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REVIEW

Synaptic Cysteine Sulfhydryl Groups as Targets of Electrophilic Neurotoxicants

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Many structurally diverse chemicals (e.g., acrylamide, 2,4-dithiobiuret, methylmercury) are electrophiles and cause synaptic dysfunction by unknown mechanisms. The purpose of this Forum review is to discuss the possibility that highly nucleophilic cysteine thiolate groups within catalytic triads of synaptic proteins represent specific and necessary targets for electrophilic neurotoxicants. Most of these toxicants share the ability to adduct or otherwise modify nucleophilic sulfhydryl groups. It is also now recognized that synaptic activity is regulated by the redox state of certain cysteine sulfhydryl groups on proteins. Electrophilic neurotoxicants might, therefore, produce synaptic toxicity by modifying these thiols. Because most proteins contain cysteine residues, target specificity is an issue that significantly detracts from the mechanistic validity of this hypothesis. However, recent research indicates that these thiolates are receptors for the endogenous nitric oxide (NO) pathway and that subsequent reversible *S*-nitrosylation finely regulates a broad spectrum of synaptic activities. We hypothesize that electrophilic neurotoxicants selectively adduct/derivatize NO-receptor thiolates in catalytic triads and that the resulting loss of fine gain control impairs neurotransmission and produces neurotoxicity. This proposal has mechanistic implications for a large class of electrophilic chemicals used in the agricultural and industrial sectors. In addition, research based on this hypothesis could provide mechanistic insight into neurodegenerative conditions such as Parkinsonism and Alzheimer's disease that presumably involve endogenous production of neurotoxic electrophiles (e.g., acrolein, 4-hydroxy-2-nonenal). The proposed mechanism of electrophilic neurotoxicants represents a new and exciting experimental framework for mechanistic research in human neuropathological conditions associated with toxicant exposure or disease-based processes.

Key Words: **neurotoxicity; nitric oxide; toxic neuropathy; protein adduct; redox signaling; *S*-nitrosylation.**

There is growing evidence that the function of chemical synapses in the peripheral nervous system (PNS) and central nervous system (CNS) can be disrupted by many structurally dissimilar electrophilic neurotoxicants, e.g., acrylamide, 2,4-dithiobiuret, methylmercury, acrolein, and diethyldithiocarbamate (Table 1; Atchison and Narahasi, 1982; Atchinson *et al.*, 1982; Danscher *et al.*, 1973; Goldstein and Lowndes, 1979, 1981; LoPachin *et al.*, 2004; Xu *et al.*, 2002). Results from corresponding research have suggested both pre- and postsynaptic sites (e.g., neurotransmitter postsynaptic receptors and presynaptic uptake, storage, and release) as possible targets for these chemicals (LoPachin *et al.*, 2004, 2006a; Lovell *et al.*, 2000; Nagendra *et al.*, 1997; Rheuben *et al.*, 2004; Vaccari *et al.*, 1998). Whereas the molecular mechanisms of these synaptic toxicants are poorly understood, they share the ability to form adducts with or otherwise modify nucleophilic sulfhydryl groups (Barber and LoPachin, 2004; Clarkson, 1972; Kruzer, 1956; Witz, 1989). Because the redox status of certain synaptic thiols represents an important regulatory determinant of neurotransmission (Ahern *et al.*, 2002; Kiss, 2000; Lipton *et al.*, 2002; LoPachin *et al.*, 2004, 2006a; West *et al.*, 2002), neurotoxic chemicals that disrupt synaptic activity might do so by reacting with these functionally important sulfhydryl groups.

In this commentary we will consider evidence suggesting that cysteine thiols are critical sites of action for both exogenous and endogenous electrophiles that mediate neurotoxicity. Low molecular weight nonprotein thiols such as glutathione (GSH) are clearly targets of toxicant action and a loss of corresponding reducing equivalents could have toxic consequences (Dickinson and Forman, 2002; LoPachin and DeCaprio, 2005; Sies, 1999; Watson *et al.*, 2004). However, substantial evidence indicates that modification of sulfhydryl groups on functionally important proteins is a primary mechanism of cell toxicity induced by many electrophilic chemicals (e.g., see Biswal *et al.*, 2002, 2003; Ku and Billings, 1986; LoPachin *et al.*, 2006a; Patel and Block, 1993; reviewed in Kehrer and Biswal, 2000). Therefore, our discussion will focus on the physiological and neurotoxicological significance of protein thiols.

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TABLE 1
Examples of Electrophilic Neurotoxicants (Metabolites)

Acrylamide (glycidamide)	Acrylonitrile (cyanoethylene)
Dithiobiuret	Sodium pyridinethione
p-Bromophenylacetylurea	Carbon disulfide
Isoniazide	Acrolein
2,5-Hexanedione	1,2-Diacetylbenzene
Styrene (styrene oxide)	Methylbromide
Acetaldehyde	Nitrogen mustard
4-HNE	

Since most proteins contain cysteine residues, it might be difficult to understand how mechanistically relevant sulfhydryl targets could be identified among the innumerable possibilities. As we will discuss, however, the specificity of synaptic neurotoxicants is likely due to selective interactions with highly reactive thiolate anions ($-S^-$) on specific cysteine residues. These residues are components of catalytic triads that regulate enzyme activities and the functions of membrane transporters, exchangers, and ion channels (e.g., see Barber and LoPachin, 2004; Forgac, 1989; Lipton *et al.*, 2002; Maekawa *et al.*, 2000; Pfister *et al.*, 1989; Voltz and Schenk, 2005). In a larger context, substantial evidence now indicates that these thiolates are effector sites for cellular redox-signaling pathways that include nitric oxide (NO) and peroxide (H_2O_2) (Martinez-Ruiz and Lamas, 2004; Matsushita *et al.*, 2005b; Stamler *et al.*, 2001). Classically, NO modulation of cellular processes was thought to be mediated by stimulation of soluble guanylyl cyclase with secondary cyclic guanosine monophosphate (cGMP) production (Ahern *et al.*, 2002). However, modification of protein thiolate residues by NO-induced nitrosylation or the formation of sulfenate by H_2O_2 has been shown to modulate many pre- and postsynaptic aspects of neurotransmission (reviewed in Esplungues, 2002; Kiss, 2000; Macarthur *et al.*, 1995; West *et al.*, 2002). These data have led to the supposition that oxidation or nitrosylation of cysteine residues regulates signal transduction in a fashion similar to the posttranslational modifications induced by protein phosphorylation (Martinez-Ruiz and Lamas, 2004). Collectively, these findings form the foundation of a new mechanistic proposal: neurotoxicants that irreversibly modify critical cysteine sulfhydryl groups produce synaptic toxicity by disrupting redox-signaling pathways. Therefore, the present Forum will conclude with a detailed discussion of this hypothesis. First, however, we will consider the unique chemistry of sulfur and the selective formation of thiolate anions in catalytic triads.

CYSTEINE THIOL CHEMISTRY: IMPORTANCE OF THE CATALYTIC TRIAD

According to the ''hard and soft acids and bases'' model (Pearson and Songstad, 1967), cysteine thiols are soft nucleophiles that readily form Michael adducts with soft electrophiles such as acrolein, acrylamide, and acrylonitrile (for detailed discussion see LoPachin and DeCaprio, 2005). The cysteine sulfhydryl group can exist in multiple oxidation states; e.g., it can be oxidized to sulfenic acid (RSOH), sulfinic acid (RSO_2H), sulfonic acid (RSO_3H), or in combination with another thiol, it can be oxidized to a disulfide bond (RSSR). This versatility is the basis of sulfur's widespread importance in biological processes. However, the formation of sulfinic or sulfonic acids is not reversible in biological systems (i.e., not readily reduced) and, therefore, these sulfur states are unlikely to be involved in mechanisms of redox signaling. Furthermore, although protein disulfides (either, intramolecular or mixed with GSH) have been considered to be a component of redox signaling (e.g., Gilbert, 1982), their formation appears to be more relevant to mechanisms of pathogenic oxidative stress (reviewed in Forman *et al.*, 2002). On a kinetic basis, cysteine sulfhydryl groups on proteins or GSH react too slowly with redox modulators (NO or H_2O_2) to be physiologically relevant. This situation changes dramatically when the sulfhydryl group (–SH) is bound to metals (e.g., Vanin *et al.*, 1997) or is in the thiolate anion ($-S^-$) state. However, the pK_a of cysteine is 8.3, consequently deprotonation of the sulfhydryl to the thiolate anion is an unfavorable event in cells. Nonetheless, thiolates with high nucleophilic reactivity are found at functionally important sites of diverse cysteine-directed proteins, e.g., skeletal muscle Ca^{2+}-release channel, creatine kinase, and hemoglobin (Miranda, 2000; Moore *et al.*, 1999; Wang *et al.*, 2001). These thiols exist in a consensus motif or catalytic triad where the deprotonation state (i.e., nucleophilic reactivity) of the cysteine residue is determined by surrounding basic (histidine, arginine, lysine) and acidic (aspartate, glutamate) amino acids (Fig. 1). Most catalytic motifs are defined by the three-dimensional associations among amino acid residues (i.e., tertiary or quaternary protein structure), e.g., the arginine$_{357}$-cysteine$_{121}$-aspartate$_{355}$ motif of methionine adenosyltransferase (reviewed in Forman *et al.*, 2002, 2004; Hess *et al.*, 2005; Stamler *et al.*, 1997, 2001). Thus, the majority of sulfhydryl groups in a given protein will exhibit relatively low reactivity, whereas those cysteine thiols in catalytic triads will be highly reactive and will, consequently, determine corresponding activity. This concept is exemplified by the ryanodine-responsive calcium-release channel of skeletal muscle, where a single cysteine thiol (Cys 3635) out of 50 possible residues is receptive to redox modulation by NO signaling (see ahead). Such site selectivity is possible because this cysteine residue is part of an acid-base motif that regulates the nucleophilic reactivity of the corresponding sulfhydryl group (Sun *et al.*, 2001). In the next section we will discuss the well-documented effector role of reactive protein thiol groups in pre- and postsynaptic processes related to neurotransmission.

THE ROLE OF PROTEIN THIOL REDOX STATES IN NEUROTRANSMISSION

Even before the concept of NO/H_2O_2 signaling was envisioned, (Dawson and Synder, 1994; Stamler, 1994), it was well

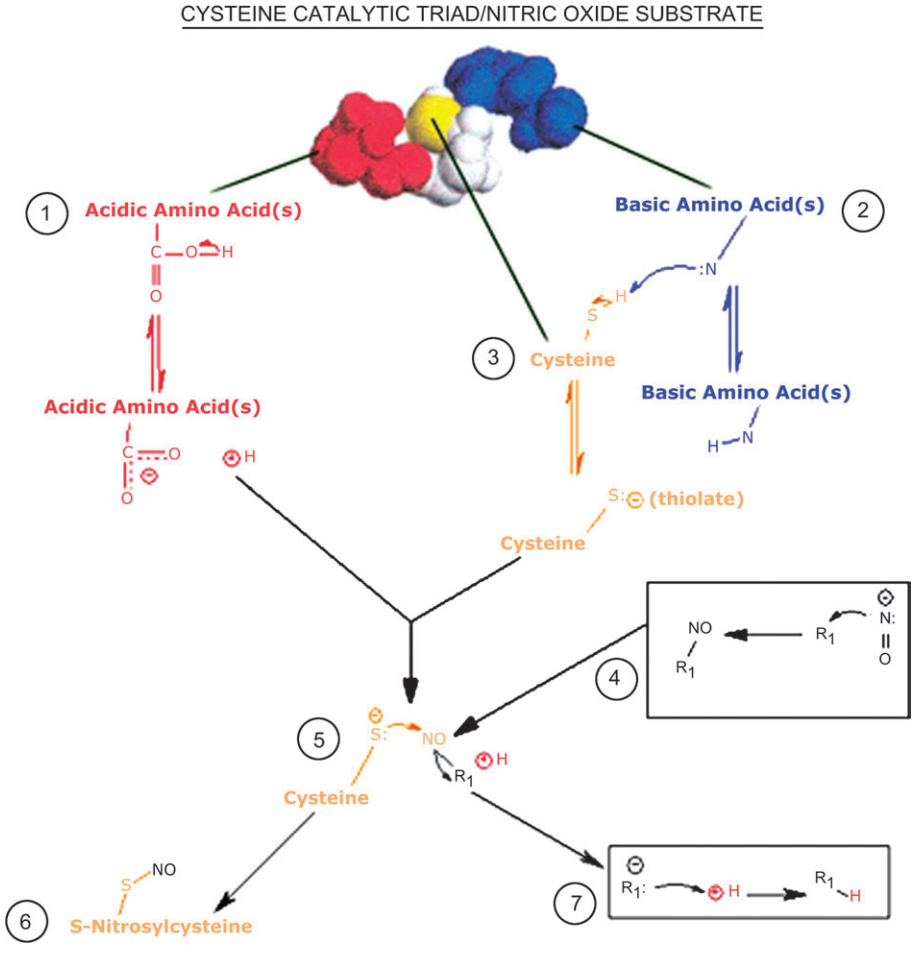

FIG. 1. A color-coded three-dimensional image of a theoretical catalytic triad is shown. The acidic amino acid residue is represented in red, the cysteine residue is presented in yellow, and the basic amino acid is presented in blue. The possible spatiotemporal chemical interactions among these triad components are illustrated schematically. (1) The acidic amino acid residue (red), which can be glutamic and/or aspartic acid(s), will undergo deprotonation to yield a proton (H^+) and a carboxylate (COO^-). (2) The basic amino acid residues (blue) can be lysine(s), histidine(s), and/or arginine(s). The, respective, nucleophilic side-chain nitrogens of these basic residues can deprotonate the sulfhydryl group of a neighboring cysteine (yellow). As the thiol group is deprotonated, the leaving hydrogen gives up electron density to the sulfur atom, which yields (3) the cysteine thiolate. (4) Before NO^- can adduct the thiolate, it must undergo a reaction with an electron pair acceptor (R_1) such as GSH. This interaction imparts electrophilic character to NO. (5) The nucleophilic thiolate can then attack the electrophilic nitrogen of the R_1-bound NO to yield (6) *S*-nitrosylcysteine. As a consequence of thiolate adduction, the nitrogen atom of NO gives up electron density to the electron acceptor (R_1), which now has nucleophilic character. The H^+ from the acidic amino acid residue (1) is an electrophile and is, therefore, susceptible to attack by the nucleophilic R_1. (7) Subsequent nucleophilic attack allows R_1 to become a better leaving group, which facilitates *S*-nitrosylation.

recognized that the redox state of sulfhydryl groups played a significant role in neurotransmission. At most chemical synapses, neurotransmitter release is achieved by a cyclic, multistep process that is initiated by the arrival of an action potential at the presynaptic terminal (Fig. 2). In general, the action potential depolarizes the presynaptic membrane, which promotes opening of voltage-gated calcium (Ca^{2+}) channels and subsequent influx of Ca^{2+} (Fig. 2, step 1). Elevation of presynaptic Ca^{2+} causes fusion of the plasma membrane with transmitter-filled synaptic vesicles (step 2). This fusion process induces transmembrane pore formation and the release of neurotransmitter into the synaptic cleft (step 3, exocytosis). The membrane of the fused vesicle is retrieved from the plasma membrane (step 4, endocytosis) and the resulting empty synaptic vesicle is refilled with neurotransmitter (step 5). Following exocytosis, the transmitter diffuses across the synaptic cleft where it binds to specific receptors located in the postsynaptic membrane. Transmitter receptor binding can directly or indirectly influence transmembrane ion flux through channels (step 6) and thereby modulate the excitability of the postsynaptic cell. The postsynaptic response is limited by presynaptic uptake of the transmitter (step 7) and by pre- and postsynaptic enzymatic metabolism (not shown). Many of the critical steps in neurotransmission, e.g., channel-mediated voltage- and ligand-gated ion flux, receptor binding, membrane fusion, presynaptic neurotransmitter uptake, and synaptic vesicle refilling, are influenced by the redox state of regulatory sulfhydryl groups on cysteine residues of functionally

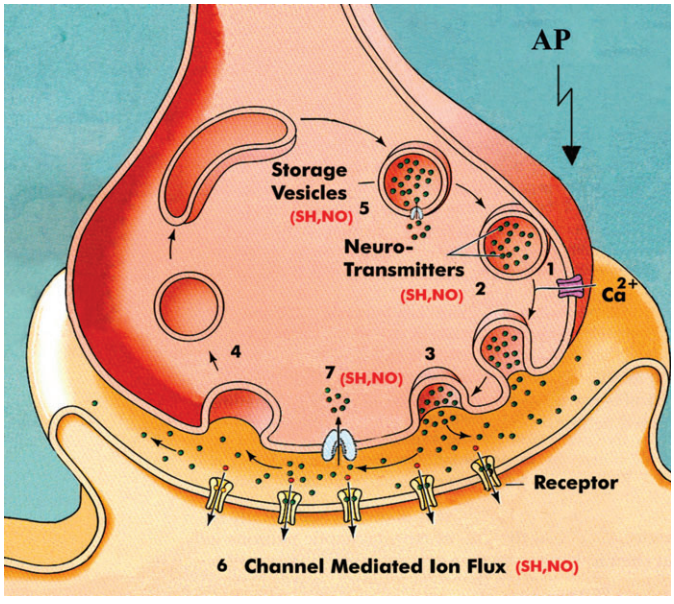

FIG. 2. Sequence of pre- and postsynaptic events involved in neurotransmission at a chemical synapse. An action potential (AP) invades the nerve terminal and the resulting depolarization of the membrane causes opening of voltage-gated Ca^{2+} channels. The resulting influx of Ca^{2+} through these channels (1) causes fusion of neurotransmitter-filled synaptic vesicles with the presynaptic membrane (2). Membrane-vesicle fusion promotes the formation of a transmembrane pore through which transmitter is released into the synapse (3). Following exocytosis, the process of endocytosis retrieves the vesicular membrane and an empty synaptic vesicle is regenerated (4). The vesicle is filled with neurotransmitter through the coupled actions of a vesicular transporter (e.g., VMAT) and a vesicular–adenosine triphosphate synthase (5). Once the transmitter has been released into the synaptic cleft, it can bind to specific receptors on the postsynaptic membrane and thereby influence channel-mediated ion flux (6). Neurotransmitter actions are terminated by postsynaptic metabolism and by presynaptic reuptake by a specific membrane transporter (e.g., DAT). The SH,NO in parentheses indicates processes that have been experimentally identified as sites of regulation by NO or thiol reagents such as NEM.

important proteins. In the following subsections, we will discuss some of the more important thiol-regulated steps in neurotransmission. A background in synaptic physiology is helpful and, therefore, the reader is referred to several recent neuroscience textbooks that provide an outstanding review of this material (i.e., Byrne and Roberts, 2004; Siegel *et al.*, 2006; Squire *et al.*, 2003).

Postsynaptic channel-mediated ion flux. Released neurotransmitter can bind to postsynaptic receptors that directly regulate channel-mediated ion flux (ionotropic receptors). Because the receptor is part of the ion channel macromolecular structure, ionotropic receptors are also known as ligand-gated ion channels. Alternatively, the transmitter can bind to postsynaptic receptors (metabotropic receptors) that are coupled to G-proteins (guanine nucleotide–binding proteins). Transmitter binding promotes dissociation of the G-protein subunits, which subsequently affect ion channel conductance either directly or indirectly through stimulation of intracellular effector proteins (e.g., adenylate cyclase). Substantial evidence indicates that both the receptor binding of transmitter and flow of ions through these types of channels (ionotropic or metabotropic) can be modulated by the redox state of sulfhydryl groups on cysteine residues of regulatory sites. A well-studied example of a thiol-regulated, ligand-gated ion channel is the N-methyl-D-aspartate (NMDA)–subtype glutamate receptor. NMDA-gated channels regulate Na^+, K^+, and Ca^{2+} conductance and are, thereby, involved in both physiological (e.g., neurodevelopment, long-term potentiation, memory) and pathophysiological (e.g., neurodegeneration) brain mechanisms. Early studies demonstrated that ion flux through the NMDA receptor was sensitive to changes in sulfhydryl redox status; e.g., application of reducing agents such as dithiothreitol (DTT) potentiated NMDA agonist responses, whereas alkylating agents such as 5-5′-dithiobis-(2-nitrobenzoic acid) (DTNB) or N-ethylmaleimide (NEM) diminished these responses (Aizenman *et al.*, 1989, 1990; Kiskin *et al.*, 1986; Laube *et al.*, 1993; reviewed in Gozlan and Ben-Ari, 1995). More recent research involving site-directed mutagenesis indicated that a total of six different cysteine residues were the basis of the observed redox sensitivity and that these cysteines formed disulfide bonds within both the NR1 receptor subunit (Cys 79 and Cys308; Cys 744 and Cys 798) and the NR2A subunit (Cys 87 and Cys 320) (Choi *et al.*, 2000, 2001; Paoletti *et al.*, 1997; see also Sullivan *et al.*, 1994). It has been postulated that the redox potential of these cysteine residues allosterically modulates high-affinity Zn^{2+} inhibition of NMDA channel opening (Choi *et al.*, 2001; Fayyazuddin *et al.*, 2000; reviewed in Lipton *et al.*, 2002).

Postsynaptic fast-acting nicotinic acetylcholine receptors (nAChR) are another example of thiol-regulated, ionotropic channels. These receptors are large protein complexes (generally five individual protein subunits) associated with muscle and nerve cells. Binding of acetylcholine or the plant alkaloid, nicotine, to the nAChR increases the channel conductance of most cations, although monovalent ions (Na^+, K^+) are preferred. Several studies have shown that agonist affinity, ion conductance, and structural stability of the nAChR are dependent upon the redox state of protein sulfhydryl groups in the receptor complex (e.g., see Bouzat *et al.*, 1991; Gysin and Flanagan, 1987; Steinacker and Zuazaga, 1981; Walker *et al.*, 1981; reviewed in Karlin, 2002). The inhibitory synapses mediated by the ionotropic $GABA_A$- γ-aminobutyric acid and glycine-gated channels are also regulated by the redox status of resident thiol groups. Thus, for example, in rat retinal ganglion cells redox modulation differentially affected $GABA_A$ and glycine neurotransmission; i.e., chemical oxidation or alkylation (DTNB, NEM) of thiol groups inhibited GABA-activated currents, whereas glycine currents were enhanced (Pan *et al.*, 1995; see also Allan and Baier, 1992).

Like their ionotropic counterparts, metabotropic receptor function can be modified by changes in protein thiol redox potential. The acetylcholine muscarinic receptor (mAChR) in nerve and heart cells is linked directly to a specific population of K^+ ion channels by G-protein signaling. In nerve cells,

mAChR (m4 subtype) stimulation by acetylcholine or the plant alkaloid, muscarine, promotes K^+ channel opening that is mediated by direct G subunit ($\beta\gamma$) interaction with the channel macromolecule. Early research showed that thiol alkylation (NEM) reduced channel opening (Braun and Sperelakis, 1998; Doods *et al.*, 1986). Results from subsequent studies suggested that NEM alkylation of the GTP-binding proteins uncoupled receptor-channel signal transduction (Nakajima *et al.*, 1990, 1991). Striatal dopamine (DA) D1 receptors are metabotropic; i.e., agonist receptor binding stimulates adenylate cyclase activity through activation of G_s. Research involving NEM and certain thiol-reactive heavy metals (Hg^{2+}, Cu^{2+}, Cd^{2+}) has shown that agonist binding to the D1 receptor is regulated by essential sulfhydryl groups located at or near the ligand recognition site (e.g., see Braestrup and Andersen, 1987; Sidhu *et al.*, 1986). Considered together, these data indicate that modification of sulfhydryl groups on certain proteins can influence ligand affinity and channel function of postsynaptic ionotropic and metabotropic receptors.

Presynaptic plasma membrane neurotransmitter uptake. Transport or reuptake of released neurotransmitter back into the nerve terminal is an important mechanism for terminating chemical signaling. Most of the major neurotransmitter systems (e.g., glutamate, GABA, glycine, catecholamine, serotonin, histamine) possess specific transport mechanisms in presynaptic membranes for removal of synaptic transmitter. These reuptake processes are energy dependent, saturable, and rely upon Na^+ cotransport, as well as requiring extraneuronal Cl^-. Ample evidence now suggests that the function of these transporters is regulated by the redox state of critical protein sulfhydryl groups (e.g., see review by Voltz and Schenk, 2005). For example, glutamate neurotransmission is inactivated primarily by uptake of the amino acid into glial and nerve cells via high-affinity, Na^+-dependent transport. Five glutamate transporter subtypes have been identified, the majority of which carry conserved cysteinyl residues (Arriza *et al.*, 1994; Kanai and Hediger, 1992). That the redox states of the corresponding sulfhydryl groups have a functional significance is indicated by substantial evidence which shows that chemical thiol modification (e.g., NEM, DTNB, iodoacetic acid) reduces the rate of glutamate uptake in several *in vitro* model systems, i.e., synaptosomes, transfected HeLa cells, and liposomes (Berman and Hastings, 1997; LoPachin *et al.*, 2004; Reis *et al.*, 2000; Trotti *et al.*, 1996, 1997). Monoaminergic neurotransmission is also terminated primarily by presynaptic transmitter uptake (Gainetdinov and Caron, 2003; Zahniser and Doolen, 2001). Thus, DA, norepinephrine (NE), and serotonin (5-HT) serotonin; each have perisynaptic transporters, i.e., respectively, DA transporter (DAT), norepinephrine transporter (NET), and serotonin transporter (SERT), that regulate the synaptic concentrations of these monoamines. These monoamine transporters are localized primarily to neurons and are, like the glutamate transporters, Na^+, Cl^- dependent. Among these transporters, thiol regulation of DAT has been described in detail. Both rat and human DAT contain 13 cysteine residues arrayed throughout the 12 transmembrane domains and corresponding intracellular and extracellular loops (Chen and Reith, 2000). Research has shown that the binding of DA agonists and transport antagonists is significantly inhibited by NEM alkylation, suggesting that reduced thiol groups are critical components of these recognition sites (Heron *et al.*, 1994; Johnson *et al.*, 1992; Schweri, 1990; Xu *et al.*, 1997). Results from additional studies using DAT mutants and chemical oxidation have suggested that the expression and activity of this transporter are also dependent upon the redox status of specific cysteine residues (Cao *et al.*, 1989; Meiergerd and Schenk, 1994; Schweri, 1990; Wang *et al.*, 1995). In this regard, Cys 342 located in the third intracellular loop, appears to be particularly important for both ligand binding and transport (Park *et al.*, 2002; Whitehead *et al.*, 2001). These data suggest that presynaptic neurotransmitter reuptake is regulated by the redox status of certain cysteine thiol groups.

Presynaptic neurotransmitter storage. Both classical (acetylcholine, biogenic amines, and amino acids) and peptide neurotransmitters are stored in synaptic vesicles where they are protected from enzymatic degradation and are available for exocytosis. Most transmitters are synthesized in the cytosol and, therefore, storage in a protective vesicle requires transporter-mediated uptake. The vesicular transport of catecholamines (e.g., DA, NE) and indolamines (e.g., 5-HT) involves the activity of vesicular monoamine transporter 2 (VMAT2), which is a Mg^{2+}-dependent transporter in brain. Other classic neurotransmitter systems also have transporters that mediate entry into corresponding storage vesicles, e.g., GABA/glycine, vesicular inhibitory amino acid transporter (VIAAT); glutamate, VGluT; and acetylcholine, VAChT (Fernandez-Chacon and Sudhof, 1999; Liu *et al.*, 1996). All neurotransmitter uptake into synaptic vesicles is coupled to an electrochemical transmembrane gradient that is established by the activity of a proton (H^+)–adenosine triphosphate synthase (ATPase) of the vacuolar class (v-ATPase; Forgac, 1989). The proton gradient represents an energy source for VMAT2 and other transporters. Presynaptic transmitter storage could, therefore, be regulated by direct inhibition of transporter function or by dissolution of the vesicular pH gradient (see ahead). The sensitivity of cysteine residues in VMAT2 and in other transporters and changes in redox status have not been defined. However, it is well recognized that v-ATPase activity is highly sensitive to inhibition by NEM (1–2μM) and other sulfhydryl reagents (Cidon and Sihras, 1989; Feng and Forgac, 1992a,b). Thus, several studies have shown that the cysteine residues responsible for NEM sensitivity reside in subunit A of the v-ATPase (Aria *et al.*, 1987; Moriyama and Futai, 1990). Among the several cysteines found in this subunit (eight total cysteines), oxidation of Cys 254 appears to specifically mediate

NEM inhibition of v-ATPase activity (Feng and Forgac, 1992a,b, 1994). This suggests that the redox state of this particular cysteine residue plays a critical role in regulating vesicular transport of neurotransmitter.

Presynaptic neurotransmitter release. The release of neurotransmitter in response to an action potential involves fusion of synaptic vesicles with a specific area of the presynaptic plasma membrane—the active zone. Because active nerve cells release neurotransmitter in a constant, ongoing fashion, the membrane fusion process must occur quickly and in sequence with an uncoupling mechanism for rapid recycling of individual constituents. In this subsection, we will discuss the molecular machinery involved in presynaptic membrane fusion and the constituent thiol-directed proteins that regulate this process. Given the speed of neurotransmission, most evidence suggests that vesicles at the release site form fusion-ready complexes with the presynaptic membrane. Therefore, the channel-mediated influx of Ca^{2+} in response to membrane depolarization needs only a trigger of simple conformational change in this complex to open a transmembrane (vesicle-plasma membrane) fusion pore for transmitter release (Fig. 3). The fusion pore is formed by the interaction of three soluble NSF attachment protein receptors (SNARE) proteins: a vesicle-associated (v-SNARE) protein called synaptobrevin (VAMP) and two target membrane (t-SNARE) proteins: soluble NSF attachment protein (SNAP)-25 and syntaxin. The interaction of these proteins creates a remarkably stable protein complex referred to as the SNARE core complex (Fig. 3). Although not completely understood, it appears that pore formation by the SNARE complex is coupled to Ca^{2+} influx via the actions of synaptotagmin. This is a Ca^{2+}-binding integral protein of the synaptic vesicle membrane that in effect acts as a Ca^{2+} sensor for pore formation. Once neurotransmitter release has occurred, the vesicle-membrane association must be uncoupled so that the synaptic vesicle can be recycled, i.e., reloaded with neurotransmitter. This energy-dependent process is accomplished by soluble NEM-sensitive factor (NSF). NSF is an ATPase that, in conjunction with the cofactor α-SNAP, causes dissociation of vesicular VAMP from membrane SNAP-25 and syntaxin.

Many studies have provided evidence that presynaptic release of neurotransmitter is a thiol-dependent process (e.g., see Barber and LoPachin, 2004; LoPachin *et al.*, 2004; Maekawa *et al.*, 2000; Matsushita *et al.*, 2003; Nedvetsky *et al.*, 2000). Indeed, the SNAREs and many of the accessory proteins (e.g., cysteine string proteins, GAP-43 = 43 kDa GTPase-activating protein, rab3A) that participate in the synaptic vesicle cycle are cysteine rich (reviewed in Calakos and Scheller, 1996; LoPachin *et al.*, 2002) and, as such, are potential sites of redox regulation. However, NSF dissolution of the SNARE core complex is a rate-limiting process in vesicle recycling and, therefore, is likely to be a primary redox regulatory step in exocytosis (Matsushita *et al.*, 2003; Morrell *et al.*, 2005; reviewed in Whiteheart *et al.*, 2001). The activity of NSF, as the name implies, is highly sensitive to inhibition by NEM and other thiol reagents (Beckers *et al.*, 1989; Glick and

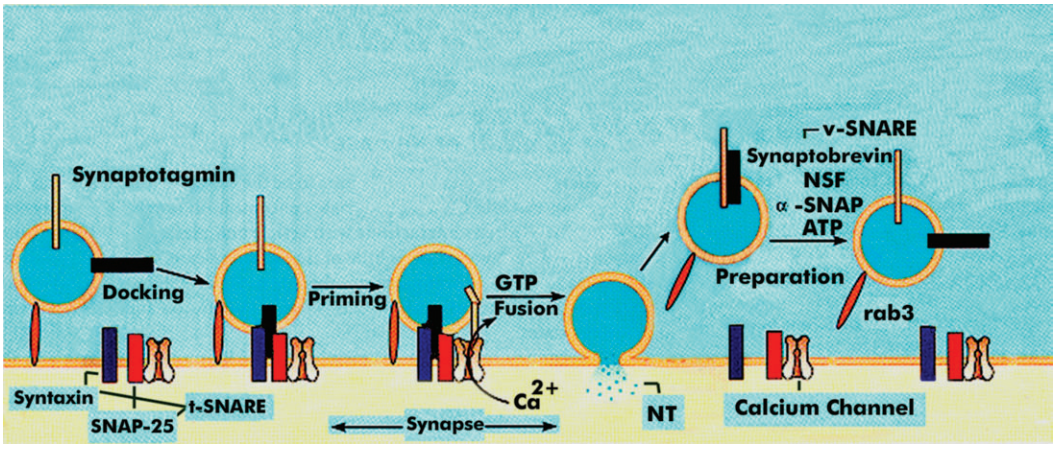

FIG. 3. Essential elements of the SNARE hypothesis are presented. According to this hypothesis, the selective docking of a synaptic vesicle with its presynaptic membrane target is mediated by the formation of a complex (the SNARE complex) between v-SNARE and cognate t-SNAREs. Once formed the SNARE complex leads to vesicle-membrane fusion and pore-mediated release of vesicular contents. As presented in Fig. 3, the synaptic vesicle protein rab3 (a low molecular weight guanosine triphosphatase [GTPase]) permits reversible vesicle attachment to a membrane target within the presynaptic active zone. This allows formation of a SNARE core complex composed of VAMP/synaptobrevin (v-SNARE) and the two t-SNAREs, syntaxin and SNAP-25. This is an irreversible step that brings the vesicle into the docked position. Before the contents of the docked vesicle can be released a priming reaction is necessary. Although the precise mechanism is unknown, priming appears to involve the recruitment of synaptotagmin to the SNARE complex. During nerve terminal depolarization, the entry of Ca^{2+} through voltage-gated channels triggers the completion of membrane-vesicle fusion. This step requires GTP and results in the formation of a pore through which neurotransmitter escapes. Following release of transmitter, the actions of NSF and α-SNAP dissociate the SNARE core complex into its individual components, i.e., VAMP/synaptobrevin, syntaxin and SNAP-25. This step requires adenosine triphosphate hydrolysis by NSF and prepares the synaptic vesicle for the next cycle of exocytosis.

Rothman, 1987). Previous research has shown that exposure of brain synaptosomes to NEM alters neurotransmitter release (LoPachin *et al.*, 2004; Nedvetsky *et al.*, 2000) and increases intrasynaptosomal SNARE core complexes (Lonart and Sudhof, 2000; Meffert *et al.*, 1996), which is consistent with decreased NSF activity. Molecular research suggests that NEM inhibition of NSF is mediated by oxidation of Cys 264 located within domain I (amino acids 255–266) of the nucleotide-binding consensus sequence. NEM adduction of this residue is likely to inhibit NSF function since this residue is critically involved in ATP binding/hydrolysis (Matsushita *et al.*, 2003; Tagaya *et al.*, 1993; Whiteheart *et al.*, 1994).

ESSENTIAL PROTEIN THIOLS ARE RECEPTORS FOR NO-SIGNALING PATHWAYS

Clearly, chemicals (e.g., NEM, DTNB) that alkylate or otherwise modify essential cysteine thiol groups can disrupt pre- and postsynaptic processes. For synaptic activities such as transmembrane pore formation or vesicular transport, specific cysteine residues have been identified that govern the actions of corresponding rate-limiting or regulatory proteins, e.g., Cys 264 of NSF, Cys 254 of v-ATPase. Although the presence of functionally essential sulfhydryl groups could reflect biochemical chance or ancestry, a growing body of evidence suggests that these thiols participate as ''receptors'' in redox-signaling pathways (e.g., NO, H_2O_2) that modulate synaptic activity and other nerve cell functions (Table 2; see also Dawson and Synder, 1994; Forman *et al.*, 2002, 2004; Hess *et al.*, 2005; Mannick and Schonhoff, 2002; Martinez-Ruiz and Lamas, 2004; Stamler, 1994; Stamler *et al.*, 1997, 2001). Adduction of these thiol receptors by electrophilic neurotoxicants could interfere with or block the signaling of these redox pathways (see ahead). In the following sections, we will discuss the best known of these redox pathways, the NO system. This discussion will include mechanisms of NO-receptor interactions and effector consequences.

TABLE 2
Thiol-Regulated Proteins and Protein Complexes Targeted by NO

Target	NO effect
NMDA receptor channel complex	I
Skeletal Ca^{2+}-release channel (ryanodine receptor)	A/I
GAP-43	Inhibits targeting
SNAP-25	Inhibits targeting
NSF	Inhibits SNARE complex dissociation
Ca^{2+}-ATPase	I
Na^+/K^+-ATPase	I
v-ATPase	I
Heavy neurofilament subunit	?
Protein kinase C	I
Methionine adenosyl transferases	I
Guanylate cyclase	A
Caspases 1–8	I
Mitochondrial complex 1	I
Thioredoxin	I
NOS	I
Cytochrome P450	I

A partial list of NO targets is presented with possible neurotoxicological significance. I, inhibition; A, activation.

NO signaling: neuromodulatory actions and target specificity. Although initially identified as the endothelial-derived relaxing factor, NO is now recognized as a major messenger molecule with diverse biological functions (Bredt, 2003; Esplungues, 2002; Foster *et al.*, 2003; Gaston *et al.*, 2003). It was originally thought that elevated intracellular cGMP levels following stimulation of soluble guanylyl cyclase mediated the neurophysiological effects of NO. However, accumulating evidence suggests that NO can modify protein function through *S*-nitrosylation of thiol side chains on cysteine residues (Ahern *et al.*, 2002; Broillet, 1999; Gaston, 1999; Hess *et al.*, 2005; Martinez-Ruiz and Lamas, 2004). In fact, it appears that *S*-nitrosylation is an equally important mode of NO signal transduction (Mannick and Schonhoff, 2002; Martinez-Ruiz and Lamas, 2004). Unlike classic chemical neurotransmitters, NO is generated on demand (see ahead) and is not stored in presynaptic vesicles or released by exocytosis. Endogenous NO is produced from L-arginine by nitric oxide synthase (NOS), of which there are at least three different isoforms: endothelial, inducible, and neuronal NOS (nNOS). The latter isotype is primarily responsible for NO production in brain. nNOS is a highly regulated, constitutive enzyme that is localized to relatively few neuronal subpopulations (e.g., cortex, striatum, and cerebellum) scattered throughout the rodent and primate brain. Although nNOS is present in only 1% of CNS neurons, these cells project so extensively that nearly every neuron in the brain is a target of NO regulation (Bredt *et al.*, 1991; Fujiyama and Mauko, 1996; Vincent and Kimura, 1992). Production of NO by nNOS is a calmodulin-stimulated process and, consequently, requires increased intraneuronal Ca^{2+} levels (reviewed in Griffith and Stuehr, 1995). As indicated above, NO is not released discretely and can, therefore, influence presynaptic nerve terminals (see ahead), as well as other neuronal regions and glial cells in the immediate vicinity (within a 40–300 μm diameter; Esplungues, 2002; Garthwaite and Boulton, 1995). During NO signaling, *S*-nitrosylation of the thiolate anion of catalytic triads involves complex adduct chemistry (Fig. 1), although it should be pointed out that the precise *in vivo* mechanism has not been determined (reviewed in Ahern *et al.*, 2002; Forman *et al.*, 2004). NO inactivation does not involve reuptake or enzymatic degradation and is, instead, passive; i.e., given the lability of the nitrosothiol product, denitrosylation is likely to be nonenzymatic and to involve cellular reductants, local shifts in pH or pO_2, or transnitrosation

of metallo- or thioproteins (Forman *et al.*, 2004; Mannick and Schonhoff, 2002; Stamler and Toone, 2002).

Given the information reviewed above, NO-mediated nitrosylation as a signaling pathway seems to lack the inherent specificity expected of a neuromodulatory or neurotransmitter system. That is, most proteins in both neuronal and glial cells contain cysteine residues, which means that the potential targets for nitrosylation are virtually ubiquitous. Furthermore, NO is not released discretely, but rather diffuses to its cellular targets (see above) and, therefore, has a theoretically broad sphere of spatial action. Despite this seemingly diluted nonsynaptic nature, it is now recognized that NO can affect individual cells and that *S*-nitrosylation can be restricted to a precisely defined subset of cellular protein targets (Stamler *et al.*, 2001). This level of target specificity is achieved through the presence of catalytic triads that increase the nucleophilicity of specific protein cysteine residues (discussed above) and by subcellular compartmentalization of subsequent nitrosylation reactions (Bredt, 2003; Esplungues, 2002; Ziolo and Bers, 2003). Compartmentalization is a product of NO-signaling modules, where the nitrosylation targets of a pathway (specific catalytic triads) and Ca^{2+}/calmodulin(CaM)-dependent nNOS are tethered through scaffolding proteins or specific protein domains. For example, the postsynaptic NMDA receptor complex (see above) is constructed so that the nNOS-CaM complex is linked via the postsynaptic density protein 95 (PSD95) to corresponding NO substrates, e.g., the regulatory subunit (NR2A) of the ionotropic receptor. Ligand (glutamate)–gated Ca^{2+} entry through the NMDA receptor therefore stimulates nNOS production of NO, which subsequently nitrosylates a specific cysteine thiolate (Cys 399) on the NR2A subunit. Nitrosylation of this regulatory subunit decreases receptor-mediated Ca^{2+} entry and thereby acts as built-in negative feedback processes to regulate postsynaptic glutamate actions (reviewed in Ahern *et al.*, 2002; Esplungues, 2002; Garthwaite and Boulton, 1995; Gozlan and Ben-Ari, 1995; Lipton *et al.*, 1996, 2002). Thus, the specificity and functional independence of NO-synaptic effects is based on signaling modules that act like neuronal microprocessors; i.e., stimulus-induced generation of NO via nNOS occurs in close proximity to the effector element, thiolate anions of catalytic triads. The resulting shift in sulfhydryl redox status specifically modulates activity of the corresponding protein.

NO modulation of synaptic strength. In attempting to define the neuromodulatory role of NO, researchers used various methodological approaches and *in vivo*/*in vitro* model systems. For example, the effects of NO on pre- and postsynaptic function were explored in synaptosomes, brain slices, cultured cell systems and in whole animal brain. In many studies, the NO content of these preparations was manipulated experimentally; i.e., NO was increased by the use of NO gas, NO donors (e.g., SNAP, 2-(N,N-diethylamino)-diazenolate-2-oxide [DEA/NO]), and NO precursors (e.g., L-arginine), whereas the amount of NO was reduced by NO scavengers (e.g., oxyhemoglobin, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide [carboxy-PTIO]) or nNOS inhibitors (e.g., L-nitorarginine methyl ester [L-NAME]). As a result of inherent methodological problems (for methodological critique see Kiss, 2000), and differences in experimental approaches and scientific viewpoints, the data from these studies are often contradictory. A goal of the following discussion is to identify these conflicts and to provide possible explanations.

As we discussed above, the redox state of cysteine sulfhydryl groups on many postsynaptic receptors (e.g., NMDA, nAChR, $GABA_A$) can modulate both neurotransmitter binding and ligand-mediated ion channel conductance. These regulatory thiols are likely sites of modulation by the endogenous NO-signaling pathway (Table 2). In this respect, the NMDA-type glutamate receptor is the best characterized of the possible NO-regulated synaptic receptors. This research focus is clearly based on the preeminent roles of the NMDA receptor in physiological and pathophysiological processes. As discussed above, it now appears that *S*-nitrosylation of Cys 399 of the NR2A subunit acts as a gain control for Ca^{2+} flux through NMDA receptor–operated channels and, thereby, represents a molecular device to limit potential excitotoxicity. There is some evidence that NO generated in the postsynaptic element can act transsynaptically as an orthograde messenger to similarly effect presynaptic NMDA receptors that regulate transmitter release (Casado *et al.*, 2002; Stout and Woodward, 1995; also see Esplungues, 2002). Despite a voluminous supporting literature (see references cited above), however, results from some previous studies have questioned the neurobiological relevance of NO regulation of NMDA receptor function (e.g., see Aizenman and Potthoff, 1999; Gbadegesin *et al.*, 1999; Hopper *et al.*, 2004). Clearly, continued research is needed to resolve this controversy and to determine whether other receptors (e.g., nAChR, mAChR, $GABA_A$) are similarly modulated by direct NO influences. In addition to these direct mechanisms, NO signaling can also affect receptor function indirectly. For example, Huang *et al.* (2005) have recently shown that, amino-3-hydroxy-5-methylisoxazole-proprionic acid receptor recycling during the induction of synaptic plasticity, is mediated by NO signaling. NO generated by NMDA receptor stimulation (see above) nitrosylates a specific cysteine residue (Cys 91) on NSF. This leads to binding of NSF to the amino-3-hydroxyl-5-methylisoxazole-proprionic acid (AMPA) GluR2 subunit, which in turn promotes surface expression of the AMPA receptor (see also Steinberg *et al.*, 2004). NO signaling can, therefore, affect synaptic strength by influencing the function and possibly the expression of pre- and postsynaptic neurotransmitter receptors.

Reuptake of released transmitter and subsequent vesicular storage are also critical components of neurotransmission and several lines of evidence suggest that NO signaling modulates these presynaptic processes. Thus, Wolosker *et al.* (1996) showed that several NO donors (S-nitroso-N-acetyl-DL-penicillamine,

S-nitroso-L-glutathione [GSNO]) produced concentration-dependent inhibition of ^{3}H-glu uptake into synaptic vesicles isolated from whole rat brain homogenates. NO scavengers (oxyhemoglobin, methemoglobin) or DTT reversed NO inhibition of vesicular transport, whereas dcGMP did not influence transport. Based on these results the authors suggested that endogenous NO signaling regulates transmitter synaptic vesicle storage through *S*-nitrosylation of functionally important cysteine residues (e.g., Cys 254; see above) on the vesicular H^+-ATPase. With respect to presynaptic membrane transport, early studies (Lonart and Johnson, 1994; Pogun *et al.,* 1994a,b) showed that NO gas and certain NO donors (e.g., sodium nitroprusside [SNP], SNAP) reduced ^{3}H-glu and ^{3}H-DA uptake into synaptosomes isolated from rat hippocampus and striatum, respectively. Pogun *et al.* (1994a,b) showed that, for both transmitter systems, the inhibitory effects were mediated by changes in K_m (increased) and V_{max} (decreased). The molecular mechanism of NO-induced transport inhibition was not examined directly in these studies. However, since there were no consensus cGMP-dependent protein kinase sites on the rat glu- or DAT (Kanai and Hediger, 1992; Shimada *et al.,* 1991) and, since sulfhydryl reagents such as NEM were known to inhibit these transporters (Pogun *et al.,* 1994a,b; Schweri, 1990), Pogun *et al.* surmised that nitrosylation of regulatory transporter thiol groups was involved in this NO effect. These initial findings were supported by subsequent studies, which also showed that NO reduced presynaptic membrane transport (Cao and Reith, 2002a,b; Chaparro-Huerta *et al.,* 1997; Cook *et al.,* 1996; Kaye *et al.,* 2000; Kiss *et al.,* 1999). In contrast, an early study by Lin *et al.* (1995) provided indirect evidence (electrochemical detection of rat striatal DA flux) that endogenous NO accelerated DA uptake. A similar finding was reported when DA transport was measured in rat striatal suspensions by rotating disk electrode voltammetry (Voltz and Schenk, 2004). In this study, increasing the NO content of the tissue suspensions with the precursor, L-arginine, antagonized NEM inhibition of the DAT. Based on these data, the authors concluded that NO and NEM compete for the same functionally important DAT cysteine residue(s); i.e., reversible NO nitrosylation of cysteine residues increases DAT activity, whereas irreversible thiol adduction by NEM (Cys 342?; Park *et al.,* 2002) decreases transporter activity. Although contradictory data exist, the weight of evidence suggests that endogenous NO signaling reduces neurotransmitter uptake and subsequent vesicular storage. This appears not to be a product of cGMP actions, but is rather mediated by *S*-nitrosylation of functionally critical cysteine thiol groups on proteins that play a regulatory role in these presynaptic processes, e.g., Cys 254 of the vesicular H^+-ATPase, Cys 342 of DAT.

Inhibition of presynaptic uptake and storage would be expected to reduce the releasable pool of neurotransmitter and thereby decrease Ca^{2+}-dependent exocytosis. Consistent with this expectation, numerous laboratories have reported NO inhibition of neurotransmitter release (Boulton *et al.*, 1994; Daniel *et al.*, 1993; Guzman-Guevara *et al.*, 1994; Lindgren and Laird, 1994; Kamisaki *et al.*, 1995; Nedvetsky *et al.*, 2000; Pan *et al.*, 1995; Sequeira *et al.*, 1997; Shibiki and Okada, 1991; Silva *et al.*, 1998; Sun *et al.*, 1995; reviewed in Kiss, 2000). In contrast, results from other investigators have suggested that NO facilitates, rather than inhibits, neurotransmission (Bon and Garthwaite, 2001; Li *et al.*, 2000; Maekawa *et al.*, 2000; Montague *et al.*, 1994; Southam *et al.*, 1996; West and Galloway, 1997a,b, 1999; West and Grace, 2000; Zhu and Luo, 1992; reviewed in West *et al.*, 2002). The reasons for these contradictory findings are uncertain, but might be related to differences in methodological approaches and/or the difficult pharmacological/toxicological nature of experimental NO application. For example, NO and its precursor chemicals are known to produce dose-dependent effects, as well as nonspecific actions (Cao and Reith, 2002a,b; Sequeira *et al.*, 1997; Silva *et al.*, 1998; Stout and Woodward, 1995). Moreover, the experimental outcome can be influenced by the redox state of the donated NO moiety, e.g., NO^- versus NO^+ (Pan *et al.*, 1995) and by differences in the physiological complexity of the model (e.g., synaptosomes vs. brain slices) and corresponding tissue oxidation state (Macarthur *et al.*, 1995; Trabace and Kendrick, 2000). Although certain technical aspects might contribute to the contradictory nature of this database, the previously defined role of protein thiols in nerve terminal function is a rational basis for deciphering the often-conflicting influences of NO signaling on neurotransmission. Thus, it should be recalled from our preceding discussions that transmitter release is impaired by chemical modification of sulfhydryl groups with NEM and other alkylating chemicals. Since these thiols are likely the same acceptors involved in NO signaling, it is reasonable to expect that corresponding *S*-nitrosylation would lead to reduced release. This possibility is supported by results from several well-designed studies in neuronal and nonneuronal systems, which showed that NO signaling decreased exocytosis (Matsushita *et al.*, 2003; Morrell *et al.*, 2005; Pan *et al.*, 1996). Furthermore, it is important to reiterate previously discussed findings that chemical alkylation and *S*-nitrosylation of presynaptic thiols decreased neurotransmitter uptake and storage, which would also be expected to diminish release. Together, these lines of evidence suggest that nitrosylation of presynaptic thiol groups inhibits exocytosis.

Whereas changes in the uptake and storage capacity of nerve terminals are likely components of NO modulation (see above), Pan *et al.* (1995) suggested that the primary regulatory mechanism was direct *S*-nitrosylation of proteins involved in membrane fusion (reviewed in Esplungues, 2002; Hess *et al.*, 2005; LoPachin *et al.*, 2003). Fusion of synaptic vesicles with the presynaptic plasma membrane occurred through the formation of transmembrane SNARE core complexes (Fig. 3). These complexes mediate subsequent pore formation and neurotransmitter release. Following exocytosis, NSF

disassembles the SNARE complexes into their individual components (i.e., SNAP-25, syntaxin, VAMP), which permits continued vesicular trafficking. In this scenario, NSF is the most likely NO target, since SNARE disassembly is a rate-limiting step in the vesicle cycle and since the ATPase activity of this protein is highly sensitive to inhibition by thiol alkylation (reviewed in Lowenstein *et al.*, 2005; Whiteheart *et al.*, 2001). Indeed, results from recent research (Matsushita *et al.*, 2003; Morrell *et al.*, 2005) showed that NO signaling inhibited NSF activity and secondarily reduced exocytosis. NO inhibition of NSF function was mediated by *S*-nitrosylation of Cys 91 and Cys 264. These residues play a critical role in dissolution of the membrane fusion complexes. As an expected consequence of reduced NSF activity, the investigators reported a build-up of 7S SNARE complexes (see also Matsushita *et al.*, 2005a; Meffert *et al.*, 1996; Morrell *et al.*, 2005). These data indicate that endogenous NO signaling reduces neurotransmitter release by inhibiting presynaptic NSF activity. These regulatory actions of NO are mediated by *S*-nitrosylation of specific cysteine residues on NSF.

The literature reviewed in this section indicates that the formation of NO-thiol adducts (*S*-nitrosylation) dampens synaptic activity by reducing receptor function and by limiting presynaptic transmitter uptake, storage, and release. These NO-induced changes parallel the synaptic effects caused by chemicals such as NEM, iodoacetamide, or DTNB (see above) that alkylate or otherwise modify protein thiol groups. This concordance suggests that these experimental chemicals and NO act at common thiol sites. Accordingly, based on the electrophilic reactivity of the neurotoxicants listed in Table 1, it is possible that these agents produce synaptic toxicity by also acting at NO sulfhydryl sites. Therefore, in the final section of this commentary, we discuss the possibility that electrophilic neurotoxicants adduct synaptic thiol receptors of the endogenous NO pathway and, thereby, inhibit neurotransmission.

ELECTROPHILIC NEUROTOXICANTS DISRUPT NO REGULATION OF NEUROTRANSMISSION: A HYPOTHESIS

As discussed in the preceding sections, the functional status of many synaptic processes is determined by proteins whose activities are regulated by the redox state of highly nucleophilic sulfhydryl groups located in corresponding catalytic triads. Based on their nucleophilic reactivity, these sulfhydryl groups could be adducted or derivatized by electrophilic neurotoxicants such as those listed in Table 1. Toxicant derivatization of these regulatory thiols is likely to disrupt the participation of the affected proteins in their respective synaptic pathways, which could, in turn, lead to a loss of synaptic strength and to the development of neurotoxicity. Whereas this hypothetical mechanism of electrophilic neurotoxicants could end here, it is important to recognize that triad sulfhydryl groups are acceptors for NO and other redox regulators (e.g., H_2O_2). The potential disruption of redox regulation by sulfhydryl adduction must, therefore, be included in any proposed mechanism of electrophile neurotoxicity. Thus, as discussed in the preceding section, NO-mediated *S*-nitrosylation of thiolate receptors is normally transient and localized to individual signaling modules that become activated, e.g., Ca^{2+} activiation of nNOS in the NMDA receptor unit. This results in regulatory changes that are specific to that module (e.g., reduced channel-mediated ion flux). In contrast, we propose that toxicant-thiolate interactions will not be compartmentalized and will occur in parallel with subcellular toxicant distribution; e.g., for a chemical with wide nerve terminal distribution, thiolate moieties of diverse catalytic triads will be adducted. This is exemplified by our recent findings that acrylamide intoxication is associated with presynaptic inhibition of neurotransmitter reuptake (membrane DAT), release (SNARE core complex), and vesicular storage (v-ATPase activity; see ahead). Toxicant interactions will be either irreversible (covalent adduction) or, at least, slowly reversible (ionic) depending upon the respective chemical characteristics, e.g., α,β-unsaturated carbonyl derivatives versus methylmercury. As a consequence, the rapid, fine gain control of synaptic activity provided by NO signaling will be lost and, instead, toxicant-thiolate adduction will produce a persistent NO-type effect. Based on the functional responses to NO-signaling electrophilic chemical adduction of the corresponding receptor thiolates would result in reduced neurotransmission and loss of synaptic strength. Decreased activity at PNS and CNS synapses could lead to the induction of cognitive, behavioral, and/or neurological dysfunction. Whereas NO signaling is clearly involved in the physiological function of nonneuronal organ systems, the vulnerability of synapses to such toxicant action is related to the extraordinarily high rate of NO modulation and the relatively slow turnover of NO-regulated proteins in this nerve region (reviewed in Brenman and Bredt, 1997; Esplungues, 2002; LoPachin *et al.*, 2002).

Conceptually, the proposed mechanism of electrophilic neurotoxicants is analogous to that of narcotic analgesics. The initial finding that morphine produced analgesia by acting at specific brain receptors implied the existence of an endogenous pathway that modified the perception of pain. Indeed, subsequent research identified several classes of peptides (endorphins, enkephalins, dynorphins) that acted as ligands for an endogenous opioid pathway. Morphine, therefore, is an exogenous agonist that binds these receptors and, thereby, mimics the effect of endogenous ligands. In a similar fashion, electrophilic neurotoxicants can be viewed as exogenous agonists that bind (adduct) receptor thiols and, accordingly, mimic the actions of endogenous NO signaling. Although this hypothesis is largely untested, a substantial body of evidence discussed in this Forum suggests that it is rational and could be a basis for new areas of research in *Neurotoxicology*. Moreover, evidence from ongoing studies has begun to connect thiol

adduct formation with toxicant-induced synaptic dysfunction. This provides an experimental rationale for research evaluating the effects of electrophilic neurotoxicants on NO signaling at the synapse. Thus, for example, acrylamide (ACR) is an α,β-unsaturated carbonyl derivative and is a member of a large class of electrophilic chemicals with multiple industrial applications (e.g., acrolein, acrylonitrile, methyl vinyl ketone). As a soft electrophile, ACR will form adducts with soft nucleophilic sulfhydryl groups on protein cysteine residues (Barber and LoPachin, 2004; LoPachin *et al.,* 2004; reviewed in LoPachin and DeCaprio, 2005). Early electrophysiological studies indicated that ACR inhibited neurotransmission at peripheral and central synapses (reviewed in LoPachin *et al.*, 2002, 2003). To determine the mechanism of impaired transmission, functional studies conducted in our laboratory focused on possible presynaptic sites of action. Corresponding findings have shown that ACR decreased neurotransmitter release, uptake, and storage when measured following *in vivo* intoxication or *in vitro* exposure (Barber and LoPachin, 2004; LoPachin *et al.*, 2004, 2006). Corroborative proteomic data have suggested that these inhibitory actions are mediated by ACR-sulfhydryl adduction of specific cysteine residues on functionally critical proteins, e.g., Cys 264 of NSF, Cys 342 of DAT, and Cys 254 of the vesicular H^+-ATPase (Barber and LoPachin, 2004; Barber *et al.*, unpublished). These sites of cysteine-ACR adduction are also sites of NO nitrosylation (Jaffrey *et al.*, 2001; Stamler *et al.*, 2001), which suggests that ACR produces synaptic toxicity by disrupting NO signaling.

Acrolein, like ACR, is an α,β-unsaturated carbonyl and is a ubiquitous environmental pollutant. In addition, acrolein and other α,β-unsaturated carbonyl derivatives (e.g., malondialdehyde, 4-hydroxy-2-nonenal [HNE]) are produced during lipid peroxidation that is presumably involved in Alzheimer's disease (AD) and other neurodegenerative conditions (Picklo *et al.*, 2002). As soft electrophiles, acrolein and its derivatives will preferentially form thiol adducts with cysteine residues on proteins (Beauchamp *et al.*, 1985; Esterbauer *et al.*, 1991; Witz, 1989). Research from our laboratory and others (LoPachin *et al.*, submitted; Lovell *et al.*, 2000; Pocernich *et al.*, 2001) indicates that acrolein can impair nerve terminal function through adduction of protein thiols. Recent evidence suggests that AD is characterized by early functional and structural damage to regional (e.g., hippocampus, cerebellar cortex) nerve terminals (e.g., see Coleman and Yao, 2003). The mechanism of synaptic dysfunction is not known, although it has been suggested that oxidative stress and subsequent production of acrolein and 4-HNE play a significant role. Indeed, acrolein and 4-HNE have been identified in brains of AD patients and certain transgenic mouse models (e.g., see Smith *et al.*, 1998; Williams *et al.*, 2005). These data suggest that acrolein might form adducts with protein thiol receptors of the synaptic NO pathway and thereby interfere with signal transduction. The ensuing loss of NO regulation could lead to diminished synaptic strength and cognitive impairment. This suggestion is in contrast to other hypotheses of neurodegenerative diseases, which emphasize NO excess or nitrosative stress (e.g., Chung *et al.*, 2004; Hess *et al.*, 2005). Thus, initial studies with ACR and acrolein suggest that endogenous or exogenous electrophilic neurotoxicants could inhibit synaptic activity by forming adducts with thiolate groups that participate in the NO- or H_2O_2-signaling pathways.

A goal of this Forum and the hypothesis presented herein, is to stimulate discussion and subsequent investigation into unexplored avenues of mechanistic research. We think that the proposed pathophysiological scenario is relevant to diverse classes of electrophilic chemicals, e.g., heavy metals, α,β-unsaturated carbonyls, and disulfides, that form adducts with or otherwise derivatize protein sulfhydryl groups. The hypothesis is based on the inherent adduct chemistry of different toxicants and might, therefore, represent a rational basis for classification. The structure-activity characteristics that dictate thiol adduction/interaction (e.g., conjugated α,β-unsaturated carbonyl) could be experimentally defined and used subsequently to predict the potential neurotoxic risks of agricultural and industrial chemical exposures. Electrophile disruption of NO signaling and consequential synaptic dysfunction also have pathophysiological ramifications for the molecular events that mediate neurodegenerative conditions such as Parkinsonism and AD. In these brain diseases, regional oxidative stress generates lipid peroxidation byproducts (e.g., 4-hydroxy-2-nonenal, acrolein). These endogenously produced electrophilic toxicants readily form thiol adducts and could, therefore, produce nerve damage by interfering with NO signaling. Realizing this potential mechanism could lead to the development of efficacious pharmacotherapies involving the prevention of adduct formation. Therefore, our proposal that electrophilic neurotoxicants act by disrupting NO signaling represents a new and exciting model for the design of mechanistic research in human neuropathological conditions resulting from toxicant exposure or disease-based processes.

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