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Redox modulation of homomeric ρ_1 $GABA_C$ receptors

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Abstract

The activity of many receptors and ion channels in the nervous system can be regulated by redox-dependent mechanisms. Native and recombinant $GABA_A$ receptors are modulated by endogenous and pharmacological redox agents. However, the sensitivity of $GABA_C$ receptors to redox modulation has not been demonstrated. We studied the actions of different reducing and oxidizing agents on human homomeric $GABA\rho_1$ receptors expressed in *Xenopus laevis* oocytes. The reducing agents dithiothreitol (2 mM) and *N*-acetyl-L-cysteine (1 mM) potentiated GABA-evoked Cl^- currents recorded by two-electrode voltage-clamp, while the oxidants 5-5′-dithiobis-2-nitrobenzoic acid (500 μM) and oxidized dithiothreitol (2 mM) caused inhibition. The endogenous antioxidant glutathione (5 mM) also enhanced $GABA\rho_1$ receptor-mediated currents while its oxidized form GSSG (3 mM) had inhibitory effects. All the effects were rapid and easily reversible. Redox modulation of $GABA\rho_1$ receptors was strongly dependent on the GABA concentration; dose–response curves for GABA were shifted to the left in the presence of reducing agents, whereas oxidizing agents produced the opposite effect, without changes in the maximal response to GABA and in the Hill coefficient. Our results demonstrate that, similarly to $GABA_A$ receptors and other members of the cys-loop receptor superfamily, $GABA_C$ receptors are subjected to redox modulation.

Keywords: antioxidants, chloride currents, cys-loop receptors, $GABA_C$ receptors, redox modulation, retina.

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Multiple lines of evidences indicate an important role of redox mechanisms and reactive oxygen species in the regulation of cell function in the CNS (Lipton *et al.* 2002; Safiulina *et al.* 2006; Kishida and Klann 2007; Sidlo *et al.* 2008). As redox status can significantly change during normal and/or pathological conditions, the study of the endogenous and pharmacological redox-dependent modulation is fundamental for a genuine understanding of the mechanisms controlling neuronal activity (Annunziato *et al.* 2002; Rice *et al.* 2002). Many neurotransmitter receptors, including glutamate and $GABA_A$ receptors, as well as diverse voltage-gated ion channels and transporters are subject to redox modulation (Bouzat *et al.* 1991; Ruiz-Gomez *et al.* 1991; Ruppersberg *et al.* 1991; DiChiara and Reinhart 1997; Trotti *et al.* 1997; Todorovic *et al.* 2001; Lipton *et al.* 2002; Chu *et al.* 2006). Thus, besides their participation in metabolic pathways in neurons and glial cells, redox mechanisms may regulate signaling processes thus shaping membrane excitability and modulating synaptic transmission.

Based on their pharmacology, $GABA_C$ receptors were classified as a separate class of GABA receptors (Sivilotti and Nistri 1991). $GABA_A$ and $GABA_C$ receptors are GABA-gated pentameric chloride (Cl^-) channels members of the cys-loop-containing neurotransmitter receptor superfamily (Moss and Smart 2001; Connolly and Wafford 2004). Remarkably, $GABA_C$ receptors are insensitive to the $GABA_A$ competitive antagonist bicuculline (Sivilotti and Nistri 1991) and appear to be exclusively composed of ρ subunits (ρ_1, ρ_2, and ρ_3) that yield homomeric and heteromeric receptors (Zhang *et al.* 2001). $GABA_C$ receptors are

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Abbreviations used: D–R, dose–response; DTNB, 5-5′-dithiobis-2-nitrobenzoic acid; DTT, dithiothreitol; NacC, *N*-acetyl-L-cysteine; oxDTT, oxidized dithiothreitol; tricine, *N*-tris[hydroxymethyl]methylglycine.

highly expressed in the retina and other visual areas, but ρ subunits were found to be widely distributed in the CNS (Strata and Cherubini 1994; Enz *et al.* 1995; Albrecht *et al.* 1997; Boue-Grabot *et al.* 1998; Wegelius *et al.* 1998; Enz and Cutting 1999) and their expression is developmentally regulated (Ogurusu *et al.* 1999; Didelon *et al.* 2002; Rozzo *et al.* 2002; Liu *et al.* 2004; Alakuijala *et al.* 2005). Functional $GABA_C$ receptors were localized at many different neuronal subtypes (Feigenspan *et al.* 1993; Strata and Cherubini 1994; Martina *et al.* 1997; Pasternack *et al.* 1999; Alakuijala *et al.* 2006).

Native and recombinant $GABA_A$ receptors can be regulated by redox mechanisms (Pan *et al.* 1995, 2000; Amato *et al.* 1999; Leszkiewicz and Aizenman 2003). The sulfhydryl reducing agents GSH and dithiothreitol (DTT) potentiate $GABA_A$ responses, while the oxidizing agents GSSG and 5-5′-dithiobis-2-nitrobenzoic acid (DTNB) inhibit them and the degree of redox modulation depends on the subunit composition (Amato *et al.* 1999; Pan *et al.* 2000; Wilkins and Smart 2002). Meanwhile, the modulation of $GABA_C$ receptors by redox mechanisms has not been revealed. Similarly to all the $GABA_A$ receptor subunits, ρ subunits forming $GABA_C$ receptors contain a number of aminoacidic residues capable to undergo redox modulation, including the two characteristic cysteine residues located on the N-terminus which are thought to participate in forming a disulphide bridge. Previous data suggested an apparent lack of sensitivity of $GABA\rho_1$ receptors to sulfhydryl reagents (Pan *et al.* 2000). However, Pan *et al.* (2000) did not assess the effects of redox agents on $GABA\rho_1$ receptors at different GABA concentrations. Thus, a more detailed characterization of the effects of redox agents on these receptors was lacking. We analyzed the effects of different reducing [DTT, GSH, and *N*-acetyl-L-cysteine (NacC)] and oxidizing [DTNB, GSSG, and oxidized DTT (oxDTT)] compounds on human homomeric $GABA\rho_1$ receptors expressed in *Xenopus laevis* oocytes by using two-electrode voltage-clamp recordings of GABA-evoked Cl^- currents. Our results demonstrate that, as are the $GABA_A$ receptors and other members of the cys-loop receptor group, $GABA_C$ receptors are susceptible to redox modulation.

Materials and methods

RNA preparation, oocyte isolation and cell injection

A human cDNA encoding the ρ_1 $GABA_C$ receptor subunit cloned in the *in vitro* transcription-suitable vector pGEM was used as a template to synthesize cRNAs *in vitro*. cRNA solutions (0.1–0.3 ng/nL) were prepared in Rnase-free H_2O and stored at −70°C. *X. 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 *et al.*, 1989). Briefly, frogs were anesthetized with 3-aminobenzoic-acid ethylester (∼1 mg/mL) and ovaries surgically removed. Ovaries were incubated with 200 U/mL collagenase for 30 min at 23–24°C, and isolated oocytes were maintained in an incubator at 17°C in Barth's medium (in mM: 88 NaCl; 0.33 $Ca(NO_3)_2$; 0.41 $CaCl_2$; 1 KCl; 0.82 $MgSO_4$; 2.4 $NaHCO_3$; 10 HEPES and 0.1 mg/mL 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 puller Narishige PB-7 (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 in 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 $CaCl_2$; 5 HEPES; pH 7.0). The agonist and other drugs were applied through the perfusion system (Goutman *et al.* 2005). DTT, DTNB, GSH, GSSG, NacC, and *N*-tris[hydroxymethyl]methylglycine (tricine) solutions were prepared freshly each day in normal Ringer's. The pH was always adjusted to 7.0 with NaOH (1 M). 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) 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_2O were purchased from Sigma-Aldrich (St Louis, MO, USA).

Data analysis

Data were analyzed with Prism v. 4.0 (GRAPHPAD Software, Inc. San Diego, CA, USA). Dose–response curves (D–R) for GABA were fit with a logistic equation of the following form: $I_{max} = B\{1 - 1/[1 + (A/EC_{50})^n]\}$, 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 $[(I_{GABA\rho1\ redox} \times 100/I_{GABA\rho1\ control}) - 100]$, where $I_{GABA\rho1\ redox}$ indicates the current amplitude evoked at each particular GABA concentration in the presence of the different redox modulators and $I_{GABA\rho1\ control}$ the corresponding responses in the absence of modulators. Paired-sample *t*-tests (two tailed) were employed to evaluate significant differences between parameters.

Results

Modulation of $GABA\rho_1$ receptors by reducing and oxidizing agents

In oocytes expressing homomeric $GABA\rho_1$ receptors GABA applications induced large bicuculline-insensitive inward currents which showed very little desensitization; the

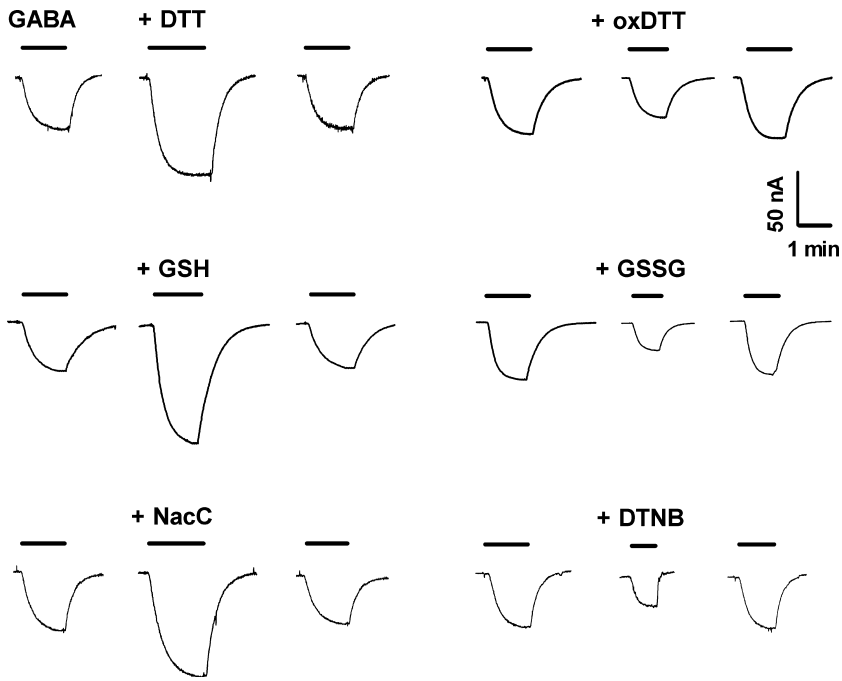

Fig. 1 Redox modulation of homomeric ρ_1 $GABA_C$ receptors expressed in *Xenopus oocytes*. Effects of reducing and oxidizing agents on GABAρ_1 receptor mediated Cl^- currents. Representative traces of the GABAρ_1 responses elicited by 0.3 μM GABA (indicated as bars) in the absence (control) or presence of redox agents. Reducing agents DTT (2 mM), GSH (5 mM), and NacC (1 mM) potentiated the GABA-evoked currents (left panel), while oxidizing agents oxDTT (2 mM), GSSG (3 mM), and DTNB (500 μM) produced inhibitory effects (right panel). For this and the subsequent figures, oocytes were voltage-clamped at −70 mV. Scale bars indicate current amplitude (*y*-axis) and time (*x*-axis).

currents showed a reversal potential about −20 mV as expected for Cl^- selective GABA channels expressed in oocytes. Figure 1 illustrates representative responses elicited by 0.3 μM GABA in voltage-clamped oocytes (−70 mV) in the absence or presence of different redox agents. Bath application of DTT (2 mM) significantly potentiated GABAρ_1 responses while oxDTT (2 mM) produced inhibition (Fig. 1). Similar results were obtained by using the reduced and oxidized forms of glutathione (GSH = 5 mM and GSSG = 3 mM, respectively) (Fig 1). The reducing agent NacC (1 mM) also enhanced GABAρ_1-receptor activity, whereas DTNB (500 μM), a potent oxidizing agent, decreased GABAρ_1 responses (Fig. 1). The concentrations selected for testing the effects of all these compounds were equivalent to those previously used to study the redox modulation of different $GABA_A$ receptor subtypes. Figure 2 summarizes the effects of redox agents on GABAρ_1 responses. The effects elicited by the redox modulators were rapid, stable, and fully reversible after washout and this is illustrated in Fig. 2 (inset), as a rapid deflection of the current trace during drug application. The effects induced on GABAρ_1-currents by on-top applications of the redox modulators (inset Fig. 2) were equivalent to those observed during the simultaneous applications of GABA and the redox agents (as shown in Fig. 1). No appreciable effects on the oocyte properties, such as membrane potential, membrane resistance or current baseline under voltage-clamp, were observed during applications of the redox agents. The exposure of mRNA injected or sham oocytes to the redox agents for 2 min in the absence of GABA produced no significant changes in the current baseline: DTT 2 mM −0.7 ± 2.1 nA; oxDTT 2 mM −6.3 ± 4.2 nA; DTNB 500 μM 6.9 ± 6.2 nA; GSH 5 mM −0.7 ± 0.4 nA; GSSG 3 mM −1.4 ± 1.9 nA, and NacC −1.1 ± 0.1 nA (n = 3–4).

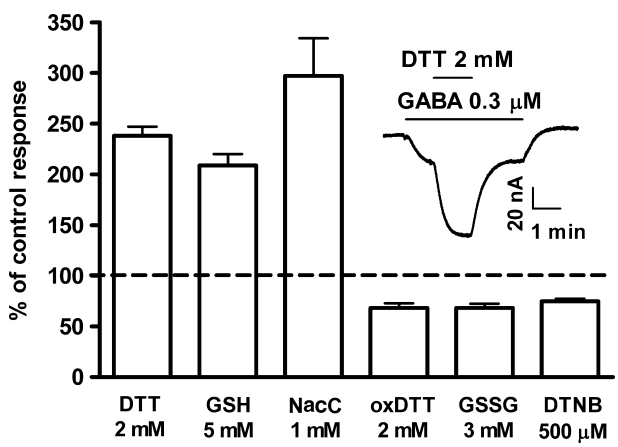

Fig. 2 Effects of reducing and oxidizing agents on the GABAρ_1 receptor. Bar graph summarizing the data obtained for redox modulation induced by the different redox agents. Responses were normalized to control current (0.3 μM GABA) (dashed line). Potentiation induced by reducing compounds was as follows: DTT 2 mM = 138.0 ± 27.8% (p < 0.003, n = 9); GSH 5 mM = 108.7 ± 25.2% (p < 0.01, n = 5) and NacC 1 mM = 197.2 ± 73.8% (p < 0.03, n = 4). Percentages of inhibition induced by oxidizing compounds were as follows: oxDTT 2 mM = 31.7 ± 9.3% (p < 0.03, n = 4); GSSG 3 mM = 31.6 ± 8.1% (p < 0.03, n = 4) and DTNB 500 μM = 25.3 ± 6.1% (p < 0.003, n = 5). The effects of redox agents on GABAρ_1 receptors were easily reversible. Inset: representative experiment illustrating the fast onset and offset showed by DTT acting on top of a GABA-evoked response.

The modulatory effects of redox agents on the GABAρ_1 receptor currents were dependent on the GABA concentration. For example, the potentiation of the GABAρ_1 responses exerted by DTT (2 mM) was minimized at GABA concentrations above 1 μM. The maximal increase was obtained at GABA 0.01 μM (183.3 ± 61.7%; $n = 3$), while potentiation was non-significant at GABA concentrations higher than 1 μM (for GABA 1 μM = 2.4 ± 1.7%, $n = 3$; for GABA 3 μM = 3.4 ± 1.3%, $n = 4$ and for GABA 10 μM = 0.6 ± 1.0%, $n = 3$). A similar situation was observed for GSH (5 mM) that increased GABAρ_1 responses a 29.5 ± 7.1% ($n = 5$) at 1 μM GABA, but GSH applications were ineffective in modulating GABAρ_1 responses evoked by higher GABA concentrations (0.0 ± 0.1% at GABA 10 μM; $n = 3$). The effects produced by oxidizing agents were also prevented by increasing GABA concentration and the results were as follows: at GABA 1 μM the percentage of inhibition was: for oxDTT (2 mM) = 7.1 ± 1.9% ($n = 4$); for GSSG (3 mM) = 33.4 ± 1.7% ($n = 3$); and for DTNB (500 μM) = 22.6 ± 4.3% ($n = 5$). Meanwhile, at GABA 10 μM the percentage of inhibition was: for oxDTT (2 mM) = 0.2 ± 0.5% ($n = 3$); for GSSG (3 mM) = 0.9 ± 0.3% ($n = 3$); and for DTNB (500 μM) = 2.7 ± 1.3% ($n = 3$).

To further study the dependence of GABAρ_1 receptor redox modulation on GABA concentration, D–R curves for GABA either in the absence (control) or the presence of different redox compounds (Fig. 3) were performed. DTT (2 mM) and GSH (5 mM) shifted the D–R curves to the left and significantly decreased the GABA EC_{50} ($^*p < 0.001$) without considerable changes in the Hill coefficients (nHill). Values obtained were as follows: $EC_{50} = 0.74 \pm 0.01$ μM, $n\text{Hill} = 1.74 \pm 0.03$ (control); $EC_{50\ DTT} = 0.58 \pm 0.01$ μM*, $n\text{Hill}_{DTT} = 1.30 \pm 0.07$; $EC_{50\ GSH} = 0.52 \pm 0.01$ μM*, $n\text{Hill}_{GSH} = 1.95 \pm 0.01$. In contrast, oxidizing compounds shifted the D–R curves to the right, increasing the GABA EC_{50}. Values obtained were: $EC_{50\ oxDTT} = 1.13 \pm 0.01$ μM* ($^*p < 0.001$) and $n\text{Hill}_{oxDTT} = 1.81 \pm 0.05$; $EC_{50\ GSSG} = 1.25 \pm 0.03$ μM and $n\text{Hill}_{GSSG} = 1.56 \pm 0.08$; $EC_{50\ DTNB} = 1.03 \pm 0.01$ μM* and $n\text{Hill}_{DTNB} = 1.88 \pm 0.03$. No changes in the maximal responses to GABA were found in the entire set of experiments.

Current–voltage relationships (I–V curves) for the GABAρ_1 receptors were carried out in the presence or absence of redox agents. The effects of redox agents 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 the redox agents. Figure 3 illustrates representative experiments performed with GSH and GSSG.

Chelation of Zn^{2+} traces present in frog Ringer's did not affect GABAρ_1 currents

It is known that the redox modulation of some $GABA_A$ receptor variants can be obscured by complexation with basal contaminating levels of Zn^{2+}, causing a partial persistent inhibition of the response to GABA (Wilkins and Smart 2002). Thus, at low concentrations of DTT, a substantial component of the potentiation induced by reducing agents occurs via Zn^{2+} chelation. In contrast, at higher concentra-

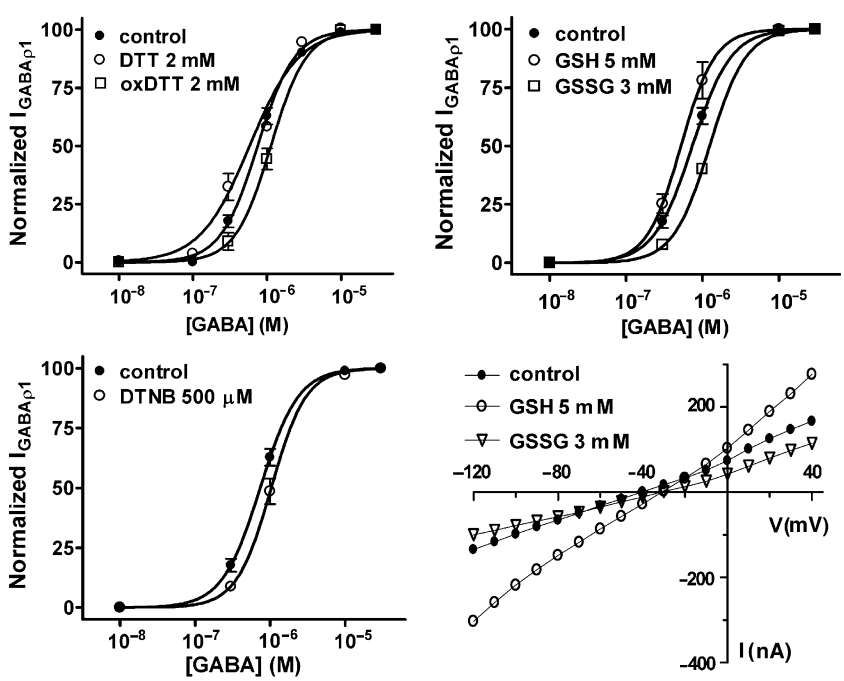

Fig. 3 Analysis of the effects of redox agents on the GABAρ_1 receptor. Dose–response curves for GABA in the absence (control ●) or presence of: (a) DTT (2 mM) (○) or oxDTT (□) (2 mM); (b) GSH (5 mM) (○) or GSSG (3 mM) (□) and (c) DTNB (500 μM) (○). Response amplitudes were expressed as fraction of 30 μM GABA-evoked currents (maximal response). Each point represents the mean and SEM of the responses obtained from 3 to 9 oocytes, notice that some of the standard error bars are hidden by the symbols. (d) Representative traces of the I–V relationships obtained for GABAρ_1 responses evoked by 0.3 μM GABA in the presence or absence of GSH or GSSG.

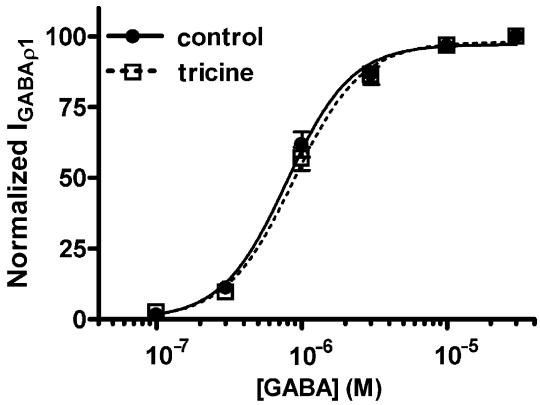

Fig. 4 GABAρ_1 receptor-mediated responses are not affected by contaminating Zn^{2+} traces. Dose–response curves for GABA in the absence (control) or presence of the heavy metal chelator tricine (10 mM). Response amplitudes were expressed as fraction of 30 μM GABA-evoked currents (maximal response). Each point represents the mean and SEM of the responses obtained from five oocytes.

tions, DTT is acting as a redox agent (Wilkins and Smart 2002). ρ_1 subunits are also inhibited by Zn^{2+} (Calvo *et al.* 1994; Chang *et al.* 1995; Wang *et al.* 1995), but they are considerably less sensitive compared with their αβ $GABA_A$ receptor counterparts (Draguhn *et al.* 1990; Pan *et al.* 2000). Therefore, traces of Zn^{2+} would have little or not effect on GABAρ_1 receptor function. Nevertheless, as DTT is a Zn^{2+} chelating agent, we studied whether or not the potentiation of $GABA_C$ receptor currents by DTT could be due, at least in part, to chelation of Zn^{2+} present in the recording solutions (as traces of around 100 nM) (Choi *et al.* 2001). To address this question, we performed D–R curves for GABA in the presence or absence of the heavy metal chelating agent tricine (10 mM) that complexes with free Zn^{2+} (Wilkins and Smart 2002). As shown in Fig. 4, D–R curves before and after the application of tricine were not significantly different. The EC_{50} for GABA was, in control conditions: 0.79 ± 0.02 μM with a *n*Hill of 1.95 ± 0.08, (n = 5), and in the presence of tricine EC_{50} was 0.88 ± 0.05 μM, with a *n*Hill of 1.8 ± 0.06 (n = 5; $p < 0.001$). These results suggest that, in our *in vitro* recording conditions, GABAρ_1 receptors are not subject to basal inhibition by Zn^{2+} or other heavy metals, and that redox modulation is not obscured by Zn^{2+} traces as in the case of $\alpha_1\beta_2$ $GABA_A$ receptors.

Discussion

Our results demonstrate that $GABA_C$ receptors formed by ρ_1 subunits were susceptible to redox modulation. The reducing reagents DTT, GSH, and NacC potentiated GABAρ_1 responses and the oxidizing reagents oxDTT, GSSG, and DTNB caused inhibition. The susceptibility of the GABAρ_1 receptors to both reducing and oxidizing agents suggests that the ρ_1 subunits are in equilibrium between fully oxidized and fully reduced forms. The overall effect induced by reducing agents on GABAρ_1 responses (an average potentiation of 150%) was greater than the effects observed for oxidizing compounds (an average inhibition of 30%). This asymmetry suggests that a large fraction of the GABAρ_1 receptors expressed in oocytes are in a relatively oxidized form.

The rate of onset and reversibility of the actions of redox agents on GABAρ_1 receptors suggest an extracellular redox modulatory site. This interpretation is supported by several observations. First, the membrane-impermeant agent GSH and the permeant agent DTT were equally effective and second, their actions were voltage independent. Moreover, the modulation of GABAρ_1 receptors by redox agents was dependent on GABA concentration. Reducing agents significantly decreased the GABA EC_{50} whereas oxidizing agents exerted the opposite effect, both without changing the cooperativity for ligand interaction (*n*Hill). As redox modulation did not affect the maximal values in the D–R curves, a change induced by redox agents in the number of receptors in the membrane surface seems unlikely. Similar to other members of the cys-loop receptor superfamily, ρ_1 $GABA_C$ receptor subunits contain two conserved cysteine residues which are separated by 13 amino acids and presumably form a disulphide bridge. This cys-loop is located in the N-terminal domain (Zhang *et al.* 2001; Connolly and Wafford 2004), but is not part of the agonist/antagonist binding pocket (Amin *et al.* 1994; Sedelnikova *et al.* 2005). Given the possibility of an external site of action for redox agents and the proposed membrane topology of the GABAρ_1 receptor (Zhang *et al.* 2001), these cysteine residues are good candidates to be the redox modulatory sites. This is in agreement with the ability of DTT to reduce disulphide bridges to sulfhydryl groups. Although ρ_1 subunits do not have additional cysteines facing the extracellular side, other aminoacidic residues such as methionine, tyrosine, phenylalanine, histidine, or lysine, could suffer redox-dependent side chain modification (Chu *et al.* 2006). In fact, the proposed agonist binding pocket of the GABAρ_1 receptor contains four residues of this type (Sedelnikova *et al.* 2005). Consequently actions in other sites of the receptor cannot be ruled out. Future site-directed mutagenesis studies could help to gain insight into these issues. However, such studies were complicated by the fact that mutagenesis of the cysteines forming the characteristic loop in this class of receptors commonly produces failure of subunit assembly (Amin *et al.* 1994).

The lateral shifts produced by redox agents on the GABA activation curve of GABAρ_1 receptors can be explained assuming that redox modulation can decrease (by reducing agents) or increase (by oxidizing agents) the energy barrier for GABA activation. This might be achieved by altering GABA binding sites in the GABAρ_1 receptors. For example, redox agents could directly modify one or more of these sites producing changes in the agonist binding affinity (Colqu-

houn 1998). Alternatively, they could allosterically influence GABA binding by changing the conformation of the binding pocket from a distant location. In addition, redox agents could induce conformational rearrangements that impact on the single-channel properties, for instance by changing the energetic of channel opening after agonist binding through an allosteric mechanism (Colquhoun 1998). However, based on a kinetic model of the ρ_1 homomeric GABA receptor, it was proposed that large changes in gating efficiency induced by site-directed mutations result in small changes in the EC_{50} (Amin and Weiss 1994; Chang and Weiss 1999). Redox modulation could as well simultaneously alter affinity and gating. At present, binding studies using thiol reducing and oxidizing agents were only performed at $GABA_A$ receptors and yielded conflicting results (Marangos and Martino 1981; Allan and Baier 1992). On the other hand, the small single channel conductance of $GABA\rho_1$ receptor channels makes the analysis of unitary $GABA\rho_1$ currents technically difficult (Martina *et al.* 1995; Wotring *et al.* 1999). Thus, further studies and different approaches will be required to get a more accurate description of the action mechanisms underlying redox modulation of the $GABA\rho_1$ receptors.

The fact that redox modulation of $GABA\rho_1$ receptors was strongly dependent on the GABA concentration is in contrast with the non-competitive mechanism shown by the same agents acting on $GABA_A$ receptors comprising $\alpha\beta$ subunits (Amato *et al.* 1999; Pan *et al.* 2000). However, redox modulation of $GABA_A$ receptors was shown to be dependent on subunit composition, for example receptors composed of $\alpha\beta$ subunits (e.g. $\alpha_1\beta_1$, $\alpha_1\beta_2$, and $\alpha_2\beta_1$) are significantly more sensitive to redox modulation than receptors containing $\alpha\beta\gamma$ subunits, particularly the γ_{2S} subunit (e.g. $\alpha_1\beta_1\gamma_{2S}$, $\alpha_1\beta_2\gamma_{2S}$, and $\alpha_1\beta_3\gamma_{2S}$) (Pan *et al.* 2000). In fact, the effects observed on GABA D–R curves for $GABA\rho_1$ receptors in the presence of reducing compounds are very similar to that previously reported for $GABA_A$ receptors from rat retinal ganglion cells after DTT treatment (Pan *et al.* 1995), and the potency shown by DTT is comparable. It was shown that retinal ganglion cells can express α_{1-3}, β_{2-3}, and γ_2 $GABA_A$ receptor subunits (Greferath *et al.* 1993; Koulen *et al.* 1996). Interestingly, even though recombinant $GABA_A$ receptors carrying γ_{2S} subunits are considerable less sensitive to redox agents than those lacking this subunit, the degree of DTT potentiation can be restored by using lower GABA concentrations (Pan *et al.* 2000).

In addition, our results suggest that $GABA\rho_1$ receptors are not subjected to basal inhibition by Zn^{2+} or other heavy metals, and that redox modulation is not obscured by Zn^{2+} traces as in the case of $\alpha_1\beta_2$ $GABA_A$ receptors (Wilkins and Smart 2002). Therefore, concerning redox modulation, homomeric $GABA_C$ receptors formed by wild-type ρ_1 subunits appear to show some parallels with $GABA_A$ receptor subtypes containing γ_{2S} subunits.

The functional regulation of neuronal $GABA_C$ receptors by redox mechanisms might be physiologically relevant considering the generation and release of endogenous redox agents in the CNS. It was shown that GSH and ascorbic acid can reach millimolar concentrations in specific areas of the CNS (Kosower and Kosower 1978; Organisciak *et al.* 1984; Slivka *et al.* 1987a,b; Yudkoff *et al.* 1990; Rice 2000), that includes the retina, hippocampus, and cerebral cortex, where $GABA_C$ receptors are selectively expressed. In addition, Ca^{2+}-dependent release of GSH from neuronal tissue (Zangerle *et al.* 1992) and glutamate–ascorbate heteroexchange at excitatory synapses (Rice 2000) were demonstrated. Redox modulation of ion channel activity was proposed as an endogenous mechanism to prevent neurotoxicity induced by oxidative stress, for example after a free radical-mediated modification of channel function (Dirksen 2002). In addition to its role in controlling the oxidative damage, redox modulation can be involved in diverse signaling pathways that regulate the normal activity of receptors that mediate excitatory and inhibitory neurotransmission, including $GABA_C$ receptors.

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References

Alakuijala A., Palgi M., Wegelius K., Schmidt M., Enz R., Paulin L., Saarma M. and Pasternack M. (2005) GABA receptor rho subunit expression in the developing rat brain. *Brain Res. Dev. Brain Res.* **154**, 15–23.

Alakuijala A., Alakuijala J. and Pasternack M. (2006) Evidence for a functional role of GABA receptors in the rat mature hippocampus. *Eur. J. Neurosci.* **23**, 514–520.

Albrecht B. E., Breitenbach U., Stuhmer T., Harvey R. J. and Darlison M. G. (1997) In situ hybridization and reverse transcription–polymerase chain reaction studies on the expression of the GABA(C) receptor rho1- and rho2-subunit genes in avian and rat brain. *Eur. J. Neurosci.* **9**, 2414–2422.

Allan A. M. and Baier L. D. (1992) Effect of thiol group modification on ion flux and ligand binding properties of the GABAA-benzodiazepine receptor chloride channel complex. *Synapse* **10**, 310–316.

Amato A., Connolly C. N., Moss S. J. and Smart T. G. (1999) Modulation of neuronal and recombinant GABAA receptors by redox reagents. *J. Physiol.* **517**, 35–50.

Amin J. and Weiss D. S. (1994) Homomeric rho 1 GABA channels: activation properties and domains. *Recept. Channels* **2**, 227–236.

Amin J., Dickerson I. M. and Weiss D. S. (1994) The agonist binding site of the gamma-aminobutyric acid type A channel is not formed by the extracellular cysteine loop. *Mol. Pharmacol.* **45**, 317–323.

Annunziato L., Pannaccione A., Cataldi M., Secondo A., Castaldo P., Di Renzo G. and Taglialatela M. (2002) Modulation of ion channels by reactive oxygen and nitrogen species: a pathophysiological role in brain aging? *Neurobiol. Aging* **23**, 819–834.

Boue-Grabot E., Roudbaraki M., Bascles L., Tramu G., Bloch B. and Garret M. (1998) Expression of GABA receptor rho subunits in rat brain. *J. Neurochem.* **70**, 899–907.

Bouzat C., Barrantes F. J. and Sigworth F. J. (1991) Changes in channel properties of acetylcholine receptors during the time course of thiol chemical modifications. *Pflugers Arch.* **418**, 51–61.

Calvo D. J., Vazquez A. E. and Miledi R. (1994) Cationic modulation of rho 1-type gamma-aminobutyrate receptors expressed in Xenopus oocytes. *Proc. Natl Acad. Sci. USA* **91**, 12725–12729.

Chang Y. and Weiss D. S. (1999) Channel opening locks agonist onto the GABAC receptor. *Nat. Neurosci.* **2**, 219–225.

Chang Y., Amin J. and Weiss D. S. (1995) Zinc is a mixed antagonist of homomeric rho 1 gamma-aminobutyric acid-activated channels. *Mol. Pharmacol.* **47**, 595–602.

Choi Y., Chen H. V. and Lipton S. A. (2001) Three pairs of cysteine residues mediate both redox and Zn^{2+} modulation of the NMDA receptor. *J. Neurosci.* **21**, 392–400.

Chu X. P., Close N., Saugstad J. A. and Xiong Z. G. (2006) ASIC1a-specific modulation of acid-sensing ion channels in mouse cortical neurons by redox reagents. *J. Neurosci.* **26**, 5329–5339.

Colquhoun D. (1998) Binding, gating, affinity and efficacy: the interpretation of structure-activity relationships for agonists and of the effects of mutating receptors. *Br. J. Pharmacol.* **125**, 924–947.

Connolly C. N. and Wafford K. A. (2004) The Cys-loop superfamily of ligand-gated ion channels: the impact of receptor structure on function. *Biochem. Soc. Trans.* **32**, 529–534.

DiChiara T. J. and Reinhart P. H. (1997) Redox modulation of hslo Ca^{2+}-activated K^+ channels. *J. Neurosci.* **17**, 4942–4955.

Didelon F., Sciancalepore M., Savic N., Mladinic M., Bradbury A. and Cherubini E. (2002) Gamma-aminobutyric acidA rho receptor subunits in the developing rat hippocampus. *J. Neurosci. Res.* **67**, 739–744.

Dirksen R. T. (2002) Reactive oxygen/nitrogen species and the aged brain: radical impact of ion channel function. *Neurobiol. Aging* **23**, 837–839.

Draguhn A., Verdorn T. A., Ewert M., Seeburg P. H. and Sakmann B. (1990) Functional and molecular distinction between recombinant rat GABAA receptor subtypes by Zn^{2+}. *Neuron* **5**, 781–788.

Enz R. and Cutting G. R. (1999) GABAC receptor rho subunits are heterogeneously expressed in the human CNS and form homo- and heterooligomers with distinct physical properties. *Eur. J. Neurosci.* **11**, 41–50.

Enz R., Brandstatter J. H., Hartveit E., Wassle H. and Bormann J. (1995) Expression of GABA receptor rho 1 and rho 2 subunits in the retina and brain of the rat. *Eur. J. Neurosci.* **7**, 1495–1501.

Feigenspan A., Wassle H. and Bormann J. (1993) Pharmacology of GABA receptor Cl^- channels in rat retinal bipolar cells. *Nature* **361**, 159–162.

Goutman J. D., Escobar A. L. and Calvo D. J. (2005) Analysis of macroscopic ionic currents mediated by GABArho1 receptors during lanthanide modulation predicts novel states controlling channel gating. *Br. J. Pharmacol.* **146**, 1000–1009.

Greferath U., Muller F., Wassle H., Shivers B. and Seeburg P. (1993) Localization of GABAA receptors in the rat retina. *Vis. Neurosci.* **10**, 551–561.

Kishida K. T. and Klann E. (2007) Sources and targets of reactive oxygen species in synaptic plasticity and memory. *Antioxid. Redox Signal.* **9**, 233–244.

Kosower N. S. and Kosower E. M. (1978) The glutathione status of cells. *Int. Rev. Cytol.* **54**, 109–160.

Koulen P., Sassoe-Pognetto M., Grunert U. and Wassle H. (1996) Selective clustering of GABA(A) and glycine receptors in the mammalian retina. *J. Neurosci.* **16**, 2127–2140.

Leszkiewicz D. N. and Aizenman E. (2003) Reversible modulation of GABA(A) receptor-mediated currents by light is dependent on the redox state of the receptor. *Eur. J. Neurosci.* **17**, 2077–2083.

Lipton S. A., Choi Y. B., Takahashi H., Zhang D., Li W., Godzik A. and Bankston L. A. (2002) Cysteine regulation of protein function–as exemplified by NMDA-receptor modulation. *Trends Neurosci.* **25**, 474–480.

Liu B., Hattori N., Jiang B., Nakayama Y., Zhang N. Y., Wu B., Kitagawa K., Taketo M., Matsuda H. and Inagaki C. (2004) Single cell RT-PCR demonstrates differential expression of GABAC receptor rho subunits in rat hippocampal pyramidal and granule cells. *Brain Res. Mol. Brain Res.* **123**, 1–6.

Marangos P. J. and Martino A. M. (1981) Studies on the relationship of gamma-aminobutyric acid-stimulated diazepam binding and the gamma-aminobutyric acid receptor. *Mol. Pharmacol.* **20**, 16–21.

Martina M., Strata F. and Cherubini E. (1995) Whole cell and single channel properties of a new GABA receptor transiently expressed in the Hippocampus. *J. Neurophysiol.* **73**, 902–906.

Martina M., Virginio C. and Cherubini E. (1997) Functionally distinct chloride-mediated GABA responses in rat cerebellar granule cells cultured in a low-potassium medium. *J. Neurophysiol.* **77**, 507–510.

Miledi R., Parker I. and Sumikawa K. (1989) Transplanting receptors from brain into oocytes, in *Fidia Research Foundation Neuroscience Award Lecture* (Smith J. J., ed.), pp. 57–89. Raven Press, New York.

Moss S. J. and Smart T. G. (2001) Constructing inhibitory synapses. *Nat. Rev. Neurosci.* **2**, 240–250.

Ogurusu T., Yanagi K., Watanabe M., Fukaya M. and Shingai R. (1999) Localization of GABA receptor rho 2 and rho 3 subunits in rat brain and functional expression of homooligomeric rho 3 receptors and heterooligomeric rho 2 rho 3 receptors. *Recept. Channels* **6**, 463–475.

Organisciak D. T., Wang H. M. and Kou A. L. (1984) Ascorbate and glutathione levels in the developing normal and dystrophic rat retina: effect of intense light exposure. *Curr. Eye Res.* **3**, 257–267.

Pan Z. H., Bahring R., Grantyn R. and Lipton S. A. (1995) Differential modulation by sulfhydryl redox agents and glutathione of GABA- and glycine-evoked currents in rat retinal ganglion cells. *J. Neurosci.* **15**, 1384–1391.

Pan Z. H., Zhang X. and Lipton S. A. (2000) Redox modulation of recombinant human GABA(A) receptors. *Neuroscience* **98**, 333–338.

Pasternack M., Boller M., Pau B. and Schmidt M. (1999) GABA(A) and GABA(C) receptors have contrasting effects on excitability in superior colliculus. *J. Neurophysiol.* **82**, 2020–2023.

Rice M. E. (2000) Ascorbate regulation and its neuroprotective role in the brain. *Trends Neurosci.* **23**, 209–216.

Rice M. E., Forman R. E., Chen B. T., Avshalumov M. V., Cragg S. J. and Drew K. L. (2002) Brain antioxidant regulation in mammals and anoxia-tolerant reptiles: balanced for neuroprotection and neuromodulation. *Comp. Biochem. Physiol. C Toxicol. Pharmacol.* **133**, 515–525.

Rozzo A., Armellin M., Franzot J., Chiaruttini C., Nistri A. and Tongiorgi E. (2002) Expression and dendritic mRNA localization of GABAC receptor rho1 and rho2 subunits in developing rat brain and spinal cord. *Eur. J. Neurosci.* **15**, 1747–1758.

Ruiz-Gomez A., Fernandez-Shaw C., Morato E., Marvizon J. C., Vazquez J., Valdivieso F. and Mayor F. Jr (1991) Sulfhydryl groups modulate the allosteric interaction between glycine binding sites at the inhibitory glycine receptor. *J. Neurochem.* **56**, 1690–1697.

Ruppersberg J. P., Stocker M., Pongs O., Heinemann S. H., Frank R. and Koenen M. (1991) Regulation of fast inactivation of cloned mammalian IK(A) channels by cysteine oxidation. *Nature* **352**, 711–714.

Safiulina V. F., Afzalov R., Khiroug L., Cherubini E. and Giniatullin R. (2006) Reactive oxygen species mediate the potentiating effects of ATP on GABAergic synaptic transmission in the immature hippocampus. *J. Biol. Chem.* **281**, 23464–23470.

Sedelnikova A., Smith C. D., Zakharkin S. O., Davis D., Weiss D. S. and Chang Y. (2005) Mapping the rho1 GABA(C) receptor agonist binding pocket. Constructing a complete model. *J. Biol. Chem.* **280**, 1535–1542.

Sidlo Z., Reggio P. H. and Rice M. E. (2008) Inhibition of striatal dopamine release by CB1 receptor activation requires nonsynaptic communication involving GABA, H(2)O(2), and K(ATP) channels. *Neurochem. Int.* **52**, 80–88.

Sivilotti L. and Nistri A. (1991) GABA receptor mechanisms in the central nervous system. *Prog. Neurobiol.* **36**, 35–92.

Slivka A., Mytilineou C. and Cohen G. (1987a) Histochemical evaluation of glutathione in brain. *Brain Res.* **409**, 275–284.

Slivka A., Spina M. B. and Cohen G. (1987b) Reduced and oxidized glutathione in human and monkey brain. *Neurosci. Lett.* **74**, 112–118.

Strata F. and Cherubini E. (1994) Transient expression of a novel type of GABA response in rat CA3 hippocampal neurones during development. *J. Physiol.* **480**, 493–503.

Todorovic S. M., Jevtovic-Todorovic V., Meyenburg A., Mennerick S., Perez-Reyes E., Romano C., Olney J. W. and Zorumski C. F. (2001) Redox modulation of T-type calcium channels in rat peripheral nociceptors. *Neuron* **31**, 75–85.

Trotti D., Nussberger S., Volterra A. and Hediger M. A. (1997) Differential modulation of the uptake currents by redox interconversion of cysteine residues in the human neuronal glutamate transporter EAAC1. *Eur. J. Neurosci.* **9**, 2207–2212.

Wang T. L., Hackam A., Guggino W. B. and Cutting G. R. (1995) A single histidine residue is essential for zinc inhibition of GABA rho 1 receptors. *J. Neurosci.* **15**, 7684–7691.

Wegelius K., Pasternack M., Hiltunen J. O., Rivera C., Kaila K., Saarma M. and Reeben M. (1998) Distribution of GABA receptor rho subunit transcripts in the rat brain. *Eur. J. Neurosci.* **10**, 350–357.

Wilkins M. E. and Smart T. G. (2002) Redox modulation of GABA(A) receptors obscured by Zn(2+) complexation(1). *Neuropharmacology* **43**, 938–944.

Wotring V. E., Chang Y. and Weiss D. S. (1999) Permeability and single channel conductance of human homomeric rho1 GABAC receptors. *J. Physiol.* **521**, 327–336.

Yudkoff M., Pleasure D., Cregar L., Lin Z. P., Nissim I., Stern J. and Nissim I. (1990) Glutathione turnover in cultured astrocytes: studies with [15N]glutamate. *J. Neurochem.* **55**, 137–145.

Zangerle L., Cuenod M., Winterhalter K. H. and Do K. Q. (1992) Screening of thiol compounds: depolarization-induced release of glutathione and cysteine from rat brain slices. *J. Neurochem.* **59**, 181–189.

Zhang D., Pan Z. H., Awobuluyi M. and Lipton S. A. (2001) Structure and function of GABA(C) receptors: a comparison of native versus recombinant receptors. *Trends Pharmacol. Sci.* **22**, 121–132.

