proteins (Wenthold et al., 2003), we examined the effect of PTP inhibition on the amounts of NMDA receptor subunits contained in PSD-95 complexes. Control and treated slices were treated with SOV (3 mM; 1 h), solubilized with buffer B and subjected to immunoprecipitation with anti-PSD-95 antibody. Western blot analysis (Fig. 5c) revealed that PTP inhibition reduces NR2A/B and NR1 subunits associated with the PSD complex to an amount of 23 ± 10 and 34 ± 9%, respectively.

The Disassembly of NR1 and NR2A/B Subunits Is a Reversible Process and Occurs through Src Family Kinases. To confirm that disassembly illustrated in Figs. 1 and 2 is due to tyrosine phosphorylation and not to unspecific drug effects, we tested two tests. First, knowing that phosphorylation is a reversible process, we tested whether SOV-induced NR1 and NR2 subunits disassembly was reversible as well. Cortical slices were first incubated with 3 mM SOV for 1 h, as described above, then quickly rinsed twice with fresh ACSF at 35°C and incubated with new ACSF for 1 h. The slices were rinsed again in ice-cold ACSF and subjected to immunoprecipitation. After drug washout, there were no differences between control and SOV-pretreated slices (Fig. 6, a and b). Tyrosine phosphorylation of NR2A/B subunits (Fig. 6a) and total amount of NR2A/B subunits (Fig. 6b) rapidly returned to base level. Second, this suggests that the effect of PTP inhibition on NMDA receptor complex disassembly is a reversible process. Because of the intricate relationship between tyrosine phosphates and tyrosine kinases and because of the importance of Src kinase family in NMDA receptors regulation, we tested whether a specific inhibitor of Src tyrosine kinase family (PP2) (Hanke et al., 1996) prevents the effect of PTP inhibition on the disasme-
bly of NR1 and NR2 subunits (Fig. 6, c and d). In this experiment, slices were preincubated with 20 μM PP2 for 10 min before adding 3 mM SOV for an additional hour (PP2 was present during SOV exposure). Results from this experiment indicate that PP2 did not only prevent SOV-induced increase in tyrosine phosphorylation but also inhibited the amount of phosphorylated NR2A/B subunit to 18% (Fig. 6c). In addition, PP2 prevented SOV-induced reduction of NR2A/B subunit (Fig. 6d). This suggests that the effect of PTP inhibition on NMDA receptor involves the activation of Src tyrosine kinases and that tyrosine phosphorylation has an important role in NR1 and NR2 subunits disassembly.

**Discussion**

An important finding in the present report is that inhibition of PTPs causes disassembly of NR1 and NR2A/B subunits of NMDA receptor complex in a dose-dependent man-
Our initial findings. Moreover, immunoprecipitation of nondenaturing Triton X-100-free buffer (buffer B) confirmed the dissociation of nonsynaptic NMDA receptor complexes. The use of a proteomic approach complemented and confirmed the coimmunoprecipitation experiments. Although proteomics of the NMDA receptor complex in the cortex has been previously investigated and resulted in the identification of 77 proteins that are part of the complex (Husi et al., 2000), our experimental design with a comparative mass spectrometry of control and treated cortex has not been reported in the past. The results from these experiments were clear, with more than 20 peptides matched for NR2A and for NR2B proteins in the control, whereas no matching peptides were found after PTP inhibition.

Solubilization of NMDA receptor subunits is an important factor in biochemical studies of the complex. In fact, NR1 and NR2 subunits display differential solubilization properties, with NR2 being weakly soluble in Triton X-100 in both microsomal and synaptic membrane fraction, whereas NR1 in the microsomal fraction is Triton X-100-soluble but is insoluble in the synaptic membrane fraction (Blahos and Wenthold, 1996). In the present report, a combination of DOC and Triton X-100 (buffer A), found to be the most effective in solubilization of NR1 and NR2 subunits (Luo et al., 1997), was used to study the effect of PTP inhibition on NMDA receptor complex. However, since receptors in the synaptic membrane are generally Triton X-100-insoluble, there was a concern that our study included only a population of nonsynaptic NMDA receptor complexes. The use of a non-denaturing Triton X-100-free buffer (buffer B) confirmed our initial findings. Moreover, immunoprecipitation of PSD-95 complexes using buffer B indicates that the synaptic NMDA receptor population is also included in this study and shows that PTP inhibition reduced the interaction between NR1 and NR2A/B subunits with the PSD-95 complex. This is in agreement with a recent study showing that inhibition of PTPs decreases the interaction between NR2A, PSD-95, and Fyn kinase (Chen et al., 2003).

The NMDA receptor subunit disassembly, observed after PTP inhibition, was accompanied by an increase in tyrosine phosphorylation of the remaining NR2A/B subunits in the receptor. Although tyrosine phosphorylation has been implicated in several processes related to trafficking (Dunah and Standaert, 2001), there is no evidence of its involvement in the assembly of the NMDA receptor subunits. Here, we show that PTP inhibition-induced disassembly of NMDA is sensitive to PP2 (specific Src kinase inhibitor), which implicates the Src tyrosine kinase family as a mediator in this process. In addition to NR2 subunits, several proteins associated with the NMDA receptor are subject to phosphorylation by Src family kinases such as PTP-95, within the PSD-95 family, which has been identified as a substrate for the Src family tyrosine kinase Fyn and has been shown to play a role in the regulation of Fyn-mediated modification of NMDA receptor function (Nada et al., 2003). Thus, tyrosine phosphorylation of one or several proteins associated with NMDA receptor could be responsible for the dissociation of NR1 and NR2A/B subunits.

 Src kinase-induced tyrosine phosphorylation has been shown to stimulate NMDA receptor function, resulting in Ca²⁺ influx in a variety of systems (Ali and Salter, 2001), and Ca²⁺ entry through NMDA receptors has been reported to induce disassembly and redistribution of postsynaptic density PSD-Zip45 (Homer 1c) (Okabe et al., 2001). Thus, it is possible that by stimulating tyrosine phosphorylation, inhibition of PTPs could lead to the activation of NMDA receptors resulting in Ca²⁺ influx, which in turn disassembles the NMDA receptor subunits. However, this is unlikely since in the present report, NMDA, which is known to mediate excitotoxicity, induced a significant decrease in neuronal viability, whereas SOV did not affect the viability of these cells even at high concentrations. Several reports from the literature suggest that inhibition of tyrosine phosphatases reduces synaptic plasticity and NMDA-mediated currents. Indeed, intracellular application of antibody against tyrosine phosphatase RPTPα into CA1 neurons has been reported to reduce the amplitude of the long-lasting excitatory postsynaptic current after tetanic stimulation, whereas RPTPα intracellular domain enhanced it (Lei et al., 2002), and absence of synaptic plasticity in brain slices of mice lacking RPTPα has also been observed (Petrone et al., 2003). Furthermore, inhibition of synaptic plasticity induced by PTPase inhibitors PAO and SOV was due to a decrease in NMDA-mediated synaptic transmission (Coussens et al., 2000). Together, this suggests that activation of NMDA receptors may not be required in PTP inhibition-induced disassembly of NMDA receptor subunits.

Activation of Src family kinases has previously been shown to enhance NMDA receptor function (Kohr and Seeburg, 1996; Yu et al., 1997) but has never been associated with a decrease in NR2A/B subunit levels. In most of these studies, endogenous PTPs were still active and constantly down-regulating tyrosine phosphorylation. However, concentrations used in the present study have been reported to inhibit 90%...
of PTP activity (Gordon, 1991), and the consequence is a constant increase in tyrosine phosphorylation with no down-regulation. This extreme tyrosine phosphorylation resulted in the disassembly of NMDA receptor subunits. Using similar concentrations, a study reported a down-regulation of NMDA-mediated synaptic potential (Coussens et al., 2000). Furthermore, lower concentration of 2.5 μM PAO increases tyrosine phosphorylation without change in the levels of NR2A/B subunits (Ferrani-Kile et al., 2003). Together, these findings suggest that moderate levels of tyrosine phosphorylation stimulate NMDA receptor function, whereas extreme levels of tyrosine phosphorylation disassemble NMDA receptor subunits and down-regulate the receptor function.

These findings define a novel role for PTPs in the NMDA receptor assembly and the disruption of the complex. Since coassembly of NR1 and NR2 subunits is required to form a functional channel (Cull-Candy et al., 2001) and PTP blockage has been shown to display neuroprotective properties (Kawano et al., 2001; Lu et al., 2002), dissociation of NR1 and NR2 mediated by PTP inhibition could be of importance in NMDA-related disorders of the central nervous system.

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References


Address correspondence to: Dr. Karima Ferrani-Kile, Division of Pharmacology and Toxicology, College of Pharmacy and the Wagringer Center for Alcohol and Addiction Research, The University of Texas at Austin, 1 University Station, A1915, Austin, TX 78712. E-mail: ferrani@mail.utexas.edu