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Long-lasting inhibition of presynaptic metabolism and neurotransmitter release by protein S-nitrosylation

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Abstract

Nitric oxide (NO) and related reactive nitrogen species (RNS) play a major role in the pathophysiology of stroke and other neurodegenerative diseases. One of the poorly understood consequences of stroke is a long-lasting inhibition of synaptic transmission. In this study, we tested the hypothesis that RNS can produce long-term inhibition of neurotransmitter release via S-nitrosylation of proteins in presynaptic nerve endings. We examined the effects of exogenous sources of RNS on the vesicular and non-vesicular L-[³H]glutamate release from rat brain synaptosomes. NO/RNS donors, such as spermine NONOate, MAHMA NONOate, S-nitroso-L-cysteine, and SIN-1, inhibited only the vesicular component of glutamate release with the order of potency that closely matched levels of protein S-nitrosylation. Inhibition of glutamate release persisted for >1 hr after RNS donor decomposition and wash out, and strongly correlated with decreases in the intrasynaptosomal ATP levels. Post-NO treatment of synaptosomes with thiol-reducing reagents decreased the total content of S-nitrosylated proteins but had little effect on glutamate release and ATP levels. In contrast, post-NO application of the end-product of glycolysis pyruvate partially rescued neurotransmitter release and ATP production. These data suggest that RNS suppress presynaptic metabolism and neurotransmitter release via poorly reversible modifications of glycolytic and mitochondrial enzymes, one of which was identified as glyceraldehyde-3-phosphate dehydrogenase. Similar mechanism may contribute to the long-term suppression of neuronal communication during nitrosative stress *in vivo*.

Keywords

nitric oxide; S-nitroso-L-cysteine; S-nitrosylation; S-nitrosation; neurotransmitter release; energetic metabolism; brain

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Introduction

The biological active molecule nitric oxide (NO) and related reactive nitrogen species (RNS) regulate cellular functions via interactions with metalloproteins, such as guanylyl cyclase and cytochrome c oxidase, but also via a variety of chemical modifications of biomolecules, such as oxidation, nitration, and *S*-nitrosylation¹ [1–4]. NO and RNS can be harmful if produced in excessive quantities or if normal intracellular redox homeostasis is impaired. In the context of brain pathophysiology, NO and RNS have been implicated in a variety of neural disorders, such as stroke, brain, trauma, Alzheimer and Parkinson's diseases, multiple sclerosis, and others [5–7].

Pathological significance of NO and RNS are particularly well studied in cerebral ischemia (stroke). Transient or permanent disruption of blood supply triggers rapid damage and death of neuronal cells which is initially restricted to the ischemic core, the region where blood flow is reduced by ~80% or more [8]. However, over a few hours to 1–3 days, the initial infarction spreads to neighboring tissue regions of ischemic penumbra, where blood flow remains partially preserved due to collateral circulation [8–10]. NO and RNS are major players in both acute and delayed forms of ischemic brain damage. In ischemia and reperfusion, cytoplasmic Ca²⁺ overload leads to uncontrolled activation of neuronal nitric oxide synthase (nNOS), which is followed by delayed inflammatory upregulation of the inducible NOS (iNOS) activity. There is a good evidence that both nNOS and iNOS mediate ischemic tissue damage since their genetic deletion or pharmacological inhibition strongly reduce infarction volume in animal models of cerebral ischemia [5,7,11–13].

Unlike the much studied mechanisms of cells death, the potential pathological impact of NO and RNS on neuronal communication is poorly understood. Long-term changes in neurotransmission are seen even in the remote periinfarct areas where cellular viability is not affected. Chronic modifications of synaptic transmission are associated either with reductions in release of the inhibitory neurotransmitter GABA [14,15], or with suppression of release of the excitatory neurotransmitter glutamate [16–18]. The *in vivo* mechanisms contributing to the defects in synaptic communication are not well defined but may involve impaired neurotransmitter release [16], downregulation of postsynaptic receptors [14], and/or degeneration of neuronal processes [19]. Importantly, synaptic transmission may be particularly vulnerable to ischemia. In one recent study, neuroprotective treatments that prevented neuronal death did not prevent the loss of synaptic function and the structural loss of neuronal projections [20].

In the present work, we explored the idea that NO and RNS can produce lasting changes in neurotransmitter release via protein *S*-nitrosylation, the covalent attachment of NO to the thiol groups of protein cysteine residues. *S*-nitrosylation has recently emerged as a widespread and important mechanism regulating protein and cellular functions in many tissues including the central nervous system [4,21–24]. Levels of *S*-nitrosothiols (RSNOs) are strongly elevated under various pathological conditions inside and outside of the brain and appear to contribute to etiology of diseases [25–31], although this is poorly defined mechanistically. Nevertheless, several *in vitro* studies established that NO-generating compounds depolarize presynaptic nerve endings, and potently inhibit the vesicular neurotransmitter release in a manner that is dependent on modification (including *S*-nitrosylation) of intrasynaptosomal sulfhydryl groups [32–34]. In non-neuronal tissue, physiological levels of *S*-nitrosylation have been shown to suppress exocytosis via inhibition

¹The term “*S*-nitrosation” is defined in chemical terms as the addition of a nitrosonium (NO⁺) equivalent to a sulfhydryl group to form an *S*-nitrosothiol (RSNO). The term “*S*-nitrosylation” is commonly used to denote the post-translational modification of proteins through the formation of RSNOs at cysteine residues. The term “*S*-nitrosylation” will be used throughout this paper.

of the *N*-ethylmaleimide fusion protein (NSF) [35]. Therefore, our initial hypothesis was that vesicular neurotransmitter release can be suppressed via an identical mechanism [36]. However, our present findings in isolated nerve endings (synaptosomes) strongly suggest that protein *S*-nitrosylation potently and persistently inhibits neurotransmitter release primarily via inhibition of energetic metabolism. These observations may be of relevance to the pathophysiology of stroke and other neurodegenerative disorders that are associated with nitrosative stress.

Experimental procedures

Reagents

Spermine-nitric oxide complex (spermine NONOate), 6-(2-Hydroxy-1-methyl-2-nitrosohydrazino)-*N*-methyl-1-hexanamine (MAHMA NONOate), lactate dehydrogenase, pyruvate kinase, glyceraldehyde-3-phosphate, 2-phosphoglycerate, and ATP luciferin-luciferase assay kit, were purchased from Sigma-Aldrich (Saint Louis, MO). 3-Morpholinylsydnoneimine Cl (SIN-1) and DL-threo- β -benzyloxyaspartic acid (DL-TBOA) were from Tocris Bioscience (Ellisville, MO). Synthetic peroxynitrite (ONOO⁻) was purchased from EMD-Calbiochem (La Jolla, CA). Thiol-labeling reagent $N\alpha$ -(3-maleimidylpropionyl)biocytin was from Invitrogen (Carlsbad, CA). L-[³H]glutamate (51 Ci/mmol), was obtained from Amersham-GE Healthcare (Piscataway, NJ). ⁴⁵CaCl₂ (0.91 Ci/mmol) was purchased from PerkinElmer (Boston, MA). And all other salts and reagents were purchased from Sigma-Aldrich, and were of highest purity available.

Preparation of rat forebrain synaptosomes

Presynaptic nerve endings (synaptosomes) were isolated from forebrains of male Sprague-Dawley rats (150–200 g), according to the method of Hajos [37] with modifications as outlined below. All animal procedures were approved by the Institutional Animal Use and Care Committee. Animals were euthanized by rapid decapitation; whole brains were rapidly removed and transferred into ice-cold sucrose medium. Cerebellum and brain stem were dissected, and remaining tissue was homogenized in 0.32 M sucrose/5 mM HEPES (pH 7.4) using a Teflon-glass homogenizer. The homogenate was centrifuged for 10-min at 900g (2°C) to remove nuclei and cell debris. The first pellet was discarded and supernatant was further centrifuged for 20 min at 9,000 (2°C). The resulting pellet (P2) was resuspended in 0.32 M sucrose, layered over 0.8 M sucrose, and additionally centrifuged for 20 min at 9,000g (2°C). The myelin-containing layer at the 0.32–0.8 M sucrose interface was aspirated, 0.8 M-sucrose supernatant containing synaptosomes was removed, and the 0.8 M-sucrose pellet containing predominantly mitochondria was discarded. Synaptosomal suspension in 0.8 M sucrose was slowly diluted with an equal volume of the phosphate-buffered basal medium containing (in mM): 125 NaCl, 5 KCl, 1.2 MgSO₄, 1 CaCl₂, 2.5 NaH₂PO₄, 7.5 Na₂HPO₄, and 10 D-glucose (pH 7.4). The resulting suspension was centrifuged for 20 min at 9,000g (2°C). The final synaptosomal pellet was resuspended in the same phosphate-buffered basal solution and used in the subsequent experiments. In each experiment synaptosomal suspension (~1.5–2 mg protein/ml) was initially preincubated for 1 h at 37°C with constant agitation to restore normal metabolic status and transmembrane ion gradients.

Treatment with exogenous RNS donors

Synaptosomal suspensions were allowed to recover metabolically as described above and then were divided into several aliquots and treated for 30 min at 37°C in the dark with the NO donors, spermine NONOate (half-life at 37°C ~39 min), MAHMA NONOate (half-life at 37°C ~1.5 min), the peroxynitrite donor SIN-1 (half life at 37°C ~90–230 min), or the nitrosothiol compound *S*-nitroso-L-cysteine. All agents were added from freshly prepared

stock solutions to the final concentration of 100 μM , except for SIN-1, which was used at both 100 and 500 μM . 100 mM spermine NONOate and 100 mM MAHMA NONOate were prepared in 10 mM NaOH. 500 mM SIN-1 stock solution was prepared immediately before experiment in H_2O and stored on ice. 200 mM *S*-nitroso-L-cysteine was freshly synthesized for each experiment from L-cysteine and NaNO_2 under acidic conditions as previously described [34]. All stock solutions were diluted 1,000-fold and tested for potential changes in pH (none found). Vehicles were routinely added to the control samples. In our previous work [34] we performed control experiments with light-decomposed *S*-nitroso-L-cysteine and found no significant effects on vesicular neurotransmitter release up to the concentration of 1 mM.

After the initial 30-min treatment with NO donors, we used two different experimental designs for studying the functional impact of NO on synaptosomal functions. In the first type of experiments, after NO treatment, synaptosomes were pelleted by 2-min centrifugation at 10,000 g in an excess of basal phosphate medium, washed twice with 2 mL of HEPES-buffered Basal medium containing (in mM) 135 NaCl, 3.8 KCl, 1.2 MgSO_4 , 1.3 CaCl_2 , 1.2 KH_2PO_4 , 10 D-glucose, and 10 HEPES (pH 7.4), and used for the subsequent assays (see below). These experiments were conducted to reveal the lasting functional impact of NO after its removal. In the second type of experiments, synaptosomes were washed of extracellular NO donors as described above, and then were allowed to recover for one additional hour in the basal HEPES-buffered medium. The recovered synaptosomes were washed three additional times and then used for functional assays. This second experimental design was employed to study the reversibility of NO effects upon metabolic recovery. To study if functional recovery after the NO treatment can be accelerated, in some experiments we supplemented the recovery media with thiol-reducing reagents or metabolic substrates as indicated in the text and figure legends. The concentration of synaptosomal protein during all treatments was maintained between 0.9 and 1.4 mg/mL.

L-[^3H]Glutamate release assay

To measure vesicular and non-vesicular glutamate release we employed a radiotracer assay. Synaptosomes were loaded with L-[^3H]glutamate (10 $\mu\text{Ci}/\text{ml}$) at 37°C for 30 min with constant agitation. The high specific activity of [^3H]-tracer was critical to achieve significant labeling of the vesicular L-glutamate pool. To terminate loading and remove the extracellular isotope, 10 volumes of ice-cold sucrose stop solution (medium S) was added to the suspension, and synaptosomes were centrifuged for 20 min at 9,000g (2°C). The medium S contained (mM): 243 sucrose (isoosmotic replacement for 135 mM NaCl); 3.8 KCl, 1.2 MgSO_4 , 1.2 KH_2PO_4 , 10 HEPES, 10 D-Glucose (pH 7.4). The resulting pellets were stored on ice and resuspended in 8 mL ice-cold medium S immediately before neurotransmitter release measurements. This procedure prevents spontaneous synaptosome depolarization and neurotransmitter release during storage on ice [34].

To start efflux assay, 400- μL aliquots of L-[^3H]glutamate-loaded synaptosomes were dispensed into tubes, containing 4.5 ml of warm basal medium, or high K^+ , or Ca^{2+} -free high K^+ medium. In high K^+ medium $[\text{K}^+]_o$ was elevated to 43 mM (final $[\text{K}^+]_o=40$ mM) by equimolar replacement of Na^+ , leaving all other components the same as in basal medium. The composition of Ca^{2+} -free high K^+ medium was similar, except 1 mM CaCl_2 was replaced with 1 mM MgCl_2 and 50 μM EGTA was added. After 5 min incubation at 37°C , the glutamate efflux was terminated by a rapid vacuum filtration through the GF/B filters (Whatman-GE Healthcare, Florham Park, NJ). The filters were placed overnight into scintillation vials with 4 ml Ecoscint A scintillation cocktail (National Diagnostics, Atlanta, GA) and counted for radioactivity that remained in synaptosomes. Fractional release of L-[^3H]glutamate was calculated in relationship to total isotope content in synaptosomes at time 0. In control experiments we determined that at the beginning of the experiment $>85\%$ of L-

[³H]glutamate was contained inside synaptosomes and that nonspecific binding of L-[³H]glutamate to the filters was negligible (<1–2% of the measured values).

Measurements of intrasynaptosomal ATP content

Intrasynaptosomal levels of ATP were measured using commercially available luciferin-luciferase ATP assay kit (Sigma). Synaptosomes were treated with NO donors, and subjected to similar wash procedures as in the neurotransmitter release experiments. 100 µL of solution containing 100 mM perchloric acid plus 50 mM EDTA was added to 1-mL synaptosomal aliquots, and they were immediately boiled for 30 sec, followed by sedimentation of the denatured proteins (10,000 g at room temperature, 22°C). Supernatants were neutralized by adding 25 µL aliquots of 3 M KOH plus 1 M Tris. 25 µL of lysate samples or the freshly prepared ATP standards were added to 2 mL of luciferin-luciferase mix diluted 1:200 with the ATP assay dilution buffer (Sigma). ATP levels were quantified as light production in a TriCarb TR1900 scintillation counter (PerkinElmer, Waltham, MA), and compared to standards with known ATP content. ATP levels were then normalized to protein content in each sample as determined using standard bicinchoninic acid (BCA) assay kit and bovine serum albumin as a standard (Pierce-Thermo Scientific, Rockford, IL).

Chemiluminescence assay of intrasynaptosomal S-nitrosothiols

Total amount of protein and lipid-bound intrasynaptosomal NO and intrasynaptosomal RSNO levels were measured using triiodide-dependent reductive release of bound NO followed by detection of ozone-based chemiluminescence as described elsewhere [25,38]. Briefly, synaptosomal samples were washed with phosphate-buffered basal medium, sedimented at 10,000 g, and then lysed in 2 mL of 4 mM phosphate buffer (pH 7.5) containing 100 µM EDTA and 10 mM NEM. Samples were then pretreated in the dark with acidified sulfanilamide (final concentration 10 mM, 100 mM sulfanilamide stock prepared in 1 N HCl) for 15 min to remove nitrite with or without HgCl₂ (final concentration 4.9 mM) to evaluate for the presence of RSNOs. Hg²⁺ selectively decomposes RSNOs [25] such that RSNO concentrations may be determined from the difference in NO signal with or without pretreatment of paired samples with HgCl₂. 400 µL of each sample were injected in a purge vessel that contained 4.5 ml of glacial acetic acid and 500 µL of an aqueous mixture of 450 mM potassium iodide and 100 mM iodine. The vessel was kept at 70°C, and the solution was constantly purged with nitrogen and changed every four injections. The amounts of NO released from the purge vessel were quantified by gas phase chemiluminescence (NOA 280, Sievers Instruments, Boulder, CO). Peak integration was performed, and the results were converted to NO concentrations using authentic NO as a standard, and then normalized to the protein content in each sample.

Biotin-switch assay of intrasynaptosomal nitrosothiols

In order to visualize the extent of protein S-nitrosylation we employed a biotin-switch technique developed by Jaffrey et al. [23] with several modifications as described below. Two main changes were as following. (1) We used NEM (final concentration ~23 mM) instead of the originally proposed MMTS. This modification allowed for more consistent blocking at room temperature (22°C) and strongly reduced nonspecific background biotinylation of the masked free thiols. (2) We used a different biotinylating agent, Na-(3-maleimidylpropionyl)biocytin, that in our hands worked more consistently and produced lower background signal than the biotin-HPDP proposed in the original method. Synaptosomes were washed from NO donors three times with HEPES-buffered basal medium, and then incubated for 1 hour at 25°C in the dark in 1 mL of blocking solution, containing 1 volume of 0.25 M NEM, 9 volumes of HEN buffer pH 7.7, and 1 volume of 25% SDS. HEN buffer was composed of (mM): 250 HEPES, 1 EDTA, 0.1 neocuproine. To remove NEM, the proteins were precipitated by adding 2 mL of pre-chilled acetone for 15

min at -20°C , followed by sedimentation. After removing the acetone, the RSNOs were labeled for 1 hour at room temperature (22°C) in the dark with 0.5 mL of reducing/labeling solution with final concentration of 3 mM ascorbate and 1 mM $\text{N}\alpha$ -(3-maleimidylpropionyl)biocytin in HEN buffer, pH 7.0. The samples were desalted with acetone one more time, pelleted and resuspended in the Basal medium. Small aliquots were used for determination of protein concentration by a colorimetric BCA assay kit (Pierce-Thermo Scientific), the rest was diluted with 2x Laemmli reducing buffer (BioRad) and used for Western blot analysis. The proteins were resolved by SDS-polyacrylamide gel electrophoresis (10%) and transferred on to Immun-Blot PVDF membrane (BioRad). After blocking with 5% milk in phosphate-buffered saline buffer, containing 0.05% Tween 20 (PBST), the membrane was incubated with polyclonal anti-biotin antibody (1:50,000 dilution, Bethyl laboratories Inc, Montgomery, TX) for 1 hour at 25°C , followed by five washes for 5 min with 1% milk PBST. The membrane was incubated for 1 hr with secondary anti-rabbit horseradish peroxidase-conjugated antibodies (GE Healthcare/Amersham Biosciences, Piscataway, NJ; 1:20,000 dilution), followed by four PBST washes. Chemiluminescence was detected using ECLplus kit (GE Healthcare-Amersham Biosciences) and CL-Xposure film (Pierce).

GAPDH activity assay

The activity of GAPDH was measured by monitoring the enzymatic reduction of NAD^{+} to NADH in the presence of the GAPDH substrate, glyceraldehyde-3-phosphate (GA-3-P). Washed and pelleted synaptosomes were lysed in 0.5 mL of ice-cold lysis buffer (4 mM phosphate buffer, 500 μM EDTA, Roche protease inhibitor cocktail, pH 7.5) and homogenized using hypodermal syringe. The lysates were clarified by 10 min centrifugation at 10,000 g (2°C). 50 μL of clarified lysates were added to 950 μL of the GAPDH reaction mix containing (in mM): 100 glycine, 100 KH_2PO_4 , 5 EDTA, 1 NAD^{+} , and 1.5 GA-3-P (pH 8.9, adjusted with NaOH). NAD^{+} and GA-3-P were added to the mix immediately before the assay. The GAPDH reaction was initiated by addition of synaptosomal lysates and carried on for 5 min at 25°C . Enzymatic production of NADH was measured as increase in the optical density at 340 nm using a ELx800 plate reader (Bio-Tek Instruments, Winooski, VT) and calculated using the NADH molar extinction coefficient of $6,300\text{ cm}^{-1}\text{ M}^{-1}$. Total protein concentration in the samples was determined using BCA assay. Results were expressed as nmol NADH produced/mg total protein per 5 min.

Enolase activity assay

The neuron specific enolase (NSE) activity was assayed in a coupled enzymatic assay by monitoring the conversion of NADH to NAD^{+} resulting in a decrease of absorbance at 340 nm. The lysates were prepared in the same manner as for GAPDH assay (see above). 50 μL of clarified lysates was added to 950 μL of the NSE reaction mix containing (in mM): 100 HEPES, 25 MgSO_4 , 100 KCl, 0.2 NADH, 1.3 ADP, 2 2-phosphoglycerate, 10 U lactate dehydrogenase, 7 U pyruvate kinase, (pH 7.4, adjusted with NaOH). Reaction was carried on for 5 min at 25°C . Enzymatic production of NAD^{+} was measured as a decrease in the optical density at 340 nm using a ELx800 plate reader and calculated using the NADH molar extinction coefficient of $6,300\text{ cm}^{-1}\text{ M}^{-1}$. Total protein concentration in the samples was determined using a BCA assay. Results were expressed as μmol NADH consumed/mg total protein per 5 min.

Ca^{2+} uptake via the voltage-gated Ca^{2+} channels

Depolarization-induced Ca^{2+} uptake was measured using $^{45}\text{Ca}^{2+}$ as a radiotracer [39]. Synaptosomes were incubated with NO donors and subjected to the same procedures as in all other assays, and then pelleted in the excess of ice-cold sucrose stop solution S (for composition see above) and transferred onto ice. Before measurements the pellets were

resuspended in the same sucrose solution (protein concentration 3–4 mg/mL). 100- μ L aliquots of synaptosomes were injected into 900 μ L of warm basal HEPES-buffered medium or 80 $[K^+]_o$ media, additionally containing 0.5 μ Ci/mL of $^{45}\text{CaCl}_2$. Basal medium consisted of (mM) 135 NaCl, 5 KCl, 1.2 MgSO_4 , 0.1 CaCl_2 , 10 HEPES, 10 D-Glucose (pH 7.4). In high K^+ medium, $[K^+]_o$ was elevated to 88.9 mM (final concentration 80 mM) by equimolar replacement of Na^+ . The uptake reaction was carried for 2 min at 37°C, and was terminated by vacuum filtration through the GF/B paper (Whatman) followed by two washes with ice-cold washing buffer containing (in mM): 125 LiCl, 5 KCl, 10 MgSO_4 , 10 Tris (pH 7.4). The Ca^{2+} uptake values were corrected for the nonspecific adsorption on filters. The rates of $^{45}\text{Ca}^{2+}$ uptake were calculated as $V=A/(act)$ where A is radioactivity (cpm) of a sample containing c mg of protein, a is a specific radioactivity of $^{45}\text{Ca}^{2+}$ related to the total content of Ca^{2+} incubation medium (cpm/nmol), and t is the time of incubation.

Statistical analysis

All data are presented as mean values $\pm$ S.E. Statistical difference between experimental groups was calculated using Student's *t*-test or one-way analysis of variance with *a priori* Newman-Keuls test for multiple comparisons. Probability values of less than 0.05 were considered significantly different. Origin 6.0 (OriginLab, Northampton, MA) and GraphPad Prism 5.0 (GraphPad Software, San Diego, CA) were used to perform statistical analyses.

Results

Effects of nitrosative stress and N-ethylmaleimide on the Ca^{2+} -dependent (vesicular) neurotransmitter release from rat cortical synaptosomes

In order to measure presynaptic neurotransmitter release, we preloaded rat brain synaptosomes with radiolabeled L- $[^3\text{H}]$ glutamate and compared the rates of neurotransmitter release in basal media (5 mM KCl), and in the media containing 40 mM KCl with or without extracellular Ca^{2+} . High K^+ -induced depolarization strongly increased L- $[^3\text{H}]$ glutamate release in both Ca^{2+} containing and Ca^{2+} -free media (Fig. 1A). The Ca^{2+} -dependent component of the release represents vesicular neurotransmitter release, while Ca^{2+} -independent release is most likely due to the reversal of membrane glutamate transporters [40]. To verify the vesicular nature of Ca^{2+} -dependent release in our preparation, we performed two types of controls. We first treated synaptosomes with sulfhydryl-modifying reagent N-ethylmaleimide (NEM), which blocks exocytosis [41]. As seen in Fig. 1B, NEM suppressed the Ca^{2+} -dependent (vesicular) L- $[^3\text{H}]$ glutamate release in a dose-dependent fashion with a complete inhibition seen at ~ 100 μM , but was totally ineffective against Ca^{2+} -independent (transporter-mediated) neurotransmitter release. We next pretreated synaptosomes for 1 hr with 20 nM tetanus toxin, a bacterial toxin that enzymatically cleaves the vesicle-associated SNARE protein synaptobrevin and thereby prevents exocytosis [42]. Tetanus toxin inhibited the Ca^{2+} -dependent neurotransmitter release by ~ 55 –60% without any effect on the Ca^{2+} -independent component of the release (Fig. 1C). Incomplete inhibition of the Ca^{2+} -dependent L- $[^3\text{H}]$ glutamate release was likely due to partial cleavage of the intrasynaptosomal synaptobrevin. Taken together, these data validate the use of Ca^{2+} -dependent L- $[^3\text{H}]$ glutamate efflux as a measure of vesicular neurotransmitter release.

We next exposed synaptosomes to several NO-generating compounds during the whole 30-min duration of the L- $[^3\text{H}]$ glutamate loading. After 30-min incubation, NO donors were washed from the extracellular media together with extracellular isotope. This experimental design emphasized long-lasting rather than acute effects of NO treatment, since the short-lived NO donors completely decompose during incubation while the long-lived donors are removed during washes. The levels of L- $[^3\text{H}]$ glutamate loading were not significantly affected by treatments with “pure” NO donors and SIN-1 (loading ranged between 85–115%

of controls in various preparations), but were increased in a number experiments after treatment with *S*-nitroso-L-cysteine (range 100–160% of control values). Since L-[³H]glutamate release values were calculated as % of the total loading (see Methods), this allowed to negate any potential impact of variations in loading levels.

In order to explore if there is a difference in actions between different RNS, we compared the effects of the short-lived NO donor MAHMA NONOate (half-life ~1.5 min), the slower decomposing NO donor spermine NONOate (SpNO, half life ~39 min), the NO/ peroxyxynitrite generator SIN-1 (half life ~90–230 min), and the nitrosothiol *S*-nitroso-L-cysteine (CysNO). CysNO rapidly decomposes in solution with formation of NO, however, its major biological effects are thought to be mediated by transmembrane transport into the intracellular compartment followed by direct transnitrosylation of intracellular thiols [43,44]. Under our experimental conditions, MAHMA NONOate did not affect the neurotransmitter release measured 30 mins after NO donor addition, while more slowly decomposing spermine NONOate suppressed the Ca²⁺-dependent L-[³H]glutamate release by ~30% (Fig. 2A). The peroxyxynitrite donor SIN-1 was ineffective when tested at 100 or 500 μM concentrations (Fig. 2A). CysNO was the most effective blocker of the vesicular neurotransmitter release, causing ~90% inhibition (Fig. 2A). In striking contrast to the Ca²⁺-dependent component, the Ca²⁺-independent L-[³H]glutamate release was not significantly different among treatment groups (Fig. 2B). Therefore, in all the subsequent experiments we present only the data on the Ca²⁺-dependent (vesicular) L-[³H]glutamate release. Since NO and CysNO may act via modification of protein sulfhydryl moieties, we additionally performed treatment of synaptosomes with the sulfhydryl-modifying reagent NEM (100 μM). As in the preceding experiments, preincubation with NEM completely inhibited vesicular but not the transporter-mediated glutamate release (Fig. 2A, B).

Effect of NO donors on intrasynaptosomal ATP levels

To study if inhibition of neurotransmitter release is due to suppression of presynaptic metabolism, we measured intrasynaptosomal ATP levels using a luciferin-luciferase assay. Because in this assay control ATP levels varied among different synaptosomal preparations (in the range of 1.4–3.0 nmol ATP/mg synaptosomal protein), we normalized the data to the control ATP levels in each preparation. Similar to the results on inhibition of vesicular neurotransmitter release, we found that CysNO and NEM strongly reduced the intrasynaptosomal ATP content by ~70% and ~80%, respectively (Fig. 2C). SpNO caused smaller reduction of ATP levels (~40%), while MAHMA-NO was ineffective (Fig. 2C). The order of potency of NO/RNS donors in inhibiting vesicular L-[³H]glutamate release and ATP production was identical, suggesting that inhibition of neurotransmitter release is likely due to inhibition of presynaptic metabolism. Similarity between the effects of CysNO and NEM indicates that modification of thiol groups may be involved.

Since MAHMA NONOate was not effective we verified the predicted levels of NO produced by this compound using an NO-selective electrode. 100 μM MAHMA NONOate produced rapid and transient increase in [NO] with complete signal decline within 12 minutes. The peak NO signal was approximately 10-fold higher than the steady-state [NO] registered upon addition of 100 μM SpNO (n=3, Supplemental fig. 1A). Consistent with its decomposition kinetics MAHMA NONOate transiently decreased the intrasynaptosomal ATP levels by ~30% at 5 minutes, that was followed by near complete recovery within 30 minutes (n=2, Supplemental fig. 1B). The most likely explanation for this phenomenon is reversible inhibition of mitochondrial cytochrome oxidase and the ATP production, as it was previously found in various cell preparations [45].

Similar control experiments were performed for SIN-1. In solutions, SIN-1 produces NO and superoxide anion, which rapidly combine into ONOO⁻. Since ONOO⁻ is a potent

oxidant, the lack of SIN-1 effect was particularly surprising. In our phosphate-buffered medium, 500 μM SIN-1 produced low steady-state levels of NO that were approximately five-fold lower than those measured in the presence of 100 μM SpNO ($n=3$, Supplemental fig. 2). Simultaneous or delayed addition of superoxide dismutase (100 U/ml) increased NO production by ~two-fold indicating that substantial fraction of NO was indeed converted into ONOO⁻ (Supplemental Fig. 2). The slow rate of NO/ONOO⁻ production is consistent with a long SIN-1 half-life in solutions (90–230 min). However, SIN-1 decomposition may be drastically accelerated by some media components leading to much stronger effects observed at the same donor concentrations in other preparations (see for example [46,47]).

Effect of NO donors on intrasynaptosomal nitrosothiols

We next quantified total levels of NO that remained in the intrasynaptosomal compartment after removal of the extracellular NO donors, using a chemiluminescence-based assay. The intrasynaptosomal NO content was measured in samples with or without preincubation with HgCl₂, an agent that selectively decomposes RSNO [Feelisch, 2002 2151/id]. Therefore, the HgCl₂-sensitive NO content reflects levels of RSNOs [25,38]. In three independent synaptosomal preparations, we found no NO signal in the control samples (Fig. 3). Treatment with NO donors produced strong increases in total intrasynaptosomal bound NO with the following order of potency: CysNO>>SpNO>MAHMA-NONOate (Fig. 3). The NO signal was reduced by 70–90% after pre-treatment with HgCl₂, indicating that the majority of the intrasynaptosomal NO is present in the form of RSNOs (Fig. 3). Samples treated with 500 μM SIN-1 also showed trace amounts of RSNOs (~20 pmols NO/mg protein; compare to 275, and 3,580 pmols NO/mg protein for SpNO, and CysNO, respectively, Fig. 3B). This is likely due to the preferential ability of this compound to oxidize rather than S-nitrosylate thiol groups, but also due to apparent limited access of ONOO⁻ to the intrasynaptosomal compartment.

CysNO efficacy is determined by its transmembrane transport

As mentioned above, previous studies found that biological activity of CysNO and other low molecular weight nitrosothiols involves their transport across the plasma membrane followed by the direct transnitrosylation of thiol groups [43,44,48,49]. The sharp differences between the effects of CysNO and other NO donors on neurotransmitter release, metabolism, and RSNO levels prompted us to evaluate the involvement of several plasmalemmal transport systems in the CysNO activity. We used the competitive inhibitor of the amino acid transport system L, L-leucine, the competitive inhibitor of the alanine-serine-cysteine transporter system ASC, L-serine, and non-transportable inhibitor of the Na⁺-dependent glutamate transporters, TBOA. Each of these targeted transporters has been implicated in L-cysteine and/or S-nitroso-L-cysteine transport [43,50,51]. L-Serine and L-leucine significantly inhibited CysNO-mediated increase of intrasynaptosomal NO RSNOs by 26% and 35% respectively, while 100 μM TBOA produced no effect (Fig. 4A). The combination of all three agents was more effective than individual inhibitors and reduced the intrasynaptosomal NO by 44% (Fig. 4A). Incomplete inhibition of S-nitrosylation by combination of transport inhibitors suggests that a fraction of CysNO may be transported into synaptosomes via alternative pathway(s), such as γ -glutamyl transpeptidase [52]. In parallel ATP assays, we tested how inhibitors of cysteine/CysNO-transporting systems protect against CysNO-induced metabolic inhibition. As in the preceding experiments, 100 μM CysNO caused strong, 77% inhibition of ATP production (Fig. 4B). Treatment with L-leucine increased intracellular ATP levels from 23% to 68% of the control values, while the combination of all three inhibitors additionally recovered [ATP]_i to 85%; this latter value was not statistically different from the vehicle-treated controls (Fig. 4B). TBOA was completely ineffective, L-serine showed a trend for preserving [ATP]_i levels but its effect was not statistically significant (Fig. 4B). Individual transporter inhibitors or their

combination did not affect ATP levels in controls samples not treated with CysNO (n=3, data not shown). Overall, RSNO and ATP assays indicate that CysNO effects require its transport across the plasma membrane, and that the substantial fraction of the transport involves the amino acid system L.

Sustained effects of CysNO on the vesicular neurotransmitter release and intrasynaptosomal ATP levels

Next, we tested if inhibition of the synaptic neurotransmitter release by NO and CysNO is persistent, similar to the sustained suppression of presynaptic activity in the post-ischemic brain [16,17]. Synaptosomes were treated with NO donors for 30 minutes as in preceding experiments, washed from NO donors, and then allowed to recover for an additional one hour at 37°C. Under these experimental conditions, we found that vesicular L-[³H]glutamate release remained unaffected after the SIN-1 treatment, largely recovered in synaptosomes treated with SpNO, but stayed strongly suppressed (~50% inhibition) after treatment with CysNO (Fig. 5A). Inhibition of vesicular neurotransmitter release closely matched sustained changes in the intrasynaptosomal ATP levels: among NO donors only the CysNO treatment produced strong and sustained suppression of ATP production (~60%), while the effect of SpNO was much weaker albeit statistically significant (Fig. 5B). This inhibition may be due to the long-lasting modification of protein sulfhydryl groups since treatment with the sulfhydryl-modifying reagent NEM produced essentially irreversible effects on the vesicular L-[³H]glutamate release (>90% acutely, >80% after metabolic recovery; Fig. 5A, compare to the data in Fig. 2A) and ATP levels (~80%, both acutely and after metabolic recovery; Fig. 5B, compare to the data in Fig. 2C). To further verify that transmembrane transport of CysNO is responsible for its long-term effects, we added 200 µM L-cysteine during initial incubation with SpNO and CysNO. L-Cysteine significantly increased the potency of SpNO and showed a trend for increasing the potency of CysNO (Fig. 5C), consistent with the idea of extracellular formation and transmembrane transport of additional CysNO molecules [43].

Long-term suppression on neurotransmitter release by CysNO is not due to inhibition of the voltage-gated Ca²⁺ channels

Although decreased ATP levels seemed sufficient to explain CysNO-induced inhibition of neurotransmitter release, several studies found that NO may block voltage-gated Ca²⁺-channels [53,54], and thereby suppress Ca²⁺-dependent neurotransmitter release. In order to test this possibility, we measured depolarization-induced Ca²⁺ (⁴⁵Ca²⁺) uptake in control and CysNO-treated synaptosomes. We found no evidence for the long-term suppression of the presynaptic Ca²⁺ channels by CysNO under the same treatment conditions when neurotransmitter release was strongly suppressed (Supplemental Fig. 3).

Sustained S-nitrosylation of intrasynaptosomal proteins by CysNO

Consistent with the idea that the effects of CysNO are due to long-term protein nitrosylation, we found detectable levels of RSNOs (HgCl₂-sensitive component of the total bound NO) in the SpNO-treated synaptosomes, and much larger nitrosylation in the CysNO treated synaptosomes that was preserved even 1 hr after NO donor removal and metabolic recovery (Fig. 6A). Since the RSNO “signal” may originate both from low molecular weight nitrosothiols (such as S-nitroso-glutathione) and nitrosylated proteins, in parallel experiments we visualized protein S-nitrosylation using a biotin-switch assay. As in the chemiluminescence data, the levels of RSNOs were increased significantly in CysNO-treated synaptosomes, and to a lesser degree in the SpNO-treated synaptosomes (Fig. 6B).

Effects of N-acetylcysteine and dithiothreitol on protein nitrosylation and recovery of vesicular neurotransmitter release and intrasynaptosomal ATP levels

In order to determine if CysNO-mediated effects are reversible, we subsequently tested two membrane-permeable reducing agents, *N*-acetylcysteine (NAC) and dithiothreitol (DTT), for their ability to reverse the effects of protein nitrosylation. Both agents were not present during exposure to NO donors, and were added during the one-hour recovery period only. With this experimental design, 5 mM NAC and 1 mM DTT strongly decreased the levels of nitrosothiols in CysNO-treated synaptosomes as measured by a chemiluminescence assay (Fig. 6A). In the biotin-switch assays, we found qualitatively similar effects seen as decreases in S-nitrosylation of various proteins. Interestingly, it appeared that, in the presence of sulfhydryl-reducing agents, protein RSNOs decompose at different rates. Thus, one immunopositive band of ~45–47 kD remained strongly biotinylated (S-nitrosylated) even after 1-hr treatment with 1 mM DTT, which reduced the total amount of RSNOs by >85% (Fig. 6B). These findings are consistent with the recent reports on substantial differences in half-life of individual endogenously S-nitrosylated proteins [55,56]. Because NAC was less effective in reducing nitrosothiol levels (Fig. 6A) and did not restore ATP levels (data not shown), in the subsequent experiments we employed DTT only. 0.3 and 1 mM DTT showed a trend for modest recovery of the Ca²⁺-dependent L-[³H]glutamate release from CysNO-treated synaptosomes, but this effect was not statistically significant (Fig. 7A). Likewise, 0.1–1 mM DTT did not protect against CysNO-induced suppression of ATP production, although there was a trend for dose-dependent metabolic recovery (Fig. 7B). Thus, the reversal of bulk protein S-nitrosylation does not allow for effective metabolic recovery and restoration of neurotransmitter release. At this stage it is difficult to determine if the persistent effects of CysNO are due to poorly reversible S-nitrosylation or other types of modifications (such as oxidation or S-glutathionylation) of the intrasynaptosomal enzymes.

Treatment with pyruvate leads to recovery of the vesicular neurotransmitter release and ATP levels in CysNO-treated synaptosomes

In the next series of experiments, we attempted to identify which part of synaptosomal energetic metabolism is affected by the treatment with CysNO. With this purpose, we supplemented the recovery media with pyruvate, the end product of glycolysis and a precursor substrate for the mitochondrial Krebs cycle. After one hour recovery in the presence of 0.5–5 mM pyruvate, intrasynaptosomal ATP levels were significantly but not completely restored (Fig. 8A). We selected 2 mM pyruvate, the most effective concentration, for further testing in neurotransmitter release experiments. At this concentration, pyruvate restored Ca²⁺-dependent L-[³H]glutamate release by more than 50% (Fig. 8B). Thus, CysNO-mediated long-term inhibition of neurotransmitter release in synaptosomes may be reversed by bypassing glycolysis. Under control conditions >80% of synaptosomal ATP is derived from oxidative phosphorylation, however, the mitochondrial/glycolytic ATP ratio may be strongly altered by depolarization and other treatments [57,58]. In our control experiments, replacement of D-glucose with D-deoxyglucose completely inhibited vesicular neurotransmitter release but this effect was completely reversed by adding 5 mM pyruvate, indicating that in intact synaptosomes ATP levels can be sustained by oxidative phosphorylation without glycolysis (Supplemental fig. 4). In D-glucose-containing media, when mitochondrial metabolism was suppressed with the complex I inhibitor rotenone (10 μM), vesicular glutamate release was reduced by ~50% (Supplemental fig 4). Thus, glycolysis can only partially sustain vesicular neurotransmitter release and ATP production.

Effects of CysNO, Pyruvate and DTT on the activity of GAPDH and enolase

GAPDH is a crucial glycolytic enzyme that is inhibited by NO via S-nitrosylation or covalent linkage of the NAD moiety [59,60]. Therefore, we assessed how DTT or pyruvate affect GAPDH activity in synaptosomes after incubation with CysNO. Synaptosomes were treated in the same fashion as in the preceding experiments including one-hr recovery. Pyruvate and DTT were present only in the recovery media. GAPDH activity was measured in synaptosomal lysates by monitoring the reduction of NAD⁺ to NADH in the presence of the GAPDH substrate, glyceraldehyde-3-phosphate. As expected, the CysNO treatment blocked GAPDH activity by ~80%, and this effect persisted after one-hr metabolic recovery (Fig. 9A). As expected, the inhibition was not reversed by post-CysNO treatment with pyruvate (Fig. 9A). However, treatment with 1 mM DTT completely reversed the CysNO effect and even increased GAPDH activity to the levels higher than in control samples. Additionally, DTT strongly elevated GAPDH activity on its own (Fig. 9A). This latter finding is consistent with the literature report on a partially oxidized state of GAPDH even in the absence of oxidative/nitrosylative stress [61]. However, in contrast to restoration of GAPDH activity, DTT did not reverse the CysNO-induced suppression of neurotransmitter release and ATP synthesis (see Fig. 7). Thus, the reduction of SH groups in the GAPDH is not sufficient for restoration of cellular functions in CysNO-treated synaptosomes and additional glycolytic enzyme(s) is (are) likely irreversibly blocked. Recently, it has been found that neuron-specific enolase is one of the prominently nitrosylated enzymes in an animal model of multiple sclerosis [31]. Therefore, we measured enolase activity in the lysates of CysNO-, DTT-, and pyruvate-treated synaptosomes. Unlike GAPDH activity in the same samples, the enolase activity was unchanged regardless of type of treatment (Fig. 9B).

Discussion

It is well established that NO and RNS originating from nNOS and iNOS participate in the acute and delayed forms of brain damage in stroke and other neuropathologies [5,7,13,62]. In this study we explored molecular mechanisms that may link nitrosative stress to the long-term inhibition of synaptic communication. Such inhibition has been found in animal models of stroke [16–18] but its mechanisms remain poorly understood. We established that in synaptosomal preparation NO donors potently suppress neurotransmitter release via inhibition of energetic metabolism, with the order of potency correlating with S-nitrosylation of intrasynaptosomal proteins. In contrast, the total amount of released NO was not related to the inhibition of synaptic metabolism. Global changes in ATP levels did not allow us to test our original hypothesis on nitrosative inhibition of the NSF protein, the activity of which is sustained by ATP hydrolysis [36]. Overall, our findings are in agreement with early observations by Brorson *et al.* who have found long-term inhibition of energetic metabolism by authentic NO and NO donors in cultured hippocampal neurons [63]. However, in synaptosomes, energetic metabolism and neurotransmitter release appear to be more sensitive to inhibition of glycolysis than in intact neuronal preparations, and could be rescued by addition of the end product of glycolysis, pyruvate.

Protein S-nitrosylation causes long-term inhibition of vesicular neurotransmitter release

The major finding of our study is that S-nitrosylation of intrasynaptosomal proteins produces potent inhibition of the vesicular neurotransmitter release that persisted after wash out of extracellular RNS donors and additional metabolic recovery. The efficacy of RNS-generating compounds was not related to the total amount of released NO or the kinetics of NO donor decomposition. Instead, we found a very close correlation between the inhibitory potency of individual RNS-generating compounds, the decreased levels of intrasynaptosomal ATP, and the increased levels of intrasynaptosomal RSNOs. NO itself

possesses very limited reactivity towards SH-groups as compared to other NO metabolites such as NO_2 , N_2O_3 , RSNOs or peroxynitrite, all of which oxidize and/or nitrosylate thiols much more readily than NO itself [38,64–66]. This may explain weak potency of the “pure” NO donors, spermine NONOate and MAHMA NONOate in our experiments.

S-nitroso-L-cysteine was the most effective among tested compounds. Our analysis of bound intrasynaptosomal NO indicated that among possible modifications more than 85% of the CysNO-mediated increase in the intrasynaptosomal NO was represented by RSNOs. Thus, protein S-nitrosylation, or related RSNO-driven modifications, such as S-glutathionylation, produce a long term impact on presynaptic metabolism and ATP-dependent vesicular neurotransmitter release. Persistent ATP depletion was not related to the reversible inhibition of mitochondrial cytochrome oxidase by NO [67,68], which in our experiments has only been seen during acute treatment with NO donors. Instead, inhibition of the ATP production involved more complex mechanisms.

Consistent with previous studies [43,44,48,69], the high efficacy of CysNO was related to its transmembrane transport, that was likely followed by direct protein transnitrosylation. Similar to the results obtained in non-neuronal preparations (see for example [43]), extracellularly added L-cysteine increased the potency of the “pure” NO donor SpNO, likely by producing extracellular CysNO. Previous pharmacological analysis and siRNA knockdown studies established an essential role for the amino acid transport system L for CysNO uptake and its biological activity in several cell types [44,48]. In our experiments, L-leucine, a competitive inhibitor of the amino acid transport system L, strongly suppressed formation of the intrasynaptosomal thiols. Additional effect of L-serine, which blocks the amino acid transport system ASC, indicates that the latter system may also contribute to CysNO transfer into synaptosomes. When both the L and the ASC transporter inhibitors were combined, intracellular RSNO formation was only partially prevented, suggesting that other transport mechanisms may additionally contribute. Based on the pharmacological evidence, it has been proposed that γ -glutamyl transpeptidase may serve as an alternative route for transmembrane S-nitrosothiol transfer [52].

Mechanisms and reversibility of nitrosative inhibition of energetic metabolism

Although the effects of SpNO and CysNO on synaptic function were long-lasting, we did observe slow spontaneous recovery of neurotransmitter release and ATP levels after removal of RNS donors. We attempted to hasten the recovery from nitrosative stress by including in the recovery media the thiol-reducing agent DTT or the membrane-permeable glutathione precursor *N*-acetylcysteine. While both agents strongly accelerated RSNO decomposition, seen as reduction of the total RSNO signal and decreased biotinylation of individual protein bands in the biotin-switch assays, they were paradoxically ineffective in restoring ATP levels or neurotransmitter release. One potential explanation is relative resistance of some CysNO-modified thiol groups to the reducing agents. Consistent with this idea, we observed the DTT-resistant nitrosylation of an unidentified protein band with an apparent molecular weight of ~45–48 kD. In the brain, many enzymes belonging to the glycolytic pathway and mitochondrial electron transfer chain may be modified and inhibited by nitrosative stress [7,68]. Paige *et al.* identified in brain cytosolic lysates ten intracellular proteins, the S-nitrosylation of which was highly resistant to reducing reagents; these included two metabolic enzymes—GAPDH and pyruvate kinase [56]. In our experiments, supplementation of the recovery media with the end product of glycolysis pyruvate strongly recovered both ATP levels and neurotransmitter release, pointing to the important role of glycolysis in the observed metabolic inhibition. The glycolytic enzyme GAPDH is the known intracellular target for nitrosative inhibition is [55,59,70]. NO and RSNOs may reversibly inhibit GAPDH via S-nitrosylation or irreversibly block its activity via covalent NAD attachment [59,60,71,72]. In our study, strong and persistent reduction of GAPDH

activity was reversed after treatment with DTT. This finding likely points to *S*-nitrosylation, but inhibition via *S*-glutathionylation has also been reported in both cellular and cell-free assay systems [73]. Strong suppression of the GAPDH activity seen in our experiments is quantitatively important for the CysNO-mediated inhibition of synaptosomal ATP production. However, since activity of the GAPDH was restored by the thiol-reducing agents without concurrent recovery of ATP levels, our findings suggest that activity of (an) additional unidentified glycolytic enzyme(s) should be persistently suppressed in a DTT-resistant manner. Neuron-specific enolase was recently found to be prominently nitrosylated *in vivo* in an animal model of multiple sclerosis [31] but its activity was not affected in the CysNO-treated samples in our experiments.

Since the effect of pyruvate on synaptosomal ATP levels was incomplete, this finding points to impaired mitochondrial function. Previously, Erecinska *et al.* showed that CysNO strongly inhibits mitochondrial O₂ consumption and decreases synaptosomal ATP levels with additional moderate inhibition of glycolysis [74], but >25, 80, and 70% decreases in the activities of mitochondrial complexes I, III, and IV, respectively, had to be reached before any changes in synaptosomal ATP production were observed [75]. In mitochondria, complexes I, II, and IV and the tricarboxylic acid cycle enzyme aconitase are potently inhibited by RNS, particularly by peroxynitrite (reviewed in [68]). Modification via *S*-nitrosylation strongly suppresses the activity of complex I [76,77] and inhibits aconitase [78].

Pathological and translational relevance of the present findings

Although chronic inhibition of synaptic transmission has been reported in both focal and global animal ischemia models, its mechanisms remain poorly understood. The defects in synaptic transmission may last for several weeks, and are seen in spite of completely recovered membrane potentials and metabolism in neuronal bodies [17,79]. It is plausible that because of the length of neuronal projections, the “remote” metabolic and functional properties, particularly at the presynaptic sites, are disproportionately affected in the post-ischemic tissue. Under this scenario, local nitrosative and oxidative inhibition of metabolic enzymes and disruption of mitochondrial function in synaptic endings (and dendrites) would require *de novo* protein synthesis and long-distance axonal delivery of mitochondria. One recent study found chronic degeneration of neuronal processes and disruption of synaptic communication in the CA1 hippocampal region 10 weeks after brief global ischemia [20]. Importantly, in this latter work a combinatorial pharmacological treatment that afforded nearly complete protection of neuronal bodies did not preserve neuronal projections and synaptic function.

Our study proposes one potential mechanism responsible for functional and metabolic deficits at the presynaptic sites. Presented here *in vitro* results have to be considered and interpreted with caution. Our model offers very limited representation of the multifaceted processes in the ischemic brain and does not reflect the complete spectrum of pathologically produced ROS and RNS. Although in our work and in cultured neurons [63] the peroxynitrite donor SIN-1 showed very low potency, this may be due to low steady-state ONOO⁻ levels and limited permeability of neuronal membranes to ONOO⁻, perhaps due to the low expression of the anion exchanger that is necessary for peroxynitrite transport [80]. Another important question that requires additional work is whether pathological levels of nitrosylation reach those attained in our experiments. As mentioned in the introduction, measuring the RSNO levels *in vivo* is a challenging task. Nevertheless, numerous brain proteins are nitrosylated even under physiological conditions by the nNOS-derived RNS [23], and strong increase in protein nitrosylation has been identified in the CNS in various animal models of neurodegenerative diseases and in human patients [27–29,31]. The associated *in vivo* chemistry and the identity of *S*-nitrosylating species are still being

debated. However, decreased O₂ levels, as expected in the ischemic tissue, seem to enhance the RSNO formation [38,81].

In spite of all the above mentioned limitations our work provides some important clues for developing novel therapeutic approaches for preservation and restoration of the synaptic function in various neuropathologies that are associated with nitrosative stress. Application of pyruvate or its derivatives may allow bypassing the persistently inhibited steps in the glycolytic pathway. As seen in our experiments, if mitochondrial function is partially preserved, pyruvate, when added *after nitrosative stress*, restores intrasynaptosomal ATP levels and synaptic neurotransmitter release. Incidentally, pyruvate and its derivative ethyl pyruvate, which is more stable in solutions, have been already tested in animal ischemia models. Systemic delivery of pyruvate or ethyl pyruvate affords potent neuroprotection in rodent models of global and focal ischemia with extended therapeutic window [82–84], as well as against ischemia-reperfusion injury in several other tissues (reviewed in [85]). It remains to be tested if pyruvate may effectively protect against loss of synaptic activity or be used for restoration of synaptic communication in the post-ischemic brain *in vivo*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

BCA	bicinchoninic acid
CysNO	S-Nitroso-L-cysteine
DTT	dithiothreitol
GA-3-P	glyceraldehyde-3-phosphate
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
NAC	N-acetylcysteine
NEM	N-ethylmaleimide
NO	nitric oxide
NSF	N-ethylmaleimide-sensitive fusion factor
RNS	reactive nitrogen species
RSNO(s)	nitrosothiol(s)
SpNO	spermine NONOate
TBOA	DL-threo-β-benzyloxyaspartic acid

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Fig. 1. Depolarization of rat brain synaptosomes in high K⁺ media triggers vesicular and non-vesicular L-[³H]glutamate release

A, Kinetics of L-[³H]glutamate release in Basal medium and 40 mM K⁺ media in the presence or absence of extracellular Ca²⁺. Ca²⁺-free medium contained 50 μM EGTA. Reaction of L-[³H]glutamate release was terminated by vacuum filtration and L-[³H]glutamate remaining in synaptosomes was determined as described in Materials and methods. Data are the mean values of two independent measurements. Representative of 3 experiments.

B, Effects of N-ethylmaleimide on Ca²⁺-dependent and Ca²⁺-independent neurotransmitter release. Synaptosomes were preloaded with L-[³H]glutamate for 30 minutes, washed from the extracellular isotope and then exposed to 40 mM [K⁺]_o media in the presence or absence of extracellular [Ca²⁺]. NEM was added to the loading media only. The data represent the mean values ±SEM of Ca²⁺-dependent and Ca²⁺-independent components of L-[³H]glutamate release in 4 independent experiments. **p<0.001, ***p<0.001, vs control.

C, Effect of 20 nM tetanus toxin on the Ca²⁺-dependent and Ca²⁺-independent L-[³H]glutamate release. The data represent mean ±SEM in 3 independent experiments. **p<0.001, vs. control.

Fig. 2. The effects of NO donors on vesicular and non-vesicular neurotransmitter release and intrasynaptosomal ATP levels

A, The effects of NO donors MAHMA NONOate (MAHMA, 100 μ M), spermine NONOate (SpNO, 100 μ M), *S*-nitroso-L-cysteine (CysNO, 100 μ M), 3-morpholindolimine (SIN-1, 500 μ M) and the sulfhydryl-modifying agent *N*-ethylmaleimide (NEM, 100 μ M) on the Ca²⁺-dependent (vesicular) L-[³H]glutamate release. Synaptosomes were treated with all agents for 30 min. Neurotransmitter release was measured after wash out of NO donors and NEM. n=6; ***p<0.001, vs. control.

B, The effects of NO donors on the Ca²⁺-independent (non-vesicular) L-[³H]glutamate release. These values were obtained in the same experiments as in A. n=6.

C, The effects of NO donors on the intrasynaptosomal ATP levels measured using a luciferin-luciferase assay. Design of these experiments was similar to that shown in A and B. n=10. **p<0.01, ***p<0.001, vs control.

Fig. 3. Determination of the total bound NO and S-nitrosothiols retained in synaptosomes after NO donor decomposition and wash out

A, Representative chemiluminescence signals after injection of synaptosomal lysates into NO analyzer. Synaptosomes were pretreated with NO donors, SpNO, MAHMA NONOate, and CysNO (all at 100 μM) for 30 min. Extracellular NO donors were washed out, and synaptosomes were lysed. Lysates were separated into two aliquots and additionally incubated with sulfanilamide with or without HgCl_2 . Under these experimental conditions HgCl_2 selectively decomposes RSNOs.

B, Quantification of the total and mercury-resistant NO content in synaptosomes treated with NO donors. The difference between total and mercury-sensitive component represents RSNOs. No NO levels were detected in control samples. $n=3$.

Fig. 4. Intracellular S-nitrosylation and metabolic depletion by CysNO require its transmembrane transport

A, Effects of the inhibitors of glutamate (and cysteine) transporters TBOA (100 μM), the amino acid transport system ASC, L-serine (SER, 1 mM), and the amino acid transport system L, L-leucine (LEU, 8 mM), on the total and mercury-resistant NO content in synaptosomes. Synaptosomes were pretreated with 100 μM CysNO for 30 min in the presence or absence of the indicated inhibitor concentrations. After NO donor wash out, synaptosomes were lysed and the NO content was determined by a chemiluminescence assay. The difference between total and $HgCl_2$ -sensitive signal represents RSNOs. $n=4$. ** $p < 0.01$, *** $p < 0.001$, vs. CysNO.

B, Inhibition of transmembrane CysNO transport protects synaptosomes from CysNO-induced metabolic depletion. Treatment conditions were identical to those presented in A. n=4. **p<0.01, ***p<0.001, vs. control. ###p<0.001, vs. CysNO.

Fig. 5. Inhibitory effects of CysNO are retained after 1-hr metabolic recovery

A, The effects of 100 μM SpNO, 100 μM CysNO, 500 μM SIN-1, and 100 μM NEM on the Ca^{2+} -dependent (vesicular) L-[³H]glutamate release. Synaptosomes were pretreated with NO donors and NEM for 30 min, washed from extracellular donors, and then allowed to recover for additional 1-hr in basal medium containing 10 mM D-glucose. n=6. ***p<0.001, vs. control.

B, The effects of NO donors and NEM on the intrasynaptosomal ATP levels after 1-hr metabolic recovery. Concentrations of all agents were the same as in A. n=10–12. **p<0.01, ***p<0.001, vs. control.

C, The effects of SpNO and CysNO on intrasynaptosomal [ATP] are enhanced in the presence of L-cysteine. Cells were treated with SpNO and CysNO as indicated in (A) and (B) but 200 μM L-cysteine was additionally added where indicated. n=5; ***p<0.001 vs. control; #p<0.05 vs. SpNO without L-cysteine.

Fig. 6. Persistent protein S-nitrosylation by CysNO measured after 1-hr recovery

A, Total intrasynaptosomal NO and the HgCl₂-sensitive NO content measured after 30-min treatment with 100 μ M CysNO or 100 μ M SpNO, followed by 1-hr recovery. In some samples after NO donor wash out, the recovery media were supplemented with 5 mM *N*-acetylcysteine (NAC) or 1 mM dithiothreitol (DTT). $n=3$. *** $p<0.001$, vs control.

B, Representative Western blot showing visualization of protein S-nitrosylation using a biotin switch assay (see Methods for detail). The upper panel shows anti-biotin (RSNO) signal. In the lower panel, the membrane was stripped and re-probed with anti-actin antibody to verify equal protein loads. Numbers on the right represent positions of molecular weight markers.

Fig. 7. Acceleration of RSNO decomposition with DTT does not rescue neurotransmitter release and energetic metabolism in the CysNO-treated synaptosomes

A, The effects of 0.3 and 1 mM DTT on Ca^{2+} -dependent (vesicular) L-[3 H]glutamate release measured following 30-min treatment with 100 μ M CysNO and 1-hr recovery. The last bar on the right shows the effect of 1 mM DTT on neurotransmitter release in synaptosomes that were not treated with CysNO. $n=5-7$. ** $p<0.01$, *** $p<0.001$, vs control. **B,** The effects of 0.1–1 mM DTT on the intrasynaptosomal ATP levels. Experimental design was similar to that in A. The last bar on the right shows the effect of 1 mM DTT on ATP levels in synaptosomes that were not treated with CysNO. $n=4-5$. *** $p<0.001$, vs control.

Fig. 8. Post-treatment with pyruvate partially rescues neurotransmitter release and ATP production in the CysNO-treated synaptosomes

A. The effect of 0.5–5 mM pyruvate on the intrasynaptosomal ATP levels measured following 30-min treatment with 100 μ M CysNO and 1-hr recovery. The last bar on the right shows the effect of 5 mM pyruvate in synaptosomes that were not treated with CysNO. $n=5$. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, vs control. ## $p<0.01$, ### $p<0.001$ vs. CysNO.

B. The effects of 2 mM pyruvate on Ca^{2+} -dependent (vesicular) L-[3 H]glutamate release measured following 30-min treatment with 100 μ M CysNO and 1-hr recovery. The last bar on the right shows the effect of 2 mM pyruvate on neurotransmitter release in synaptosomes that were not treated with CysNO. $n=6-7$. * $p<0.05$, *** $p<0.001$, vs control. ## $p<0.01$, vs. CysNO.

Fig. 9. Treatment with CysNO causes long-lasting inhibition of synaptosomal GAPDH activity but does not affect the enolase activity

A, Measurements of GAPDH activity. Synaptosomes were treated for 30 min with 100 μ M CysNO and then allowed to recover for additional 1 hr in the basal medium containing 10 mM D-glucose, or 10 mM glucose +2 mM pyruvate. GAPDH activity was measured in synaptosomal lysates as described in Methods. $n=4$. * $p<0.05$, *** $p<0.001$, vs control. ### $p<0.001$, vs. CysNO.

B, Measurements of enolase activity. Experiments conditions were identical to those presented in A. Enolase activity was measured in lysates as described in Methods. $n=3$.