

Published in final edited form as:

*Trends Neurosci.* 2010 November ; 33(11): 493–502. doi:10.1016/j.tins.2010.07.004.

## Protein modifications involved in neurotransmitter and gasotransmitter signaling

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### Abstract

Covalent modifications of intracellular proteins, such as phosphorylation, are generally thought to occur as secondary or tertiary responses to neurotransmitters, following the intermediation of membrane receptors and second messengers such as cyclic AMP. By contrast, the gasotransmitter nitric oxide directly S-nitrosylates cysteine residues in diverse intracellular proteins. Recently, hydrogen sulfide has been acknowledged as a gaso-transmitter, which analogously sulfhydrates cysteine residues in proteins. Cysteine residues are also modified by palmitoylation in response to neurotransmitter signaling, possibly in reciprocity with S-nitrosylation. Neurotransmission also elicits sumoylation and acetylation of lysine residues within diverse proteins. This review addresses how these recently appreciated protein modifications impact our thinking about ways in which neurotransmission regulates intracellular protein disposition.

### Introduction

Neurotransmitter influences on cellular function have classically been ascribed to second and tertiary messengers such as cyclic AMP and protein phosphorylation. The advent of gasotransmitters, particularly nitric oxide (NO), but including carbon monoxide (CO) and hydrogen sulfide (H<sub>2</sub>S), has markedly altered conceptualizations about the path from the synapse to intracellular protein modification. Gases do not bind to cell surface receptors, hence do not require the intermediation of conventional membrane receptors and second messenger machinery such as G-proteins and adenylyl cyclase. Instead, the gases directly interact with targets, such as guanylyl cyclase [1]. Probably more prevalent is the S-nitrosylation (hereafter designated ‘nitrosylation’) by NO of cysteine residues in a wide range of target proteins. Specificity of signaling derives from NO synthase (NOS) binding to its targets directly or via scaffolding proteins such as CAPON (carboxy-terminal PDZ ligand of nNOS) [2]. H<sub>2</sub>S also modifies cysteines in target proteins, forming persulfide bonds, a process designated sulfhydration [3] (Glossary). In addition to nitrosylation and sulfhydration, cysteines in a wide range of proteins can be modified by fatty acids. Most of these alterations, such as prenylation, farnesylation and geranylation, are semi-permanent, serving to anchor proteins to membranes. By contrast, palmitoylation is dynamic and turns over with a half-life as short as 1–2 h [4].

Lysine residues in a variety of intracellular proteins are also modified in response to neurotransmitter signaling systems. Acetylation of nuclear histones has long been known to

regulate transcription [5]. More recently, many non-nuclear, non-histone proteins have been shown to be acetylated, with this process being regulated by neuro-transmission [6]. Sumoylation involves the attachment of the 11-kDa protein SUMO (small ubiquitin-like modifier) to lysines in target proteins in a fashion analogous to ubiquitination. Recent studies implicate sumoylation in neural events, including neurological disorders such as Huntington's disease (HD) [7].

This review will focus upon recent advances in neural signaling via protein modification. Because of space constraints and many excellent previous reviews on phosphorylation and ubiquitination, we will not deal with these modifications here, but focus instead on alterations of cysteine and lysine residues by nitrosylation, sulfhydration, palmitoylation, sumoylation and acetylation.

## S-nitrosylation

Stamler and associates [8] pioneered the concept of nitrosylation as a signaling system. The biotin-switch technique, which can monitor basal levels of nitrosylation, has permitted demonstration that many brain proteins are physiologically nitrosylated, because nitrosylation is lost in neuronal NOS (nNOS)-deleted mice [9]. We will focus on a limited number of nitrosylation targets that illustrate specific themes (Table 1).

The NR2A subunit of the NMDA receptor (NMDAR), an ionotropic glutamate receptor, was one of the first characterized nitrosylation targets. Nitrosylation of cysteine residue 399 within NR2A inhibits NMDAR activity, which affords a negative feedback system for glutamate neuro-transmission [10,11]. NMDARs are linked via the scaffolding postsynaptic density protein 95 (PSD95) to nNOS. The PDZ ligand of NMDAR binds with a PDZ domain of PSD95 which, in turn, associates with the PDZ domain of nNOS [12]. This linkage affords a pathway whereby calcium ions, entering cells through NMDARs, bind to calmodulin, which is tightly associated with nNOS. Calmodulin activates nNOS, which leads to the generation of NO, which stimulates cyclic GMP formation. NO also feeds back to nitrosylate and inhibits NMDARs [13].

NMDARs function in close concert with another subtype of the ionotropic glutamate receptor family, AMPA receptors (AMPA). NMDARs and AMPARs are physically linked by transmembrane AMPA receptor regulatory proteins (TARPs) and PSD95 [14]. Synaptic plasticity, evident in long-term potentiation (LTP) and long-term depression (LTD), is regulated by the cycling of AMPARs into and out of the plasma membrane [15,16], a process which is modulated by several proteins. One of these, *N*-ethylmaleimide sensitive factor (NSF), enhances surface expression of AMPARs [16]. Its binding to the GluR2 subunit of AMPARs is disrupted by ATP degradation, consistent with the known ATPase function of NSF [16]. NSF is basally nitrosylated, with its nitrosylation abolished in nNOS-deleted mice [17]. NMDAR activation stimulates NO formation, with associated nitrosylation of NSF augmenting the binding of NSF to the GluR2 subunit, resulting in an enhancement of the surface expression of AMPARs [17].

Stargazin is an auxiliary subunit of AMPARs that is crucially involved in their surface expression [18]. As occurs with NSF, NMDAR transmission leads to nitrosylation of stargazin, increasing its binding to AMPARs (in this instance the GluR1 subunit), whose surface expression is thereby enhanced [19]. Thus, nitrosylation impacts crosstalk between ionotropic glutamate transmission at multiple levels.

Serine racemase (SR) generates D-serine, a coagonist with glutamate at NMDARs. Postsynaptic stimulation of NO formation feeds back to presynaptic cells to nitrosylate SR

and decrease D-serine availability to postsynaptic NMDARs [20]. SR knockout mice are less susceptible to NMDAR-mediated neurotoxicity and stroke [20].

NMDAR transmission is also influenced by nitrosylation of a small G-protein, Dexras1, a member of the Ras subfamily identified as a protein induced by the glucocorticoid dexamethasone [21]. Dexras1 binds to neuronal nNOS via the adaptor protein CAPON, eliciting nitrosylation and activation of Dexras1 [22]. Nitrosylation of Dex-ras1 physiologically stimulates iron uptake by DMT1 (Divalent Metal Transporter 1), which is linked to Dexras1 via a scaffolding protein PAP7 (PBR associated protein 7). Because selective iron chelation prevents NMDA neurotoxicity in cortical cultures, the NMDA-NO-Dexras1-PAP7-DMT1-iron uptake signaling cascade also appears to influence NMDA neurotoxicity [23].

In addition to its role in the physiological transmission of information, NO in excess is pathophysiological. NO is generated in the periphery largely in response to activation of inducible NOS (iNOS), whereas in the brain such stress involves NMDARs activating nNOS. The large amount of NO generated by iNOS in response to cell stressors facilitates the inflammatory response initiated by macrophages and other cells and can also lead to cell death [24]. Overactivation of nNOS by NMDAR activation during cerebral ischemia triggers NO formation which is cytotoxic [24]. Cell death can be mediated by a novel signaling cascade involving nitrosylation. The generated NO nitrosylates the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) at cysteine-150, abolishing catalytic activity and conferring upon GAPDH the ability to bind to the E-3 ligase Siah1 [25]. The nuclear localization signal of Siah1 enables it to ferry GAPDH to the nucleus where events involving another protein modification, acetylation (to be discussed below), lead to cell death. GOSPEL, a 52-kDa cytosolic protein, physiologically binds GAPDH in competition with Siah, retaining GAPDH in the cytosol and preventing its nuclear translocation (Figure 1). GOSPEL is neuroprotective, because its overexpression prevents NMDA-glutamate excitotoxicity, whereas its depletion enhances death in primary neuron cultures [26]. S-nitrosylation of GOSPEL at cysteine 47 enhances GAPDH-GOSPEL binding and the neuroprotective actions of GOSPEL [26].

The GAPDH-NO signaling system might participate in the pathophysiology of neurological diseases. GAPDH nitrosylation in the brain is augmented in the mouse MPTP model of Parkinson's disease (PD) [25]. MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) is a neurotoxin that causes symptoms of PD by destroying dopamine neurons in the substantia nigra. Deprenyl, a monoamine oxidase inhibitor used to treat PD, is neuroprotective independently of monoamine oxidase inhibition. Its neuroprotective actions reflect its potent inhibition of GAPDH nitrosylation, which is demonstrable in the brains of MPTP-treated mice [27].

Nitrosylation could also impact PD via parkin, an ubiquitin E3 ligase whose mutation elicits an autosomal-recessive form of PD [28]. The E3 ligase activity of parkin, which is neuroprotective, stems from the ring finger domain of the protein, which contains several conserved cysteines. In MPTP parkinsonism and in brains of patients with PD, parkin is nitrosylated, which impairs its E3 ligase activity and leads to neuroprotective effects [29].

Several enzymes exert denitrosylase activity, one of which is superoxide dismutase 1 (SOD1) [30]. Mutations of SOD1 are responsible for some familial cases of amyotrophic lateral sclerosis (ALS) via a toxic gain-of-function. Also, thioredoxin/thioredoxin reductases can elicit both basal and stimulus-coupled protein denitrosylation, a process regulated dynamically by a thioredoxin-interacting protein (Txnip), which inhibits thioredoxin activity [31].

Protein-disulfide isomerase (PDI) prevents protein mis-folding by preserving appropriate alignment of crucial disulfide bonds. Nitrosylation of PDI inhibits this process, and increased levels of nitrosylated PDI accumulate in brains of patients with PD and Alzheimer's disease (AD) [32]. NO might also influence mitochondrial fission with neuropathological consequences. Nitrosylation of Drp-1 enhances its dimerization and GTPase activity, which facilitates mitochondrial fission [33]. Increased nitrosylation of Drp-1 has been associated with neuronal injury and has been observed in AD patients [33].

NO generated by iNOS plays a prominent role in inflammation. It also regulates prostaglandin formation by cyclo-oxygenase-2 (COX2) via nitrosylation [34]. Thus, iNOS binds selectively to COX2 with the generated NO nitrosylating COX2 and activating prostaglandin formation [34]. NMDA neurotoxicity appears to involve an analogous process, because NMDA stimulation augments nNOS-COX2 binding and prostaglandin formation. Selective disruption of nNOS-COX2 binding prevents NMDA neurotoxicity [35].

Nuclear factor-kappa B (NF- $\kappa$ B) is a dimeric transcription factor composed of two members, p50 and RelA/p65 [40]. It is essential for neuronal survival and its activation could protect neurons against oxidative-stresses or ischemia-induced neurodegeneration [36–38], although its activation can contribute to inflammatory reactions and apoptotic cell death after brain injury and stroke [37,39]. Nitrosylation of the p65 subunit inhibits NF- $\kappa$ B-dependent gene transcription, and nuclear levels of nitrosylated p65 correlate with decreased DNA binding of the p50–p65 heterodimer [40].

## Sulfhydration

Because of its high chemical reactivity, H<sub>2</sub>S has long been studied for its toxic actions [41–43]. Until recently, there was no definitive evidence for its physiological formation in mammalian tissues. H<sub>2</sub>S can be generated from cysteine by the actions of cystathionine  $\gamma$ -lyase (cystathionase; CSE) [42–45] or cystathionine  $\beta$ -synthase (CBS) [46]. In mice with targeted deletion of CSE, H<sub>2</sub>S formation is abolished in numerous peripheral tissues but not the brain [47]. In brain, CBS is highly expressed in the hippocampus and the cerebellum and appears primarily responsible for H<sub>2</sub>S formation [48].

Here, we will focus on the biosynthesis of H<sub>2</sub>S, its signaling mechanisms and on a limited number of neural H<sub>2</sub>S targets. Influences of H<sub>2</sub>S upon neuronal activity in the brain have been explored extensively by Kimura and colleagues [49]. This group noted that physiological concentrations of H<sub>2</sub>S enhance LTP, and that this effect is abolished by NMDA antagonists [48]. In addition to its actions upon neurons, H<sub>2</sub>S also appears to influence astrocytes [50].

H<sub>2</sub>S might also serve as a neuroprotectant. Glutamate neurotoxicity in brain cultures involves, at least in part, inhibition of cystine uptake [51]. The cystine/glutamate anti-porter couples influx of cystine, an H<sub>2</sub>S precursor, with efflux of glutamate. H<sub>2</sub>S can inhibit monoamine oxidase, leading to norepinephrine elevations in the hippocampus, striatum and brainstem but not in the cortex and cerebellum [52]. H<sub>2</sub>S ameliorates hippocampal damage caused by febrile seizures [53] and it has been demonstrated to be neuroprotective in a 6-hydroxydopamine model of PD [54]. H<sub>2</sub>S appears to signal predominantly by sulfhydrating cysteines in its target proteins, analogous to S-nitrosylation by NO [3,55]. For instance, GAPDH, whose catalytic activity is abolished by nitrosylation at C150, is sulfhydrated at the same position, with sulfhydration resulting in a 700% enhancement of catalytic activity [3,55]. Actin is also sulfhydrated physiologically. H<sub>2</sub>S donors augment actin polymerization more than 30% [3,55], an effect reversed by dithiothreitol (DTT), which abolishes sulfhydration. Sulfhydration also leads to rearrangement of the actin cytoskeleton [55]. These actions could regulate the influence of actin on synaptic dynamics.

## Palmitoylation

Palmitoylation is one of four principal types of lipid modifications of proteins. The other three lipid modifications are isoprenylation [56] and myristoylation [57] of cytosolic proteins and modifications of membrane proteins by glycosphosphatidyl inositol (GPI). However, because palmitoylation is quantitatively much more prominent than these other modifications, most authors prefer to use the term 'palmitoylation' to refer to all four of these lipid modifications, rather than the more accurate term of S-acylation.

Here, we will address only palmitoylation reactions of particular relevance to synaptic transmission (Table 2). Palmitoylation impacts several proteins of the synaptic vesicle fusion apparatus. Vesicular exocytosis involves interactions of a family of SNARE (soluble N-ethylmaleimide-sensitive fusion protein-attachment protein receptor) proteins present on the membranes of vesicles and on the plasma membrane. The most prominent vesicular SNARE is termed synaptobrevin-2 (VAMP2) [58], whereas SNAP23/25 (Synaptosomal-associated protein 23/25) and syntaxin 1 [58] are membrane-associated SNAREs. All of these exocytotic proteins appear to be palmitoylated [58–62]. Whereas syntaxin 1 and VAMP2 have membrane-spanning domains, SNAP25 is a soluble protein that requires palmitoylation on a cluster of cysteine residues to facilitate stable membrane attachment [59,60].

Voltage-dependent potassium ion channels and G-proteins have been demonstrated to be palmitoylated [63]. Activation of beta-adrenergic receptors markedly accelerates the depalmitoylation of  $G\alpha_s$ , which translocates the G-protein from the membrane to a cytosolic localization. This increases its interactions with RGS proteins (Regulators of G-protein Signaling), whose GTPase-stimulating activity further diminishes G-protein signaling [64,65]. Adrenergic agonists elicit depalmitoylation of beta-adrenergic receptors, which promotes their phosphorylation and internalization via  $\beta$ -arrestin [66]. The influence of palmitoylation on G-protein coupled receptors can vary, with depalmitoylation of alpha-adrenergic receptors preventing the reduction in number of receptors associated with desensitization [67].

Ionotropic glutamate receptors are also regulated by palmitoylation. The NMDAR subunits NR2A and NR2B have two separate clusters of palmitoylation sites. Palmitoylation in the first cluster has been shown to enhance tyrosine phosphorylation by Src kinases, leading to increased surface expression [68,69]. Palmitoylation of the second cluster, evidently by distinct enzymes, elicits receptor accumulation in the Golgi apparatus, with reduced NMDAR surface expression [68,69].

AMPA receptors are also palmitoylated in two domains: one in the second transmembrane domain at cysteine-610 and another in the juxtatransmembrane region of the C-terminal cytoplasmic tail at cysteine-836 of GluR2 [69]. Juxta-membrane palmitoylation does not directly affect AMPAR trafficking to the cell surface but does decrease interaction with the protein band 4.1N [69]. Depalmitoylation of the second site of GluR1 stabilizes surface expression through interactions with protein 4.1N [70].

Palmitoylation is catalyzed by a family of palmitoyl acyl transferases (PATs), most if not all of which display a consensus sequence aspartate-histidine-histidine-cysteine (DHHC) [71]. One of these, DHHC3, is a potential PAT for palmitoylating the transmembrane domain site of AMPAR, which could negatively regulate AMPAR trafficking from the Golgi complex to the plasma membrane [69]. Palmitoylation of PSD95 is particularly dynamic and regulates the surface expression of AMPARs [71]. PSD95 is palmitoylated at cysteines-3 and -5 at the N-terminus of the protein. Their mutation, which prevents palmitoylation, markedly diminishes surface expression of AMPAR [72]. Turnover of palmitate on PSD95 during

synaptic activity is rapid with a half-life of under an hour [72], consistent with a role in regulating synaptic plasticity. By contrast, palmitate associated with synaptotagmin-2 and SNAP25 turns over very slowly and presumably functions primarily as a structural signal [73]. DHHC3 and DHHC2 are both required for postsynaptic accumulation of PSD95, but only DHHC2 mediates palmitoylation of PSD95 in response to reduced synaptic activity [74]. Suppression of neuronal activity rapidly mobilizes dendritic DHHC2 close to the postsynaptic membranes, thereby inducing robust palmitoylation and enhancing synaptic accumulation of PSD95. The increase in synaptic AMPARs elicited by blockade of synaptic activity requires DHHC2-mediated PSD95 palmitoylation [74].

DHHC3 has also been implicated in GABA<sub>A</sub> receptor (GABA<sub>A</sub>R)-mediated inhibitory synaptic transmission. Palmitoylation of the  $\gamma 2$  subunit of GABA<sub>A</sub>Rs occurs on cysteine residues in the large intracellular loop and is required for efficient postsynaptic clustering of GABA<sub>A</sub>R at inhibitory synapses [75,76]. Gephyrin, a scaffolding protein for glycine receptors and GABA<sub>A</sub>Rs, is expected to be palmitoylated, as indicated by synaptic palmitoyl proteome analysis [71,77]. Similarly to PSD95 at excitatory synapses [71], palmitate cycling on gephyrin might modulate the efficacy of GABA<sub>A</sub>R-mediated synaptic transmission in an activity-dependent manner.

Palmitoylation might also impact synaptic transmission through direct effects on dendritic spine morphology. Glutamatergic stimulation induces rapid depalmitoylation of Cell Division Control Protein-42 Homolog (CDC42), a member of the Rho family implicated in dendritic spine disposition. Depalmitoylation of CDC42 leads to its dislocation from dendritic spines in cultured hippocampal neurons, possibly contributing to rapid spine collapse [60,84].

DHHCs have also been implicated in neurological and psychiatric disorders. DHHC17 palmitoylates huntingtin and regulates its trafficking and other functions [83]. Moreover, palmitoylation of huntingtin is diminished in a mouse model of HD [83]. DHHCs have also been associated with schizophrenia. Microdeletions of the 22q11.2 locus, a common chromosomal abnormality, are associated with a high incidence of emotional problems, with approximately 30% involving schizophrenia or related disorders [78,79]. This area of the genome possesses 27 genes, one of which is DHHC8, a genetic variant of which is associated with schizophrenia [80]. Thus, psychiatric disturbances associated with 22q11.2 microdeletion might reflect a loss of DHHC8 and associated palmitoylation [81]. PSD95 appears to be a physiological substrate of DHHC8, because deletion of the enzyme markedly reduces endogenous palmitoylation of PSD95 [81]. The enzymology of depalmitoylation is as yet unclear, although candidate depalmitoylating enzymes have been reported [82].

## Sumoylation

Early studies focused on sumoylation of nuclear proteins, particularly those in nuclear promyelocytic leukemia protein (PML) bodies, whereas it is now appreciated that proteins in all intracellular compartments can be sumoylated [85]. Poly-ubiquitination in almost all instances targets proteins for degradation, whereas sumoylation has diverse functions including protecting proteins from ubiquitination, regulating protein-protein interactions, and transporting proteins between cytosol and the nucleus [86–88]. The sumoylation enzymatic pathway is analogous to ubiquitination, with SUMO attachment to proteins involving a series of enzymatic steps (Figure 2) [89–91].

Although regulation of nuclear events by sumoylation might have bearing on neural function, the best characterized neuronal targets are non-nuclear. Neural sumoylation targets are largely membrane proteins including transporters, neurotransmitter receptors, regulators of neuronal development, and proteins involved in the pathophysiology of

neurodegenerative diseases [92]. In the interest of brevity, we will focus only on a few examples, which highlight particular themes (Table 3).

In cerebellar neuronal preparations, myocyte enhancer factor-2 (MEF2) is required for dendritic development, specifically of dendritic claws, the branched tips of granule cell dendrites where glutamate inputs synapse [93]. Sumoylation of MEF2 enhances synapse formation, whereas replacement of the sumo group by an acetyl moiety reduces synaptic density [94].

At least two neuronal potassium channels are regulated by sumoylation. K2P1 channels are background plasma membrane leak channels whose sumoylation under basal conditions abolishes basal channel function [95], although there is some controversy about the importance of sumoylation for K2P1 [96]. The voltage-gated Kv1.5 potassium channel participates in microglial generation of NO following brain injury [97]. Selective mutations blocking its sumoylation markedly alter channel function [98].

Sumoylation influences a variety of glutamate receptors, including metabotropic receptors and the ionotropic kainate receptors. Sumoylation of the GluR6 subunit of kainate receptors is triggered by synaptic activation with glutamate or kainate [99]. Sumoylation leads to rapid internalization of GluR6-containing receptors, resulting in decreased synaptic activity. This is a physiological process, because the SENP1 desumoylating enzyme, which removes SUMO from proteins, increases spontaneous miniature excitatory post-synaptic currents [100]. Internalization of GluR6 is also enhanced by ubiquitination. However, GluR6 ubiquitination and sumoylation involve distinct lysine residues, although they are only three amino acids apart [99].

Sumoylation plays a role in neurodegenerative disorders, particularly those involving polyglutamine repeats, such as in HD and spinocerebellar ataxia (SCA). HD is caused by genetically dominant mutations in the gene encoding the protein huntingtin (Htt). HD is brain-specific, with profound abnormal movements related to selective, gross degeneration of the corpus striatum and lesser damage to the cerebral cortex eliciting dementia [100]. Sumoylation reduces pathology in *Drosophila* HD models, where it is more prominent than ubiquitination [101]. The striatal selectivity of HD pathophysiology could be explained by interactions of mHtt with the small G-protein Rhes (Ras Homologue Enriched in Striatum) [102]. Rhes binds mHtt with much greater avidity than wild-type Htt. Moreover, Rhes functions as an E3 ligase to stimulate sumoylation of mHtt [103]. Such sumoylation augments the neurotoxicity of mHtt by decreasing its aggregation [102]. Rhes is a major determinant of protein sumoylation in the striatum, because sumoylation of multiple proteins is markedly and selectively diminished in the striatum of Rhes-deleted mice [102]. Earlier studies identifying aggregation of mHtt had assumed that such aggregation was associated with neurotoxicity, whereas subsequent studies have established that the disaggregated soluble form of mHtt is most probably the pathogenic species [104]. Synaptic NMDAR activity induces mHtt inclusions via a T complex-1 (TCP-1) ring complex (TRiC)-dependent mechanism, rendering neurons more resistant to mHtt-mediated cell death. By contrast, stimulation of extrasynaptic NMDARs increases the vulnerability of mHtt-containing neurons to cell death by impairing the neuroprotective CREB-PGC-1 $\alpha$  cascade and increasing levels of Rhes [104].

SCA is a dominantly inherited progressive condition with atrophy of the Purkinje cell layer of the cerebellum associated with expanded polyglutamines in ataxin1. Ataxin1 is sumoylated at several lysines, with sumoylation diminished by the expansion of polyglutamines [105]. SCA type 3 patients and mouse models of SCA all display substantial levels of SUMO1 in affected tissues [106].

PD might also be impacted by sumoylation. Alpha-synuclein is mutated in certain familial forms of the disease. It is known to be sumoylated, although the role of sumoylation in alpha-synuclein function has not been clarified [107]. Parkin is an E3 ubiquitin ligase associated with autosomal-recessive juvenile parkinsonism [107]. SUMO interacts non-covalently with parkin and increases its ubiquitin-E3-ligase activity [108]. Parkin regulates the turnover of the SUMO E3 ligase RanBP2 by catalyzing its ubiquitination and proteasomal degradation [109]. DJ-1 is a multifunctional protein involved in the transcriptional regulation of genes [110]. Loss-of-function in DJ-1, either by deletion or point mutation, leads to early onset of PD [111]. DJ-1 is modified by SUMO1 and also interacts with members of the PIAS family [111], which act as SUMO E3 ligases.

## Acetylation and deacetylation

Protein acetylation in discrete brain areas can affect behavior. For instance, chronic social defeat stress in mice leads to a selective increase of acetylated histone H3 in the nucleus accumbens (Nac) [112]. The increased histone H3 acetylation is associated with decreased levels of histone deacetylase2 (HDAC2), and similar effects are detected in the nucleus accumbens of post-mortem depressed patients [112]. Infusion of HDAC inhibitors into the Nac elicits anti-depressant effects in the social defeat model [112]. Many other beneficial therapeutic effects have been suggested for HDAC inhibitors [113,114].

Brain-derived neurotrophic factor (BDNF) influences multiple genetic programs that are important for synaptic plasticity [115]. Recently, it was shown that nitrosylation of HDAC2 induces its release from chromatin, which increases acetylation of histones surrounding neurotrophin-dependent gene promoters and promotes transcription [116].

There has been increasing appreciation that many non-nuclear proteins are acetylated. A broad screen identified 3500 acetylation sites in 1700 acetylated proteins with the resultant 'acetylome' approaching the size of the 'phosphoproteome' [117]. Most of the acetylated proteins were found to be non-nuclear. Cell death, which is influenced by histone acetylation, is also regulated by acetylation of the non-nuclear protein GAPDH. As described above, a NO-GAPDH-Siah1 signaling cascade leads to nuclear translocation of GAPDH and cell death. What nuclear events elicit the associated apoptosis? In the nucleus, the acetylating enzymes p300/CBP acetylate GAPDH, causing it to activate auto-acetylation of p300/CBP, which, in turn, acetylate and activate p53 (Figure 1) [118]. Thus, it appears that the 'executioner' of the NO/GAPDH pathway is acetylated and activated p53.

Acetylation directly regulates actions of neurotoxic proteins. Acetylation of mHtt at lysine-444 facilitates its trafficking into autophagosomes, leading to clearance of mHtt by autophagy, thereby reversing the toxic effects of mHtt in rat primary striatal and cortical neurons, as well as in a *Caenorhabditis elegans* model of HD [119]. mHtt that is resistant to acetylation at this position accumulates to high levels and leads to neurodegeneration [118]. In SCA, polyglutamine proteins inhibit transcription, either by directly inhibiting histone acetylase co-activator activity, or as a consequence of histone 'masking' by binding to histones or blocking the sites that are available for acetylation [120].

## Concluding remarks

It has long been known that neurotransmitters signal through a variety of secondary and tertiary messengers to impact a myriad of cellular functions including nuclear ones. The advances described here show that transmitters themselves, particularly gasotransmitters such as NO and H<sub>2</sub>S, directly modify target proteins. The extent of such modifications is substantial, with sulfhydrylation impacting up to 25% of certain proteins and with a major



portion of all proteins examined being sulfhydrated [55]. Thus, S-nitrosylation and sulfhydration could be as prevalent and as functionally important as phosphorylation.

Increasingly appreciated are interactions between the various post-translational modifications which could have bearing on neurotransmission (Box 1). For example, cysteines are modified by palmitoylation as well as nitrosylation and sulfhydration, and there is early evidence that nitrosylation and palmitoylation are reciprocal processes. Modifications of lysines, particularly acetylation and sumoylation, might also display reciprocity, although there is thus far only modest evidence. The prevalence of sumoylation and acetylation of non-nuclear proteins is striking, as is the occurrence of these modifications in disease-related proteins, as exemplified by the role of mHtt sumoylation in the neurotoxicity of HD. As these modifications are further characterized, clinical relevance is probable, both for understanding pathophysiology and for the development of new therapeutic agents.

### Box 1

#### Outstanding questions

- Excessive activation of NMDARs has been implicated in neuronal damage in many neurological disorders, ranging from acute hypoxic-ischemic brain injury to chronic neurodegenerative diseases that include Parkinson's disease, Huntington's disease, Alzheimer's disease, amyotrophic lateral sclerosis, and HIV-associated dementia. Might post-translational modifications of NMDARs, particularly nitrosylation, play a role in these diseases?
- Nitrosylation and sulfhydration generally involve the same cysteines in proteins and seem to be functionally counterbalanced. Are the two modifications dynamically and reciprocally regulated in the brief time span of synaptic events?
- Post-translational modifications occur on individual cysteine, serine, and lysine residues within proteins. Because multiple modifications can occur on each of these amino acids, how do the combinational interactions between the different modifications regulate synaptic signaling?
- A striatal specific protein, Rhes, sumoylates mutant Huntington (mHtt) and enhances its cytotoxicity by disaggregating and solubilizing mHtt. Might drugs that prevent Rhes-mHtt binding retard the progression of Huntington's disease?

## Acknowledgments

This work was supported by United States Public Health Services (USPHS) grants DA-000266 and MH-18501 and Research Scientist Award DA-00074 to S.H.S.

## Glossary

### Acetylation

histone acetylation involves the attachment of acetyl groups to lysine residues in the N-terminal tails of histone proteins. It is believed that the acetylation of lysine residues decreases the affinity of histones for DNA, thereby making DNA more accessible for transcription. The opposite reaction (deacetylation) removes acetyl groups from lysine residues in the N-terminal tails of histone proteins. A large number of non-histone proteins are also acetylated to control diverse cell processes

<b>Biotin Switch assay</b>	demonstration of physiological nitrosylation of numerous proteins under basal conditions by endogenously generated NO was rendered feasible by development of the biotin switch assay. In this procedure free thiols are blocked by the sulfhydryl-reactive compound, methyl methane thioisulfonate; the nitrosylated thiols are then exposed by treatment with ascorbate, labeled with biotin, coupled to streptavidin, and nitrosylated proteins are separated by gel electrophoresis
<b>Palmitoylation</b>	the most prominent modification of cysteines in proteins is palmitoylation, Protein palmitoylation is an enzymatic reaction, which is mediated by palmitoyltransferases (PAT). Palmitate, a 16-carbon fatty acid, forms an ester linkage with free thiols of cysteines. Other fatty acids, such as oleate, arachidonate, myristate and stearate can also attach in a similar manner to cysteine residues in proteins, however, these forms of modifications are beyond the scope of the current review
<b>S-nitrosylation</b>	nitric oxide (NO) is produced enzymatically in most or all cell types and tissues. Addition of an NO group to the thiol side chain of cysteine residues within proteins and peptides is termed S-nitrosylation. Nitrosylation provides an NO 'cap' to the reactive sulfhydryl of cysteines in proteins, thus limiting chemical reactivity of this side chain
<b>Sulfhydration</b>	sulfhydration (SHY) is a physiological process wherein hydrogen sulfide (H <sub>2</sub> S) attaches an additional sulfur to the thiol (-SH) groups of cysteine residues within proteins, yielding a hydropersulfide group (-SSH). The process is mediated by H <sub>2</sub> S enzymatically generated from L-cysteine by cystathione $\gamma$ -lyase (CSE) or cystathionine $\beta$ -synthase (CBS). This is not to be confused with S-thiolation or S-thionylation, in which a protein thiol forms a mixed disulfide with a small-molecular weight thiol such as glutathione or cysteine. S-thiolation blocks the protein thiol rendering it non-reactive, whereas S-sulfhydration yields an -SSH moiety which has enhanced chemical reactivity
<b>Sumoylation</b>	SUMO (small ubiquitin-related modifier), also known as 'centrin', is a 101 amino acid protein. In a similar fashion to ubiquitin, SUMO can be covalently attached to the $\Sigma$ -amino group of lysine side chains on proteins via an enzymatic reaction known as sumoylation. Four mammalian SUMO isoforms have been identified (SUMO 1-4), although their differential functions are not altogether clear yet

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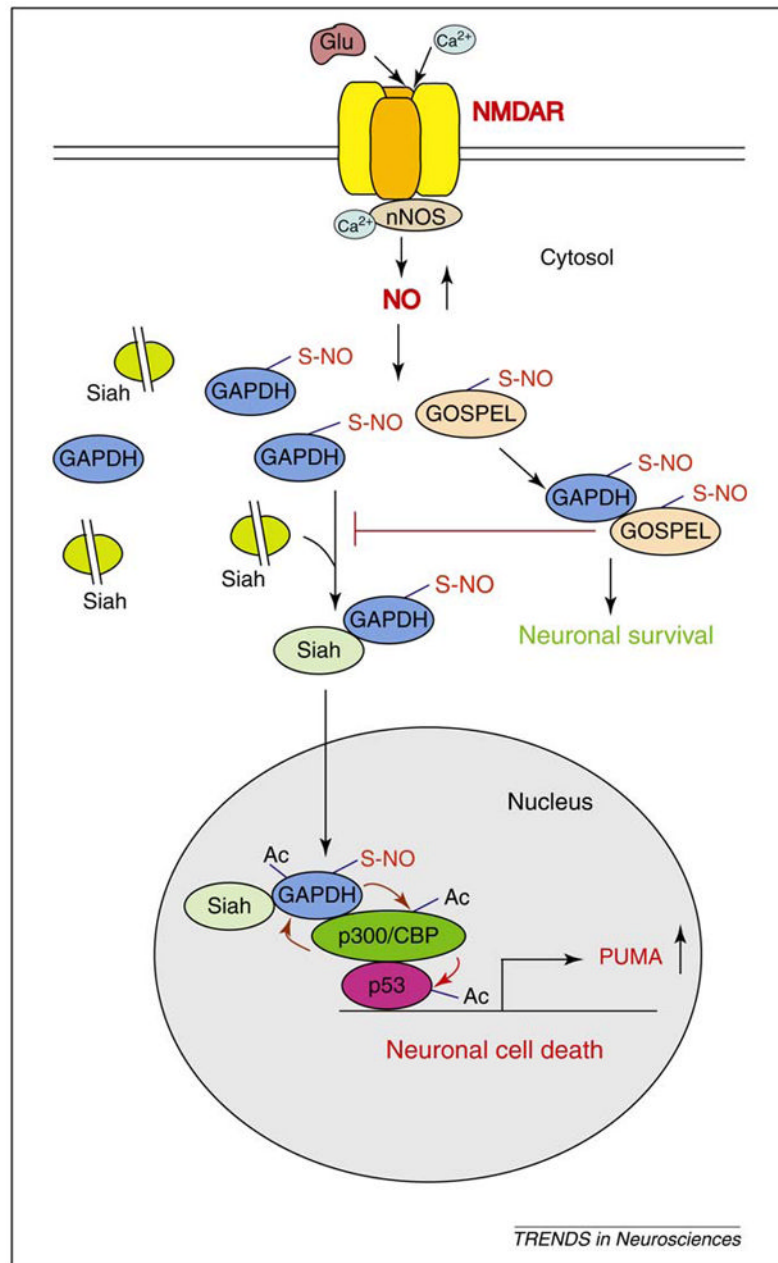
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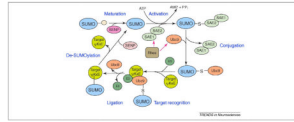


**Figure 1.**

An apoptotic cell death cascade involving S-nitrosylation (S-NO) of GAPDH (glyceraldehyde-3-phosphate dehydrogenase), its nuclear translocation and acetylation of nuclear targets. Diverse apoptotic stimuli, including the pathological overactivation of NMDARs, can lead to the activation of nNOS. The subsequent generation of NO results in the S-NO of GAPDH, which abolishes its catalytic activity and confers on it the ability to bind to Siah1 (seven in absentia homolog 1), an E3-ubiquitin-ligase with a nuclear localization signal (NLS). Siah1, a protein with a rapid turnover rate, is degraded by the ubiquitin proteasome system (represented by Siah with dashed line in between two parts) so that under basal conditions tissue levels are extremely low. GAPDH stabilizes the rapidly turning over Siah1 (oval shaped without dashed line), augmenting its nuclear levels. The GAPDH-Siah1 protein complex, in turn, translocates to the nucleus, where GAPDH is



acetylated at lysine residue 160 by the acetyltransferase p300/CREB binding protein (CBP) through direct protein interaction, which, in turn, stimulates the acetylation and catalytic activity of p300/CBP. Consequently, downstream targets of p300/CBP, such as p53, are activated and cause neuronal cell death through the transcriptional activation of apoptotic-inducing genes such as PUMA (p53 upregulated modulator of apoptosis). PUMA is a pro-apoptotic member of the Bcl-2 protein family which mediates apoptosis associated with p53 activity. GOSPEL (GAPDH's competitor Of Siah Protein Enhances Life), physiologically binds GAPDH, in competition with Siah, retaining GAPDH in the cytosol and preventing its nuclear translocation. S-nitrosylation of GOSPEL enhances GAPDH-GOSPEL binding and the neuroprotective actions of GOSPEL.



**Figure 2.**

The SUMO modification cycle. SUMO precursor proteins contain a C-terminal extension which is cleaved by SUMO-specific carboxyl-terminal hydrolase activity of a sentrin-specific protease (SENP) enzyme to produce a carboxyl-terminal diglycine motif in a process called ‘SUMO maturation’. The mature SUMO is activated by an E1 SUMO activating enzyme (SAE1/2) which utilizes ATP and links the C-terminal glycine of SUMO by a thioester bond to a cysteine in the E1 activating enzyme. During the conjugation step, the SUMO moiety is transferred from the E1 activating enzyme to Ubc9, the SUMO E2 enzyme, which then attaches the SUMO to a lysine residue in the target protein that is typically, but not always, found within the consensus sequence  $\Psi$ KxE ( $\Psi$  represents hydrophobic amino acids). SUMO E3 proteins stimulate protein sumoylation by associating with both UBC9 and substrates to increase the efficiency of the modification reaction by a process called target recognition and ligation. The SUMO E3 proteins identified to date include members of the family of Protein Inhibitor of Activated STAT (PIAS) family of proteins (PIAS1, PIAS3, PIASx and PIASy), the polycomb protein 2 (Pc2), and Ran-binding protein 2 (RANBP2). SUMO groups are removed from proteins by SENPs, of which there are six human forms. Rhes, a small G-protein, is selectively localized to the corpus striatum and binds directly to both E1 and Ubc9, enhancing cross-sumoylation as well as thioester transfer from E1 to Ubc9.

**Table 1**

## Neural roles of nitrosylation

Protein targets	Alteration in function	Physiological/pathophysiological relevance	Refs
COX2	Increase in activity, binds nNOS	Decrease in NMDA-mediated neurotoxicity	[34,35]
Dexas	Increase in activity	Enhanced iron uptake and NMDA-mediated neurotoxicity	[23]
Drp1	Increase in GTPase activity and mitochondrial fission	Increase in cell death in Alzheimer's disease	[33]
GAPDH	Decrease in activity, nuclear translocation	Increase in cell death in MPTP parkinsonism	[25,27]
GOSPEL	Increase in binding to GAPDH, prevent nuclear translocation of GAPDH	Decrease in cell death in NMDA-mediated neurotoxicity	[26]
NMDAR	Decrease in activity	Decrease in NO production	[10]
NSF	Increase in activity and decrease in platelet granules	Increase in AMPAR surface expression	[17]
Parkin	Decrease in activity	Increase in cell death in Parkinson's disease and Alzheimer's disease	[28]
Stargazin	Increase in interaction with AMPAR	Increase in surface expression of AMPAR	[19]
Serine racemase	Decrease in activity	Increase in stroke	[20]

COX2, cyclo-oxygenase-2; Drp1, dynamin related protein-1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GOSPEL, GAPDH's competitor of Siah protein enhances life; NMDAR, *N*-methyl-D-aspartate receptor; NSF, *N*-ethylmaleimide sensitive factor; AMPAR,  $\alpha$ -amino-3-hydroxyl-5-methyl-4-isoxazole-propionate receptor; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine.

**Table 2**

## Neural features of palmitoylation

Protein targets	Protein family	Palmitoylating enzyme	Physiological/pathophysiological relevance	Refs
$\beta_2$ -adrenergic receptor	G-protein coupled receptor	ND	Regulates phosphorylation and internalization of $\beta$ -arrestin	[66]
CDC42	Small GTPase	ND	Neurite outgrowth and dendritic spine formation	[60,62]
GABA <sub>A</sub> R ( $\gamma 2$ subunit)	Neurotransmitter	DHHC 3,7	Inhibition of synaptic transmission	[76]
AMPA (GluR1, GluR2 subunits)	Ion channel	DHHC 3	Regulates AMPAR surface expression	[69]
G <sub>aq</sub> , G <sub>as</sub>	G-protein signaling	DHHC 7	Regulates G-protein assembly and activity	[64,65]
Huntingtin	Polyglutamine	DHHC 17	Huntington's disease	[83]
NMDAR	Neurotransmitter receptor	ND	Enhances tyrosine phosphorylation and increases surface expression	[68]
PSD95	Scaffolding protein	DHHC 2, 15, 3, 7	Synaptic targeting and postsynaptic protein assembly	[71]
SNAP25	Vesicle trafficking	DHHC 17	Neurotransmitter release, Huntington's disease	[59]
Synaptobrevin	Vesicle trafficking	ND	Multimerization of synaptobrevin	[60]

GABA<sub>A</sub>R, gamma-aminobutyric acid receptor; DHHC, aspartate-histidine-histidine-cysteine; NMDAR, *N*-methyl-D-aspartate receptor; PSD95, postsynaptic density protein 95; SNAP25, synaptosomal-associated protein 25; CDC42, cell division control protein 42; AMPAR,  $\alpha$ -amino-3-hydroxyl-5-methyl-4-isoxazole-propionate receptor; ND, not determined.

**Table 3**

Impact of sumoylation on neuronal activity

<b>Protein targets</b>	<b>Physiological/pathophysiological relevance</b>	<b>Refs</b>
$\alpha$ -Synuclein	Pathophysiology of Parkinson's disease	[105]
Ataxin1	Pathophysiology of SCA type 1	[104]
DJ-1	Pathophysiology of Parkinson's disease	[109]
Kainate receptor (GluR6 subunit)	Regulates kainate receptor endocytosis and synaptic activity	[98]
mHtt	Solubilization of huntingtin and increase in toxicity	[101,102]
MEF2	Regulates cerebellar granule cell differentiation	[94]
Parkin	Pathophysiology of Parkinson's disease	[106]
Potassium channel (K2P1)	Microglial cell proliferation, production and release of NO following brain injury	[96]

mHtt, mutant Huntingtin; MEF2, myocyte enhancer factor-2; SCA type 1, spinocerebellar ataxia type 1.