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Glutathione and redox signaling in substance abuse

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Abstract

Throughout the last couple decades, the cause and consequences of substance abuse has expanded to identify the underlying neurobiological signaling mechanisms associated with addictive behavior. Chronic use of drugs, such as cocaine, methamphetamine and alcohol leads to the formation of oxidative or nitrosative stress (ROS/RNS) and changes in glutathione and redox homeostasis. Of importance, redox-sensitive post-translational modifications on cysteine residues, such as *S*-glutathionylation and *S*-nitrosylation could impact on the structure and function of addiction related signaling proteins. In this commentary, we evaluate the role of glutathione and redox signaling in cocaine-, methamphetamine- and alcohol addiction and conclude by discussing the possibility of targeting redox pathways for the therapeutic intervention of these substance abuse disorders.

Keywords

Oxidative stress; Redox; *S*-glutathionylation; Glutathione-*S*-transferase Pi; Glutathione; Addiction

1. Oxidative-nitrosative stress signaling

Post-translational modification (PTM) of proteins leads to the formation of sub-proteomes that have evolved a high degree of functional redundancy through reversible cycling reactions. Among the most widely studied PTM is phosphorylation and as such, kinase pathways have been generally accepted as the cornerstones of signal transduction. Cellular homeostasis is regulated through reduction and oxidation (redox) reactions resulting from the transfer of electrons from one species to another. The formation of oxidative and nitrosative species (ROS/RNS) are a consequence of redox reactions and are important to physiology and their dysregulation is attributed to pathology [1,2]. Cysteine residues within

proteins are subject to PTM following exposure to ROS/RNS. Fig. 1 represents cysteine modifications attributed to ROS/RNS known to date. Drugs of abuse are known to lead to the formation of ROS/RNS and alter neuronal function and thereby behavior. The impact of redox-mediated PTMs is poorly understood in the drug addiction field. In this review, the consequences of *S*-glutathionylation and *S*-nitrosylation on redox sensor proteins will be evaluated in models of substance abuse, specifically cocaine-, methamphetamine (METH)-, and alcohol addiction. In addition, we will discuss the possibility of targeting redox pathways for the pharmacotherapeutic intervention of addiction.

2. Redox-mediated post-translational modifications

2.1. *S*-glutathionylation

S-glutathionylation is a redox-mediated PTM whereby glutathione (GSH) is conjugated to cysteine residues (P-SSG), leading to an increase of 305 Da and a net negative charge that can impact on protein structure and function. Not all cysteine residues are targets for *S*-glutathionylation. Rather, cysteine residues with a low pK_a values have the nucleophilicity that can be oxidized under oxidative/nitrosative conditions to a thiyl radical ($RS^{\bullet}$) with strong reactivity toward oxygen followed by the addition of GSH, Fig. 1. *In vitro* studies have shown that *S*-glutathionylation reactions can occur spontaneously with ROS/RNS, generating compounds in the presence of GSH. In cells, a small number of enzymes has been shown to promote the forward reaction of *S*-glutathionylation, *e.g.* thiolase activity.

A large proportion of *S*-glutathionylated proteins that have been identified are compartmentalized within the cell and subcellular organelles [1-3]. However, evidence suggests that extracellular and cell surface proteins are *S*-glutathionylated and impacts on the proliferative/apoptotic balance. Glutathione is unable to diffuse through the cell membrane. Gamma-glutamyl-transpeptidase (GGT) is a cell surface enzyme that cleaves GSH into glutamate and cysteinyl-glycine (CG) that can more readily enter cells. GGT impacts on intracellular redox homeostasis through GSH biosynthesis and also plays an active role in the *S*-glutathionylation cycle [4,5]. Both GSH and CG have been shown to form mixed-disulfides in proteins in the presence of activated GGT [4,5]. Acivicin, a GGT inhibitor, decreased the formation of protein mixed disulfide formation. The specificity and targets of GGT-mediated *S*-glutathionylation remains poorly understood despite the key role that GGT plays in the liver and at the blood brain barrier (BBB). Psychostimulants, including methamphetamine and cocaine, and chronic alcohol [6] compromise the BBB, antioxidant capacity and potentially redox signaling [7].

Glutathione-*S*-transferase (GST) is a family of phase II detoxification enzymes that promote the conjugation of GSH to electrophilic compounds. The GST superfamily is composed of 7 classes which share > 70% homology and have overlapping substrate specificity [8]. The pi class of GSTs (GSTP1) was the first to emerge as regulators of redox-mediated kinase signaling [9-11]. Specifically, it was shown that GSTP binds directly to *c*-Jun *N*-terminal kinase (JNK) and prevents kinase activity, thereby modulating proliferation and apoptosis. The protein binding function of GSTP regulates ROS/RNS-induced kinase signaling in a mechanism distinct from *S*-glutathionylation reactions [12,13]. The catalytic activity is required for *S*-glutathionylation of a wide range of proteins in cells and *in vivo*. GSTP

appears to have a multitude of molecular targets and thereby may function as a promiscuous *S*-glutathionylase.

S-glutathionylation of peroxiredoxin (Prdx) and Fas ligand is attributed to GSTP-thiolase activity [14-16]. Peroxiredoxins are antioxidant enzymes that detoxify hydrogen peroxide through oxidation–reduction reactions with Thioredoxin (Trx), as follows:

Peroxiredoxin VI (Prdx6) is a bifunctional enzyme with two cysteine containing active sites that function to reduce hydrogen peroxide and short chain organic, fatty acids and phospholipid hydrogen peroxides. Prdx6 regulates phospholipid turnover and protects the cell against oxidative stress induced injury. Manevich et al. (2004) showed that heterodimerization of GSTP1 with Prdx6 results in *S*-glutathionylation of the sulfinic acid and restoration of peroxidase activity [16]. Polymorphisms within GSTP (GSTP1*A–D) exist that have functional consequences thereby affecting disease susceptibility and response to oxidative and nitrosative stress, (review [8]). Manevich et al. (2012) was the first to show that polymorphisms within GSTP impact *S*-glutathionylation and lipid peroxidation [13]. Specifically, it was shown that GSTP1*A and GSTP1*C had a lower binding affinity (Kd) for Prdx6 and altered lipid peroxidation. The clinical implication of polymorphisms of GSTP are recognized and attributed to its role in phase II metabolism. Polymorphisms of GSTP regulate drug response to compounds that are not substrates for phase II metabolism. These findings suggest that the protein thiolase activity of GSTP may impact on an individual's response to ROS/RNS-mediated signal transduction. The role of GSTP on the *S*-glutathionylation cycle remains poorly understood clinically but likely supersedes its role in metabolism.

S-glutathionylation reactions control opposing pathways including but not limited to apoptotic, proliferative and protective. While *S*-glutathionylation of Prdx6 is protective, the second protein has been identified as a GSTP target amplifies apoptosis. The death receptor Fas is *S*-glutathionylated on Cys294 (Fas-SSG) prior to the onset of apoptosis [14,17]. Fas is a member of the tumor necrosis receptor superfamily such that the binding of ligand leads to the downstream activation caspase 8- or 10- mediated apoptosis. While GSTP is considered a cytosolic protein, Anathy et al. (2012) showed that GSTP forms a protein complex with Fas and ERp57 in the endoplasmic reticulum and leads to Fas-SSG and subsequent mobilization from the ER to the cytosol [14]. Inhibition of GSTP with the specific inhibitor TLK199 diminished the thiolase activity of GSTP, decreased Fas-SSG and caspase activation in epithelial cells. Pharmacologic and genetic modulation of GSTP showed that GSTP-mediated *S*-glutathionylation of Fas plays a role in a bleomycin model of acute lung injury [14]. These studies are important and unveil a mechanism through which non-cytosolic proteins are redox regulated.

Redox signaling events are triggered under physiological levels of oxidative and nitrosative stress and the temporal fluxes in homeostasis are restored through de-glutathionylation of redox sensors and increased GSH/GSSG levels. In the forward reaction of the *S*-glutathionylation cycle, GSTP plays a role as a generalist with a multitude of target proteins while Grx and GGT may have more specialized roles. This paradigm follows through in the reverse reaction, de-glutathionylation. Glutaredoxin (Grx) mediates the removal of GSH through direct thiol-disulfide exchange reactions that lead to the formation of GSSG that is subsequently reduced by glutathione reductase. Grx is a generalist de-glutathionylase with a broad spectrum of substrates known to be dysregulated multiple pathologies, (review [18]).

2.2. S-nitrosylation

Nitric oxide (NO) is an important messenger molecule that participates in a broad range of physiological processes. NO is generated in mammalian cells through enzymatic oxidation of *L*-arginine by nitric oxide synthase (NOS) in the presence of NADPH and oxygen. NOS exist in three isoforms, inducible (iNOS), endothelial (eNOS) and neuronal (nNOS) that is present in one or more forms in all cells. NO transduces its biological activity through redox reactions that lead to the formation of protein and non-protein thiols, including *S*-nitrosocysteine, *S*-nitrosohomocysteine and *S*-nitrosoglutathione (GSNO). Elevated levels of NO species (RNS) lead to nitrosative stress and play a role in physiology as well as human disease, (review [19]). Cysteine residues within proteins are also subject to the addition of an NO moiety leading to *S*-nitrosylation (P-SNO). To date, approximately 233 proteins have been identified as *S*-nitrosylated, of which 171 occur under physiologic conditions; 28 under pathological conditions and 34 are ascribed to both [20]. Targets for *S*-nitrosylation overlap with *S*-glutathionylation because both PTMs have selectivity toward a charged/low pK_a cysteine [21]. However, redox-mediated P-SSG and P-SNO are more complex than mere acid-base proximity as it is estimated that only ~ 19% are overlapping [20]. However, as analytical methods improve, the number of co-regulated targets will likely increase.

S-nitrosylation is dynamically regulated through nitrosylases and denitrosylases, (review [22]). *S*-nitrosylation reactions occur through multiple mechanisms that include trans-nitrosylation, acidic nitrosylation and catalysis by metalloproteins. Unlike phosphorylation, the *S*-nitrosylation cycle can be regulated by a single enzyme depending upon its oxidation state. Thioredoxins (Trxs) are a family of antioxidant enzymes that catalyze thiol-disulfide oxidoreduction reactions using active site cysteine residues in a CXXC motif that cycle between oxidized and reduced states [23,24]. The Trx system plays a role in trans-nitrosylation and has more recently been shown to denitrosylate proteins as well as low molecular mass RNS, including GSNO, CysNO and HCysNO [25]. As such, Trx plays an important role in nitrosative stress. On a biochemical level, the impact of P-SNO is documented, however, the relative ratio of P-SNO that is required to exhibit a cellular change or contribute to disease initiation or progression is poorly understood. Dysregulation of the enzymes that lead to the forward and reverse reactions of P-SNO as well as the protein targets themselves are implicated in disease and pathology. The *S*-nitrosylation cycle is important to redox signaling and plays a role in drug response. In this review, we will discuss the impact of P-SNO in models of drug abuse.

3. Drugs of abuse and oxidative/nitrosative stress

3.1. Cocaine

Cocaine is used by millions of people and it is estimated that in 2009, 4.8 million Americans age 12 and older had abused cocaine in any form. There is currently no FDA approved pharmacotherapy for cocaine usage and our understanding of its impact on neuronal signaling is not thoroughly characterized. Although research on GSH and redox regulation in cocaine addiction is very limited, recent work suggests that there may be a link between cocaine and redox-mediated pathways. Neurons are able to synthesize GSH through the intracellular constitutive amino acids, glutamate, cysteine, and glycine, or the dipeptide cysteinyl–glycine that is supplied by astrocytes [26]. Astrocytes are able to use cystine (cysteine disulfide) for GSH synthesis through the cystine– glutamate exchanger, system Xc- [27]. The cystine–glutamate exchanger is able to exchange one glutamate for each cystine and was shown to be downregulated by daily cocaine administration [28,29].

As an extrapolation of these results, there are now limited data on the effects of chronic cocaine treatment on redox-sensitive enzymes and protein S-glutathionylation in the brain. Most studies have focused on the hepatotoxicity of the drug and corresponding GSH reductase measurements in liver or spleen [30–34]. Studies focusing on GSH in the brain found that daily cocaine administration reduces GSH levels in the hippocampus and restoration of GSH was associated with normalizing cocaine-induced memory impairments [35]. In contrast, an acute cocaine overdose increased the GSH concentration in the prefrontal cortex and striatum of mice [36]. In addition, an acute and chronic cocaine injections (20 mg/kg for 10 days) increased ROS, hydrogen peroxide and lipid peroxide generation in the rat frontal cortex and striatum [37]. This was accompanied by reduced functioning of mitochondrial complex I and compensatory increases in the antioxidant enzymes, superoxide dismutase and glutathione peroxidase [37]. A recent study examined the redox potential, levels of modified protein thiols and protein levels of GSH reductase, glutaredoxin, and glutathione-S transferase Pi (GSTP) in the nucleus accumbens (NAc) of cocaine-withdrawn rats before and after a subsequent acute cocaine injection [38]. While there were no differences in GSH reductase, cocaine-withdrawn rats showed an increase in glutaredoxin and GSTP, 45 min after a cocaine injection. In addition, there was a decrease in the redox potential (i.e. an increase in the oxidative state) and in unmodified protein thiols [38]. Taken together, these results suggest that cocaine-withdraw in rats have an increase in S-glutathionylation after a cocaine injection (Fig. 2). It is possible that the cocaine-induced effects on S-glutathionylation and GSTP are compensatory biological actions to limit excessive neuroplasticity. This seems to be a reasonable interpretation, since further studies using GSTP knockout (GSTP KO) mice and the general GST inhibitor ketoprofen found that both genetic knockout and pharmacological inhibition resulted in an increase in cocaine sensitization as measured by locomotor activity, while GSTP KO also showed an increase in conditioned place preference [38]. GSTP KO animals did not differ from WT mice with respect to basal locomotor activity, suggesting that GSTP KO's mice are not naturally hyperactive [38].

There are also early indications that GSTP polymorphisms might influence cocaine addiction through *S*-glutathionylation, not phase II drug metabolism. As outlined earlier, GSTP exists as four functional allelic variants in humans: wild-type GSTP1*A (Ile105/Ala114), GSTP1*B (Val105/Ala114), GSTP1*C (Val105/Val114) and GSTP1*D (Ile105/Val114) [39] with GSTP1*B and GSTP1*C having lower catalytic activity than GSTP1*A [40]. The contribution of GSTP-mediated *S*-glutathionylation of cocaine-induced neuroplasticity proteins remains unexplored. For example, cAMP-dependent protein kinase (PKA) has been shown to play a role in cocaine addiction [41] and can be *S*-glutathionylated resulting in decreased PKA activity [42]. However, it is unknown if PKA *S*-glutathionylation is GSTP-mediated and/or altered in cocaine addiction. Another redox-sensitive protein is cyclin-dependent kinase 5 (Cdk5), which has been implicated in cocaine self-administration [43] and cocaine sensitization [44]. A recent study showed the regulation of Cdk5 kinase activity by GSTP [45], suggesting a potential link between cocaine addiction and GSTP-mediated regulation of Cdk5. Obviously, this field is emerging and further preclinical and clinical studies should help to clarify the cause–effect relationships of GSTP involvement with cocaine addiction – an analysis that may eventually lead to novel therapeutic interventions. Pharmacotherapeutic intervention through targeting redox homeostasis has generally focused on increasing GSH levels by *N*-acetylcysteine (NAC) administration in preclinical models and clinical studies of cocaine addiction [46–52]. A recent clinical study has shown that NAC treatment normalizes brain glutamate levels in the dorsal anterior cingulate cortex of cocaine-dependent patients [53]. While initial studies have been promising, work remains to be done by targeting redox pathways for cocaine addiction.

3.2. Methamphetamine

Amphetamine-type stimulants (including METH) is estimated to be used by 14 million people worldwide, more than cocaine and the second most abused drug after cannabis [54]. It is well known that METH induces extensive oxidative stress through numerous intracellular mechanisms. Due to the similarity in the structure between dopamine (DA) and METH, METH can enter the presynaptic cellular compartment through the dopamine reuptake transporter (DAT), where it can subsequently enter synaptic vesicles through VMAT2 and resultant DA release into the cytoplasm [55–57]. This cytoplasmic dopamine can rapidly be auto-oxidized to toxic products, such as superoxide radicals, hydrogen peroxide and DA quinones [58,59]. Furthermore, DA quinones can then bind to cysteinyl residues on proteins [58], while hydrogen peroxide can interact with transition metals via the Haber–Weiss/Fenton reactions to form hydroxyl radicals [60]. Superoxide can react with NO to form toxic peroxynitrite, which can further exacerbate METH-induced oxidative stress [60]. In addition to non-enzymatic mechanisms of DA oxidation, enzymatic mechanisms can also contribute to dopamine metabolism and subsequent oxidative stress. The mitochondrial enzyme, monoamine oxidase, which is present on the cytoplasmic side, catalyzes the deamination of dopamine producing 3,4-dihydroxyphenylacetic acid (DOPAC) and hydrogen peroxide [60]. Hydrogen peroxide inactivation involves glutathione peroxidase and catalase and can be generated through detoxification of superoxide. Intracellular redox homeostasis is dependent on these enzymes, with hydrogen peroxide levels having a bidirectional effect on cellular resistance [61] or apoptotic cell death to

hydrogen peroxide toxicity [62], respectively. Taken together, these data show that METH-induced oxidative stress can occur through multiple redox-sensitive mechanisms, which results in a change in redox homeostasis. Given the important role of GSH in redox homeostasis, it is not surprising that changes in GSH and redox-sensitive enzymes have been found *in vitro* and preclinical and clinical studies of METH addiction. METH-induced autophagy and apoptotic cell death in the N27 dopaminergic neuronal cell model were accompanied by GSH depletion and increases in 3-nitrotyrosine and 4-hydroxynonenal [63]. Mice treated with METH have shown a decrease in GSH in the striatum, amygdala, hippocampus and frontal cortex [64]. Data from rat studies suggest that METH administration selectively induces changes in GSH systems, but not other antioxidant systems, such as vitamin E or ascorbate [65]. Clinical studies in deceased METH users with severe dopaminergic loss in the caudate have shown a 35% decrease in caudate GSH and a 58% increase in the oxidized form of GSH, GSSG [66]. While studies on METH-induced *S*-glutathionylation are scarce, the allelic variant GSTP1*B has been associated with methamphetamine-induced psychosis in clinical studies [67]. Given the role for GSTP in catalyzing *S*-glutathionylation, this interesting observation merits further investigation. One study has found an increase in *S*-nitrosylated VMAT2 *in vivo* 1 h after repeated high dose METH treatment (10 mg/kg every 2 h for a total of 4 injections) [68] with a decrease in total VMAT2. This decrease persisted 7 days post-treatment and was attenuated by pretreatment with the nNOS inhibitor, *S*-methyl-L-thiocitrulline (SMTC). In addition, a decrease in DAT immunoreactivity was observed 7 days post-treatment, which was also attenuated by SMTC pretreatment [68]. NAC has provided protection against METH-induced dopaminergic neurodegeneration *in vitro* [63] and improved memory consolidation in METH-treated mice [64]. The NAC derivative and novel antioxidant, *N*-acetylcysteine amide (NACA), has shown to be protective against METH-induced oxidative stress *in vitro* [69]. Immortalized human brain endothelial cells were used as a model for blood brain barrier integrity through permeabilization and trans-endothelial electrical resistance studies after METH treatment. Preclinical evidence has also suggested a role for NAC in METH addiction. NAC pretreatment in cocaine-sensitized rats not only attenuated the METH-induced reduction in striatal DA, but also, it blocked the development of behavioral sensitization [70]. Clinical studies in METH-dependent patients treated with NAC have been limited in scope and success [71]. However, given the extensive impact of METH on redox pathways, additional research may provide better avenues for therapeutic intervention.

3.3. Chronic alcohol abuse

Heavy alcohol consumption produces a reduction in brain volume, loss of neurons in cortical and sub-cortical structures, and shrinkage of grey and white matter [72,73]. Chronic alcohol-associated neurodegeneration is caused by direct effects of alcohol during heavy or binge consumption patterns and impaired nutritional utilization or nutritional deficiency [72-74]. While the mechanisms of neuronal loss are complex, substantial evidence from clinical studies and animal models has demonstrated a critical role for oxidative-nitrosative stress and activation of inflammatory cascades in mediating alcohol-induced neurodegeneration (Crews and Nixon, [74]). In rodent models, prolonged or binge alcohol exposure activates nuclear factor kappa-B (NF-KB) pathways and increases lipid peroxides, nitrite levels, NADPH oxidase (NOX), cytochrome *c* oxidase II, and reactive O₂⁻ and O₂

derived oxidants in brain [74-76]. Binge alcohol exposure of rodents resulted in persistent alterations in brain pro-inflammatory cytokines (i.e., tumor necrosis factor- α , interleukin 1 β) and enhanced cytokine signaling leading to DNA fragmentation, microglial activation, and ultimately neuronal loss [74,76]. These maladaptive changes in oxidative-nitrosative stress signaling have also been reported in the frontal cortex of post-mortem brains from alcoholics [76]. Pharmacologically targeting, these pathways have proved useful in preclinical models of chronic alcohol exposure. Indeed, antioxidants and NOX inhibitors prevented oxidative damage and neuroinflammatory cascades in brain and attenuated cognitive impairments produced by chronic and binge ethanol treatment [74]. Markers of oxidative stress in alcoholics are typically considered as part of late stage signs of brain toxicity. However, recent compelling evidence has demonstrated that young drinkers (age 18–23 years old) show oxidative damage biomarkers [77]. In comparison with age-matched non-drinking controls, young adults who have been drinking for 4–5 years displayed reductions in GSH peroxidase levels and increases in lipid peroxidation and damaged DNA in blood without clinical evidence of hepatic damage [77]. Together, these data strongly implicate oxidative damage in early and late stages of alcohol dependence as a contributing factor to brain damage induced by heavy alcohol consumption.

3.3.1. Chronic alcohol, glutathione and S-glutathionylation—Like biomarkers of oxidative-nitrosative stress, acute and chronic effects of alcohol on GSH levels have been studied in clinical and rodent studies. While acute alcohol exposure does not appear to regulate GSH or GSH peroxidase levels [78], chronic alcohol consistently reduces GSH and GSH peroxidase levels in the brain and plasma from rodents and alcoholics. In rodents, long-term intragastric alcohol administration (2 g/kg/day) markedly reduced GSH and GSH activity and enhanced oxidized GSH measured in whole brain [79]. Similarly, 10 weeks of intragastric alcohol exposure (10 g/kg/day) impaired performance on the Morris water maze task and significantly reduced GSH levels in cerebral cortex and hippocampus [75]. Decreases in GSH peroxidase levels and increases in lipid peroxidase were reported in cerebral cortex and hippocampus after 3 weeks of intragastric administration of high doses of alcohol [80]. Lower doses of alcohol administered in an intermittent fashion across 90 days also produced reductions in total GSH levels and increases in oxidized proteins and lipids in cortex and cerebellum [81]. Evidence has demonstrated that individuals with alcohol use disorders (AUDs) have decreased GSH activity and reduced plasma and salivary content of GST [82,83]. However, some studies have reported increased GST activity in blood of alcoholics [84,85]. The reason for the discrepancy in these findings is unclear, but may relate to duration, pattern, or amount of alcohol consumption or the time of measurement in relation to the number of withdrawals or the time since withdrawal. In rodents, transcript and proteins levels the GST- $\alpha 4$ isoform were lower in the amygdala, but higher in the hippocampus of alcohol-preferring compared with non-preferring rats [86,87], suggesting a possible role for GST in regulating voluntary alcohol consumption. Indeed, null mutations in *GST μ 1* are associated with an increased risk for AUDs [88]. Curiously, the expression level of GST μ class was increased in the medial prefrontal cortex of alcohol-preferring rats [86]. Together, findings from these studies support the contention that chronic alcohol exposure in humans and rodents can decrease GSH and GSH reductase levels in brain and alter GST in brain, blood and saliva.

While these studies clearly demonstrate perturbations in GSH and GST in brain of rodents chronically exposed to alcohol and in blood and saliva of alcoholics, *S*-glutathionylation of proteins by acute or chronic alcohol exposure has not been studied. We hypothesize that *S*-glutathionylation represents a post-translational modification that could have profound implications for alcohol addiction and dependence. For example, once thought to be only important for regulating firing rates and neuronal excitability, emerging evidence has identified a significant role for small-conductance calcium-activated K^+ (K_{Ca2}) channels in synaptic plasticity, cognition, and alcohol and drug addiction [89]. $K_{Ca2.2}$ channels are solely activated by transient elevations of intracellular Ca^{2+} and form functional heteromeric complexes with calmodulin that acts as a high-affinity Ca^{2+} sensor [89]. In hippocampus, amygdala and prefrontal cortex, $K_{Ca2.2}$ channels are highly expressed and are localized to the postsynaptic density (PSD) of neuronal dendritic spines where they form a Ca^{2+} -mediated negative feedback loop with synaptic NMDA receptors. In comparison, $K_{Ca2.3}$ channels are highly expressed in the NAc and ventral tegmental area. $K_{Ca2.3}$ channels contribute to action potential after hyperpolarization and modulate activity patterns of midbrain DA neurons. Recent evidence has demonstrated that $K_{Ca2.3}$ channels in DA neurons also functionally couple to synaptic NMDA receptor activity [90]. Activation of this feedback loop is thought to act as a postsynaptic shunt to decrease spine Ca^{2+} transients and synaptic depolarization. We reported that chronic alcohol exposure reduced $K_{Ca2.2}$ channel surface expression and increased the synaptic expression of GluN1 and GluN2B subunits of the NMDA receptor [91]. These bidirectional neuroadaptations lead to a functional uncoupling of the feedback loop between K_{Ca2} channels and NMDA receptors in dendritic spines after chronic alcohol exposure. Restoration of K_{Ca2} channel activity prevented cellular injury produced by alcohol withdrawal and attenuated the severity of handling-induced convulsion in alcohol-dependent mice. In the NAc and VTA, chronic alcohol exposure reduced K_{Ca2} channel function and facilitated NMDA-induced burst firing [92,93]. These functional adaptations in K_{Ca2} channels induced by alcohol self-administration were associated with a significant reduction in $K_{Ca2.3}$ channel expression in NAc core. In opposition, 3-week withdrawal from repeated cocaine exposure in rats increased K_{Ca2} channel function and produced a reduction in intrinsic excitability of NAc medium spiny neurons [94]. Thus, these data suggest that withdrawal from chronic alcohol and cocaine treatment can alter the function of K_{Ca2} channels in the hippocampus, VTA, and NAc core, and that these adaptations are important for regulating NMDA receptor overactivation and membrane excitability.

We then inspected the GluN1 subunit of NMDA receptors and $K_{Ca2.2}$ and $K_{Ca2.3}$ channels for candidate *S*-glutathionylation sites. Cysteine residues with basic amino acids (i.e., lysine, histidine, or arginine) in their near vicinity are prone to *S*-glutathionylation [95]. As shown in Fig. 3A, inspection of the GluN1 subunit revealed four cysteine residues resembling potential motifs for *S*-glutathionylation. Three of these cysteine residues are located extracellularly, two in the *N*-terminus (Cys308, Cys420) and one in the S2 region (Cys744). The potential intracellular cysteine residue for GluN1 is located near the end of the *C*-terminus (Cys932). In contrast, inspection of $K_{Ca2.2}$ and $K_{Ca2.3}$ channels did not reveal any extracellular cysteine residues resembling a consensus motif for *S*-glutathionylation. However, multiple cysteine residues were identified on the intracellular *N*-terminus for both

K_{Ca}2.2 (Cys40, Cys83, Cys93, Cys122, Cys125, Cys213, and Cys269; Fig. 3B) and K_{Ca}2.3 channels (Cys21 and Cys179). Both isoforms of K_{Ca}2 channels had potential *S*-glutathionylation sites in the M2 region (K_{Ca}2.2: Cys432; K_{Ca}2.3: Cys322; Fig. 3C) and near the pore of the channel (K_{Ca}2.2: Cys592; K_{Ca}2.3: Cys482). While it remains unknown if NMDA receptors or K_{Ca}2 channels can be *S*-glutathionylated, inspection identified 4 or more candidate cysteine residues on each of these proteins. This supports the suggestions that *S*-glutathionylation may alter their function. If so, studies should examine how *S*-glutathionylation of NMDA receptors or K_{Ca}2 channels influences alcohol, METH, and cocaine addiction.

3.3.2. Chronic alcohol abuse and S-nitrosylation—Despite its known function as a PTM that critically regulates proteins involved in synaptic transmission and cellular death [96], *S*-nitrosylation of proteins in brain has received no attention in clinical or preclinical models in the alcohol research field. The lack of investigation in area is quite surprising, especially given the prominent role of NMDA receptors in the plasticity of alcohol addiction and alcohol withdrawal hyperexcitability and neuro-toxicity. Synaptic targeting of GluN1- and GluN2B-containing NMDA receptors by chronic alcohol and their subsequent overstimulation during withdrawal produces excessive Ca²⁺ influx and promotes pathological NO signaling and cellular injury. The production of NO and the increased activity of NOS contributes to the alcohol withdrawal-induced anxiety-like behaviors [97,98], and inhibitors of NOS attenuate the severity of alcohol withdrawal and reduce alcohol consumption in rodents [97-99]. Endogenous NO-induced *S*-nitrosylation of GluN1 and GluN2A subunits of NMDA receptors at extracellular cysteine residues leads to an inhibition of NMDA-evoked currents [96]. It has been proposed that *S*-nitrosylation of NMDA receptors during hyperactive conditions may be a neuroprotective mechanism that limit excessive Ca₂₊ influx [96]. Although it has yet to be tested, this suggests that the ability of *S*-nitrosylation to reduce NMDA receptor activity during alcohol withdrawal may be disrupted. It is unknown if NO can *S*-nitrosylate the GluN2B subunit of NMDA receptors. It is possible that the GluN2B subunit is insensitive to *S*-nitrosylation, and the synaptic targeting of GluN2B receptors by chronic alcohol exposure renders NO unable to reduce NMDA receptor hyperactivity during withdrawal. Alternatively, perhaps NO is unable to *S*-nitrosylate enough of the synaptic NMDA receptors, regardless of subunit composition, to attenuate the hyperactivity. The potential importance of *S*-nitrosylation in the alcohol field is not just limited to synaptic NMDA receptors. NO induces *S*-nitrosylation of additional proteins that have also been implicated in models of alcohol dependence. These alcohol- and *S*-nitrosylated sensitive proteins include NF-κB, L-type Ca²⁺ channels, and heat-shock proteins and are involved in neuro-transmission, protein quality control, and transcription. Thus, the effect of chronic alcohol exposure and withdrawal on *S*-nitrosylation of these proteins represents an exciting area of investigation. Such studies will lead to a better understanding of the molecular neuroadaptations that contribute to alcohol dependence and may, in turn, identify novel targets that could be explored as therapeutic agents.

As described above, evidence suggests that there may be a possible link between alcohol consumption and the GST-α4 and GSTμ classes. These findings from the human genetic and

alcohol-preferring rat studies on GST are intriguing, but a direct relationship between alcohol consumption and GST has not been empirically tested. Two recent studies have demonstrated that the antioxidants α -lipoic acid and ebselen significantly reduce alcohol consumption and reinstatement of alcohol-seeking behavior in rats and mice [100,101]. In both studies, saccharin intake was not affected by α -lipoic acid or ebselen suggesting a selective role for this system in alcohol reward. Ledsema et al. (2013) argued that antioxidants might reduce drinking by attenuating acetaldehyde-mediated DA transmission in mesolimbic pathway. Although the mechanisms are unknown, these findings provide compelling evidence that oxidative stress signaling induced by alcohol may not only contribute to cellular injury, but also influences the motivational states that drive alcohol consumption. This suggests that future studies are aimed at characterizing the role of GST and GST modulators on voluntary alcohol drinking in rodents are warranted and may lead to novel therapeutic targets for treating AUDs.

4. Conclusion

Oxidative and nitrosative stress play a key role in normal physiology and imbalance is attributed to pathology and disease progression (review [1,2]). Redox signaling through oxidation and reduction reactions plays an essential role in numerous cell-signaling cascades, including those with opposing cellular consequences, proliferation and apoptosis. Our current understanding of the molecular targets of ROS/RNS and the features of S-glutathionylation that regulate cell-signaling pathways is limited, especially in the substance abuse field. A growing number of disease states are associated with S-glutathionylation of proteins. Hence, a more detailed understanding of redox-mediated events may provide the platform to develop therapeutic agents or diagnostic tools.

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Fig. 1.

Reactive oxygen and nitrogen species lead to thiyl radical formation and target cysteine residues. Subsequent oxidative and nitrosative stress leads to *S*-glutathionylation and/or *S*-nitrosylated proteins. Homeostasis is restored through the removal of the PTM.

Fig. 2.

Model depicting how cocaine changes redox potential ($E_{h(mV)}$), GSTP protein expression and unmodified protein thiols (Cys-SH) in response to chronic cocaine pretreatment in rats. A single cocaine challenge (15 mg/kg ip) results in a decrease in the redox potential (i.e., more oxidative state) in the nucleus accumbens 45 min post-injection, independent of chronic saline or cocaine pretreatment. GSTP levels drop in chronic cocaine treated rats, but are increased after a cocaine challenge, suggesting that chronic cocaine treatment induces long lasting alterations in GSTP protein expression. Increased GSTP protein expression coincides with a decrease in unmodified protein thiols (Cys-SH), which together imply an increase in GSTP-mediated protein S-glutathionylation.

Fig. 3.
Putative *S*-glutathylation residues on (A) the GluN1 subunit of the NMDA receptor and (B, C) K_{Ca}2.2 and K_{Ca}2.3 channels. Possible locations of putative *S*-glutathionylated cysteine residues are shown in red. Closely localized lysine (K), arginine (R), and histidine (H) residues to these putative *S*-glutathionylated residues are shown in green.