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MINIREVIEW—A LATIN AMERICAN PERSPECTIVE ON ION CHANNELS

Dynamic Regulation of the $GABA_A$ Receptor Function by Redox Mechanisms [S]

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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 $GABA_A$ 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 $GABA_A$ 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 $GABA_A$ 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 $GABA_A$ receptors ($GABA_A$Rs) and metabotropic $GABA_B$ receptors (Alexander et al., 2013a,b). The $GABA_A$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 $GABA_A$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 $GABA_A$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_2O_2), and nitric oxide (NO), could potentiate or inhibit $GABA_A$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 $GABA_A$Rs. Thus, redox signaling may represent a mechanism by which the physiologic and pharmacological properties of the $GABA_A$Rs could be changed during variations in the physiologic conditions and pathologic states.

$GABA_A$R Properties

$GABA_A$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 $GABA_A$Rs. To date, 19 $GABA_A$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

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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; $GABA_A$R, $GABA_A$ receptor; GSH, glutathione; GSSG, oxidized glutathione; H_2O_2, hydrogen peroxide; NO, nitric oxide; NOS, nitric oxide synthase; O_2^-; superoxide; OH; hydroxyl; ROS, reactive oxygen species; RNS, reactive nitrogen species; M, transmembrane domain.

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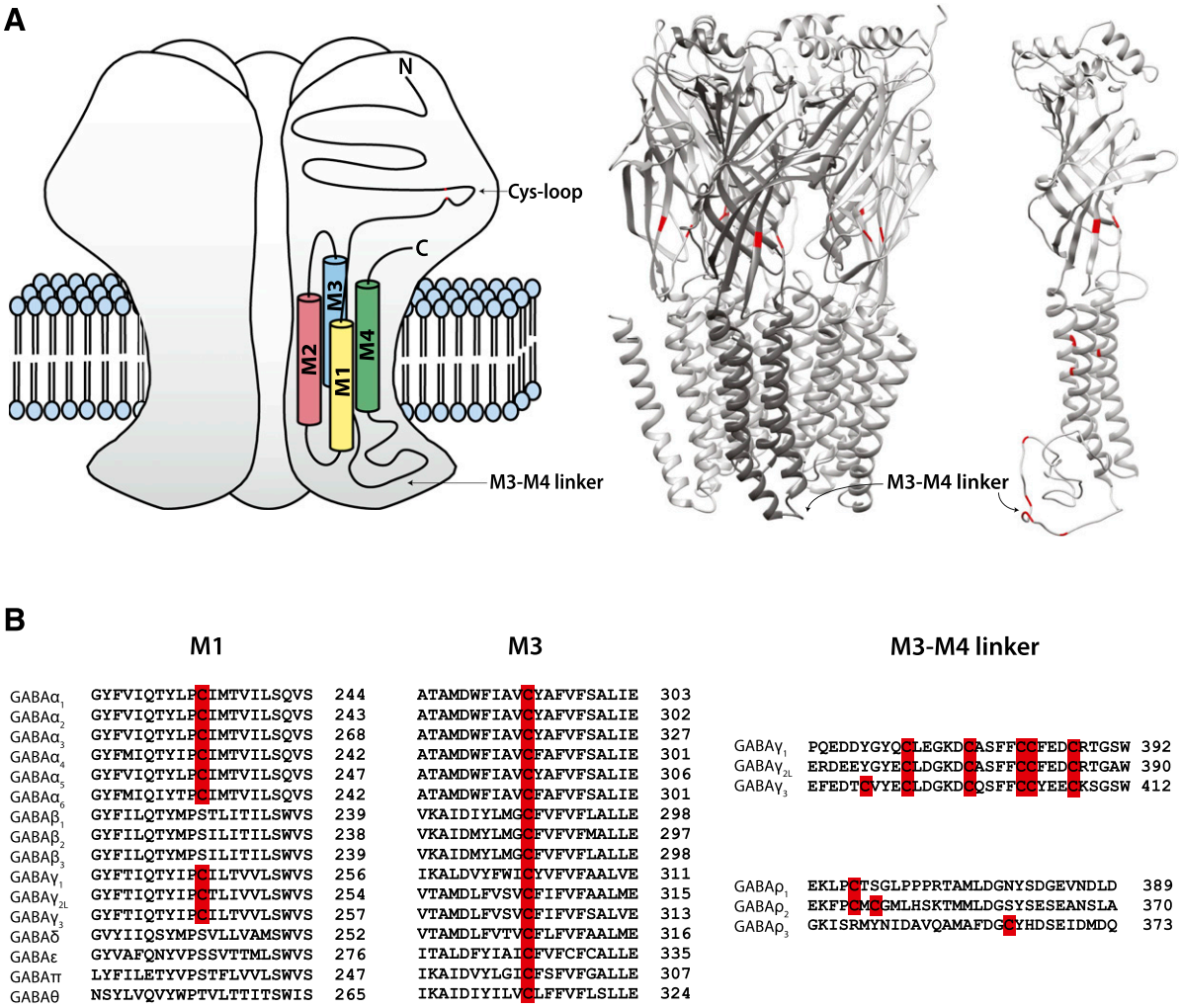

Fig. 1. GABA$_A$R structure, subunit topology, and identification of cysteine residues located in the different receptor domains. (A, left) Schematic representation showing the transmembrane topology of a single GABA$_A$R subunit. (A, middle) Crystal structure of the human GABA$_A\beta$3R homopentamer (Protein Data Bank ID, 4COF.pdb). Cysteine residues at the Cys-loop are marked in red. Note that crystallized GABA$_A\beta$3Rs have truncated M3-M4 linkers (Miller and Aricescu, 2014). (A, right) Model of γ2 subunit generated with Phyre[2]. Cysteine residues at the Cys-loop, the transmembrane domains, and the intracellular M3-M4 linker are marked in red. Molecular graphics and analyses performed with Chimera, package developed by the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (https://www.cgl.ucsf.edu/chimera/docs/credits.html). (B) Comparison of the primary sequences in the M1, M3, and M3-M4 GABA$_A$R domains showing the conservation of several cysteine residues at specific positions. Alignments were performed with Clustal Omega.

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), a bulky intracellular loop (M3-M4 linker), and an extracellular C-terminus (Fig. 1). Two cysteine residues located at the external N-terminus of the GABA$_A$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$_A$R (Fig. 1A) (Pan et al., 1995; Amato et al., 1999; Calero and Calvo, 2008). Most of the GABA$_A$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$_A$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$_A$R-Mediated Neurotransmission

Activation of GABA$_A$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$_A$R subtypes (Farrant and Nusser, 2005). Phasic GABAergic synaptic transmission is mediated by the most abundant heteromeric GABA$_A$R subtype expressed in brain containing the γ2 subunit (e.g., $\alpha1\beta2\gamma2$). 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$_A$Rs by ambient GABA during spillover can induce slower, persistent, and less spatially and temporally restricted

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"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 $GABA_A$Rs of defined subunit compositions (e.g., $\alpha5\beta\gamma2$ and $\alpha4\beta\delta$ in the hippocampus; $\alpha6\beta\delta$ in the cerebellum; homomeric $\rho1$ 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 $GABA_A$R subtypes, add extra complexity to the integrative mechanisms of dynamic signaling in neurons. Thus, redox-based regulation of phasic and tonic $GABA_A$ 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_2O_2, the free radicals hydroxyl ($OH^\bullet$), nitrosium, nitroxyl, and superoxide ($O_2^{-\bullet}$)]; and reactive nitrogen species (RNS) (e.g., NO, which reacts with $O_2^{-\bullet}$ to form the highly toxic peroxynitrite). The brain is particularly vulnerable to oxidative damage due to its 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_2O_2, $O_2^{-\bullet}$, $OH^\bullet$, 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_2O_2 and lipid peroxides (as cofactor of the GSH peroxidase), scavenging of $OH^\bullet$ 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 $GABA_A$Rs are targets for the actions of all the above-mentioned redox agents. Thus, the study of the redox-dependent modulation of $GABA_A$Rs is fundamental for understanding the physiologic and pharmacological mechanisms that control neuronal inhibition.

Redox Modulation of the $GABA_A$R Function

Redox modulation of $GABA_A$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

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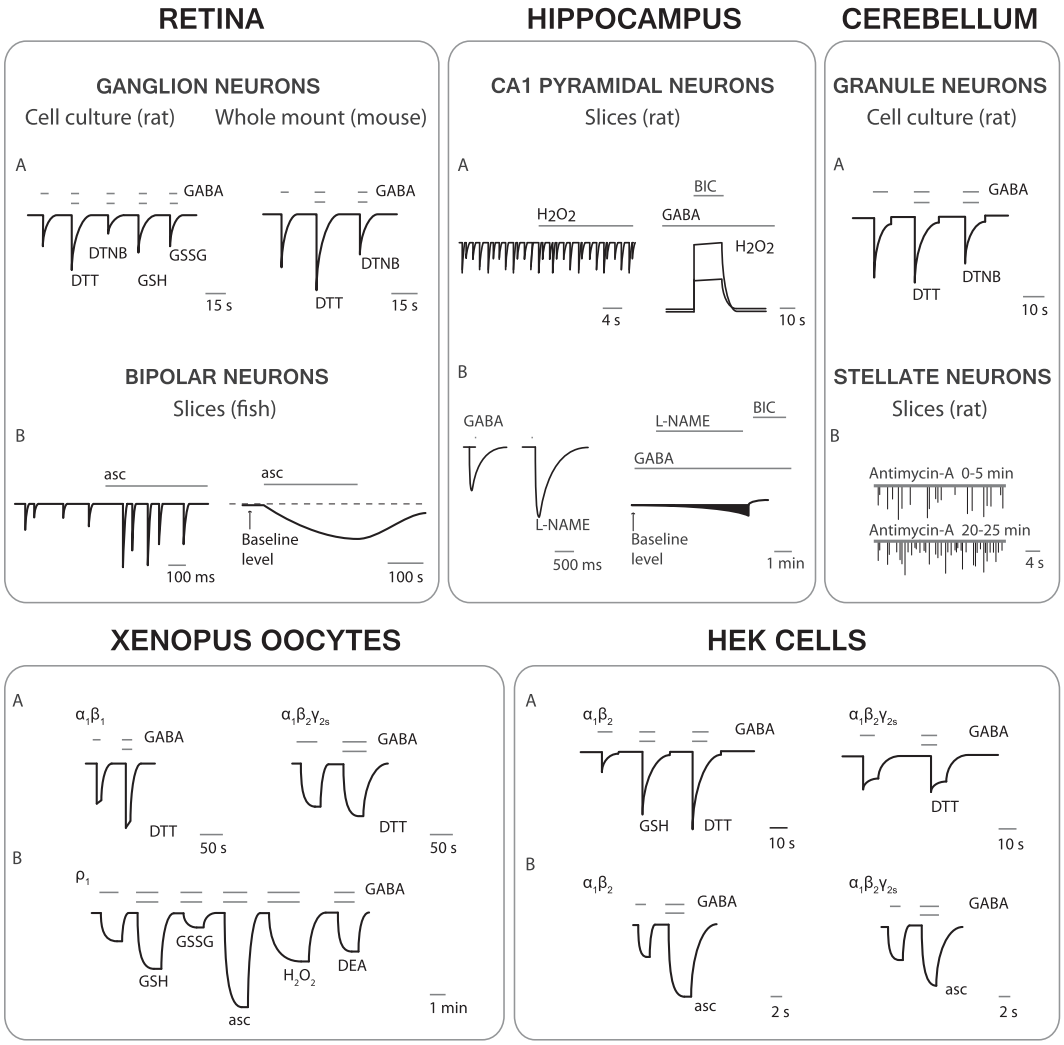

Fig. 2. Redox modulation of $GABA_AR$-mediated responses. Retina: (A) Effects of thiol reagents on phasic $GABA_AR$ currents in ganglion neurons (GABA 5 μM; DTT, GSH and GSSG 5 mM; DTNB 500 μM) (Pan et al., 1995). (B) Effects of ascorbate (3 mM) on phasic currents (miniature synaptic currents, left) and tonic currents (induced by bath application of GABA, right) (Calero et al., 2011). $GABA_AR$ currents recorded in synaptic terminals of bipolar neurons. In (A) and (B) and following records, the membrane potential is −60 mV. Hippocampus: (A) H_2O_2 (200 μM) produced no changes in spontaneous synaptic $GABA_A$ currents (left), but enhanced tonic $GABA_A$ currents [right, GABA 0.5 μM; without bicuculline (BIC) 100 μM] in pyramidal neurons (Penna et al., 2014). (B) NOS inhibition by L-NAME (100 μM) potentiated both phasic currents (left, induced by GABA puffs) and tonic currents (right, GABA 5 μM, without bicuculline 100 μM) $GABA_AR$ responses (Gasulla and Calvo, 2015). Cerebellum: (A) $GABA_A$ responses (GABA 10 μM) in acutely dissociated granule cells showed very low sensitivity to DTT (5 mM) and DTNB (500 μM) (Amato et al., 1999). (B) After an increase in ROS in granule cells, induced by mitochondrial uncoupling (bottom trace) (Accardi et al., 2015), a higher frequency of low-amplitude postsynaptic responses was detected. Expression in oocytes: (A) Effects of DTT (2 mM) on responses mediated by human $GABA_AR$ variants (GABA 10 μM) (Pan et al., 2000). (B) Effects of GSH (5 mM), GSSG (3 mM), ascorbate (3 mM), H_2O_2 (500 μM), and the NO donor 1,1-diethyl-2-hydroxy-2-nitroso-hydrazine sodium (DEA) (100 μM) on responses mediated by human $GABA_A\rho$1Rs (GABA 0.3 μM) (Calero and Calvo, 2008; Calero et al., 2011; Beltrán González et al., 2014; Gasulla et al., 2012). Expression in human embryonic kidney (HEK) cells: (A) Effects of GSH (5 mM) and DTT (2 mM) on responses mediated by murine $GABA_ARs$ (GABA 10 μM). $GABA_A\alpha1\beta2\gamma2$Rs exhibited lower redox sensitivity than $GABA_A\alpha1\beta2$Rs (Amato et al., 1999). (B) Effects of ascorbate (1 mM) on responses mediated by human $GABA_A\alpha1\beta2$Rs and $GABA_A\alpha1\beta2\gamma2$sRs (recorded at the corresponding EC_{50}, and GABA 1 and 5 μM respectively) (Calero et al., 2011). The $\gamma2$ subunit containing $GABA_ARs$ showed a decreased sensitivity to ascorbate.

responses to GABA and glycine (referenced in Pan et al., 1995); however, the functional modulation of $GABA_ARs$ 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 $GABA_A$ 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 $GABA_AR$ 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 $GABA_ARs$ 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.,

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2014) (Fig. 2). Redox modulation of $GABA_AR$ responses typically depended on GABA concentration; receptors were more sensitive to redox reagents at low GABA levels, and differences in redox sensitivity were associated with different $GABA_AR$ subtypes. For example, $GABA_AR$-mediated responses in cerebellar granule neurons showed much less susceptibility to redox modulation by thiol reagents than in retinal ganglion neurons (Fig. 2) (Pan et al., 1995, Amato et al., 1999). These results were consistent with the fact that diversity in subunit composition causes variation in the functional properties of $GABA_ARs$. Additional studies showed that diverse recombinant $GABA_AR$ subtypes, expressed in heterologous systems, also displayed differential susceptibilities to redox agents (Fig. 2 and Supplemental Table 2). As a rule, $GABA_A\alpha\beta Rs$ or $GABA_A\rho 1Rs$ were more vulnerable to regulation by redox agents than $GABA_A\alpha\beta\gamma 2Rs$ (Amato et al., 1999; Pan et al., 2000; Calero and Calvo, 2008).

It is important to mention that basal contaminating levels of Zn^{2+} can exert a standing inhibition of the responses mediated by $GABA_A\alpha\beta Rs$ by acting on specific histidine residues (Pan et al., 2000; Wilkins and Smart, 2002). Zn^{2+} inhibition is diminished in the presence of redox agents that chelate this cation, such as DTT, and this results in a potentiating effect on $GABA_AR$ currents that is not related to the actual redox mechanism that normally involves sulfhydryl residues. Thus, selective Zn^{2+} chelating agents such as tricine and *N*,*N*,*N*9,*N*9-tetrakis(2-pyridylmethyl)ethane1,2-diamine, which do not interact with amino acidic residues at the $GABA_AR$ and do not interfere with redox modulation, were needed to avoid overestimation of redox modulation (Pan et al., 2000; Wilkins and Smart, 2002). Other receptor subtypes, such as the $GABA_A\alpha 1\beta 2\gamma 2R$ and $GABA_A\rho 1R$, are less sensitive to ambient Zn^{2+}, and redox modulation induced by DTT was not affected by tricine (Pan et al., 2000; Wilkins and Smart, 2002; Calero and Calvo, 2008). It remains unknown whether endogenous Zn^{2+} can also mask the modulation exerted on $GABA_ARs$ by endogenous redox agents.

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. H_2O_2 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 homomeric $GABA_A\rho 1Rs$ expressed in oocytes (Beltrán González et al., 2014), where H_2O_2 applications produced reversible increases in the amplitude of $GABA_A\rho 1R$-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, H_2O_2 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 $GABA_A$ currents by H_2O_2 in these neurons was very selective (Fig. 2). ROS action do not appear to be mediated by the extrasynaptic $GABA_AR$ subtypes formed by $\alpha 5$ or δ subunits, which are commonly involved in hippocampal tonic $GABA_A$ responses, but rather by multiple subtypes containing $\alpha\beta$, $\alpha 1\beta\delta$, $\alpha 3\beta\gamma 2$, $\alpha 4\delta$, $\alpha 6\delta$, and $\alpha\beta\varepsilon$ subunits (Mortensen and Smart, 2006; Penna et al., 2014). Modulatory actions of H_2O_2 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 H_2O_2 effects only partially, suggesting that in addition to the $OH^{\bullet}$ radical and other ROS eventually involved, H_2O_2 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 $GABA_A$ 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 $\alpha 3$-containing $GABA_ARs$ into discrete postsynaptic sites, without affecting the resident $\alpha 1$-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 $\alpha 6$-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 $GABA_A$ 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 $GABA_A$ responses in frog pituitary melanotrophs observed in the presence of NO donors (Castel and Vaudry, 2001). NO donors also enhanced responses mediated by $GABA_A\rho 1Rs$ expressed in oocytes (Fig. 2). Experiments combining differentially acting selective thiol reagents, specific NO scavengers, and site-directed mutagenesis of the $\rho 1$ subunits indicated that NO induced *S*-nitrosylation of cysteine residues that are critical for receptor function (Gasulla et al., 2012). Tonic and phasic $GABA_A$ currents recorded

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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, $GABA_A$Rs 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 $GABA_A$Rs, 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 $GABA_A$Rs 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 $GABA_A$Rs.

Effects of Ascorbate on $GABA_A$R-Mediated Responses. $GABA_A$R 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^+-driven ascorbate extrusion via the sodium-dependent vitamin C transporter significantly enhanced both tonic and phasic $GABA_A$R responses (Fig. 2). In addition, tonic $GABA_A$ 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 $GABA_A$R function can also be regulated by the intracellular levels of ascorbate (Calero et al., 2011). Meanwhile, synaptic $GABA_A$ 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 $GABA_A$ responses were reproduced in heterologous systems where the corresponding recombinant receptor subtypes were expressed to reproduce native retinal $GABA_A$R-mediated responses (Fig. 2). These data suggested that ascorbate may act as an endogenous agent capable of potentiating $GABA_A$R 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 $GABA_A$R 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 $GABA_A$Rs

Many of the specific structural components of the $GABA_A$R that may contribute to redox sensitivity have not been determined yet. Mutational analysis of the diverse $GABA_A$R 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 cysteines at the N-terminal domain was suggested using chemical modification of $GABA_A$R with thiol reagents. The conserved cysteine residues at the Cys-loop participate in GSH potentiation of $GABA_A$R-mediated responses in retinal ganglion neurons (Fig. 2) (Pan et al., 1995), recombinant $GABA_A\alpha1\beta2$R expressed in human embryonic kidney cells (Fig. 2) (Amato et al., 1999), and $GABA_A\rho1$Rs expressed in oocytes (Fig. 2) (Calero and Calvo, 2008). Cys-loop cysteines were also essential for the potentiating effects of ascorbate and NO on $GABA_A\rho1$R expressed in oocytes (Fig. 2) (Calero et al., 2011; Gasulla et al., 2012). As site-directed mutagenesis affecting the disulphide bond disturbed the capacity of the $GABA_A$R subunits to assemble (Amin et al., 1994), this approach was avoided during heterologous expression of $GABA_A$Rs (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 $GABA_A$R was also proposed to mediate the increase in tonic $GABA_A$R responses produced by H_2O_2 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 $GABA_A$R 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. $GABA_A$R subunits can have additional cysteines at the N-terminal domain aside from those involved in the Cys-loop, including $\alpha4$ (C^9), $\beta3$ (C^{37}), ε (C^{110}), π (C^4), and $\rho3$ (C^{11} and C^{19}) (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 $GABA_A$R subunit subtypes, except in ρ1-3 (Fig. 1B). The role of this residue has only been studied in homomeric $GABA_A\beta3$Rs, which yield spontaneous currents when expressed in oocytes (Pan et al., 2000). Cysteine replacement by alanine in the mutant $GABA_A\beta3^{C288A}$Rs produced a significant change in redox sensitivity, but the heterologous expression of heteromeric $GABA_A\alpha1\beta3^{C288A}$R or $GABA_A\beta3^{C288A}\gamma2$SR 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^{260} in M2), $\gamma2$ (C^{415} in M4), and ε (C^{481} 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 $GABA_A$Rs. A cysteine located at the M3-M4 linker (Fig. 1, A and B) of each $\rho1$ subunit (C^{364}) acts as an intracellular sensor for the actions of ROS on homomeric $GABA_A\rho1$Rs expressed in oocytes. This cysteine residue is also present in $\rho2$ subunits, but not in $\rho3$ subunits. Meanwhile, γ-subunits have a group of

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five cysteines whose relative positions are conserved at this domain, with two of them contiguous, in sharp contrast with those exhibited by the ρ-subunits that show a different arrangement (Fig. 1B). The M3-M4 linker of the Cys-loop receptors is a large intracellular loop (~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_2O_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_2O_2 application was equivalent to that observed by using an oxygen-glucose deprivation protocol, which produces high endogenous levels of H_2O_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 $GABA_A$Rs 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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