Communication

Peroxynitrite Inhibits Glutamate Transporter Subtypes*

Davide Trotti‡§, Daniela Rossi‡, Ola Gjesdal¶, Line M. Levy¶, Giorgio Racagni‡, Niels C. Danbolt¶, and Andrea Volterra‡∥

From the ‡Center of Neuropharmacology, Institute of Pharmacological Sciences, University of Milan, via Balzaretti 9, 20133 Milan, Italy and the ¶Department of Anatomy, Institute of Basic Medical Sciences, University of Oslo, N-0317 Oslo, Norway

     The reuptake of glutamate in neurons and astrocytes terminates excitatory signals and prevents the persistence of excitotoxic levels of glutamate in the synaptic cleft. This process is inhibited by oxygen radicals and hydrogen peroxide (H_2O_2). Here we show that another biological oxidant, peroxynitrite ($ONOO^-$), formed by combination of superoxide ($\cdot O_2^-$) and nitric oxide (NO), potently inhibits glutamate uptake by purified or recombinant high affinity glutamate transporters reconstituted in liposomes. $ONOO^-$ reduces selectively the V_{max} of transport; its action is fast (reaching 90% within 20 s), dose-dependent (50% inhibition at 50 μM), persistent upon $ONOO^-$ (or by product) removal, and insensitive to the presence of the lipid antioxidant vitamin E in the liposomal membranes. Therefore, it likely depends on direct interaction of $ONOO^-$ with the glutamate transporters. Three distinct recombinant glutamate transporters from the rat brain, GLT1, GLAST, and EAAC1, exhibit identical sensitivity to $ONOO^-$. H_2O_2 also inhibits reconstituted transport, and its action matches that of $ONOO^-$ on all respects; however, this is observed only with 5–10 mM H_2O_2 and after prolonged exposure (10 min) in highly oxygenated buffer. NO, released from NO donors (up to 10 mM), does not modify reconstituted glutamate uptake, although in parallel conditions it promotes cGMP formation in synaptosomal cytosolic fraction. Overall, our results suggest that the glutamate transporters contain conserved sites in their structures conferring vulnerability to $ONOO^-$ and other oxidants.

     Glutamate uptake in neurons and astrocytes is essential to maintain resting extracellular glutamate concentration below levels inducing significant activation of excitatory amino acid (EAA)[1] receptors (1). Thereby it provides a high signal to noise ratio for excitatory neurotransmission and prevents harmful receptor overstimulation. Altered transport function has been associated with neuronal damage in ischemia/reperfusion injury (2) and amyotrophic lateral sclerosis (ALS) (3, 4). The uptake process is mediated by glycoproteins located in the plasma membrane of neuronal and glial cells. At least four different transporters are now cloned, *i.e.* GLAST (5), GLT1 (6), EAAC1 (7), and EAAT4 (8), constituting a gene family with specialized brain distributions (9–11). There is increasing evidence that glutamate transport is regulated, *e.g.* via protein kinase C-mediated phosphorylation (12) and arachidonic acid (13, 14). Oxygen radicals and H_2O_2 induce persistent inhibition of glial glutamate uptake, probably via direct interaction with the transport process (15). Sodium nitroprusside, a NO generator, decreases uptake into synaptosomes (16). When generated simultaneously, $\cdot O_2^-$ and NO react together at a diffusion-limited rate to form the strong oxidant $ONOO^-$ (17). Several biological or toxic effects originally attributed to either NO or $\cdot O_2^-$ are now thought to be mediated by $ONOO^-$ (18–22). In the present study, we address the possibility that $ONOO^-$ affects glutamate uptake by direct interaction with the glutamate transporters.

EXPERIMENTAL PROCEDURES

     Materials—L-[³H]Glutamic acid (50 Ci/mmol) was from Amersham, while ⁸⁶Rb (120 mCi/mmol) from DuPont NEN; Sephadex G-50 fine from Pharmacia; CHAPS, wheat germ agglutinin (free lectin), vitamin E from Sigma; H_2O_2, GTP, and 3-isobutyl-1-methylxanthine from Aldrich; and $ONOO^-$ from U.B.I. (Lake Placid, NY). $ONOO^-$ was stored at −80 °C, and its concentration in the stock solution was determined spectrophotometrically following the manufacturer's protocol. The NO releasers DEA/NO (diethylamine/NO adduct) and MAHMA/NO (*N,N'*-dimethyl-1,6-hexanediamine/NO adduct) were kindly provided by Dr. L. K. Keefer, NIH, Laboratory of Comparative Carcinogenesis (Frederick, MD); (±)-*S*-nitroso-*N*-acetylpenicillamine (SNAP) was from Biomol.

     Purification and Reconstitution of Glutamate Transporters into Liposomes—This was done as described previously (23). Briefly, crude rat brain plasma membranes were solubilized with CHAPS and centrifuged. The supernatant (CHAPS extract) was passed through a wheat germ agglutinin-agarose column, and the glycoproteins eluted with *N*-acetylglucosamine (partially purified transporters). For reconstitution, this fraction (20 mM CHAPS and 0.1–0.2 mg of protein/ml) was mixed with 1.5 volumes of a reconstitution mixture consisting of phospholipid, cholate, and salt, incubated on ice, and gel-filtered on spin columns (13) equilibrated with KP_i to remove detergent and sodium ions. The liposomes form spontaneously during this gel filtration, and KP_i becomes their internal medium. This preparation (10, 24)[2] contains immunoreactivity to the 3 rat brain glutamate transporters cloned to date: GLT1, GLAST, and EAAC1. In some experiments, vitamin E (1:100 or 1:25 w/w) was added to the reconstitution mixture. To test membrane integrity, liposomes were formed in the presence of ⁸⁶Rb, exposed to $ONOO^-$ or H_2O_2, and blotted on Millipore filters (0.45-μm pores). Radioactivity retained was compared to control.

     Cloning and Sequencing of the Glutamate Transporters—Rat GLT1 and EAAC1 clones were the same as published previously (6, 24). For GLAST, a cDNA library (in the pCD2 vector) from the cerebral cortex

     § Present address: Renal Division, Dept. of Medicine, Brigham and Women's Hospital, Harvard Medical School, 75 Francis St., Boston, MA 02115.

     ∥ To whom correspondence should be addressed. Tel.: 39-2-2048-8206; Fax: 39-2-2940-4961; E-mail: volterra@isfunix.farma.unimi.it.

     [1] The abbreviations used are: EAA, excitatory amino acid; ALS, amyotrophic lateral sclerosis; DEA, diethylamine; MAHMA, *N,N'*-dimethyl-1,6-hexanediamine; SNAP, (±)-*S*-nitroso-*N*-acetylpenicillamine; DOTAP, *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium methyl-sulfate, KP_i, potassium phosphate buffer; NaP_i, sodium phosphate buffer; PBS, phosphate-buffered saline; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate.
     [2] N. C. Danbolt, unpublished observation.

was screened with a 160-nucleotide probe from the N-terminal (5). The probe was obtained by polymerase chain reaction-amplification from cDNA of rat brain using synthetic primers. Positive clones were isolated, and plasmid DNA was prepared from 500 ml of Luria-Bertani broth cultures using the Wizard Maxiprep DNA isolation system (Promega Biotech Inc.). The cDNA fragment was subcloned into the pBluescript vector and amplified by transformation of the *Escherichia coli* strain JM103. The plasmid DNA was isolated as described above. DNA sequencing was performed by the dideoxy chain termination method (26) using Sequenase (U. S. Biochemical Corp.) and primers derived from the GLAST cDNA sequence as well as from the pBluescript T7 and T3 promoters. The sequences were analyzed with the DNA analysis software (Geneting, Lillestrøm, Norway) and found to be identical to GLAST.

Transfection of Glutamate Transporter Plasmid cDNAs in HeLa Cells and Reconstitution into Liposomes—HeLa cells infected with vTF-7 recombinant vaccinia virus (6, 25, 26) were transfected with pT7-GLT1, pT7-EAAC1, or pT7-GLAST plasmids (1.5 μg/cm² flask) by means of DOTAP (Boehringer Mannheim) following the manufacturer's instructions. Control cells were treated with DOTAP without plasmid. Transfected and control cells were washed, collected with PBS (pH 7.4), and centrifuged (200 × g × 5 min, 4 °C). Pellet was resuspended with 4 volumes of 0.5 M NaCl, 10 mM NaP$_i$ (pH 7.4), 1.5% cholate buffer and reconstituted with 1.5 volumes of reconstitution mixture using spin columns as above.

Glutamate Transport Assay—Glutamate transport in liposomes was measured using an inwardly directed Na⁺ gradient in the presence of a negative membrane potential, as described (13, 23). Briefly, liposomes were diluted 1 + 3 with 130 mM NaCl, 20 mM NaP$_i$ (pH 7.4), 1% glycerol (PBSG) plus ONOO⁻ or NO releasers (buffer a) or with Krebs/HCO₃⁻, pH 7.4 (oxygen-saturated) plus H₂O₂ (buffer b), incubated at room temperature for 70 s (ONOO⁻, unless otherwise specified), 2 min (NO releasers), or 10 min (H₂O₂) and gel-filtered to remove the compounds and their by-products. 80 μl of liposomes recovered from spin columns were diluted 1 + 5.5 volumes with PBSG, and the uptake assay was started by adding 1.4 μCi of [³H]glutamate + 2.8 μM valinomycin and stopped 70 s later. In the kinetic experiments, uptake assay was run for 2 s as described previously (13). Then, liposomes were collected on filters and counted for radioactivity. Statistical analysis was done by Student's *t* test (2-tailed).

Lipid Peroxidation—Lipid peroxidation was measured on liposomal suspensions treated with ONOO⁻ or H₂O₂ as in the glutamate uptake assay, using an LPO-586 kit from Bioxytech S.A.

Monitoring of NO Release and NO₂⁻ Accumulation—Stock solutions of DEA/NO and MAHMA/NO in 10 mM NaOH were added to NaP$_i$ (0.1 M, pH 7.4) in a standard UV cell to give final concentrations of 0.1, 1, and 10 mM. The rate of NO release was determined by monitoring the disappearance of the characteristic UV absorption (λ_{max} = 250–252 nm) exhibited by the NO adducts (27). Nitrite accumulation was detected following the method of Ignarro *et al.* (28).

Cyclic GMP Formation in Cortical Crude Synaptosomal Cytosol—Synaptosomal cytosol was prepared according to Knowles *et al.* (29) without the final chromatographic step. 150 μl of cytosol were added to 50 μl of PBSG buffer + (in mM) 5 MgCl₂, 5 GTP, 1 isobutylmethylxanthine (final concentration). Incubation at room temperature was started by addition of 0, 0.1, or 1 mM DEA (control) or DEA/NO and terminated 2 min later with 20 μl of 20% HClO₄. After centrifugation (12000 × g, 1 min), the cyclic GMP formed was determined by radioimmunoassay using the ¹²⁵I-cGMP kit (RPA 525, Amersham) on supernatant aliquots diluted 1:100 or 1:1000 with PBSG + 4 mM EDTA. The cyclic GMP content, expressed as nanomoles/sample, was calculated by extrapolation from a standard curve constructed following the kit manual.

RESULTS

The possible direct interaction of ONOO⁻ with glutamate uptake was first studied on a preparation of partially purified brain glutamate transporters reconstituted in liposomes. Liposomes were exposed to ONOO⁻ or to decayed ONOO⁻ as control (see legend to Fig. 1). The agents were then removed by gel filtration and the uptake assay run. ONOO⁻ (but not decayed ONOO⁻) inhibited uptake dose dependently (Fig. 1*A*). A 70-s exposure to 50 μM ONOO⁻ reduced uptake by 50 ± 6%. Threshold inhibition (−10 ± 6%) was seen with 5 μM ONOO⁻, while nearly complete inhibition (−85 ± 5.1%) required 250 μM ONOO⁻. The ONOO⁻ effect developed almost immediately, reaching ≥90% within 20 s and then increasing slightly in the
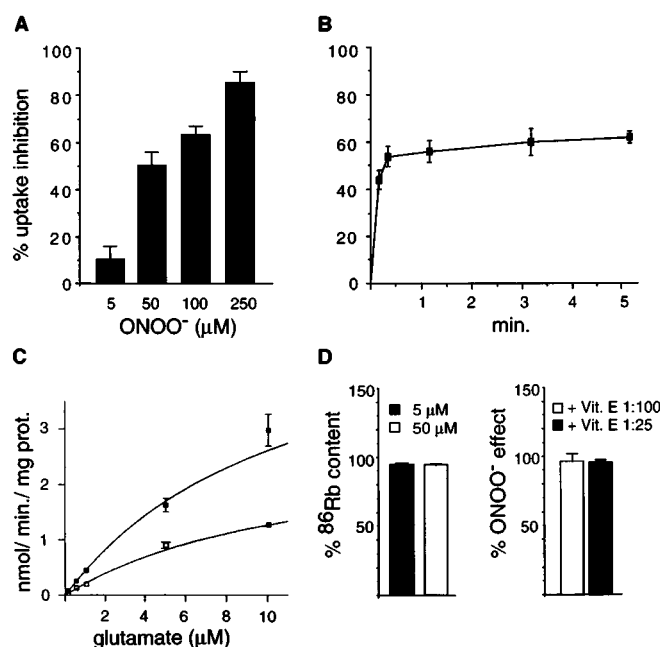

FIG. 1. **Characteristics of the inhibitory effect of ONOO⁻ on reconstituted glutamate transport.** [³H]Glutamate uptake was run on partly purified glutamate transporters reconstituted in liposomes and exposed to ONOO⁻ or decayed ONOO⁻ as control (see "Experimental Procedures" for details). Decayed ONOO⁻ is an ONOO⁻ solution left to decompose for at least 1 min in PBSG, pH 7.4. Time course of decay indicates that the species active on glutamate transport disappear within 20 s from addition of ONOO⁻ to PBSG. *A*, dose dependence of ONOO⁻ inhibition. Data are expressed as % inhibition ± S.D. of control uptake (*n* = 3 in triplicate). ONOO⁻ inhibition was significant at all tested concentrations (*p* < 0.01). *B*, time course of ONOO⁻ inhibition. Liposomes were exposed for 10, 20, 70, 190, or 310 s to 50 μM ONOO⁻ and uptake assay run in the last 15 s, except for the 10-s point where uptake was run during ONOO⁻ exposure. Data are expressed as % inhibition ± S.D. of control (*n* = 3 in triplicate). *C*, effect of ONOO⁻ on the kinetics of glutamate uptake. Assay run for 2 s on liposomes preexposed to control (■) or 50 μM ONOO⁻ (□): (*n* = 2 in quadruplicate, see Ref. 13 for details). Kinetic analysis was performed with the Enzpack 3.0 program and the direct linear plot method (13). *D, left*, effect of ONOO⁻ on the ⁸⁶Rb content of liposomes. Radioactivity in liposomes with KP$_i$ + ⁸⁶Rb as internal medium was measured after exposure to either control (=100%) or ONOO⁻ (5 or 50 μM). No significant difference was observed (*n* = 2 in triplicate). *D, right*, effect of ONOO⁻ on glutamate transport reconstituted in liposomes with different vitamin E content. The effect of ONOO⁻ (50 μM, 70 s) was compared in liposomes formed without (=100%) or with vitamin E as a membrane constituent (1:100 or 1:25 (w/w) with phospholipid). No significant difference was observed (*n* = 3 in triplicate). The effective presence of vitamin E in the membranes was confirmed by chloroform extraction from liposomes blotted on filters after uptake assay and fluorescence detection (30).

next 2–3 min (Fig. 1*B*). Conversely, 50 μM ONOO⁻ preincubated for 20 s at pH 7.4 before addition to liposomes was totally devoid of effect. Characterization of uptake kinetics without ONOO⁻ gave a K_m value of 11 ± 0.8 μM and a V_{max} of 5.7 ± 1 nmol/min/mg of protein. ONOO⁻ (50 μM, 70 s) reduced V_{max} by 50% without affecting K_m (Fig. 1*C*). Different from ONOO⁻, 3 fast NO donors, failed to significantly modify reconstituted glutamate uptake. In a few cases, MAHMA/NO (1 mM) or SNAP (1 mM) were used, while in most experiments we utilized DEA/NO (0.1–10 mM). Uptake assay was run after preincubation (2 min) and removal of DEA/NO or in its presence. In either case, the compound produced weak inhibition (≤10%) that was similar at 0.1, 1, and 10 mM (Fig. 2*A*). However, in the same condition, NO release from DEA/NO, observed spectrophotometrically (see "Experimental Procedures"), was found to proceed dose dependently, with a $t_{1/2}$ of 2 min. NO₂⁻ accumulated accordingly. Finally, we confirmed that active NO was indeed released from DEA/NO. Thus, the compound-induced
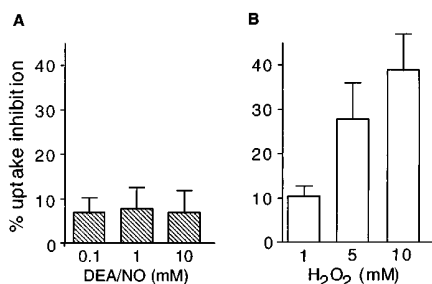
FIG. 2. **Concentration-dependent inhibition of reconstituted glutamate transport by H_2O_2 but not by NO.** [^{3}H]glutamate uptake was run on partly purified glutamate transporters reconstituted in liposomes and exposed to either DEA/NO (*hatched bars*) or H_2O_2 (*hollow bars*) (see "Experimental Procedures"). Data are expressed as percent inhibition ± S.D. of control uptake ($n = 4$ in triplicate). Control for DEA/NO was DEA (diethylamine hydrochloride). Other controls tested and without effect on uptake included $NaNO_2$ at pH 7.4, $NaNO_3$, and a decomposed DEA/NO solution (for 24 h). H_2O_2 effect, but not that of DEA/NO, was significantly different from control at all the reported concentrations ($p < 0.01$).

TABLE I

$ONOO^-$ and H_2O_2 inhibit uptake by recombinant rat GLT1, GLAST, and EAAC1 reconstituted in liposomes

Data are expressed as % inhibition ± S.E. of uptake with respect to control (see legend to Fig. 1). Liposomes were inlaid with glutamate transporters from HeLa cells transfected with one of the 3 rat glutamate transporter cDNAs. Pre-exposures to $ONOO^-$ and H_2O_2 and uptake assay were performed as described under "Experimental Procedures" ($n = 3$ in quadruplicate). All reported inhibitions were significantly different from control ($p < 0.01$).

		GLT1	GLAST	EAAC1
$ONOO^-$	5 μM	9.0 ± 0.6	9.9 ± 2.8	10.0 ± 3.2
	50 μM	60.4 ± 1.0	50.3 ± 5.1	58.3 ± 4.0
H_2O_2	1 mM	15.4 ± 0.2	16.8 ± 3.5	18.2 ± 2.3
	10 mM	50.4 ± 2.7	50.8 ± 7.2	48.8 ± 6.6

dose-dependent accumulation of cGMP in synaptosomal soluble fraction from rat cortex cGMP was 0.1 ± 0.03 nmol/sample in controls (2 min, PBSG), 0.08 ± 0.03 in 1 mM DEA, 0.17 ± 0.02 in 0.1 mM DEA/NO (+117%), and 0.33 ± 0.05 in 1 mM DEA/NO (+312%) ($n = 3$ in duplicate). Different from NO and similar to $ONOO^-$, H_2O_2, another biological oxidant, inhibited reconstituted glutamate transport. Like $ONOO^-$, H_2O_2 selectively reduced uptake V_{max}; however, it induced a comparable level of inhibition only at mM concentrations (−38.9 ± 8.2% at 10 mM), after long exposure (10 min) and in highly oxygenated buffer (Fig. 2B).

Glutamate uptake inhibition with $ONOO^-$ or H_2O_2 could be due to peroxidation of the liposome membranes resulting either in loss of the ion gradients fueling the uptake process or changes in the lipidic environment of the transporter proteins. To exclude the first possibility, liposomes were preloaded with ^{86}Rb and exposed to $ONOO^-$ (5–50 μM). No release of radioactivity was observed at times parallel to uptake inhibition (Fig. 1D, *left*). To address the second possibility, we prepared liposomes containing the lipophilic antioxidant vitamin E among the lipid constituents (1:100 or 1:25 w/w). The effect of $ONOO^-$ (50 μM, 70 s) was compared in liposomes with and without vitamin E, finding the same extent of uptake inhibition in all types of liposomes, independent of their vitamin E content (Fig. 1D, *right*). Moreover, by use of a standard assay, we failed to detect any significant signal of lipid peroxidation in conjunction with uptake inhibition. H_2O_2 (10 mM, 10 min) behaved identically to $ONOO^-$ in the above experiments. We then tested the effect of $ONOO^-$ and H_2O_2 on uptake by recombinant transporter subtypes. HeLa cells were transfected with cDNAs encoding the glutamate transporters GLT1, GLAST, or EAAC1 and their cell membranes reconstituted into liposomes. Uptake capacity was found about 100- (GLT1), 20- (GLAST), and 50-fold (EAAC1) higher with respect to mock-transfected cells similarly reconstituted. As shown in Table I, μM $ONOO^-$ inhibited uptake by any transporter subtype dose-dependently and with equivalent potency. Again, mM H_2O_2 behaved comparably.

DISCUSSION

$ONOO^-$, a biological oxidant and the combination product of $^\cdot O_2^-$ and NO, potently inhibits purified and recombinant glutamate transporters reconstituted in liposomes. NO alone appears unable to directly modify glutamate transport. Thus, 3 fast NO releasers, MAHMA/NO (1 mM), SNAP (1 mM), and DEA/NO (0.1, 1, and 10 mM), failed to inhibit reconstituted transport. To be sure that active NO indeed was released, we

checked that the dose- and time-dependent NO disappearance from the DEA/NO adduct paralleled NO_2^- accumulation and that DEA/NO dose-dependently enhanced cGMP levels in synaptosomal soluble fraction.

We reported previously that oxygen radicals and H_2O_2 induce long-lasting decreases of glutamate transport in astrocytic cultures, probably due to protein oxidation (15). In agreement, here we find that H_2O_2 directly affects reconstituted glutamate transport. Its mode of action is superimposable to that of $ONOO^-$. Thus, both agents selectively reduce uptake V_{max}. This effect involves some persistent modification of proteoliposomes, because it is observed after removal of the compounds and their by products via gel filtration. However, it unlikely depends on oxidation of the lipid component. Thus, (*a*) $ONOO^-$ or H_2O_2 inhibition is not attenuated in liposomes containing vitamin E among the membrane constituents (up to ≈1:10 molar ratio with phospholipids) and (*b*) a standard lipid peroxidation assay does not reveal detectable levels of malonaldehyde or 4-hydroxyalkenals paralleling uptake changes. Generalized membrane damage is also ruled out because no radioactive leakage is observed from ^{86}Rb-filled liposomes exposed to 5–50 μM $ONOO^-$ or to 10 mM H_2O_2. Therefore, uptake inhibition likely depends on direct interaction of $ONOO^-$ and H_2O_2 with the glutamate transporter proteins resulting in covalent modification of their structure. Recombinants of the 3 major cloned rat brain subtypes, GLT1, GLAST (both glial), and EAAC1 (neuronal) are all similarly inhibited by $ONOO^-$ (or H_2O_2). Lack of differential sensitivity suggests that one or more "oxidant-vulnerable site(s)" are present in conserved regions of these proteins.

Although similar in the mode of action, $ONOO^-$ is significantly more potent and rapid than H_2O_2 in inhibiting reconstituted glutamate transport. Thus, while H_2O_2 effect is seen at mM concentrations and after several minutes, $ONOO^-$ acts in the μM range and within seconds (≥90% of inhibition at 20 s, paralleled by disappearance of the active species), in line with its reported half-life and rate of decomposition at pH 7.4, resulting in the formation of potent oxidant intermediates with reactivity of hydroxyl radical ($^\cdot$OH) and other reactive species such as nitronium ion and nitrogen dioxide (31). Moreover, $ONOO^-$ is effective in normal air-equilibrated buffer, while H_2O_2 only in a hyperoxygenated buffer, suggesting that its transformation into OH$^\cdot$ via $^\cdot O_2^-$-driven Fenton reaction may be required (32). A common primary target for $ONOO^-$, H_2O_2, or downstream products such as $^\cdot$OH is oxidation of cysteine sulfhydryl groups. Thiol oxidation by $ONOO^-$ proceeds 10^3-fold faster than with H_2O_2 (32). In addition, $ONOO^-$ could induce nitrosylation and/or nitration of aromatic amino acids. Targeting of transporter SH groups by H_2O_2 and $ONOO^-$ would be consistent with our previous observation that glial uptake, inhibited by oxygen radicals, is significantly restored with dithiothreitol, a disulfide-reducing agent (15).

Inhibition of glutamate uptake by ONOO⁻ may be of pathological significance, contributing to the build up of excitotoxic extracellular glutamate. The conditions for local formation of ONOO⁻ at glutamate synapses exist. Thus, both $\cdot O_2^-$ and NO can be generated as a result of activation of EAA receptors (33, 34). If formed in conjunction, $\cdot O_2^-$ and NO react together to give ONOO⁻ at a diffusion-limited rate (17). Several pathological situations would favor this process, *e.g.* because NO levels are enhanced (via activation of inducible NO synthase (35, 36)), because $\cdot O_2^-$ catabolism is reduced, or superoxide dismutase (SOD) activity is altered, as proposed for mutant SOD1 in familial ALS (37, 38). Indeed, enhanced protein tyrosine nitration, a marker for ONOO⁻ formation, was recently reported in mutant SOD1 transgenic mice (39) as well as in other animal models of neurodegenerative diseases thought to involve excitotoxicity (40, 41). The case of ALS is intriguing, as this pathology has been associated with defect of glutamate transport (2). μM ONOO⁻ is highly neurotoxic to cultured neurons (21). Due to its half-life, ONOO⁻ can travel quite a distance from the site of production to damage critical neuronal constituents, such as the neurofilament proteins (37, 38) or the mitochondrial enzyme aconitase (19, 20). The present study indicates that the glutamate transporters could be other important targets of ONOO⁻ toxicity.

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Davide Trotti, Daniela Rossi, Ola Gjesdal, Line M. Levy, Giorgio Racagni, Niels C. Danbolt and Andrea Volterra

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