

## RESEARCH PAPER

# Nitric oxide potentiation of the homomeric $\rho 1$ GABA<sub>C</sub> receptor function

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### Keywords

GABA receptor; GABA<sub>C</sub> receptor; nitric oxide; S-nitrosylation; retina

### Received

18 November 2011

### Revised

7 May 2012

### Accepted

19 June 2012

## BACKGROUND AND PURPOSE

NO is a highly diffusible and reactive gas produced in the nervous system, which acts as a neuronal signal mediating physiological or pathological mechanisms. NO can modulate the activity of neurotransmitter receptors and ion channels, including NMDA and GABA<sub>A</sub> receptors. In the present work, we examined whether GABA<sub>C</sub> receptor function can also be regulated by NO.

## EXPERIMENTAL APPROACH

Homomeric  $\rho 1$  GABA<sub>C</sub> receptors were expressed in oocytes and GABA-evoked responses electrophysiologically recorded in the presence or absence of the NO donor DEA. Chemical protection of cysteines by selective sulfhydryl reagents and site-directed mutagenesis were used to determine the protein residues involved in the actions of NO.

## KEY RESULTS

GABA $\rho 1$  receptor responses were significantly enhanced in a dose-dependent, fast and reversible manner by DEA and the specific NO scavenger CPTIO prevented these potentiating effects. The  $\rho 1$  subunits contain only three cysteine residues, two extracellular at the Cys-loop (C177 and C191) and one intracellular (C364). Mutations of C177 and C191 render the  $\rho 1$  GABA receptors non-functional, but C364 can be safely exchanged by alanine (C364A). NEM, N-ethyl maleimide and (2-aminoethyl) methanethiosulfonate prevented the effects of DEA on GABA $\rho 1$  receptors. Meanwhile, the potentiating effects of DEA on mutant GABA $\rho 1$ <sub>C364A</sub> receptors were similar to those observed on wild-type receptors.

## CONCLUSIONS AND IMPLICATIONS

Our results suggest that the function of GABA<sub>C</sub> receptors can be enhanced by NO acting at the extracellular Cys-loop.

## Abbreviations

CPTIO, 2-(4-carboxyphenyl)-4,4,5,5 tetramethylimidazoline-1-oxyl-3-oxide potassium salt; DEA, 1,1-diethyl-2-hydroxy-2-nitroso-hydrazine sodium; GSNO, S-nitrosoglutathione; MTSEA, (2-aminoethyl) methanethiosulfonate; NEM, N-ethyl maleimide

## Introduction

NO is a very diffusible and reactive molecule produced in the CNS by the neuronal form of the Ca<sup>2+</sup>/calmodulin-dependent nitric oxide synthase (NOS) (Garthwaite, 2008;

Steinert *et al.*, 2011). Low concentrations of NO mediate physiological signalling (e.g. synaptic plasticity, proliferation, survival and differentiation), whereas higher concentrations can be neurotoxic (Moncada and Bolanos, 2006; Hall and Garthwaite, 2009).

Many neurotransmitter receptors and ion channels, including the major excitatory and inhibitory synaptic receptors in the CNS (e.g. glutamate and GABA receptors), are sensitive to NO (Wexler *et al.*, 1998; Ahern *et al.*, 2002; Lipton *et al.*, 2002). The effects of NO are classically mediated by activation of a soluble guanylyl-cyclase that produces cGMP (Garthwaite, 2008). However, the importance of cGMP-independent pathways is increasingly recognized (Hess *et al.*, 2005). S-nitrosylation can work as a reversible post-translational modification, analogous to phosphorylation, to convey redox-based cellular signals (Stamler *et al.*, 2001). For example, in NMDA receptors and other ionic channels, specific cysteine residues critical for channel function can be S-nitrosylated by NO (Bolotina *et al.*, 1994; Broillet and Firestein, 1996; Ahern *et al.*, 1999; Choi *et al.*, 2000; Eu *et al.*, 2000; Poteser *et al.*, 2001; Yoshida *et al.*, 2006). Thus, S-nitrosylation of synaptic receptors and ion channels was proposed as a signalling mechanism to physiologically regulate neurotransmission and neuronal excitability (Yoshida *et al.*, 2006; Takahashi *et al.*, 2007). Remarkably, the modulation of Cys-loop receptors by S-nitrosylation has not been substantiated experimentally.

Based on their pharmacology, the ionotropic GABA receptors are commonly classified in two classes denominated GABA<sub>A</sub> and GABA<sub>C</sub>. They are pentameric chloride (Cl<sup>-</sup>) channels members of the Cys-loop containing neurotransmitter receptor superfamily (Moss and Smart, 2001; Farrant and Nusser, 2005). GABA<sub>C</sub> receptors mediate responses, which are typically insensitive to the GABA<sub>A</sub> competitive antagonist bicuculline (Zhang *et al.*, 2001). They appear to be exclusively composed of  $\rho$  subunits ( $\rho$ 1,  $\rho$ 2 and  $\rho$ 3) that are widely distributed in many areas of the CNS, but highly concentrated in the retina and other visual areas (Enz *et al.*, 1995; Boue-Grabot *et al.*, 1998; Wegelius *et al.*, 1998). Interestingly, NOS is also highly expressed in the retina and NO is part of a light-activated signalling pathway that influences the physiology of all retinal neuronal types (Eldred and Blute 2005; Hoffpauir *et al.*, 2006; Wang *et al.*, 2007). In the inner retina, NO is generated at high levels and local production of NO can modulate GABAergic neurotransmission (Groppe *et al.*, 2003; Hoffpauir *et al.*, 2006; Maggesissi *et al.*, 2009).

GABA<sub>A</sub> and GABA<sub>C</sub> receptors are sensitive to many endogenous and exogenous redox agents (Amato *et al.*, 1999; Pan *et al.*, 2000; Calero and Calvo, 2008; Calero *et al.*, 2011). Several studies have also shown that NO can modulate the activity of GABA<sub>A</sub> receptors through pathways both dependent and independent, of cGMP (Fukami *et al.*, 1998; Wexler *et al.*, 1998; Castel *et al.*, 2000; Castel and Vaudry, 2001; Wall, 2003). However, the modulation of ionotropic GABA receptors by S-nitrosylation was still not demonstrated, mainly because studies combining the use of differentially acting selective thiol reagents, specific scavengers for NO and mutational analysis, were lacking. Based on the above findings, we analysed whether GABA<sub>C</sub> receptor function can also be regulated by NO. GABA<sub>C</sub> receptor-mediated Cl<sup>-</sup> currents were electrophysiologically recorded from *Xenopus laevis* oocytes expressing recombinant homomeric  $\rho$ 1 GABA<sub>C</sub> receptors. We found that GABA $\rho$ 1 receptor responses were significantly enhanced in the presence of NO. Experiments involving the chemical modification of sulfhydryl groups and site-directed mutagenesis at the  $\rho$ 1 subunits indicated that C177 and

C191, which form the Cys-loop located in the N-terminal extracellular domain, are critical for NO modulation of GABA $\rho$ 1 receptors.

## 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. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010).

### RNA preparation, oocyte isolation and cell injection

Human cDNA encoding the  $\rho$ 1 GABA<sub>C</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 the QuickChange Site-Directed Mutagenesis Kit (Stratagene). cRNA solutions (0.3–1 ng·nL<sup>-1</sup>) 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 anaesthetized with 3-aminobenzoic-acid ethylester (-1 mg·mL<sup>-1</sup>) and ovaries surgically removed. Ovaries were incubated with 400 U·mL<sup>-1</sup> 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<sup>-1</sup> gentamycin; 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 M $\Omega$ . 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  $\mu$ L) continuously superfused (12 mL·min<sup>-1</sup>) 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). S-nitrosoglutathione (GSNO), 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazole-1-oxyl-3-oxide potassium salt (CPTIO), N-ethyl maleimide (NEM) and (2-aminoethyl) methanethiosulfonate (MTSEA) solutions were prepared freshly before each experiment in normal Ringer's.

1,1-Diethyl-2-hydroxy-2-nitroso-hydrazone sodium (DEA) stock solution (10 mM) was prepared in 0.01 M NaOH and stored on ice for no more than 2 h. The pH was adjusted to 7.0 with NaOH (1 M) or HCl (1 M). Expired DEA was prepared by depletion of 10 mM DEA at room temperature for 24 h. All the experiments were carried out at room temperature (23–24°C).

## 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), except for MTSEA, which was from Toronto Research Chemicals (North York, Ontario, Canada) and GSNO from Tocris Bioscience (Ellisville, MO, USA).

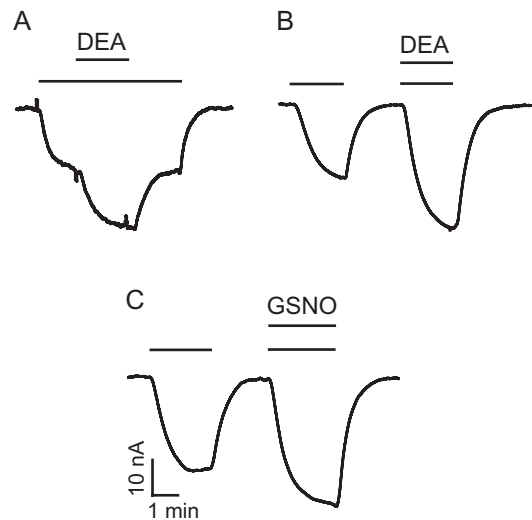
## Data analysis

Data were analysed with Prism v. 5.0 (Graphpad Software, Inc. San Diego, CA, USA). Dose–response curves (D–R) for GABA and dose–effect curve for DEA were fit with a logistic equation of the following form:  $I_{\text{GABA}}/B = [A^{n_H}/(A^{n_H} + EC_{50}^{n_H})] \times 10$  where  $A$  is the agonist concentration,  $B$  the maximal response,  $EC_{50}$  the concentration of agonist that elicits half-maximal responses and  $n_H$  the Hill coefficient. Percentage of potentiation ( $P$ ) was calculated as  $[(I_{\text{GABA}1\text{DEA}} \times 100/I_{\text{GABA}1\text{control}}) - 100]$ , where  $I_{\text{GABA}1\text{DEA}}$  indicates the current amplitude evoked at each particular GABA concentration in the presence of the different NO donor and  $I_{\text{GABA}1\text{control}}$  the corresponding responses in the absence of modulators. Student's  $t$ -tests (two-tailed) were employed to evaluate significant differences between parameters. In all cases errors are expressed as SEM.

## Results

### Functional modulation of GABA<sub>A</sub>1 receptors expressed in *Xenopus laevis* oocytes by NO donors

The application of GABA to oocytes expressing homomeric  $\rho 1$  GABA<sub>C</sub> receptors induced large inward Cl<sup>-</sup> currents displaying all of the features of retinal GABA<sub>C</sub> receptor-mediated responses. For example, they were insensitive to bicuculline, sensitive to TPMPA and picrotoxin, non-desensitizing and displayed the same pharmacological profile for agonists (Zhang *et al.*, 2001; Hull *et al.*, 2006). Figure 1 illustrates representative responses elicited by 0.3  $\mu$ M GABA recorded at -70 mV in the absence or presence of two different NO donors. DEA applications (100  $\mu$ M) produced a significant potentiation of the GABA-evoked responses. In order to characterize DEA effects, we used two different procedures with equivalent results, namely, DEA applications made on-top of the plateau of GABA responses (Figure 1A), or co-application of DEA and GABA (Figure 1B). The effects induced by DEA washed out relatively fast (1–3 min) and a second application of the drug produced similar results (data not shown). The S-nitrosothiol GSNO, which is capable of liberating both NO

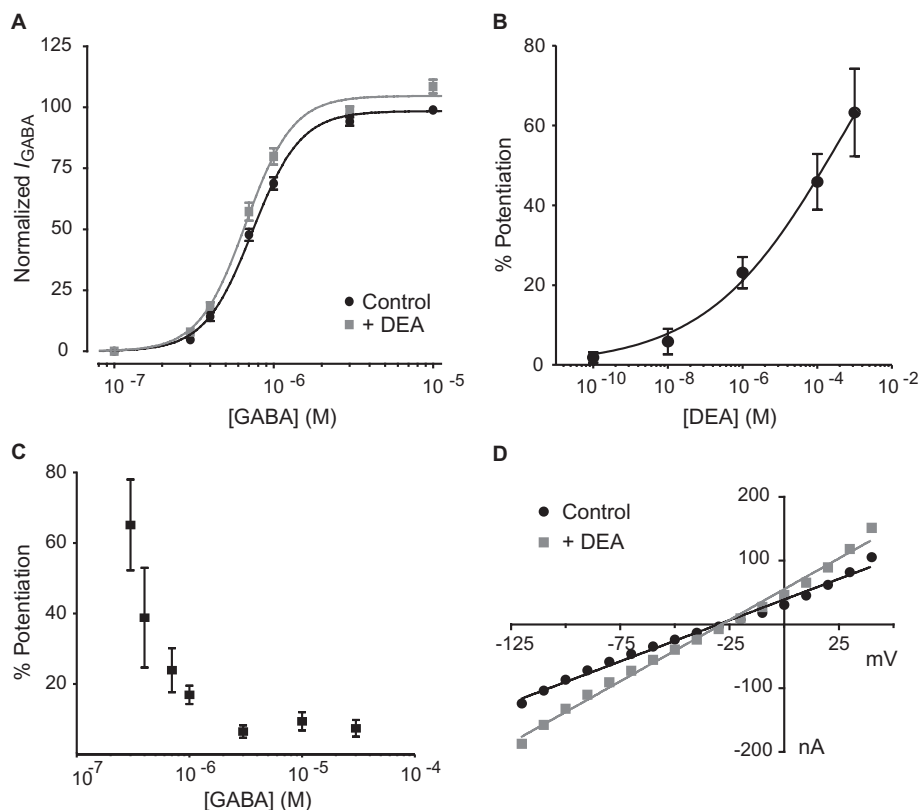


**Figure 1**

Potentiating effects of NO donors on responses mediated by GABA<sub>A</sub>1 receptors expressed in *Xenopus laevis* oocytes. Representative traces of GABA<sub>A</sub>1 receptor-mediated Cl<sup>-</sup> currents elicited by 0.3  $\mu$ M GABA applications (indicated as bars) in the absence (control) or presence of NO donors. DEA (100  $\mu$ M) was either delivered on top of the GABA-evoked responses (A) or co-applied with GABA (B). GSNO (1 mM) produced similar effects on GABA<sub>A</sub>1 receptor responses (C). 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).

and trans-nitrosylate protein thiols (Fernhoff *et al.*, 2009), also produced a significant potentiation of the 0.3  $\mu$ M GABA-evoked responses (%  $P = 58.5 \pm 17.0\%$ ;  $n = 11$ ;  $P < 0.01$ ; Figure 1C). GSNO effects were not steady among the different oocyte batches, possibly because some products of the GSNO hydrolysis can directly modify GABA<sub>A</sub>1 receptor function (Calero and Calvo, 2008). Thus, for the next experiments we only used DEA as the NO donor. The fast onset observed in the potentiation of GABA-evoked responses by DEA suggests a direct modulation exerted on the GABA<sub>A</sub>1 receptor.

D–R curves for GABA either in the absence (control) or the presence of DEA (Figure 2A) were performed. DEA (100  $\mu$ M) produced a slight but significant potentiation of the maximal GABA responses ( $7.4 \pm 2.3\%$ ;  $P < 0.03$ ) concomitantly with a leftward shift in GABA  $EC_{50}$  without considerably affecting the  $n_H$  ( $EC_{50}^{\text{GABA}} = 0.74 \pm 0.02 \mu\text{M}$ ,  $n_H = 2.9 \pm 0.1$ ;  $EC_{50}^{\text{GABA} + \text{DEA}} = 0.67 \pm 0.03 \mu\text{M}$ ,  $n_H = 2.9 \pm 0.2$ ;  $P < 0.005$ ). Potentiation was significant over the range of GABA concentrations studied ( $P < 0.05$ ). In order to determine the concentration range for effective modulation, we tested increasing levels of DEA and obtained a dose–effect curve (Figure 2B). Effects were significant, even for DEA concentrations as low as 1  $\mu$ M (%  $P = 23.1 \pm 3.9\%$ ;  $P < 0.005$ ), which is equivalent to NO levels in the nM range (Kim *et al.*, 1999). The dose–effect curve was fitted using a sigmoid equation (see Methods) although saturation could not be reached at the maximal DEA concentration tested (1 mM), the estimated  $EC_{50}$  was 400  $\mu$ M. In agreement with the effects displayed by other GABA<sub>A</sub>1 receptor redox modulators previously studied, such as DTT or GSH, the



## Figure 2

Analysis of DEA effects on GABA<sub>A</sub>1 receptors. (A) Dose–response curves for GABA in the presence or absence (control) of 100 μM DEA. Response amplitudes were expressed as fraction of maximal current values evoked by 30 μM GABA. (B) Potentiation of GABA<sub>A</sub>1 receptor responses (0.3 μM GABA) by increasing concentrations of DEA. (C) GABA concentration-dependence of the potentiation of GABA<sub>A</sub>1 receptor responses induced by DEA (100 μM). (D) *I*–*V* relationship for GABA<sub>A</sub>1 receptor responses evoked by 0.3 μM GABA in the presence or absence (control) of 100 μM DEA.

degree of potentiation exerted by NO donors on GABA<sub>A</sub>1 receptor responses decreased as GABA concentration increased (Figure 2C). For example, in the presence of DEA, the amplitude of currents evoked by 0.3 μM GABA was enhanced by  $65.1 \pm 12.9\%$  ( $n = 13$ ), whereas potentiation of the currents evoked by 30 μM GABA was  $7.4 \pm 2.3\%$  ( $n = 10$ ). Current–voltage relationships (*I*–*V* curves) for the GABA<sub>A</sub>1 receptors performed in the presence or absence of the NO donor indicated that DEA effects were independent of the membrane potential; a significant change in the slope without alteration in the linearity of the *I*–*V* relationship or the reversal potential, in the range between –120 and +40 mV, was observed in the presence of DEA (100 μM;  $n = 6$ ;  $P = 0.3$ ; Figure 2D). Therefore, the effects of DEA were voltage-independent and not due to a variation in intracellular Cl<sup>–</sup> levels.

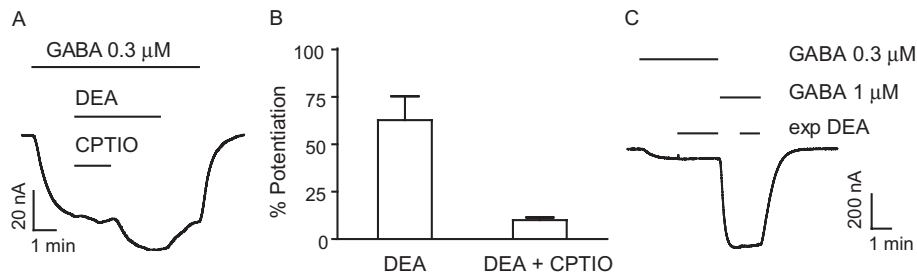
NO donors were safely used in this kind of pharmacological study; however, it is still possible that derivatives of DEA hydrolysis, or alternatively intact DEA molecules, exert some effects on the receptor. To remove these possibilities, we co-applied DEA with CPTIO, a specific scavenger that quickly inactivates NO and found that CPTIO (500 μM) dramatically attenuated the effects of DEA. Figure 3A shows that DEA potentiation reappeared immediately after CPTIO was washed out. Although CPTIO significantly prevented DEA

effects, the current potentiation was not completely abolished ( $\% P_{\text{DEA}} = 62.8 \pm 12.6\%$ ;  $\% P_{\text{DEA+CPTIO}} = 10.0 \pm 1.4\%$ ;  $n = 5$ ;  $P < 0.03$ ; Figure 3B). The residual potentiation might be explained by an insufficient scavenger concentration to react fast enough with the generated NO, or because of a differential accessibility. At the concentration tested, CPTIO alone did not elicit measurable effects, either on the baseline current or on GABA-evoked currents (data not shown). As an additional control, we also tested a DEA solution, which was prepared 24 h before the experiment was performed (kept at RT at pH = 7.0). This expired DEA solution had no effects on the GABA-evoked responses (Figure 3C). These results strongly suggest that NO, itself, is capable of directly exerting a potentiating effect on the GABA<sub>A</sub>1 receptor responses and that modulation was not due to artefacts caused by the decomposition of the NO donor DEA.

### *Involvement of cysteines forming the Cys-loop in the potentiation of GABA<sub>A</sub>1 receptors by NO*

In previous studies, we have shown that reducing and oxidizing thiol agents are effective modulators of the GABA<sub>A</sub>1 receptor function. In addition, other ionic channels, which are also sensitive to redox modulation, can be chemically modified by a NO-induced S-nitrosylation of cysteine resi-



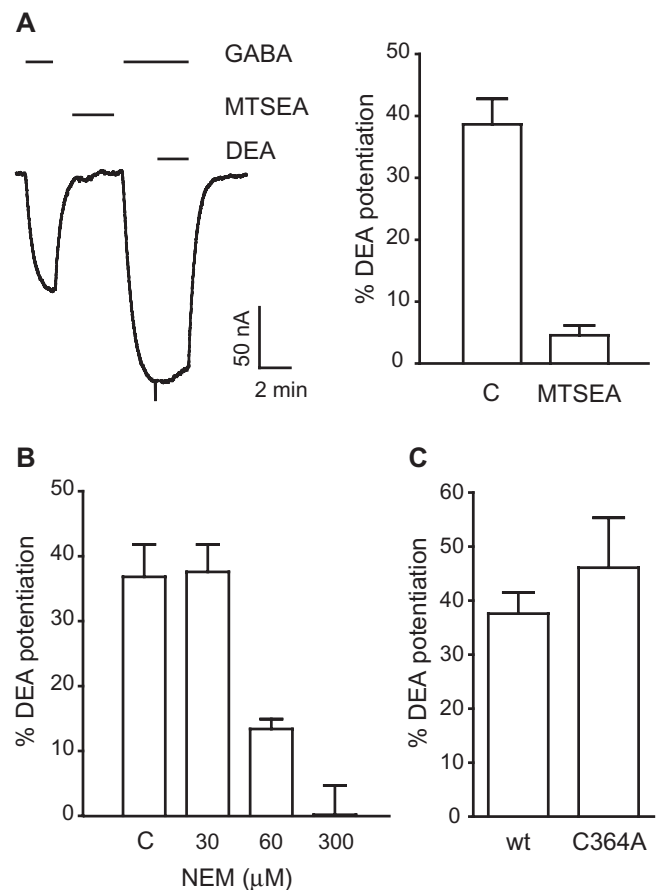


**Figure 3**

The specific NO scavenger CPTIO prevents the potentiating effects of DEA on GABA<sub>A</sub> receptor responses. (A) Potentiation of the GABA<sub>A</sub> receptor responses by DEA (100 μM) was prevented in the presence of the specific NO scavenger CPTIO (500 μM). Scale bars indicate current amplitude (y-axis) and time (x-axis). Data are summarized in (B). (C) Expired DEA had no effect on GABA<sub>A</sub> receptor responses.

dues. The rapid onset observed here for NO acting on the GABA<sub>A</sub> receptors is consistent with a direct modulatory action. To test this hypothesis, we examined NO effects in the presence of reagents that modify cysteine thiol groups. MTSEA is a very useful alkylating reagent to modify accessible cysteines, that is extensively employed to perform structure-function studies in wild-type and mutant receptors expressed in *Xenopus laevis* oocytes (Xu and Akabas, 1993; Choi *et al.*, 2000). The application of 2.5 mM MTSEA produced a significant potentiation of the 0.3 μM GABA-evoked currents (Figure 4A, left panel), which slowly disappeared 2–3 min after MTSEA washout (not shown). In addition, we observed that DEA effects on GABA<sub>A</sub> receptor responses were almost completely prevented during MTSEA potentiation (Figure 4A). Potentiation of the GABA<sub>A</sub> receptor responses by 100 μM DEA was reduced from  $38.7 \pm 4.1\%$  to  $4.6 \pm 1.6\%$  ( $n = 8-10$ ;  $P < 0.0001$ ) in the presence of MTSEA (Figure 4A, right panel). In a different group of oocytes we used the irreversible alkylating reagent NEM, which selectively forms covalent bonds with the free sulfhydryl groups preventing any further chemical reaction at these sites (at pH = 7.0; Means and Feeney, 1971). NEM concentration was kept as low as possible, and incubation periods were kept very short to prevent nonspecific effects. Pre-incubation with 300 μM NEM for 150 s completely prevented the potentiating effects of DEA on GABA<sub>A</sub> receptor responses (%  $P_{\text{control}} = 36.9 \pm 5.0\%$ ; %  $P_{\text{NEM } 300 \mu\text{M}} = 0.2 \pm 4.5\%$ ;  $n = 4$ ;  $P < 0.01$ ). This inhibitory effect of NEM was dose-dependent (%  $P_{\text{NEM } 60 \mu\text{M}} = 13.4 \pm 1.5\%$ ;  $n = 5$ ;  $P < 0.05$ ; %  $P_{\text{NEM } 30 \mu\text{M}} = 37.6 \pm 4.2\%$ ;  $n = 4$ ; *n.s.*; Figure 4B). Taken together, these results suggest that NO can modulate GABA<sub>A</sub> receptor function by interacting with one or more accessible cysteines.

GABA<sub>A</sub> receptors have only three cysteines in each of their five subunits; these are C177 and C191 located at the N-terminal extracellular domain forming the characteristic Cys-loop and C364 at the intracellular M3-M4 linker (Zhang *et al.*, 2001). Mutations of the cysteines at the Cys-loop are precluded because they render the receptors non-functional (Amin *et al.*, 1994; Sedelnikova *et al.*, 2005), but to determine whether C364 is involved in the modulation of GABA<sub>A</sub> receptors by NO, we replaced this amino acid residue with alanine using site-directed mutagenesis. Mutant GABA<sub>A</sub><sub>1C364A</sub>



**Figure 4**

Extracellular Cys-loop in the GABA<sub>A</sub> receptor subunits is essential for NO actions. (A) Potentiation of the GABA<sub>A</sub> receptor responses by MTSEA (2.5 mM; left panel). Note that the effects of MTSEA were not further increased by adding 100 μM DEA once the current peak had been reached (left panel). Potentiation of GABA<sub>A</sub> receptor responses by DEA (100 μM) was prevented if cysteine thiols were protected with MTSEA (2.5 mM; A, right panel) or NEM (B). (C) Mutation of the intracellular C364 to A at the  $\rho 1$  subunit did not affect the potentiating actions of NO on GABA<sub>A</sub> receptors.

receptors expressed in oocytes showed typical responses to GABA and  $EC_{50}$  values for GABA were slightly lower than for wild-type receptors ( $EC_{50}$  GABA $\rho 1_{C364A}$  =  $0.46 \pm 0.05 \mu\text{M}$ ;  $EC_{50}$  GABA $\rho 1_{wt}$  =  $0.61 \pm 0.07 \mu\text{M}$ ). Potentiation of the GABA $\rho 1_{C364A}$  receptor responses (GABA =  $0.3 \mu\text{M}$ ) by DEA ( $100 \mu\text{M}$ ) was not significantly different from that obtained with wild-type (wt) receptors (% P GABA $\rho 1_{C364A}$  =  $46.1 \pm 9.3$  vs. % P GABA $\rho 1_{wt}$  =  $37.6 \pm 3.9$ ;  $n = 6$ ; *n.s.*; Figure 4C). In summary, these results indicate that extracellular cysteines C177 and C191 that form the Cys-loop are targets for the NO action and that their chemical modification potentiates GABA $\rho 1$  receptor function.

## Discussion

We have shown significant potentiation of the homomeric  $\rho 1$  GABA $_C$  receptor activity by NO. The present findings are the first to support the modulation of a member of the Cys-loop receptor superfamily by NO directly acting at the Cys-loop.

### *NO actions on ionotropic GABA receptors*

Potentiation of GABA $\rho 1$  receptors by NO was fast, reversible, dose-dependent and strongly dependent on GABA concentration. The sensitivity of GABA $_C$  receptors to reactive nitrogen species was unknown, but several studies have previously shown functional modulation of native and recombinant GABA $_A$  receptors by NO. NO can induce either potentiating or inhibitory actions, depending on the neuronal type and the GABA $_A$  receptor subtype involved (Fukami *et al.*, 1998; Wexler *et al.*, 1998; Castel *et al.*, 2000; Castel and Vaudry, 2001; Zanelli *et al.*, 2009). NO effects on GABA $_A$  receptor-mediated responses can be mediated through the classic cGMP/PKG pathway as well as by PKG-independent pathways (Wexler *et al.*, 1998; Castel *et al.*, 2000; Castel and Vaudry, 2001). Castel and Vaudry proposed that S-nitrosylation of intracellular cysteines at the GABA $_A$  receptor subunits might account for a direct PKG-independent mechanism (Castel and Vaudry, 2001). However, the possible targets and actual mechanisms underlying a direct modulation of ionotropic GABA receptors, both GABA $_A$  and GABA $_C$ , by NO were not confirmed experimentally.

### *Mechanisms underlying the potentiation of GABA $_C$ receptors by NO*

A simple way to explain the fast onset of NO actions on GABA $\rho 1$  receptors is a direct reaction between NO and one or more accessible amino acid residues in the  $\rho 1$  subunits. Because of their reactivity and location, cysteines at the Cys-loop were good candidates. Chemical protection studies using differentially acting selective sulfhydryl reagents, such as NEM and MTSEA, as well as the replacement by alanine of the intracellular C364 using site-directed mutagenesis experiments indicated that C177 and C191 at the extracellular Cys-loop are critical for NO effects. Since NO produced identical effects on mutant GABA $\rho 1_{C364A}$  receptors or wild-type receptors, it is unlikely that intracellular C364 contributes to the modulation induced by NO. In addition to NO, other reactive nitrogen species, reactive oxygen species, glutathione, ascorbic acid, etc., can also produce cysteine modifica-

tions. Previous studies have suggested that the extracellular Cys-loop in  $\rho 1$  subunits spontaneously fluctuates between oxidized (disulfide bond) and reduced (free sulfhydryls) states, and that exposure to certain redox thiol agents can influence the equilibrium between these two states (Calero and Calvo, 2008). Thus, in a similar manner NO can react producing an S-nitrosylation of thiol groups at Cys-loop C177 and C191 and, in turn, this covalent modification induces protein structural rearrangements that impact on GABA binding and channel gating (Chang and Weiss, 2002). The leftward shift and the concomitant increase in the maximal current values, observed in D-R curves for GABA in the presence of NO, are compatible with this hypothesis. This interpretation is also consistent with the effects of reducing agents that prevent Cys-loop formation and behave as GABA $\rho 1$  receptor potentiators (Calero and Calvo, 2008). Interestingly, previous studies on NMDA receptors showed that redox modulation induced by both reducing thiol agents and NO-induced S-nitrosylation is mediated through the same extracellular cysteines (Lipton *et al.*, 2002). Besides NMDA receptors, ryanodine receptors, TRP channels and many other membrane-signalling proteins are physiological targets for cysteine S-nitrosylation (Eu *et al.*, 2000; Lipton *et al.*, 2002; Yoshida *et al.*, 2006). However, the modulation of Cys-loop receptors by S-nitrosylation was still not substantiated. It was shown that the redox modulation of Cys-loop receptors, including the GABA $_C$  receptors, is typically reversible (Amato *et al.*, 1999; Pan *et al.*, 2000; Calero and Calvo, 2008). Similarly, we found that NO modulation of GABA $\rho 1$  receptors is easily reversible. Thus, the present results also suggest that other redox-sensitive amino acid residues in the  $\rho 1$  subunits, such as tryptophane, methionine and tyrosine, are not involved, mainly because these residues are normally modified by reactive nitrogen species in an irreversibly manner (e.g. by peroxynitrite, which can be produced by the reaction of NO with superoxide). Nitrosothiols are normally extremely labile in the presence of reducing reagents, but our experiments showed that NO effects on GABA $\rho 1$  receptors can also be washed out in the absence of reducing agents. A possible explanation is that chemical modification of the extracellular redox site (the disulfide bond that forms the Cys-loop) produces a transient conformational change in the receptor that, in the absence of NO, rapidly relaxes to a lower energy state by excluding the NO group. This description is compatible with the actions of MTSEA on GABA $\rho 1$  receptors. Usually, the effects of this cysteine-specific reagent require the presence of reducing agents in order to be washed out (Xu and Akabas, 1993; Choi *et al.*, 2000). In contrast, we found here that MTSEA applications produced a fast potentiation of the GABA $\rho 1$  receptor responses that spontaneously disappeared during bath perfusion with a normal Ringer's solution.

### *Pharmacological and physiological relevance of the modulation of GABA $_C$ receptors by NO*

GABA $_C$  receptors mediate several modes of inhibitory actions in the retina (Lukasiewicz *et al.*, 2004). They are highly expressed in retinal bipolar cells (Koulen *et al.*, 1998) and play an important role in the control of axon terminal excitability by mediating reciprocal synapses with amacrine cells (Matthews *et al.*, 1994; Dong and Werblin, 1998; Hartveit, 1999).

GABA<sub>C</sub> receptors also mediate tonic inhibitory currents, which can be persistently activated by low concentrations of ambient GABA, locally controlled by GABA transporters located on amacrine cells (Hull *et al.*, 2006; Jones and Palmer, 2009). Additionally, NO has been implicated in diverse retinal functions (Wang *et al.*, 2007). Furthermore, NO might play an important role as a retrograde messenger in the plasticity of GABAergic neurotransmission (Yu and Eldred, 2005; Szabadits *et al.*, 2007; Kovacs *et al.*, 2009; Zanelli *et al.*, 2009; Cserep *et al.*, 2011). It was shown that bipolar and amacrine neurons, as do many other retinal cells, express the NO-signalling machinery (Eldred and Blute 2005) and that at the inner plexiform layer, a region with high densities of GABA<sub>C</sub> receptors, NO can be generated at high (micromolar) levels (Groppe *et al.*, 2003; Hoffpaur *et al.*, 2006). Therefore, GABA<sub>C</sub> receptor function would be dependent on the local production of NO. We observed that nM concentrations of NO produced significant GABA<sub>A</sub>1 receptor potentiation. These values are lower than those required to modulate other receptors and channels (Castel and Vaudry, 2001; Choi *et al.*, 2001; Eldred and Blute 2005; Ledo *et al.*, 2005; Hall and Garthwaite, 2009). NO effects on GABA<sub>A</sub>1 receptors strongly depended on the GABA concentration. As GABA<sub>C</sub> receptors show relatively high affinity for GABA, NO modulation at low GABA concentrations could be ideally suited within the dynamic range of ion channel activation. Thus, extrasynaptic GABA<sub>C</sub> receptors, which would presumably be exposed to lower GABA concentrations, might be effectively modulated by NO. However, further studies will be required to address whether or not the functional regulation of neuronal GABA<sub>C</sub> receptors by NO, revealed by these pharmacological experiments performed in a heterologous expression system, would also be observed in native neurons *in situ*. It will also be necessary to clarify whether physiologically relevant concentrations of NO would have such actions on neuronal GABA<sub>C</sub> receptors.

In conclusion, our results suggest that NO enhances GABA<sub>C</sub> receptor function by directly acting on C177 and C191 at the  $\rho$ 1 subunit extracellular Cys-loop. These findings also raise the question of whether S-nitrosylation might represent a general mechanism regulating the activity of Cys-loop receptors.

## Acknowledgements

We thank Drs Viviana Dalamon and Marcelo Martí for discussion. This work was supported by CONICET and FONCYT grants.

## Conflicts of interest

None.

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