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Redox modulation of the NMDA receptor

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Abstract. Redox modulation has been recognized to be an important mechanism of regulation for the *N*-methyl-D-aspartate (NMDA) receptor. Sulfhydryl reducing agents enhance, whereas oxidizing agents decrease, NMDA-evoked currents. Multiple cysteine residues located in different NMDA receptor subunits have been identified as molecular determinants underlying redox modulation. The NMDA receptor is also regulated by nitric oxide (NO)-related species directly, not involving cyclic GMP, but the molecular mechanism of this action has heretofore not been entirely clear. The confusion arose at least partly due to the fact that various redox forms of NO (NO^+, $NO^\bullet$, NO^-, each having an additional electron compared with the previous) have distinct mechanisms of action. Recently, a critical cysteine residue (Cys 399) on the NR2A subunit has been shown to react under physiological conditions with NO by S-nitrosylation (transfer of the NO^+ to cysteine thiol) or by reaction with NO^- (nitroxyl anion) to underlie this form of modulation.

Key words. NMDA receptor; redox; DTT; nitric oxide; S-nitrosylation; cysteine.

Introduction

Redox modulation by covalent modification of sulfhydryl (thiol) groups on protein cysteine residues can regulate protein function. If they possess a sufficient redox potential, oxidizing agents can react to form adducts on single sulfhydryl groups or, if two free sulfhydryl groups are vicinal (in a close proximity), disulfide bonds may possibly be formed. Reducing agents can regenerate free sulfhydryl (–SH) groups by donating electron(s). About 10 years ago, our group discovered that NMDA receptor activity can be modulated by sulfhydryl reducing and oxidizing agents [1]. In this review, we will discuss the progress made in the characterization of redox modulation of the NMDA receptor by our group and colleagues. Particular attention will be given to recent studies on molecular localization of the redox site and modulation of the NMDA receptor by NO-related species.

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Redox modulation of the NMDA receptor by sulfhydryl redox agents in primary neuronal cultures

Sulfhydryl reducing agents, such as dithiothreitol (DTT), enhance NMDA-evoked currents, whereas oxidizing agents, such as 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB) decrease these currents in cerebrocortical and retinal ganglion cell cultures [1]. The observation that both reducing and oxidizing agents have an effect on NMDA-evoked currents suggests that the redox modulatory sites of the NMDA receptor exist in an equilibrium between fully reduced and fully oxidized states. Yet, in general, our experiments on neurons not previously exposed to redox agents revealed that the magnitude of potentiation by the reducing agent, DTT, was severalfold greater than the magnitude of inhibition by the oxidizing agent, DTNB. This finding suggested that the native redox state of NMDA receptors, at least in our culture system, is closer to the fully oxidized than the fully reduced state. Following reduction with DTT, alkylating agents such as *N*-ethylmaleimide (NEM) can block further effects of redox agents on the NMDA receptor, supporting the existence of critical sulfhydryl

groups on the receptor that mediate these effects. This finding is easily explained by the fact that agents such as NEM, if accessible to free thiol groups, irreversibly alkylate them and prevent further interaction between redox agents and these sites. Several endogenous redox agents, such as oxygen-free radicals [2, 3], oxidized glutathione (GSSG) [4, 5], pyrroloquinoline quinone (PQQ) [6] and lipoic acid [7] all have been shown to interact with the redox site(s) on the NMDA receptor.

Redox modulation of recombinant NMDA receptors

The cloning of complementary DNAs (cDNAs) encoding NMDA receptor subunits [8–12] affords the opportunity to investigate the molecular basis for redox modulation of recombinant NMDA receptors. Different subunit combinations display differences with respect to redox modulation by sulfhydryl reducing and oxidizing agents [13, 14]. Kohr et al. using recombinant NMDA receptors expressed in human embryonic kidney (HEK 293) cells obtained the following results. The potentiation of NR1/NR2A receptors by DTT consisted of both a rapid component, which reverses upon washout, and a slow component which is persistent upon washout and can only be reversed with oxidizing agents. Redox modulation of NR1/NR2B, NR1/NR2C and NR1/NR2D receptors, however, consisted only of a slow/persistent component. From studies of chimeric recombinant receptor subunits, the N-terminal 370 amino acid residues of NR2A was shown to mediate the rapid/reversible component [13]. Meanwhile, using site-directed mutagenesis and the *Xenopus* oocyte expression system, our laboratory in collaboration with that of S. F. Heinemann found that two cysteine residues in the NR1 subunit (Cys 744 and Cys 798) were responsible for the slow/persistent component of redox modulation of NR1/NR2B, NR1/NR2C and NR1/NR2D receptors [14]. Interestingly, mutation of either cysteine residue in a pair had the same effect as mutating both cysteine residues, leading us to postulate that the two thiols may form a disulfide bond. A recent paper describing the crystal structure of the glutamate binding site on the GluR2 subunit supports this assertion. Two cysteine residues on GluR2, which are homologous to Cys 744 and Cys 798 of NR1, were shown to form a disulfide bond [15]. In addition, mutation of these same two cysteines also eliminated potentiation by spermine and shifted the IC_{50} for proton inhibition, suggesting the effects of several modulators may be allosterically linked through these cysteines [14].

However, Paoletti et al. showed that heavy metal chelators, such as tricine, potentiate NMDA-evoked currents in NR1/NR2A receptors; this implies that trace amounts of a heavy metal (probably Zn^{2+}) contaminate standard recording solutions and tonically inhibit NR1/NR2A receptors [16]. Furthermore, it was suggested that the rapid component of DTT potentiation, which is unique to NR1/NR2A receptors, might be due to chelation of trace amounts of Zn^{2+} by DTT [16]. Our preliminary data suggest that the rapid component of DTT potentiation seen in NR1/NR2A receptors is in fact due to both redox effect and Zn^{2+} chelation. It appears that there are other pairs of cysteine residues besides Cys 744 and Cys 798 of NR1 that are important mediators of redox modulation in NR1/NR2A receptors. These cysteine residues also affect high-affinity, voltage-independent Zn^{2+} inhibition that is specific to NR1/NR2A receptors, again suggesting the possibility that multiple modulators of the NMDA receptor may share a common molecular pathway [Y.-B. Choi, Chen and Lipton, unpublished results].

Interaction of NO-related species with cysteine sulfhydryls

NO-related species have been implicated in a number of diverse physiological processes, including smooth muscle relaxation [17], neurotransmission [18] and cell-mediated immune responses [19]. NO-related species include nitric oxide ($NO^{\bullet}$), nitrosonium ion equivalents (NO^+) and nitroxyl ion (NO^-) [20]. These different redox-related forms or their functional equivalents appear to exist endogenously in the body and are important pharmacologically and physiologically, participating in distinctive chemical reactions. For example, transfer of NO^+ equivalents occurs from one nitrosothiol to another, a reaction termed transnitrosylation, i.e. $R\text{–}SH + R'\text{-}SNO \rightleftharpoons R\text{–}SNO + R'\text{–}SH$. Endogenous nitrosothiols, such as S-nitrosoglutathione, have been demonstrated to exist in brain and in lung at concentrations approaching tens of micromolar, and to react in this manner [21–23]. NO^- can exist in a high (singlet) or low (triplet) energy state, each with distinctive chemistries. In particular, singlet NO^- can react directly with thiol, whereas triplet NO^- does not [24]. However, in the triplet state, NO^- may react with O_2 to form peroxynitrite ($ONOO^-$), which in turn may oxidize free thiols to disulfide [25, 26]. $NO^{\bullet}$ preferably reacts with superoxide ($O_2^{\bullet -}$) to form peroxynitrite [27]. The distinct properties of redox-related forms of NO can be best illustrated in their interaction with the NMDA receptor. NO^+ equivalents can react with a redox modulatory site on the NMDA receptor to down-regulate the receptor's activity, which produces neuroprotection (fig. 1A) [28, 29]. $NO^{\bullet}$ reacts with superoxide anion ($O_2^{\bullet -}$) to form peroxynitrite ($ONOO^-$) and causes neuronal destruction either by itself or via its breakdown products (fig. 1B) [29, 30]. NO^- can also

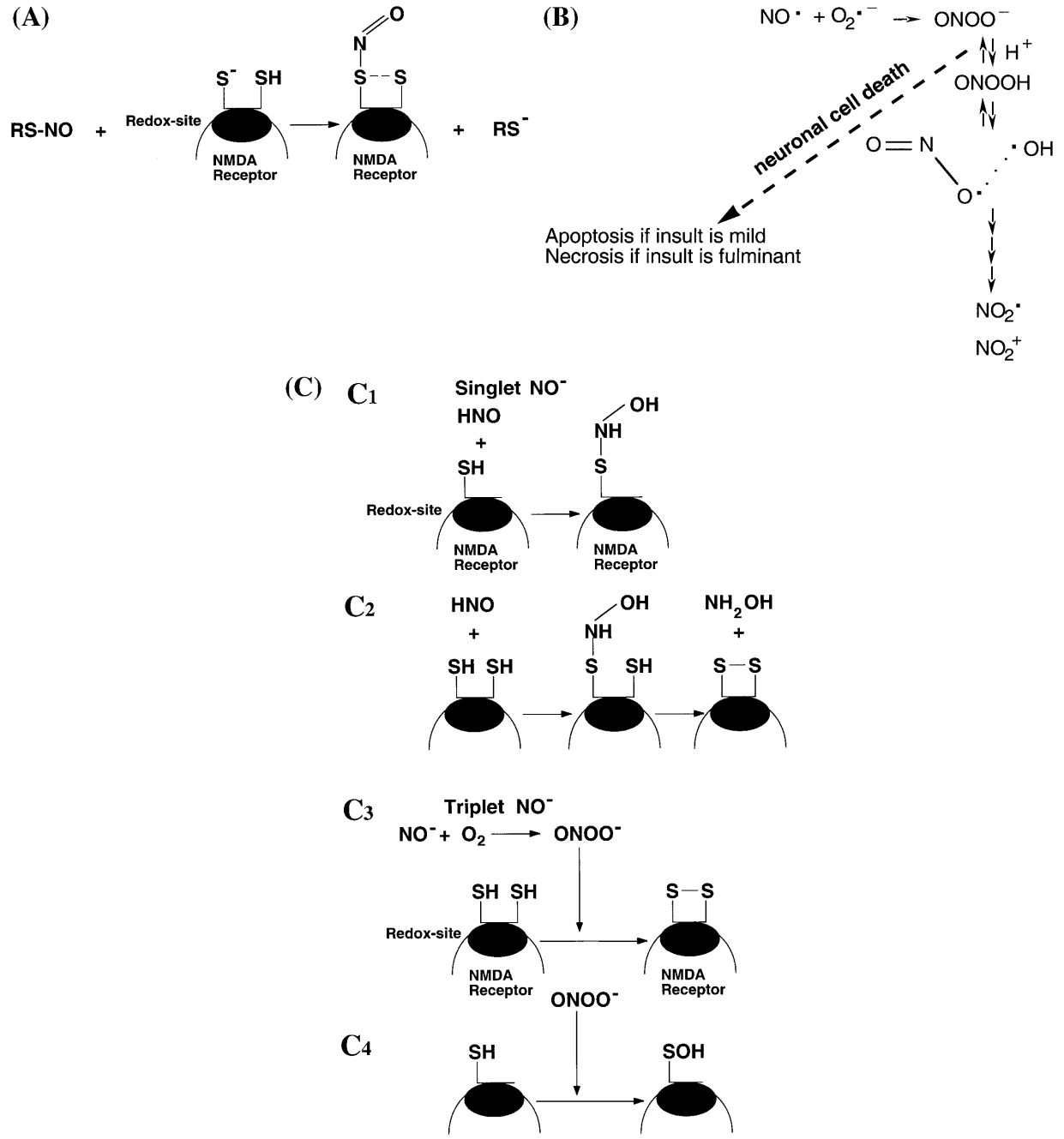

Figure 1. Proposed mechanism of the interactions of various redox forms of NO-related species with the NMDA receptor. (*A*) The redox modulatory site of the NMDA receptor is transnitrosylated by transferring the NO group (in the NO^+ form) from RS-NO to cysteine sulfhydryl groups on the NMDA receptor. This results in a decreased frequency of channel opening, and hence decreased NMDA receptor activity. (*B*) $NO^{\bullet}$ reacts with O_2^- to form peroxynitrite ($ONOO^-$) which in turn triggers neurotoxic reactions either by itself or via its breakdown products. (*C*) NO^- exists in the singlet or triplet state. In the singlet state, NO^- can react with critical thiols of the NMDA receptor to yield an R-SNH-OH derivative or disulfide, in which case hydroxylamine (NH_2OH) is also formed. These reactions would downregulate NMDA receptor activity. Triplet NO^-, rather than reacting directly with thiols, can react with molecular oxygen to form peroxynitrite, which can also oxidize thiols to disulfide or produce an –OH modification of sulfur (yielding a sulfenic acid or R-SOH).

react either directly or indirectly with thiol groups on the NMDA receptor to downregulate its activity (fig. 1C) [26].

Since our initial proposal that NMDA receptor activity may be regulated by S-nitrosylation, there is a growing list of proteins (ion channels, receptors, enzymes, and transcription factors) which undergo functional changes upon S-nitrosylation. S-nitrosylation of a single critical cysteine residue in hemoglobin [31], cyclic nucleotide-gated channels [32] and $p21^{ras}$ [33] can modulate the function of those proteins. S-nitrosylation of one or more cysteines (and possible subsequent formation of disulfide bonds) appears to be involved in modulation of Ca^{2+}-dependent potassium channels (K^{2+}_{Ca}) [34], caspase enzymes [35], protein kinase C [36] and ryanodine receptors [37], among others. In addition, our laboratory has suggested a consensus motif for S-nitrosylation [38]. Just as amino acid sequences surrounding serine or threonine residues are important for specificities of kinases, it appears that a basic or acidic amino acid in the -1 position and an acidic amino acid in the $+1$ position relative to a cysteine residue are important for facilitating S-nitrosylation of proteins. Thus, there is an emerging theme of S-nitrosylation of cysteine residues (or NO^+ group transfer) as a cellular signaling pathway analogous to phosphorylation (or phosphoryl group transfer), and this mechanism can serve as a molecular switch for the regulation of protein function [38].

Inhibition of NMDA receptor activity by S-nitrosylation

We and others previously reported that NO-related species inhibited responses mediated by the NMDA receptor via reaction with the redox modulatory site(s) [28, 29]. However, other groups have reported that NO decreases NMDA receptor activity via another mechanism, possibly related to Zn^{2+} inhibition of NMDA receptor responses [39–41]. These studies argued against direct reaction of NO-related species with redox modulatory site(s), i.e., critical cysteine sulfhydryl groups, on the NMDA receptor largely based on the observation that the alkylating agent, NEM, failed to block the inhibitory effect of NO-related species in their hands, although it was effective in ours. However, NEM must have access to the particular sulfhydryl of interest in its thiolate anion form to have an effect; this reaction can therefore be hindered for steric, kinetic, pH, redox, temperature or charge-related reasons [42]. Very recently, we definitively showed the involvement of cysteine residues in the interaction of NO-related species with the NMDA receptor [43]. First, we showed that recombinant NR1/NR2A receptors expressed in *Xenopus* oocytes could be inhibited by application of S-nitrosocysteine (SNOC) (fig. 2A). Next, methanethiosulfonate derivatives, which react specifically and rapidly with thiol groups to form mixed disulfides, occluded the action of NO-related species (fig. 2B). Finally, using site-directed mutagenesis, we identified

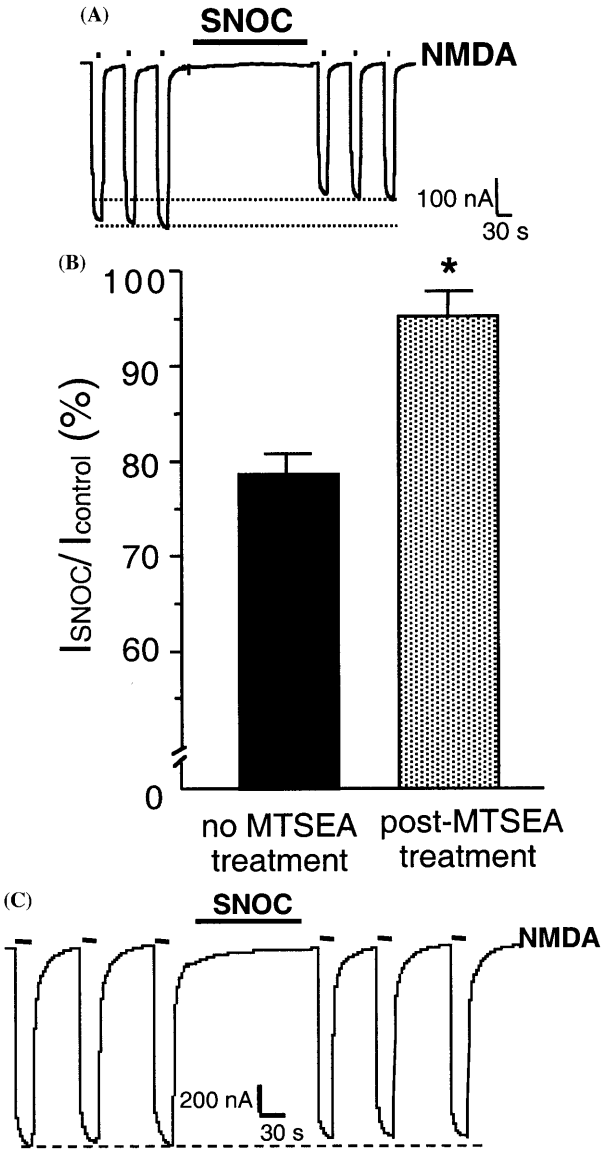

Figure 2. Interaction of NO-related species with the NMDA receptor. (*A*) Inhibition of NMDA-evoked currents (elicited by 200 μM NMDA and 100 μM glycine) after SNOC application for 3 min to *Xenopus* oocytes expressing NR1/NR2A receptors. Holding potential −80 mV, pH 7.5. (*B*) Modifying cysteine residues with (2-aminoethyl)methanethiosulfonate (MTSEA; 500 μM) for 2 min inhibited NMDA-evoked currents in oocytes expressing NR1/NR2A receptors. Exposure to MTSEA occluded further inhibition of NMDA-evoked currents by SNOC. (*C*) Virtual absence of inhibition of NMDA-evoked currents by the NO donor SNOC in oocytes expressing NR1/NR2A(C399A) receptors.

Cys 399 of NR2A as the major molecular determinant that reacts with NO species to produce inhibition of NMDA receptor activity (fig. 2C). It appears that additional cysteine residues including Cys 744 and Cys 798 of NR1 which are also involved in redox modulation of NMDA receptor activity by DTT, make a more minor contribution to NO inhibition of the NMDA receptor, but only if these cysteines are in the free sulfhydryl form and not disulfide. The report that NR1 Cys 744 and Cys 798 mutation alone did not abolish the effect of NO on NR1/NR2A receptors [44] may be explained by the fact that unless chemically reduced, these two cysteine residues would be expected to form a disulfide bond, and hence not react with NO-related species, as discussed above. Taken together, our findings indicate that the NMDA receptor undergoes modulation by polynitrosylation, similar to that demonstrated recently for the cardiac ryanodine receptor [45]. Nonetheless, the major contribution to NO modulation of NMDA receptor activity is produced by S-nitrosylation of Cys 399 on the NR2A subunit under physiological (nonreducing) conditions [43].

Joël Bockaert and co-workers suggested that the NO-induced blockage of the NMDA receptor is not related to S-nitrosylation, but to a possible interaction of NO species with the Zn^{2+} binding site [41]. Recently, we demonstrated chemically that S-nitrosylation of cysteine residues is indeed involved in NO inhibition of the NMDA receptor [43], but it is possible that Zn^{2+} and NO share cysteine residues with which they both interact on the NMDA receptor, since Zn^{2+} coordinates, among other amino acid residues, cysteine. Such competition between Zn^{2+} and NO^{+}-related species for cysteine residues, in effect producing dual modulation of protein function, has been observed previously to regulate enzyme activity [46], and may hold to some degree for the NMDA receptor as well.

Redox modulation of NMDA receptors in a physiologic context

Excessive activation of the NMDA receptor, resulting in high Ca^{2+} influx and free-radical generation, has been associated with a wide range of neurological disorders and neurodegenerative diseases, including hypoxic-ischemic brain injury, trauma, epilepsy, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis and human immunodeficiency virus (HIV)-associated dementia [47, 48]. The redox modulatory sites of NMDA receptors are of clinical interest because targeting of oxidizing agents to areas of excessive NMDA receptor activity can potentially inhibit the effects of high levels of glutamate in compromised areas of the brain while sparing normal neurotransmission in other regions of the brain [49, 50]. In support of this, the degree of neurotoxicity produced by excessive NMDA receptor activation was shown to be curtailed by oxidizing agents [51, 52] or by nitroglycerin, which is capable of donating an NO^{+}-like species [28]. Furthermore, oxidizing agents can act as an anticonvulsant and neuroprotectant in experimental models [53–55]. Additionally, redox modulation of NMDA receptors has been observed in hippocampal slices and was shown to be involved in LTP generated by a brief period of anoxia [56]. Redox modulation of NMDA receptors also appears to alter acute pain transmission; administration of DTT to the mouse spinal cord enhanced NMDA-induced nociceptive behavior, and this enhancement was blocked by the oxidizing agent DTNB [57].

Concluding remarks

Previously, several modulators of NMDA receptors had been shown to exert their effects through common molecular determinants. For example, the glycine-independent form of spermine potentiation of NMDA responses, which occurs in NMDA receptors containing an NR1 subunit that lacks exon 5 (designated NR1-1a), was reported to be due to relief from tonic proton inhibition [58]. More recently, a link between proton inhibition and zinc inhibition has been suggested [59, 60]. Mutation of NR1 Cys 744 and Cys 798 abolished not only DTT potentiation but also glycine-independent spermine potentiation and shifted the sensitivity of NR1-1a/NR2B receptors to protons [14]. Our data also suggest the existence of multiple pairs of cysteine residues that mediate modulation by both redox agents and Zn^{2+}. These findings lead one to speculate that the effects of various modulators of NMDA receptor function may be transduced via common molecular determinants. Our findings from several studies suggest that the cysteine residues of the redox sites may constitute, at least in part, these determinants. If that is indeed the case, sulfhydryl redox reagents may be uniquely capable of influencing and possibly occluding effects of other modulators by changing the redox status of these critical cysteine residues. Regulation of these molecular 'cysteine switches' may also offer a novel therapeutic approach to curtail excessive NMDA receptor activity under pathological conditions.

Redox modulation of the NMDA receptor by NO-related species via S-nitrosylation is of particular interest because the action of 'NO' in the central nervous system is closely linked to the NMDA receptor. After the initial observation that NO is produced in certain neurons following activation of the NMDA receptor, and in turn triggers an increase in cyclic GMP (cGMP) [7], it was shown that the entry of Ca^{2+} through NMDA

receptor/channels stimulates neuronal nitric oxide synthase (nNOS) activity [61]. The NMDA receptor NR1 subunit appears to be physically coupled to nNOS by an intermediary adaptor protein, PSD95, through a unique PDZ-PDZ domain interaction [62]. The fact that NO-related species can downregulate NMDA receptor activity, as discussed here, by reacting with critical cysteine thiol groups indicates that the juxtaposition of these proteins facilitates this form of feedback inhibition in the submembrane space of the postsynaptic density. Considering the range and ubiquity of proteins whose function can be regulated by S-nitrosylation has led us and our colleagues to speculate that this recently recognized reaction may be a cellular signaling pathway as fundamental as phosphorylation [29, 38].

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