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Dynamic Regulation of the GABA<sub>A</sub> Receptor Function by Redox Mechanisms

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ABSTRACT

Oxidizing and reducing agents, which are currently involved in cell metabolism and signaling pathways, can regulate fast inhibitory neurotransmission mediated by GABA receptors in the nervous system. A number of in vitro studies have shown that diverse redox compounds, including redox metabolites and reactive oxygen and nitrogen species, modulate phasic and tonic responses mediated by neuronal GABA<sub>A</sub> receptors through both presynaptic and postsynaptic mechanisms. We review experimental data showing that many redox agents, which are normally present in neurons and glia or are endogenously generated in these cells under physiologic states or during oxidative stress (e.g., hydrogen peroxide, superoxide and hydroxyl radicals, nitric oxide, ascorbic acid, and glutathione), induce potentiating or inhibiting actions on different native and recombinant GABA<sub>A</sub> receptor subtypes. Based on these results, it is thought that redox signaling might represent a homeostatic mechanism that regulates the function of synaptic and extrasynaptic GABA<sub>A</sub> receptors in physiologic and pathologic conditions.

Introduction

GABA is one of the major inhibitory neurotransmitters in the nervous system and its actions are mediated via two classes of receptors, ionotropic GABA<sub>A</sub> receptors (GABA<sub>A</sub>Rs) and metabotropic GABA<sub>B</sub> receptors (Alexander et al., 2013a,b). The GABA<sub>A</sub>Rs are critical targets for therapeutic interventions, and a site of action for typical anxiolytic, anticonvulsant, and sedative/hypnotic drugs, such as benzodiazepines, barbiturates, and neurosteroids (Sieghart, 2015). It has long been recognized that the activity of GABA<sub>A</sub>Rs can undergo changes in the presence of both exogenous and endogenous redox agents (Pan et al., 1995; Fukami et al., 1998; Amato et al., 1999; Pan et al., 2000; Sah et al., 2002; Wilkins and Smart, 2002; Wall, 2003; Calero and Calvo, 2008). However, the role of redox signaling in the regulation of the GABA<sub>A</sub>R function is poorly understood, but is recently being explored in greater detail.

Based on current evidence, it is thought that changes in the levels of some endogenous redox agents, such as ascorbic acid (vitamin C), glutathione (GSH), hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), and nitric oxide (NO), could potentiate or inhibit GABA<sub>A</sub>R function. It is also believed that redox status might couple cell metabolism to the control of neuronal inhibition by taking part in diverse forms of modulation and plasticity of synaptic and extrasynaptic GABA<sub>A</sub>Rs. Thus, redox signaling may represent a mechanism by which the physiologic and pharmacological properties of the GABA<sub>A</sub>Rs could be changed during variations in the physiologic conditions and pathologic states.

GABA<sub>A</sub>R Properties

GABA<sub>A</sub>Rs are heteropentameric GABA-gated chloride channels that belong to the Cys-loop ligand-gated ion channel superfamily (Fig. 1A) (Smart and Paoletti, 2012). Diversity in subunit composition underlies the variation in the physiologic and pharmacological properties of GABA<sub>A</sub>Rs. To date, 19 GABA<sub>A</sub>R subunits have been cloned in the mammalian central nervous system (CNS) and classified into classes based on the following sequence identity: α(1–6), β(1–3), γ(1, 2S, 2L, 3), δ, ε, π, θ, and ρ(1–3) (Alexander et al., 2013b). Each individual subunit is composed of a large extracellular N

ABBREVIATIONS: CNS, central nervous system; DEA, sodium 1,1-diethyl-2-hydroxy-2-nitroso-hydrazine sodium; DTNB, 5,5′-dithio-bis-[2-nitrobenzoic acid]; DTT, dithiothreitol; GABA<sub>A</sub>R, GABA<sub>A</sub> receptor; GSH, glutathione; GSSG, oxidized glutathione; H<sub>2</sub>O<sub>2</sub>, hydrogen peroxide; NO, nitric oxide; NOS, nitric oxide synthase; O<sub>2</sub>·−, superoxide; OH·, hydroxyl; ROS, reactive oxygen species; RNS, reactive nitrogen species; M, transmembrane domain.
terminus, four transmembrane domains (termed M1-M4, with M2 contributing to the ionic channel), a short intracellular loop (M1-M2 linker), a small extracellular loop (M2-M3 linker), and an extracellular C-terminus (Fig. 1). Two cysteine residues located at the external N-terminus of the GABA<sub>A</sub>R subunits form a disulfide bridge that delimitate the Cys-loop, a distinct domain conserved in all the members of the receptor superfamily that is a critical target for redox agents within the GABA<sub>A</sub>R (Fig. 1A) (Pan et al., 1995; Amato et al., 1999; Calero and Calvo, 2008). Most of the GABA<sub>A</sub>R subunits contain extra cysteines (1–11) that can significantly contribute to redox modulation (Fig. 1, A and B, and Supplemental Table 1) (Beltrán González et al., 2014), but the sensitivity of the diverse GABA<sub>A</sub>R subtypes to endogenous redox agents is not well characterized, and the functional role of most of these other cysteine residues is presently unknown.

**Phasic and Tonic GABA<sub>A</sub>R-Mediated Neurotransmission**

Activation of GABA<sub>A</sub>Rs, after vesicular GABA release from presynaptic terminals, increases the permeability of the postsynaptic membrane to chloride and bicarbonate ions, leading to a net inward flow of anions (Miller and Smart, 2010). GABAergic neurotransmission can take place in two modalities, each one mediated by different GABA<sub>A</sub>R subtypes (Farrant and Nusser, 2005). Phasic GABAergic synaptic transmission is mediated by the most abundant heteromeric GABA<sub>A</sub>R subtype expressed in brain containing the g<sub>2</sub> subunit (e.g., a<sub>1</sub>b<sub>2</sub>g<sub>2</sub>). This modality operates point to point, fast, and transiently at high GABA concentrations to build hyperpolarizing postsynaptic responses called inhibitory postsynaptic potentials. In addition, the activation of GABA<sub>A</sub>Rs by ambient GABA during spillover can induce slower, persistent, and less spatially and temporally restricted
“tonic” responses. Tonic GABA responses are commonly considered to be evoked by low concentrations of GABA acting on extrasynaptic receptors located beyond the synaptic cleft, on presynaptic terminals, or at neighboring synapses on the same or adjacent neurons. Tonic GABA responses are usually mediated by GABA<sub>A</sub>Rs of defined subunit compositions (e.g., α5βγ2 and α4δβ in the hippocampus; α6δβ in the cerebellum; homomeric p1 in the retina) (Jones and Palmer, 2009; Brickley and Mody, 2012). The contrasting features of phasic and tonic inhibition, mediated by one or the other GABA<sub>A</sub>R subtypes, add extra complexity to the integrative mechanisms of dynamic signaling in neurons. Thus, redox-based regulation of phasic and tonic GABA<sub>A</sub> responses could represent new elaborate forms of synaptic modulation, with a differential impact on the excitability of different neuronal types and the activity of discrete CNS circuits.

**Redox Agents and Their Role in the Nervous System**

A wide variety of redox agents are found in the nervous system, including antioxidant/reducing agents (e.g., ascorbic acid, GSH, lipoic acid, l-cysteine, l-carnitine, l-carnosine, homocysteine, retinol, α-tocopherol, ubiquinol, flavonoids, and carotenoids); enzymes (e.g., superoxide dismutase, glutathione peroxidases, catalase); oxidizing agents such as reactive oxygen species (ROS) ([e.g., H<sub>2</sub>O<sub>2</sub>, the free radicals hydroxyl (OH<sup>−</sup>), nitrosium, nitroxy, and superoxide (O<sub>2</sub>−)]; and reactive nitrogen species (RNS) (e.g., NO, which reacts with O<sub>2</sub>− to form the highly toxic peroxynitrite). The brain is a high oxygen utilization, a high content of oxidizable polyunsaturated fatty acids, and the presence of redox-active metals (Cu and Fe). Redox homeostasis is maintained in the cells by the balance between the generation and elimination of oxidants by the antioxidant systems. However, redox status can generally undergo fluctuations during both normal and pathologic conditions. Redox signaling is mediated by small and ubiquitous molecules that show high reactivity toward specific redox-sensitive thiols, such as H<sub>2</sub>O<sub>2</sub>, O<sub>2</sub>−, OH<sup>−</sup>, and NO (Thomas et al., 2008; Rice, 2011; Schieber and Chandel, 2014). These local diffusible messengers commonly produce neurotoxic effects during oxidative and nitrosative stress, and are involved in normal aging and neurodegenerative disorders (Thomas et al., 2008).

ROS are primarily generated as byproducts of mitochondrial oxidative metabolism (Schieber and Chandel, 2014). The damaging effects of ROS can be counteracted by the specific detoxifying enzymes and high levels of antioxidant agents in a sophisticated way such that their local concentration and distribution can be rapidly and precisely controlled to allow dynamic cell signaling (Rhee, 2006; Bao et al., 2009; Woo et al., 2010; Rice, 2011; Finkel, 2011). ROS effects in the nervous system can include transient changes in neuronal activity and synaptic plasticity (Klann, 1998; Bao et al., 2009), as well as diverse actions on both excitatory and inhibitory neurotransmission (Frantseva et al., 1998; Sah and Schwartz-Bloom, 1999; Kamsler and Segal, 2003), including regulatory changes on neurotransmitter receptors (Aizenman et al., 1990; Chu et al., 2006; Coddou et al., 2009; Accardi et al., 2014, 2015; Beltrán González et al., 2014; Penna et al., 2014).

RNS are also involved in the regulation of neuronal excitability and induce several forms of synaptic plasticity (Garthwaite, 2008; Steinert et al., 2010). NO is an unstable free radical gas, produced from L-arginine by the NO synthase (NOS) that acts as a short-lived cell-signaling molecule. However, at high concentrations NO can be a neurotoxic agent producing neuronal injury and apoptotic cell death (Garthwaite, 2008; Hardingham et al., 2013). NO effects are mainly mediated by the activation of a soluble guanylyl cyclase that leads to increased cGMP levels, which in turn activates the cGMP-dependent protein kinase (Bradley and Steinert, 2016). However, NO actions can also take place through cGMP/cGMP-dependent protein kinase–independent pathways, including cysteine S-nitrosylation and S-glutathionylation, which are reversible post-translational modifications that convey redox-based cellular signals (Hess et al., 2005; Okamoto and Lipton, 2015).

Besides the role of the reactive species, the intrinsic antioxidant mechanisms can also contribute to redox signaling (Papadia et al., 2008; Harrison and May, 2009; Aquilano et al., 2014; Covarrubias-Pinto et al., 2015). Neurons and glial cells accumulate ascorbate at millimolar concentrations by specific transporters (Hediger, 2002; Harrison and May, 2009). The extracellular concentrations of ascorbate are also substantial and can transiently undergo large increases during neuronal firing, mainly due to extensive extrusion through the neuronal sodium-dependent vitamin C transporter (Portugal et al., 2009). However, the effects of ascorbate on neurotransmission, and particularly on the function of excitatory and inhibitory receptors, have not been extensively studied. Ascorbate exerts diverse neuromodulatory actions, including redox modulation of synaptic receptors (Majewska et al., 1990; Calero et al., 2011), either directly by reducing amino acidic residues or indirectly by scavenging ROS capable of modifying redox-sensitive residues (Covarrubias Pinto et al., 2015). Meanwhile, GSH, the major thiol antioxidant and redox buffer of the cell, is found at very high concentrations (millimolar) in all the neuronal compartments and in the extracellular milieu (Do et al., 2009). GSH exerts a protective role against oxidative stress, for example, in the detoxification of H<sub>2</sub>O<sub>2</sub> and lipid peroxides (as cofactor of the GSH peroxidase), scavenging of OH<sup>−</sup> and the regeneration of the most important antioxidants, vitamins C and E. GSH was also involved in the regulation of many synaptic functions and in neuronal plasticity (Do et al., 2009; Robillard et al., 2011; Aquilano et al., 2014).

Phasic and tonic GABA<sub>A</sub>Rs are targets for the actions of all the above-mentioned redox agents. Thus, the study of the redox-dependent modulation of GABA<sub>A</sub>Rs is fundamental for understanding the physiologic and pharmacologic mechanisms that control neuronal inhibition.

**Redox Modulation of the GABA<sub>A</sub>R Function**

Redox modulation of GABA<sub>A</sub>R function was established in several regions of the nervous system through the use of several different in vitro experimental models, including freshly dissociated neurons, acute slices, and whole-mount preparations (Fig. 2) (Pan et al., 1995; Amato et al., 1999; Sah et al., 2002; Calero et al., 2011; Penna et al., 2014; Accardi et al., 2014, 2015). Previous receptor-binding studies indicated that sulfhydryl and disulfide groups may play a role in the
responses to GABA and glycine (referenced in Pan et al., 1995); however, the functional modulation of GABA\(_\text{A}\)Rs by redox agents was revealed by studying the effects of endogenous and exogenous thiol reagents on inhibitory responses in retinal neurons (Pan et al., 1995) (Fig. 2). Whole-cell patch-clamp recordings in retinal ganglion cells showed that GABA\(_\text{A}\) responses were reversibly potentiated in the presence of the reducing agents DTT and GSH, and were inhibited by thiol oxidant agents such as DTNB and oxidized GSH (GSSG) (Fig. 2). Effects were surmounted by pretreatment with the irreversible thiol alkylating agent N-ethylmaleimide, which suggests that, as in other neurotransmitter receptors and ion channels, cysteine residues at the GABA\(_\text{A}\)R are likely targets for the actions of redox reagents. Thiol reagents elicited opposite effects on glycine-evoked responses evoked in these cells, indicating that other Cys-loop receptors could also be sensitive to redox modulation (Pan et al., 1995). Redox susceptibility of GABA\(_\text{A}\)Rs to diverse endogenous agents (e.g., ascorbic acid, GSH, ROS, RNS) was later corroborated in several neuronal types, including cerebellar granule cells (Amato et al., 1999; Accardi et al., 2015), superior cervical ganglionic neurons (Amato et al., 1999), retinal bipolar neurons (Calero et al., 2011), hippocampal CA1 pyramidal neurons (Penna et al., 2014), and cerebellar stellate cells (Accardi et al., 2015).
interact with amino acidic residues at the GABAAR n dd o tetrakis(2-pyridylmethyl)ethane1,2-diamine, which do not appear to be mediated by the extrasynaptic GABAAR subtypes, but rather by multiple subtypes containing αβ, αβδ, α3βγ2, αδδ, αδδ, and αδβ subunits (Mortensen and Smart, 2006; Penna et al., 2014). Modulatory actions of H2O2 on both retinal and hippocampal GABA ARs were induced even at relatively low concentrations (in the high micromolar range), and strongly depended on GABA concentration. Free radical scavengers prevented H2O2 effects only partially, suggesting that in addition to the OH* radical and other ROS eventually involved, H2O2 could induce GABA AR potentiation directly (Beltrán González et al., 2014; Penna et al., 2014).

Endogenous ROS production was also shown to be a key factor that can strengthen inhibitory neurotransmission mediated by GABA ARs in cerebellar neurons (Accardi et al., 2014, 2015). Specifically, synaptic GABA AR responses were recorded in stellate and granule cells, whereas ROS levels were enhanced, for example, by intracellular perfusion of the mitochondrial uncoupler antimycin A (Fig. 2). In both cell types, the frequency of inhibitory synaptic currents increased after increasing mitochondrial ROS generation, but there was no reported change in tonic GABA AR responses. In stellate cells, the enhancement of ROS levels promoted the emergence of postsynaptic events with small amplitude and slow kinetics, and involved a mechanism of recruitment of α3-containing GABA ARs into discrete postsynaptic sites, without affecting the resident α1-containing GABA ARs (Accardi et al., 2014). In granule cells, the enhancement of ROS levels augmented inhibitory synaptic transmission in a similar way (Fig. 2), but the new synaptic events were not distinguishable by their kinetics and were most likely associated to a recruitment of α6-containing GABA ARs (Accardi et al., 2015). The mechanisms underlying this redox-induced receptor plasticity still need to be elucidated, but data clearly suggested that ROS can regulate the degree of the GABAergic inhibitory tone in the cerebellum.

Effects of NO on GABA AR-Mediated Responses. NO was shown to potentiate presynaptic GABA release at inhibitory synapses by acting as a retrograde signaling molecule (Szabadits et al., 2007). The postsynaptic actions of NO were less well explored, but multiple lines of evidence indicate that GABA AR-mediated responses can be modulated by NO. Either potentiating or inhibitory actions were observed on both phasic and tonic GABA AR responses (Fukami et al., 1998; Castel and Vaudry, 2001; Wall, 2003; Gasulla and Calvo, 2015). S-nitrosylation has been proposed as a redox step that accounts for the direct NO actions on many synaptic receptors and ion channels, including the GABA AR. For example, S-nitrosylation was speculated as a mechanism that explained the increase in the activity of GABA AR responses in frog pituitary melanotrophs observed in the presence of NO donors (Castel and Vaudry, 2001). NO donors also enhanced responses mediated by GABA ARs expressed in oocytes (Fig. 2). Experiments combining differentially acting selective thiol reagents, specific NO scavengers, and site-directed mutagenesis of the ρ1 subunits indicated that NO induced S-nitrosylation of cysteine residues that are critical for receptor function (Gasulla et al., 2012). Tonic and phasic GABA AR currents recorded

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**Modulation of GABA AR-Mediated Responses by Physiologically Relevant Redox Agents**

**Effects of ROS on GABA AR-Mediated Responses.** Multiple studies indicate that ROS modulate GABAergic neurotransmission via both presynaptic and postsynaptic mechanisms (Frantseva et al., 1998; Sah and Schwartz-Bloom, 1999; Sah et al., 2002; Kamsler and Segal, 2003). However, the mechanisms underlying the postsynaptic effects of ROS at GABAergic synapses have only recently been revealed. H2O2 was re-examined for its actions on GABA ARs in several preparations. A number of observations now suggest that at moderate concentrations (in the high-micromolar range) it can exert a dynamic modulation of GABA AR function. The direct effects of ROS on GABA ARs were demonstrated in human homeric GABA AR1Rs expressed in oocytes (Beltrán González et al., 2014), where H2O2 applications produced reversible increases in the amplitude of GABA AR1R-mediated currents (Fig. 2). Subsequent work showed that ROS sensitivity is not a feature restricted to this particular GABA AR subtype involved in tonic GABA currents in the retina. In fact, H2O2 applications also increased tonic GABA AR currents recorded in CA1 pyramidal hippocampal cells (Fig. 2) (Penna et al., 2014). Interestingly, since synaptic responses were not affected, it is thought that the modulation of GABA AR currents by H2O2 in these neurons was very selective (Fig. 2). ROS action do not appear to be mediated by the extrasynaptic GABA AR subtypes formed by α5 or δ subunits, which are commonly involved in hippocampal tonic GABA AR responses, but rather by multiple subtypes containing αβ, α1βδ, α3βγ2, αδδ, αδδ, and αδβ subunits (Mortensen and Smart, 2006; Penna et al., 2014).
from CA1 pyramidal neurons in hippocampal slices can also be modulated by NO (Fig. 2), but the underlying mechanisms are still unknown (Gasulla and Calvo, 2015). Meanwhile, GABA<sub>AR</sub><sub>Rs</sub> in cultured hippocampal neurons were also shown to be functionally modulated by S-nitrosylation, but indirectly through the post-translational modification of gephyrin (Dejanovic and Schwarz, 2014). Gephyrin, a scaffold protein essential for clustering postsynaptic GABA<sub>AR</sub><sub>Rs</sub>, forms complexes with neuronal NOS and can be S-nitrosylated in vivo. The inhibition of neuronal NOS results in a loss of S-nitrosylation and increases in the size of the gephyrin clusters, eventually raising the number of synaptic GABA<sub>AR</sub><sub>Rs</sub> expressed at the neuronal surface and consequently enhancing the strength of GABAergic transmission (Dejanovic and Schwarz, 2014). Thus, taken together, the present data indicate that S-nitrosylation might represent a redox-based pathway that plays a role in the plasticity of inhibitory synapses by controlling diverse aspects of the function and assembly of GABA<sub>AR</sub><sub>Rs</sub>.

**Effects of Ascorbate on GABA<sub>AR</sub>-Mediated Responses.** GABA<sub>AR</sub><sub>R</sub> responses in the synaptic terminals of retinal bipolar cells can be regulated by physiologically relevant concentrations of ascorbate (Calero et al., 2011). For example, increases in the extracellular levels of ascorbate close to those associated with an intense neuronal activity after Na<sup>+</sup>-driven ascorbate extrusion via the sodium-dependent vitamin C transporter significantly enhanced both tonic and phasic GABA<sub>AR</sub><sub>R</sub> responses (Fig. 2). In addition, tonic GABA<sub>A</sub> currents showed significant rundown in the absence of ascorbate (Calero et al., 2011), a decay that was prevented by restoring ascorbate intracellular concentration to its estimated physiologic levels. These results indicated that GABA<sub>AR</sub><sub>R</sub> function can also be regulated by the intracellular levels of ascorbate (Calero et al., 2011). Meanwhile, synaptic GABA<sub>A</sub> current amplitudes in bipolar cells were also reversibly increased by ascorbate, without a significant change in the frequency of the miniature inhibitory postsynaptic current (Calero et al., 2011) (Fig. 2). The effects of ascorbate on retinal GABA<sub>A</sub> responses were reproduced in heterologous systems where the corresponding recombinant receptor subtypes were expressed to reproduce native retinal GABA<sub>AR</sub>-mediated responses (Fig. 2). These data suggested that ascorbate may act as an endogenous agent capable of potentiating GABA<sub>AR</sub> activity. A similar modulatory role could be ascribed to glutathione, another fundamental cellular antioxidant, although at present the experimental evidence is more limited (Pan et al., 1995; Amato et al., 1999; Calero and Calvo, 2008). The effects of these and other antioxidant systems on GABA<sub>AR</sub> function require further investigation, and studies need to be extended to other regions of the nervous system.

**Possible Sites of Action of Endogenous Redox Agents at the GABA<sub>AR</sub><sub>Rs**

Many of the specific structural components of the GABA<sub>AR</sub><sub>R</sub> that may contribute to redox sensitivity have not been determined yet. Mutational analysis of the diverse GABA<sub>AR</sub><sub>R</sub> subunits is still incipient, but a number of cysteine residues were already identified as targets for redox actions (Amato et al., 1999; Pan et al., 2000; Calero and Calvo, 2008; Calero et al., 2011; Beltrán González et al., 2014). Oxidation/reduction of these cysteine residues may induce allosteric transitions in the receptors that modify channel gating, causing reversible potentiation or inhibition of the GABA responses (Miller and Smart, 2010; Nemecz et al., 2016).

The contribution of extracellular cysteine at the N-terminal domain was suggested using chemical modification of GABA<sub>AR</sub><sub>R</sub> with thiol reagents. The conserved cysteine residues at the Cys-loop participate in GSH potentiation of GABA<sub>AR</sub><sub>R</sub>-mediated responses in retinal ganglion neurons (Fig. 2) (Pan et al., 1995), recombinant GABA<sub>AR</sub><sub>α1β2R</sub> expressed in human embryonic kidney cells (Fig. 2) (Amato et al., 1999), and GABA<sub>AR</sub><sub>α1Rs</sub> expressed in oocytes (Fig. 2) (Calero and Calvo, 2008). Cys-loop cysteines were also essential for the potentiating effects of ascorbate and NO on GABA<sub>AR</sub><sub>α1R</sub> expressed in oocytes (Fig. 2) (Calero et al., 2011; Gasulla et al., 2012). As site-directed mutagenesis affecting the disulfide bond disturbed the capacity of the GABA<sub>AR</sub> subunits to assemble (Amin et al., 1994), this approach was avoided during heterologous expression of GABA<sub>AR</sub><sub>Rs</sub> (Amato et al., 1999; Pan et al., 2000; Calero and Calvo, 2008; Calero et al., 2011; Gasulla et al., 2012). An extracellular oxidative reaction at the GABA<sub>AR</sub><sub>R</sub> was also proposed to mediate the increase in tonic GABA<sub>AR</sub> responses produced by H<sub>2</sub>O<sub>2</sub> in hippocampal CA1 pyramidal neurons (Penna et al., 2014), and the corresponding amino acidic residues acting as ROS targets were not identified in this case.

Redox sensitivity was even observed in other members of the Cys-loop receptor superfamily (Pan et al., 1995; Thio and Zhang, 2006), but GABA<sub>AR</sub><sub>R</sub> variants insensitive to redox modulation were also found (Amato et al., 1999; Pan et al., 2000), indicating that simply the presence of the Cys-loop is insufficient to confer redox susceptibility. GABA<sub>AR</sub><sub>R</sub> subunits can have additional cysteine at the N-terminal domain aside from those involved in the Cys-loop, including α4 (C<sup>ε100</sup>), β3 (C<sup>ε377</sup>), ε (C<sup>ε110</sup>), π (C<sup>π5</sup>), and ρ3 (C<sup>ρ11</sup> and C<sup>ρ19</sup>) (Supplemental Table 1). The potential role of these residues to act as targets for redox actions was not explored.

Transmembrane domains can contain cysteines whose positions show a relatively high degree of conservation. For example, a single cysteine at the M3 domain is present in all GABA<sub>AR</sub><sub>R</sub> subunit subtypes, except in ρ3-1 (Fig. 1B). The role of this residue has only been studied in homomeric GABA<sub>AR</sub><sub>β3Rs</sub>, which yield spontaneous currents when expressed in oocytes (Pan et al., 2000). Cysteine replacement by alamine in the mutant GABA<sub>AR</sub><sub>β3</sub>C<sup>ε110A</sup>Rs produced a significant change in redox sensitivity, but the heterologous expression of heteromeric GABA<sub>AR</sub><sub>α1β3</sub>C<sup>ε110A</sup>R or GABA<sub>AR</sub><sub>β3</sub>C<sup>ε110A</sup>γ2SR restored the original sensitivity. These results suggested that other cysteine residues in the α- and γ-subunits may be conveniently redox modulated (Pan et al., 2000) and that folding can also be a critical factor in determining redox sensitivity. A single cysteine is also conserved at the M1 domain of α (1–6) and γ (1–3) subunits (Fig. 1B), whereas M2 and M4 domains rarely contain cysteines (with a few exceptions, π (C<sup>ε120</sup> in M2), γ2 (C<sup>ε415</sup> in M4), and ε (C<sup>ε581</sup> in M4) (Supplemental Table 1). The possible role of these cysteine residues in redox modulation requires analysis.

A single study (Beltrán González et al., 2014) indicated that intracellular cysteines can also contribute to redox modulation of GABA<sub>AR</sub><sub>Rs</sub>. A cysteine located at the M3-M4 linker (Fig. 1, A and B) of each ρ1 subunit (C<sup>ε364</sup>) acts as an intracellular sensor for the actions of ROS on homomeric GABA<sub>AR</sub><sub>β1Rs</sub> expressed in oocytes. This cysteine residue is also present in ρ2 subunits, but not in ρ3 subunits. Meanwhile, γ-subunits have a group of
five cysteines whose relative positions are conserved at this domain, with two of them contiguous, in sharp contrast with those exhibited by the \( \rho \)-subunits that show a different arrangement (Fig. 1B). The M3-M4 linker of the Cys-loop receptors is a large intracellular loop (\( \sim 100-200 \) amino acids) whose structure can be predicted as a disorganized region (Fig. 1A). This loop contains motifs required for pentameric assembly; diverse protein-binding domains important for receptor clustering, sorting, targeting, and trafficking; phosphorylation sites for protein kinase A, protein kinase C, and protein tyrosine kinase; and structural determinants that are critical for single-channel conductance, desensitization properties, and interactions with other neurotransmitter receptors and the cytoskeleton (Macdonald and Botzolokis, 2009; Papke and Grosman, 2014). As the M3-M4 linker supports a variety of functions, redox modulation at this level could possibly have an important impact in many functions mediated by GABA\(_{A}\)Rs.

Variation in the number and location of cysteines in the diverse GABA\(_{A}\)R subunit subtypes, could account for differential redox sensitivities observed between receptor variants. But the potential contribution of the remaining cysteines to redox modulation needs to be further investigated. Other amino acidic residues at the GABA\(_{A}\)R subunits, such as methionine, tyrosine, phenylalanine, histidine, or lysine, could also undergo redox-dependent side chain modification. However, it is possible that the chemical modification of such residues requires highly extreme conditions. In addition, redox modulation of the GABA\(_{A}\)R function can also be mediated indirectly, compromising additional targets for endogenous agents, such as receptor associated-proteins (Wang et al., 1999). As in the S-nitrosylation–induced, gephyrin-mediated regulation of the synaptic GABA\(_{A}\)R density in hippocampal neurons (Dejanovic and Schwarz, 2014), these pathways need to be more closely analyzed using different neuronal types.

### Physiologic Significance

Endogenous redox agents are generated and/or accumulated in neural tissue under physiologic conditions and oxidative stress. Therefore, effects such as those described here for H\(_2\)O\(_2\), NO, ascorbate, and GSH on GABA\(_{A}\)Rs could be relevant for neuronal excitability. These redox agents exert a dynamic modulation on the function of diverse GABA\(_{A}\)R subtypes in various areas of the CNS, including the hippocampus, cerebellum, and retina (Pan et al., 1995; Amato et al., 1999; Calero et al., 2011; Accardi et al., 2014, 2015; Penna et al., 2014). However, the significance of these modulatory actions is not yet well understood.

In the hippocampus, the selective potentiation of tonic GABA\(_{A}\) currents in CA1 pyramidal cells induced during H\(_2\)O\(_2\) application was equivalent to that observed by using an oxygen-glucose deprivation protocol, which produces high endogenous levels of H\(_2\)O\(_2\) and other ROS (Penna et al., 2014). These results suggested a novel link between cellular metabolism and GABA\(_{A}\)R activity in hippocampal neurons. In the cerebellum, ROS were also proposed as homeostatic signaling molecules coupling cellular metabolism to the strength of inhibitory transmission, by inducing synaptic plasticity through the recruitment of nonresident GABA\(_{A}\)Rs in granule and stellate cells (Accardi et al., 2014, 2015). In the retina, there is considerable production of free radicals, and ascorbate is accumulated at high concentrations (Harrison and May, 2009). Hence, given the fact that GABA\(_{A}\)Rs mediate several modes of inhibitory actions in retinal neurons (Jones and Palmer, 2009), the modulatory effects observed for free radicals and antioxidants on tonic and phasic GABA\(_{A}\)R responses in retinal bipolar cells might be physiologically relevant (Calero et al., 2011; Gasulla et al., 2012; Beltrán González et al., 2014). Changes in GABA\(_{A}\)R function were also observed in retinal neurons during experimental diabetes (Ramsey et al., 2007) and in anoxia (Katchman et al., 1994); GABA\(_{A}\)Rs were also involved in mechanisms of cell death during ROS-induced oxidative stress (Okumichi et al., 2008), but the underlying mechanisms and the particular roles of free radicals and ascorbate in this process were not determined.

The effects of ROS and RNS on GABA\(_{A}\)Rs depended on many factors, including the CNS region and the neuronal type involved (Penna et al., 2014; Dejanovic and Schwarz, 2014; Accardi et al., 2014, 2015), in addition to structural determinants of the GABA\(_{A}\)R subunits and their functional relations with the cell machinery (e.g., redox-sensitive residues, receptor folding, and associated proteins) (Amato et al., 1999; Pan et al., 2000; Calero et al., 2011; Gasulla et al., 2012; Dejanovic and Schwarz, 2014). Other critical aspects include the interaction of the generated redox agents, or reactive species, with cellular antioxidant systems and the metabolic state of the cells in the experimental preparations (e.g., levels of ascorbate, glutathione, and antioxidant enzymes, oxidative agents such as CO\(_2\), O\(_2\), and light). All concomitant factors must be considered in future experiments to determine whether endogenous redox agents are actually acting as signaling molecules, as well as to characterize the specific pathways and mechanisms involved.

### Concluding Remarks

Modulation of tonic and phasic GABA\(_{A}\)R responses by endogenous redox agents has been demonstrated in different CNS regions using a diverse range of in vitro models. Future studies are needed to evaluate the interplay between these endogenous redox agents, the cellular antioxidant systems, as well as the possible role of multiple cysteines at the different GABA\(_{A}\)R subunits in redox modulation. In vivo studies will also be required to more precisely define the relevance that this redox modulation might have in normal physiologic conditions and during oxidative stress.

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### Authorship Contribution

Wrote or contributed to the writing of the manuscript: Calvo and Beltrán González

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