

# **An intracellular redox sensor for reactive oxygen species at the M3-M4 linker of GABA<sub>A</sub> receptors.**

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*Running title: GABA<sub>A</sub> receptors and ROS*

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This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/bph.12581

## Summary

### BACKGROUND AND PURPOSE

ROS are normally involved in cell oxidative stress but also play a role as cellular messengers in redox signaling, for example modulating the activity of neurotransmitter receptors and ion channels. However, the direct actions of ROS on GABA<sub>A</sub> receptors were not previously demonstrated. In the present work, we studied the effects of ROS on GABA<sub>Ap1</sub> receptor function.

### EXPERIMENTAL APPROACH

GABA<sub>Ap1</sub> receptors were expressed in oocytes and GABA-evoked responses electrophysiologically recorded in the presence or absence of ROS. Chemical protection of cysteines by selective sulfhydryl reagents and site-directed mutagenesis studies were used to identify protein residues involved in ROS actions.

### KEY RESULTS

GABA<sub>Ap1</sub> receptor-mediated responses were significantly enhanced in a concentration-dependent and reversible manner by H<sub>2</sub>O<sub>2</sub>. Potentiating effects were attenuated by a free radical scavenger, lipoic acid, or an inhibitor of the Fenton reaction, deferoxamine. Each p1 subunit contains only three cysteine residues, two extracellular at the Cys-loop (C<sup>177</sup> and C<sup>191</sup>) and one intracellular (C<sup>364</sup>) at the M3-M4 linker. Mutant GABA<sub>Ap1</sub> receptors in which C<sup>364</sup> was exchanged by alanine were completely insensitive

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to modulation, implying that this site, rather than a cysteine in the Cys-loop, is essential for ROS modulation.

## CONCLUSIONS AND IMPLICATIONS

Our results show that the function of GABA<sub>A</sub>ρ1 receptors is enhanced by ROS and that the intracellular C<sup>364</sup> is the sensor for ROS actions.

### Abbreviations

GABA, γ-aminobutyric acid; ROS, reactive oxygen species; NEM, N-ethylmaleimide; DFX, deferoxamine; LA, lipoic acid; CNS, central nervous system.

## Introduction

Reactive oxygen species (ROS) are highly active molecules (e.g: superoxide ( $O_2^-$ ), hydroxyl radical ( $OH^\cdot$ ) and hydrogen peroxide ( $H_2O_2$ )), that induce oxidative stress in cells (Adam-Vizi, 2005; Rhee, 2006; Halliwell, 2011). ROS are involved in brain processes underlying normal aging and the development of neurodegenerative disorders (Parkinson's and Alzheimer's disease, Schizophrenia, amyotrophic lateral sclerosis and ischemia-reperfusion injury) (Annunziato et al., 2002; Brennan and Kantorow, 2009; Do et al., 2009). Beside their role in pathological processes, some ROS act as cellular messengers in redox signaling (Bao et al., 2009; Toledano et al., 2010; Rice, 2011). ROS are primarily produced as by-products of the mitochondrial oxidative metabolism and act as local diffusible messengers that regulate neuron-glia and interneuronal communication (Giniatullin et al., 2005; Safiulina et al., 2006; Kishida and Klann, 2007). In the central nervous system (CNS) ROS can be also generated secondary to the activation of neuronal NMDA and AMPA receptors (Kishida and Klann, 2007). It was as well suggested that ROS levels modulate neurotransmission and eventually cause changes in neuronal activity and induce synaptic plasticity (Colton et al., 1989; Bernard et al., 1997; Frantseva et al., 1998; Knapp and Klann, 2002; Garcia et al., 2011). Considering the production of ROS and their effects on many ligand- and voltage-gated ion channels (Aizenman et al., 1990; Vega-Saenz De Miera and Rudy, 1992; Li et al., 1998; Annunziato et al., 2002; Dirksen, 2002; Campanucci et al., 2008; Coddou et al., 2009; Rice, 2011) we hypothesized that ionotropic  $\gamma$ -aminobutyric acid (GABA) receptors might also be targets for ROS actions. Previous work showed that diverse redox agents modulate the activity of native and cloned ionotropic GABA receptors (Amato et al., 1999; Pan et al., 2000; Calero and Calvo, 2008;

Calero et al., 2011; Gasulla et al., 2012), but direct effects of ROS on GABA receptors had not been examined before. A number of studies have indicated that GABAergic neurotransmission is sensitive to ROS (Sah and Schwartz-Bloom, 1999; Sah et al., 2002; Takahashi et al., 2007; Saransaari and Oja, 2008; Tarasenko et al., 2012) and high levels of these agents are normally generated in the retina (Brennan and Kantorow, 2009). However, the molecular targets for ROS actions on the synaptic GABAergic machinery remained so far elusive.

GABA<sub>A</sub> receptors are GABA-gated pentameric chloride (Cl<sup>-</sup>) channels, members of the Cys-loop receptor superfamily, that mediate most of the inhibitory neurotransmission in the CNS (Moss and Smart, 2001; Farrant and Nusser, 2005). GABA<sub>A</sub> receptors are made up by combination of diverse functionally distinct subunits ( $\alpha_{1-6}$ ,  $\beta_{1-3}$ ,  $\gamma_{1-3}$ ,  $\delta$ ,  $\epsilon$ ,  $\pi$ ,  $\theta$ ,  $\rho_{1-3}$ ) that commonly form heterooligomeric complexes (e.g: GABA<sub>A</sub> $\alpha_1\beta_2\gamma_2$  receptors) (Farrant and Nusser, 2005). Most of these heteromeric GABA<sub>A</sub> receptors are antagonized by bicuculline or picrotoxin and modulated by benzodiazepines and barbiturates (Moss and Smart, 2001). In contrast, GABA<sub>A</sub> $\rho$  receptors appear to be exclusively composed of  $\rho$  subunits which are widely distributed in the CNS, but are highly expressed only in the retina densely and selectively concentrated in bipolar cells (Enz et al., 1995; Boue-Grabot et al., 1998; Wegelius et al., 1998). GABA<sub>A</sub> $\rho$  receptor-mediated responses are blocked by picrotoxin but typically insensitive to bicuculline (Zhang et al., 2001). GABA<sub>A</sub> $\rho_1$  receptors display both high affinity for GABA and poor desensitization (Farrant and Nusser, 2005), these distinctive properties allow them to mediate several modes of inhibitory signaling in the retina (Matthews, 1994; Zhang and Slaughter, 1995; Dong and Werblin, 1998; Hartveit, 1999; McCall et al., 2002; Lukasiewicz et al., 2004; Hull et al., 2006; Chávez et al., 2010).

In the present work, GABA<sub>A</sub>ρ<sub>1</sub> receptor activity was measured before, during and after ROS generation using an *in vitro* cell model. GABA<sub>A</sub>ρ<sub>1</sub> receptors were heterologously expressed in *Xenopus laevis* oocytes and GABA<sub>A</sub>ρ<sub>1</sub> receptor-mediated Cl<sup>-</sup> currents electrophysiologically recorded. Our results showed that responses mediated by GABA<sub>A</sub>ρ<sub>1</sub> receptors are potentiated by ROS. Additionally, experiments involving the chemical modification of sulfhydryl groups and site-directed mutagenesis indicated that Cys<sup>364</sup>, located at the intracellular M3-M4 linker of the ρ<sub>1</sub> subunits, is essential for ROS modulation.

## Methods

All experimental procedures were carried out in accordance with the National Institutes of Health *Guidelines for the Care and Use of Laboratory Animals* and were approved by the CONICET-University of Buenos Aires Animal Care and Use Committee.

### *RNA preparation, oocyte isolation and cell injection*

Human cDNA encoding the ρ<sub>1</sub> GABA<sub>A</sub> receptor subunit, cloned in the *in vitro* transcription-suitable vector pGEM, was used as a template to synthesize cRNAs *in vitro*. Site-directed mutagenesis was achieved by the PCR overlap extension method using QuickChange Site-Directed Mutagenesis Kit (Stratagene). cRNA solutions (0.3-1 ng/μl) were prepared in Rnase-free H<sub>2</sub>O and stored at -70°C. *Xenopus laevis* (Nasco, Modesto, CA, USA) oocytes at stages V and VI were used for expression of exogenous cRNAs. Isolation and maintenance of cells were carried out as previously described (Miledi and Woodward, 1989). Briefly, frogs were anesthetized with 3-aminobenzoic-acid ethylester

(~1 mg/ml) and ovaries surgically removed. Ovaries were incubated with 400 U/ml collagenase for 4 h at 23–24°C and isolated oocytes maintained in an incubator at 18°C in Barth's medium (in mM: 88 NaCl; 0.33 Ca(NO<sub>3</sub>)<sub>2</sub>; 0.41 CaCl<sub>2</sub>; 1 KCl; 0.82 MgSO<sub>4</sub>; 2.4 NaHCO<sub>3</sub>; 10 HEPES and 0.1 mg/ml gentamicin; pH adjusted to 7.4 with NaOH). After 1 day, each oocyte was manually microinjected (microinjector Drummond Sci. Co., Broomall, PA, USA) with 50 nl of a solution containing 5–50 ng of cRNA.

### *Electrophysiological recordings*

Two-electrode voltage-clamp recordings were performed 3–7 days after oocyte injection, with an Axoclamp 2B amplifier (Axon Instruments, Union City, CA, USA). Standard glass recording electrodes were made in a Narishige PB-7 puller (Narishige Scientific Instrument Lab., Tokyo, Japan) and filled with 3 M KCl. Pipette resistance values were approximately 1 MΩ. The holding potential was set to -70 mV and current traces acquired by a PC through a Labmaster TL-1 DMA interface (Scientific solutions Inc., Solon, OH, USA) using AXOTAPE software (Axon Instruments). Cells were placed in a chamber (volume 100 μl) continuously superfused (12 ml/min) with frog Ringer's solution (in mM: 115 NaCl; 2 KCl; 1.8 CaCl<sub>2</sub>; 5 HEPES; pH 7.0). GABA and other drugs were applied through the perfusion system (Goutman et al., 2005). N-ethyl maleimide (NEM) was freshly prepared prior to each experiment in normal Ringer's. Hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) stock solution (1 M) was stored at -20 °C and its concentration was confirmed spectrophotometrically at 240 nm. pH was adjusted to 7.0 with NaOH (1 M) or HCl (1 M). All the experiments were carried out at room temperature (23–24°C) and were replicated in at least 5 different oocytes isolated from at least two different frogs.

## Materials

The transcription kit mMessage mMachine was purchased from Ambion (Austin, TX, USA), QuickChange Site-Directed Mutagenesis Kit was from Stratagene (La Jolla, CA, USA) and type I or type II collagenase from Worthington (Freehold, NJ, USA). The agonist and all the drug and salts, HEPES, 3-aminobenzoic-acid ethylester and Rnase-free H<sub>2</sub>O were purchased from Sigma-Aldrich (St Louis, MO, USA).

## Data analysis

Data were analyzed with Prism v. 5.0 (Graphpad Software, Inc. San Diego, CA, USA). Concentration–response curves for GABA and concentration-effect curve for H<sub>2</sub>O<sub>2</sub> were fit with a logistic equation of the following form:  $I_{GABA}/B = [A^n/(A^n + EC_{50}^n)] \times 100$  where  $A$  is the agonist concentration,  $B$  the maximal response,  $EC_{50}$  the concentration of agonist that elicits half-maximal responses, and  $n$  the Hill coefficient. Percentage of potentiation was calculated as  $[(I_{GABA\rho 1H_2O_2} \times 100 / I_{GABA\rho 1control}) - 100]$ , where  $I_{GABA\rho 1H_2O_2}$  indicates the current amplitude evoked at each particular GABA concentration in the presence of H<sub>2</sub>O<sub>2</sub> and  $I_{GABA\rho 1control}$  the corresponding responses in the absence of modulator. Student's t-tests (two tailed) were employed to evaluate significant differences between parameters. In all cases errors were expressed as SEM.



## Results

### *Functional modulation of GABA<sub>A</sub>ρ1 receptors by H<sub>2</sub>O<sub>2</sub>*

GABA applications to oocytes expressing homomeric GABA<sub>A</sub>ρ1 receptors induce large inward Cl<sup>-</sup> currents displaying all features of the bicuculline resistant component of the retinal GABA receptor-mediated responses (Zhang *et al.*, 2001; Hull *et al.*, 2006). For example, in addition to their bicuculline insensitivity, they are antagonized by TPMPA and picrotoxin, non-desensitizing and display the same pharmacological profile for agonists. Fig. 1 illustrates representative responses elicited by 0.3 μM GABA in the absence or presence of H<sub>2</sub>O<sub>2</sub>, recorded at -70 mV. Significant potentiation of the GABA-evoked responses was produced by H<sub>2</sub>O<sub>2</sub> (500 μM). In order to characterize H<sub>2</sub>O<sub>2</sub> effects we mainly used two different procedures with equivalent results namely, H<sub>2</sub>O<sub>2</sub> applications made *on-top* of the plateau of the GABA responses (Fig. 1A), or co-applications of H<sub>2</sub>O<sub>2</sub> and GABA (Fig. 1B). Preincubation with H<sub>2</sub>O<sub>2</sub> (up to 10 min) was also tested and gave equivalent results. H<sub>2</sub>O<sub>2</sub> effects were reversible (Fig. 1A) and a second application (not shown) produced similar results. Our previous work showed that several redox modulators of the GABA<sub>A</sub>ρ1 receptors such as ascorbic acid and glutathione act rapidly and extracellularly (Calero and Calvo, 2008; Calero *et al.*, 2011). In contrast, potentiation of the GABA<sub>A</sub>ρ1 receptor activity by H<sub>2</sub>O<sub>2</sub> showed a relatively slow onset (Fig. 1A). Considering that H<sub>2</sub>O<sub>2</sub> is membrane permeable (Desagher *et al.*, 1997), the slow time course of action might be the result of an intracellular mechanism. No appreciable effects on oocyte properties, such as membrane potential, membrane resistance or current baseline under voltage-clamp, were observed during H<sub>2</sub>O<sub>2</sub> applications (500 μM, up to 10 min) (Fig. 1C).

Concentration-response curves (C-R) for GABA were also performed in the absence (control) or presence of H<sub>2</sub>O<sub>2</sub> (Fig. 2A). H<sub>2</sub>O<sub>2</sub> (500 μM) produced a leftward shift in GABA<sub>EC50</sub> without significantly affecting the maximal responses to GABA and *n*H (EC<sub>50</sub><sub>GABA</sub> = 0.76 ± 0.03 μM, *n*H = 2.4 ± 0.3, *n* = 6; EC<sub>50</sub><sub>GABA + H2O2</sub> = 0.64 ± 0.03 μM, *n*H = 2.5 ± 0.4, *n* = 6; *p* < 0.005). In order to determine the concentration range for effective H<sub>2</sub>O<sub>2</sub> modulation, we tested increasing concentrations of H<sub>2</sub>O<sub>2</sub>. A concentration-effect curve (C-E) (Fig. 2B) was fitted to a sigmoid equation (see Methods). No saturation was observed at the maximal H<sub>2</sub>O<sub>2</sub> concentration tested (2 mM), but higher concentrations of H<sub>2</sub>O<sub>2</sub> significantly increased leak currents and were toxic to the oocyte membrane. Similarly to effects displayed by other GABA<sub>A</sub>1 receptor redox modulators previously studied (Calero and Calvo, 2008; Calero et al., 2011) the degree of potentiation exerted by H<sub>2</sub>O<sub>2</sub> on GABA<sub>A</sub>1 receptor responses decreased as GABA concentration increased (Fig. 2C). For example, the amplitude of currents evoked by 0.3 μM GABA was enhanced in a 73.6 ± 3.0 % (*n* = 10), whereas potentiation of currents evoked by 30 μM GABA was 1.3 ± 0.7 % (*n* = 5). For GABA concentrations lower than 3 μM, potentiation induced by H<sub>2</sub>O<sub>2</sub> was always significant (*p* < 0.05). Current-voltage relationships (I-V curves) were carried out, in the presence or absence of H<sub>2</sub>O<sub>2</sub>. In the presence of H<sub>2</sub>O<sub>2</sub> (500 μM) a significant change in slope without alteration in linearity of the I-V relationship or reversal potential (between -120 and +40 mV) was observed (Fig. 2D). Therefore, H<sub>2</sub>O<sub>2</sub> effects were voltage independent and not due to variations in intracellular Cl<sup>-</sup> levels.

***Potentiation of GABA<sub>A</sub>1 receptors by H<sub>2</sub>O<sub>2</sub> is mediated by an intracellular cysteine residue***

Many ionic channels sensitive to redox modulation can be chemically modified through oxidation of cysteine residues. We have previously shown that reducing and oxidizing thiol agents are also effective modulators of the GABA<sub>A</sub>ρ1 receptor function (Calero and Calvo, 2008). The reversible effect of H<sub>2</sub>O<sub>2</sub> on GABA<sub>A</sub>ρ1 receptors is consistent with a direct modulatory action, and thiol groups located at the ρ<sub>1</sub> subunits are the most reactive candidates to be oxidized by this agent. In order to elucidate if cysteines are involved in this modulation, we examined the effect of dithiothreitol (DTT), a membrane permeable reagent which reversibly reduces the thiol groups (Lauriault and O'Brien, 1991). Preincubation with DTT 2 mM for 120 s enhanced the potentiating effects of H<sub>2</sub>O<sub>2</sub> on GABA<sub>A</sub>ρ1 receptor responses (% P<sub>control</sub> = 72.3 ± 5.5 %; %P<sub>DTT 2 mM</sub> = 129.2 ± 17.5 %; n = 6; *p* < 0.03) (Fig. 3A). We further examined if H<sub>2</sub>O<sub>2</sub> actions on GABA<sub>A</sub>ρ1 receptor mediated responses were affected in the presence of a reagent that irreversibly modifies cysteine thiol groups. N-ethyl-maleimide (NEM) is a membrane permeable irreversible alkylating reagent which selectively forms covalent bonds with free sulfhydryl groups, preventing any further chemical reaction at these sites (at pH = 7) (Means and Feeney, 1971). NEM concentration was kept as low as possible, and incubation periods maintained very short to prevent non-specific effects (Calero et al., 2011). Pre-incubation with NEM 30 μM for 120 s completely prevented potentiating effects of H<sub>2</sub>O<sub>2</sub> on GABA<sub>A</sub>ρ1 receptor responses (% P<sub>control</sub> = 68.1 ± 5.1 %; %P<sub>NEM 30 μM</sub> = 2.9 ± 3.2 %; n = 8; *p* < 0.0001) (Fig. 3B). These results suggest that H<sub>2</sub>O<sub>2</sub> modulates GABA<sub>A</sub>ρ1 receptor function by interacting with one or more cysteines.

Each ρ1 subunit contains only three cysteine residues, two extracellular located in the N-terminal domain that form the characteristic Cys-loop (C<sup>177</sup> and C<sup>191</sup>) of the

GABA<sub>Ap1</sub> receptors and one intracellular (C<sup>364</sup>) located at the M3-M4 linker (Zhang et al., 2001). Mutations of the cysteines at the Cys-loop are precluded because they render non-functional receptors (Amin et al., 1994). To determine whether C<sup>364</sup> was involved in the modulation of GABA<sub>Ap1</sub> receptors by H<sub>2</sub>O<sub>2</sub>, we performed site-directed mutagenesis replacing this aminoacidic residue by alanine, a small neutral amino acid insensitive to redox modulation. Mutant GABA<sub>Ap1C364A</sub> receptors expressed in oocytes responded to GABA ( $EC_{50} \text{ GABA}_{Ap1C364A} = 0.52 \pm 0.03 \mu\text{M}$ ,  $n = 7$ ;  $EC_{50} \text{ GABA}_{Ap1wt} = 0.70 \pm 0.04 \mu\text{M}$ ,  $n = 6$ ) and showed a similar pharmacological profile. Interestingly, in sharp contrast with wild-type receptors, GABA<sub>Ap1C364A</sub> receptors were largely insensitive to H<sub>2</sub>O<sub>2</sub> applications. Potentiation of 0.3  $\mu\text{M}$  GABA responses by 500  $\mu\text{M}$  H<sub>2</sub>O<sub>2</sub> was as follows: % P  $\text{GABA}_{Ap1C364A} = 1.1 \pm 0.9$  vs. % P  $\text{GABA}_{Ap1wt} = 72.3 \pm 9.3$ ;  $n = 5$ ;  $p < 0.0001$  (Fig. 4A). Because H<sub>2</sub>O<sub>2</sub> potentiation observed in wild-type receptors could be due to the polar environment conferred by the C<sup>364</sup>, and GABA<sub>Ap1C364A</sub> receptors showed changes in GABA affinity when compared to wild-type receptors, we performed a more conservative mutation and further replaced this cysteine by serine, an amino acid of similar polarity and size. Mutant GABA<sub>Ap1C364S</sub> receptors expressed in oocytes showed typical responses to GABA and  $EC_{50}$  values for GABA were not significantly different from the  $EC_{50}$  values obtained for wild type receptors ( $EC_{50} \text{ GABA}_{Ap1C364S} = 0.69 \pm 0.04 \mu\text{M}$ ,  $n = 14$ ;  $EC_{50} \text{ GABA}_{Ap1wt} = 0.71 \pm 0.04 \mu\text{M}$ ,  $n = 7$ ; *n.s.*). H<sub>2</sub>O<sub>2</sub> (500  $\mu\text{M}$ ) also failed to potentiate GABA responses elicited by GABA<sub>Ap1C364S</sub> receptor. Potentiation of 0.3  $\mu\text{M}$  GABA responses by 500  $\mu\text{M}$  H<sub>2</sub>O<sub>2</sub> was as follows: % P  $\text{GABA}_{Ap1C364S} = 2.5 \pm 1.9$  vs. % P  $\text{GABA}_{Ap1wt} = 72.6 \pm 6.2$ ,  $n = 8$ ;  $p < 0.0001$  (Fig. 4B). To further analyze if the insensitivity of GABA<sub>Ap1C364S</sub> receptor to H<sub>2</sub>O<sub>2</sub> depended on GABA concentration, we performed C–R curves for GABA either in the absence (control) or the presence of H<sub>2</sub>O<sub>2</sub> (Fig. 4C). H<sub>2</sub>O<sub>2</sub> (500  $\mu\text{M}$ ) had no shifting effect

on GABA EC<sub>50</sub> in GABA<sub>A</sub>ρ<sub>1</sub>C364S receptor (EC<sub>50</sub> GABA<sub>A</sub>ρ<sub>1</sub>C364S = 0.71 ± 0.02 μM, nH = 2.6 ± 0.2; EC<sub>50</sub> GABA<sub>A</sub>ρ<sub>1</sub>C364S+H<sub>2</sub>O<sub>2</sub> = 0.67 ± 0.02 μM, nH = 2.7 ± 0.3; n = 5; n.s).

In summary, these results indicate that the intracellular cysteine C<sup>364</sup>, located at the intracellular M3-M4 linker of the ρ<sub>1</sub> subunits, can be a specific target for the action of ROS and that its chemical modification by ROS potentiates the function of GABA<sub>A</sub>ρ<sub>1</sub> receptors.

### ***Hydroxyl radicals are involved in the potentiation of GABA<sub>A</sub>ρ<sub>1</sub> receptor function by H<sub>2</sub>O<sub>2</sub>***

In the presence of iron (II) traces, H<sub>2</sub>O<sub>2</sub> generates hydroxyl radicals (OH) via Fenton reaction (Sah et al., 2002). In order to determine if H<sub>2</sub>O<sub>2</sub> can act indirectly through the production of hydroxyl radicals we studied the effect of H<sub>2</sub>O<sub>2</sub> on GABA responses in the presence of lipoic acid (a free radical scavenger), deferoxamine (DFX) (an iron chelator that inhibits Fenton reaction) and iron (II) sulfate (that enhances Fenton reaction). The potentiation of GABA<sub>A</sub>ρ<sub>1</sub> receptor current responses elicited by 0.3 μM GABA during *on-top* applications of 500 μM H<sub>2</sub>O<sub>2</sub> decreased in the presence of 200 μM lipoic acid (control= 82.1 ± 7.3 %, n= 9; lipoic acid= 30.5 ± 5.6 %, n= 6; p<0.001) (Fig. 5A) or 100 μM DFX (control= 68.7 ± 10.9 %, n = 3; DFX= 32.2 ± 7.4 %, n= 6; p<0.002) (Fig. 5B) and increased after pre-incubation with 100 μM FeSO<sub>4</sub> for 2 min (control = 71.3 ± 4.1 %, n = 6; FeSO<sub>4</sub> = 104.4 ± 8.1 %, n = 6; p<0.005) (Fig. 5C). Potentiation of the GABA<sub>A</sub>ρ<sub>1</sub> receptor-mediated current was reversible in the presence of iron as it was in the presence of H<sub>2</sub>O<sub>2</sub> alone (not shown). None of these agents produced significant changes in the current baseline or modulate GABA<sub>A</sub>ρ<sub>1</sub> receptor responses *per se*. These results suggest that hydroxyl radicals contribute to the potentiation of GABA<sub>A</sub>ρ<sub>1</sub> receptor function by H<sub>2</sub>O<sub>2</sub>.

## Discussion and conclusions

The present findings are the first to demonstrate the existence of a putative intracellular redox sensor at an ionotropic GABA receptor. We showed here the potentiation of the homomeric GABA<sub>A</sub>ρ1 receptor function by ROS and identified the intracellular C<sup>364</sup> residue, located at the M3-M4 cytoplasmic linker of the ρ1 subunits, as the target for ROS actions.

GABA<sub>A</sub>ρ1 receptors can be considered a simple and suitable model for studying the sensitivity of ionotropic GABA receptors to ROS for several reasons. GABA<sub>A</sub>ρ1 receptors are key players in synaptic inhibition in the retina, a tissue that produces high levels of ROS. In addition, homomeric ρ receptors present less structural complexity compared to classic heteromeric GABA<sub>A</sub>αβγ receptors. In fact, ρ1 subunits contain only two molecular sites contributing potential reactive cysteine residues (the extracellular Cys-loop and the single intracellular C<sup>364</sup>), whereas other GABA<sub>A</sub> receptor subunit subtypes can exhibit multiple intracellular cysteine residues as potential targets for ROS modulation (Sedelnikova et al., 2005; Lo et al., 2008).

### *Mechanisms underlying the potentiation of GABA<sub>A</sub>ρ1 receptors by ROS*

The sensitivity of GABA<sub>A</sub>ρ1 receptors to ROS was unknown, but previous work indicated that ROS were capable of modulating GABAergic neurotransmission, presumably via both presynaptic and postsynaptic mechanisms (Colton et al., 1986; Sah et al., 2002; Safiulina et al., 2006; Takahashi et al., 2007; Saransaari and Oja, 2008; Yowtak et al., 2011; Tarasenko et al., 2012). Sah and co-workers showed that exposure of hippocampal

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slices to H<sub>2</sub>O<sub>2</sub> concomitantly altered GABA<sub>A</sub> receptor binding characteristics and increased GABA<sub>A</sub> receptor-mediated Cl<sup>-</sup> influx in CA1 pyramidal cells (Sah and Schwartz-Bloom, 1999; Sah et al., 2002). These results raised the question if postsynaptic ROS effects were exerted at the GABAergic synapses by directly acting through redox-sensitive sites on GABA receptors, or indirectly via peroxidation of membrane lipids located near to the Cl<sup>-</sup> channel (Sah et al., 2002). We demonstrated here that ROS are capable of inducing functional changes on GABA<sub>A</sub>ρ1 receptors, a GABA<sub>A</sub> receptor subtype highly expressed in retinal bipolar cells. These changes were similar to that reported for classic GABA<sub>A</sub> receptors from different brain regions (Sah et al., 2002). However, we also provided experimental evidences indicating that GABA<sub>A</sub>ρ1 receptors are directly modulated by ROS. Potentiation of GABA<sub>A</sub>ρ1 receptors by H<sub>2</sub>O<sub>2</sub> was reversible, concentration-dependent, voltage-independent and strongly dependent on the GABA concentration. H<sub>2</sub>O<sub>2</sub> effects were partially prevented in the presence of the free radical scavenger lipoic acid or by DFX, an inhibitor of the Fenton reaction. In contrast, potentiation of GABA<sub>A</sub>ρ1 receptors by H<sub>2</sub>O<sub>2</sub> was extensively enhanced if Fenton reaction was amplified by using iron (II) sulfate. These data suggest that H<sub>2</sub>O<sub>2</sub> can act as a precursor for the generation of hydroxyl radicals that eventually exerted their effects on the GABA receptors. H<sub>2</sub>O<sub>2</sub>-induced potentiation of GABA<sub>A</sub>ρ1 receptors persisted in the presence of DFX, thus H<sub>2</sub>O<sub>2</sub> might also be acting directly on the receptor to produce these modulatory effects without reaction intermediates involved.

Due to their reactivity, protein cysteine residues were good candidates for sensing ROS. Chemical protection studies, using the selective membrane permeable sulfhydryl reagent NEM, and site-directed mutagenesis experiments, where C<sup>364</sup> was replaced by

alanine or serine, indicated that this particular intracellular residue is essential for ROS effects to take place. The slow onset for potentiation (illustrated in Fig. 1) was also consistent with an intracellular mechanism of action. External  $H_2O_2$  concentrations below the range of those normally used in previous studies (Vega-Saenz De Miera and Rudy, 1992; Rice, 2011) had significant effects on  $GABA_{A\rho 1}$  receptors. The effective intracellular concentrations sensed by the  $GABA_{A\rho 1}$  receptors are expected to be lower than bath concentrations, due to the high reducing power of the oocyte's cytoplasm. The intracellular antioxidant network that maintains redox balance of amphibian oocytes is composed by multiple enzymatic activities and metabolites, as superoxide dismutase, catalase, ascorbic acid and glutathione and other (Ferrari et al., 2008). Actually, ROS effects on  $GABA_{A\rho 1}$  receptors were completely washed out in the absence of supplementary reducing agents, probably because the intracellular environment caused potentiation to cease after  $H_2O_2$  applications were stopped. This also suggests that  $C^{364}$  may undergo a reversible chemical modification producing a transient conformational change in the receptor that, in the absence of ROS, rapidly relaxed to a lower energy state. One possible interpretation is that oxidation of the thiol group of  $C^{364}$  by ROS induced protein structural rearrangements that impact on GABA binding. The leftward shift produced in C-R curves for GABA in the presence of  $H_2O_2$  is compatible with this hypothesis. In addition, because  $H_2O_2$  treatment did not change the reversal potential of the I-V curves is unlikely that, in our experiments, ROS actions were due to a change in the intracellular  $Cl^-$  levels.



*Potential physiological relevance of the modulation of ionotropic GABA receptors by ROS*

ROS production in neurons can impact on multiple targets, including several neurotransmitter receptors (Rice, 2011). Previous studies demonstrated that modulation of nicotinic cholinergic and purinergic receptors by ROS is exerted through specific intracellular cysteines (Campanucci et al., 2008; Coddou et al., 2009) and that actions of this kind could be involved in neuropathological events (Campanucci et al., 2008, 2010). Concerning GABA<sub>A</sub> receptors, is quite remarkable that all  $\rho$  subunits display a conserved single intracellular cysteine residue at the M3-M4 linker (C<sup>364</sup> in  $\rho_2$  and C<sup>379</sup> in  $\rho_3$ ). Moreover, most of the GABA<sub>A</sub> receptor subunits contain also one or more cysteine residues at their intracellular loops. Particularly, the M3-M4 intracellular loop is known to interact directly with several cellular regulatory proteins which can be involved in GABA<sub>A</sub> receptor oligomerization, assembly, forward trafficking and clustering (Boué-Grabot et al., 2004; Lo et al., 2008). Thus, the importance that these intracellular cysteines might have for GABA<sub>A</sub> receptor function during endogenous ROS generation in neurons deserves to be further studied. Given that H<sub>2</sub>O<sub>2</sub> is generated normally during cell activity, whereas hydroxyl radical is typically generated under pathological conditions, it will be important to establish whether redox modulation of GABA receptors is physiological or pathophysiological. It will be also interesting to examine whether oxidation of these intracellular cysteines by ROS might represent a common mechanism for regulating the activity of diverse GABA<sub>A</sub> receptor subtypes and other members of the cys-loop receptor superfamily.

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Since GABA<sub>A</sub>ρ1 receptors provide significant inhibitory drive to the synaptic terminals of retinal bipolar cells, including tonic, reciprocal, and lateral inhibition (Zhang and Slaughter, 1995; Lukasiewicz et al., 2004; Hull and von Gersdorff, 2004; Chavez et al., 2010), modulation of the GABA<sub>A</sub>ρ1 receptor activity by ROS could eventually shape ganglion cell responses via control of glutamate release at these terminals. Nevertheless, if ROS modulation of ionotropic GABA receptors represents a physiologically relevant mechanism for controlling the activity of retinal neuronal circuits will need to be assessed by using both retinal slices and *in vivo* models.

#### **Acknowledgements**

We thank Dr. J. J. Poderoso and his group, Dr. Cecilia I. Calero and Dr. Marcela Lipovsek for discussion. This work was supported by CONICET and FONCyT grants.

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## Figure legends

**Figure 1.** Potentiating effects of H<sub>2</sub>O<sub>2</sub> on responses mediated by GABA<sub>A</sub>ρ1 receptors expressed in *Xenopus laevis* oocytes. Representative traces of GABA<sub>A</sub>ρ1 receptors mediated Cl<sup>-</sup> currents elicited by GABA (0.3 μM) applications (indicated as bars) in the absence (control) or presence of H<sub>2</sub>O<sub>2</sub> (500 μM). H<sub>2</sub>O<sub>2</sub> was either delivered on-top of the GABA-evoked responses (A) or co-applied with GABA (B), flanked by control responses to GABA. (C) Lack of effect of H<sub>2</sub>O<sub>2</sub> on a representative baseline current recorded from a non-transfected oocyte. For this and the subsequent figures, the oocytes were voltage-clamped at -70 mV. Scale bars indicate current amplitude (y-axis) and time (x-axis).

**Figure 2.** Analysis of H<sub>2</sub>O<sub>2</sub> effects on GABA<sub>A</sub>ρ1 receptors. (A) C-R curves for GABA in the absence (●) or presence (■) of H<sub>2</sub>O<sub>2</sub> (500 μM). Response amplitudes were expressed as fraction of 30 μM GABA-evoked currents (maximal response). (B) Potentiation of GABA<sub>A</sub>ρ1 receptors responses (0.3 μM GABA) by increasing concentrations of H<sub>2</sub>O<sub>2</sub>. (C) GABA concentration dependence of the induced H<sub>2</sub>O<sub>2</sub> (500 μM) potentiation of GABA<sub>A</sub>ρ1 receptors responses. (D) I-V relationship for GABA<sub>A</sub>ρ1 receptors responses evoked by GABA (0.3 μM) in the absence (●) or presence (■) of H<sub>2</sub>O<sub>2</sub> (500 μM).

**Figure 3.** Cysteine thiols are involved in H<sub>2</sub>O<sub>2</sub> modulation of responses mediated by GABA<sub>A</sub>ρ1 receptors. Potentiation of GABA<sub>A</sub>ρ1 receptors responses (0.3 μM GABA) by H<sub>2</sub>O<sub>2</sub> (500 μM) was enhanced if cysteine thiols were reduced with DTT (2mM) (A) and

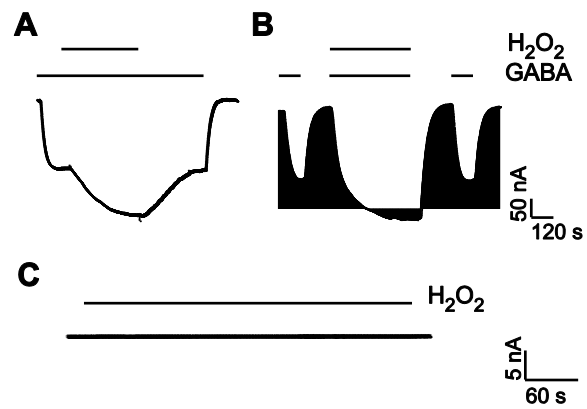
prevented if sulfhydryl residues were protected by the alkylating reagent NEM (30  $\mu$ M) (B).

**Figure 4.** Intracellular cysteine C<sup>364</sup> mediates H<sub>2</sub>O<sub>2</sub> modulation of responses mediated by GABA<sub>AP1</sub> receptors. Mutation of the C<sup>364</sup> affects the potentiating actions of H<sub>2</sub>O<sub>2</sub>. H<sub>2</sub>O<sub>2</sub> (500  $\mu$ M) failed to potentiate GABA<sub>AP1C364A</sub> (A) and GABA<sub>AP1C364S</sub> (B) receptors responses (0.3  $\mu$ M GABA). (C) C-R curves for GABA<sub>AP1C364S</sub> receptors in the absence (●) or presence (■) of H<sub>2</sub>O<sub>2</sub> (500  $\mu$ M). Response amplitudes were expressed as fraction of 30  $\mu$ M GABA-evoked currents (maximal response).

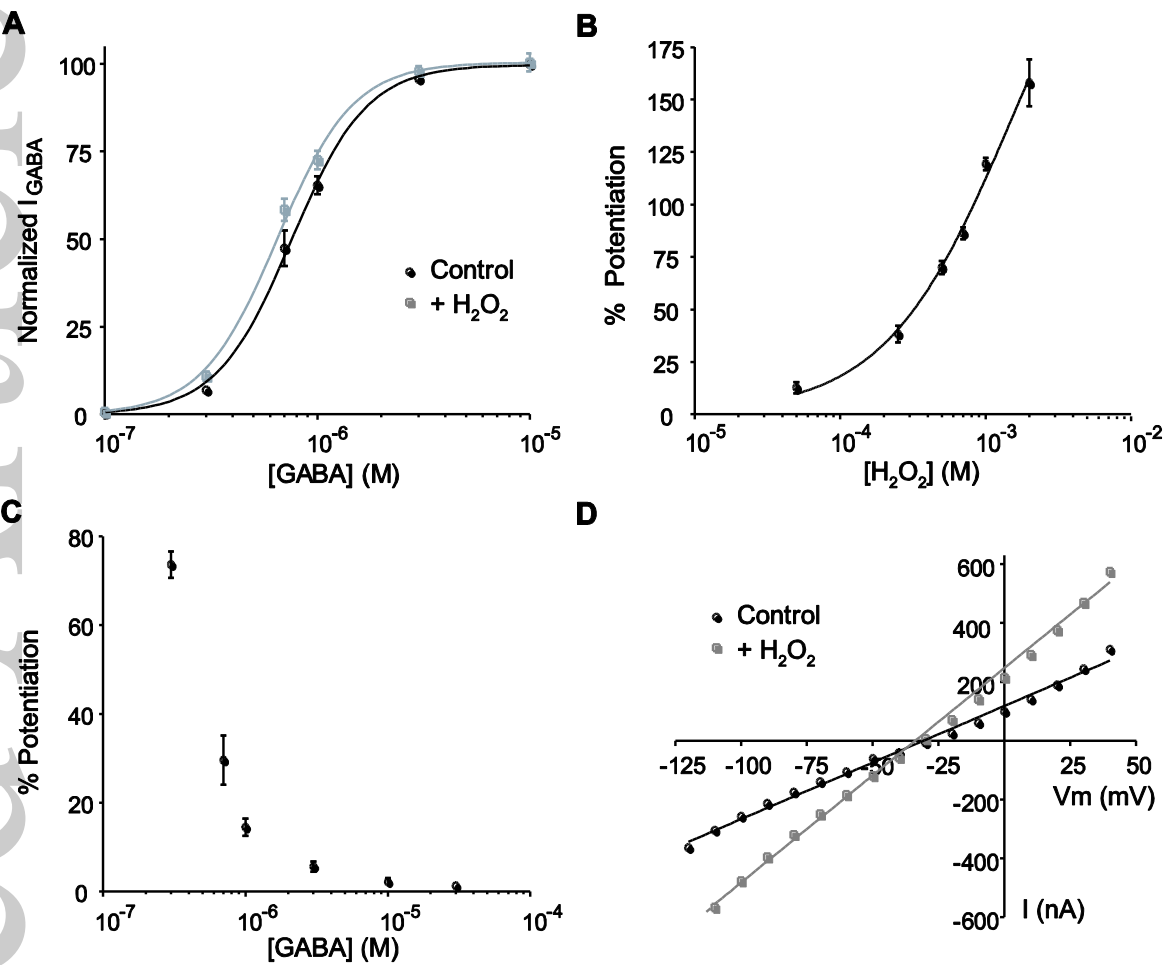
**Figure 5.** Hydroxyl radicals are involved in H<sub>2</sub>O<sub>2</sub> potentiation of GABA<sub>AP1</sub> receptors responses. Potentiation of GABA<sub>AP1</sub> receptors responses by H<sub>2</sub>O<sub>2</sub> (500  $\mu$ M) was decreased in the presence of the free radical scavenger lipoic acid (200  $\mu$ M) (A) and the iron chelator deferoxamine (100  $\mu$ M) (B) and was enhanced with pre-incubation with FeSO<sub>4</sub> (100 $\mu$ M) (C).

#### Conflict of interest

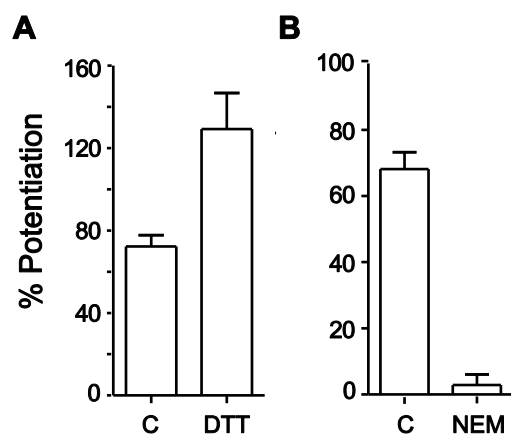
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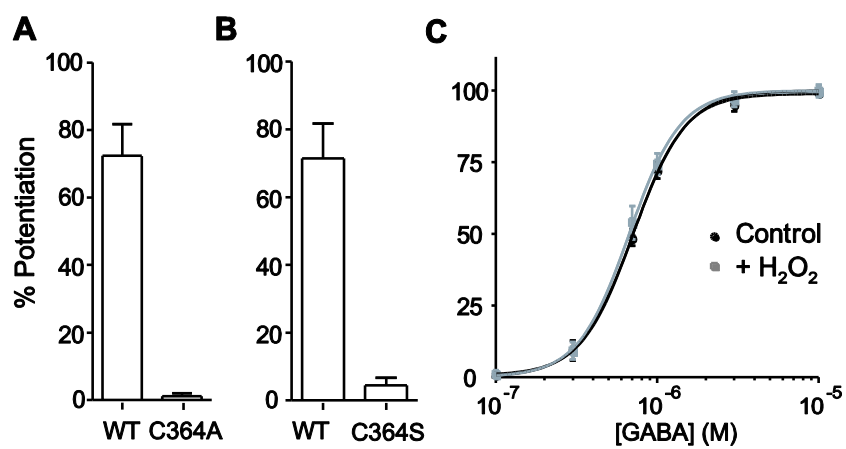
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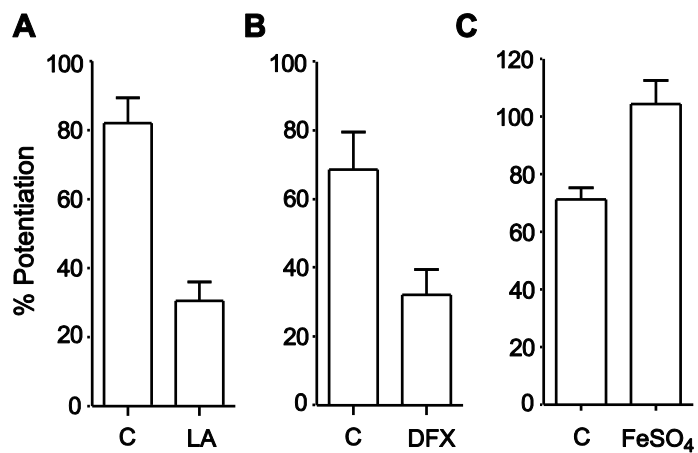
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