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SPECIAL REPORT

# A single amino acid confers barbiturate sensitivity upon the GABA $\rho_1$ receptor

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Many structurally diverse general anaesthetics enhance inhibitory neurotransmission in the central nervous system by interacting with the $GABA_A$ receptor. By contrast, GABA receptors composed of the $\rho_1$ subunit are anaesthetic-insensitive. Here, we demonstrate that both $\delta$-hexachlorocyclohexane ($\delta$-HCH; 1–100 $\mu$M), a positive allosteric modulator of the $GABA_A$ receptor, and the anaesthetic pentobarbitone (10–600 $\mu$M) have no effect on GABA-evoked currents mediated by wild-type $\rho_1$ recombinant receptors (expressed in *Xenopus laevis* oocytes). By contrast, these agents produce up to a 10 fold enhancement of GABA responses transduced by a $\rho_1$ receptor in which a transmembrane located isoleucine residue is replaced by serine. However, not all general anaesthetics were similarly influenced by this mutation, because propofol and 5$\beta$-pregnan-3$\alpha$-ol-20-one (5$\beta$3$\alpha$) remained ineffective. These data are discussed in relation to the specificity of general anaesthetic action.

**Keywords:** $GABA_A$ receptor; $\rho$-subunit; general anaesthetic; pentobarbitone; $\delta$-hexachlorocyclohexane

**Abbreviations:** $\delta$-HCH, $\delta$-hexachlorocyclohexane; $EC_{10}$, the concentration of GABA which produced a response 10% of the GABA maximum; $E_{max}$, the amplitude of the response evoked by GABA at $EC_{10}$ in the presence of a maximally effective concentration of the modulator; HEPES, N-2-Hydroxyethylpiperazine-N′-2-ethanesulphonic acid; 5$\beta$3$\alpha$, 5$\beta$-pregnan-3$\alpha$-ol-20-one

**Introduction** Although general anaesthetics have been utilized in clinical practice for over 150 years, the molecular mechanisms by which they cause a rapid depression of central nervous system function remain unknown. The traditional view, held for much of this century, hypothesized that anaesthetics act primarily to perturb neuronal membrane structure. However, a number of recent findings have challenged this concept and have encouraged the assessment of membrane proteins, and transmitter-gated ion channels in particular, as anaesthetic targets (Franks & Lieb 1994). Structurally, general anaesthetics range from chemically inert gases to complex steroidal agents. In view of this chemical diversity, it is surprising that many general anaesthetics used clinically or as experimental agents share, at relevant concentrations, the effect of potentiating the actions of GABA acting at the $GABA_A$ receptor (Franks & Lieb, 1994; Belelli *et al*., 1996a). Although not precluding alternative ion channels as mediators of anaesthetic action, positive allosteric modulation of the $GABA_A$ receptor appeals logically as a mechanism by which the various components of the anaesthetic state may occur.

The mammalian $GABA_A$ receptor is composed of five subunits drawn from the products of a multigene family ($\alpha_{1-6}$, $\beta_{1-3}$, $\gamma_{1-3}$, $\delta$, $\varepsilon$ and $\pi$) that exhibit a distinct distribution within the CNS (Barnard *et al*., 1998). The identification of recombinant GABA receptors that respond differentially to general anaesthetics may aid the identification of protein domains which are essential for anaesthetic activity. Utilizing this strategy, our own studies and those of others have recently identified a single amino acid residue located in the second transmembrane region (TM2) of $GABA_A$, glycine and invertebrate GABA (RDL) receptors that affects the anaesthetic pharmacology of these inhibitory amino acid receptors (Belelli *et al*., 1997; Mihic *et al*., 1997; Krasowski *et al*., 1998; McGurk *et al*., 1998; Pistis *et al*., 1999). Of particular interest are the pharmacological properties of homo-oligomeric GABA receptors formed from the $\rho$ subunit ($\rho_{1-3}$). In contrast to $GABA_A$ receptors, homo-oligomeric $\rho_1$ GABA receptors are not positively modulated by benzodiazepines, barbiturates, steroids or volatile anaesthetic agents (Shimada *et al*., 1992; Mihic & Harris, 1996). Here, we report that the barbiturate pharmacology of the $\rho_1$ GABA receptor is governed by the nature of a single amino acid which occupies a position homologous to that identified in related inhibitory amino acid receptors. Pentobarbitone, acting at $\rho_1$ GABA receptors in which a transmembrane located isoleucine residue (wild type) is mutated to serine (the homologous amino acid in $GABA_A$ receptor $\alpha_{1-6}$, $\beta_1$ and $\gamma_{1-3}$ subunits) now produced a large concentration-dependent enhancement of GABA-evoked responses. Whether this amino acid contributes directly to a binding pocket for the anaesthetic on the receptor protein, or alternatively influences transduction of anaesthetic binding, is discussed.

**Methods** The cDNA encoding the human $\rho_1$ GABA subunit contained within the eukaryotic expression vector pcDNA3 (InVitrogen; CA, U.S.A.), under the control of the cytomegalovirus (CMV) promoter was kindly provided by Dr D. Weiss. For site-directed mutagenesis, single stranded template cDNAs were synthesized from the M13 origin of replication and mutation of the isoleucine residue at position 307 to serine ($\rho_{1I307S}$) was generated using standard procedures (Kunkel *et al*., 1987). Briefly, the following oligonucleotide encoding the mutated sequence was used to prime the mutagenesis reaction: 5′-CACGCCCGT**GCT**GATGGTGGACATGGTTAGCAC-CGTTG-3′. The mutated codon is highlighted in bold whereas the underlined sequence represents a silent mutation removing

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a *Th111*I restriction site to facilitate the rapid screening of mutants by restriction analysis. The fidelity of the mutagenesis reaction was confirmed by standard dideoxynucleotide sequencing (*fmol* DNA Sequencing System Promega, Southampton, U.K.) of both wild type and mutated $\rho_1$ cDNAs.

cDNA (for either the $\rho_1$ or mutated $\rho_1$ subunit $\rho_{1I307S}$) was injected (20 nl of 0.2 mg ml$^{-1}$) into *Xenopus laevis* oocytes (Stage V–VI) defolliculated by pre-treatment with collagenase (see Belelli *et al*., 1996a, for details). The cDNA was injected intranuclearly using the 'blind method' (Colman, 1984). Injected oocytes were individually maintained for up to 12 days as previously described and used for experimentation 2–12 days after cDNA injection. Oocytes were held in a recording chamber (0.5 ml) and continuously superfused (7–10 ml min$^{-1}$) with frog Ringer solution (composition in mM: NaCl 120, KCl 2, $CaCl_2$ 1.8, N-2-Hydroxyethylpiperazine-N′-2-ethanesulphonic acid (HEPES) 5; adjusted to pH 7.4 with NaOH). Electrical recordings were made from oocytes voltage-clamped at a holding potential of −60 mV using a Gene Clamp 500 amplifier (Axon Instruments, U.S.A.) in the two-electrode voltage-clamp mode. Voltage-sensing and current-passing electrodes were filled with 3 M KCl and had resistances of 1–2 MΩ when measured in frog Ringer solution. Current signals were analysed using the Win WCP program, a Dell pentium computer and an Axon 1200 Digidata A to D converter (Dempster, 1997; Mair *et al*., 1998). Timed pulses of drugs dissolved in Ringer solution were applied to oocytes *via* a BPS-4 bath perfusion system (Adams & List Associates, New York) with a four-way manifold. Drug application was computer controlled (see Mair *et al*., 1998). In all experiments investigating potentiation by modulators, a concentration of GABA which produced a response 10% of the GABA maximum ($EC_{10}$) was utilized, whereas the inhibitory actions of propofol and 5$\beta$3$\alpha$ were assessed against the GABA $EC_{50}$. The GABA $EC_{10}$ or $EC_{50}$ was determined for each oocyte. Concentration-response data for GABA and the enhancement of GABA responses by pentobarbitone and $\delta$-HCH were fitted iteratively with the Hill equation as previously described (McGurk *et al*., 1998). From such fits, for both pentobarbitone and $\delta$-HCH, the $E_{max}$ (the amplitude of the response to a GABA $EC_{10}$ concentration in the presence of a maximally effective concentration of the modulator which is expressed as a percentage of the maximum response to GABA) and the $EC_{50}$ (the concentration of modulator required to produce a response 50% of the $E_{max}$) were derived. Quantitative results are expressed as the arithmetic mean ± s.e.mean. Statistical significance of the difference between agonist-evoked response in the absence and presence of antagonists in wild type and mutant receptors was determined by repeated measures ANOVA followed by Newman-Keuls test when warranted. Drugs were obtained from the following sources: pentobarbitone, 5$\beta$3$\alpha$, $\delta$-HCH (Sigma), propofol (Aldrich).

**Results** Concentrations of pentobarbitone (10–300 $\mu$M) known to produce a large enhancement of $GABA_A$ receptor mediated responses (e.g. Belelli *et al*., 1996a) had no effect on

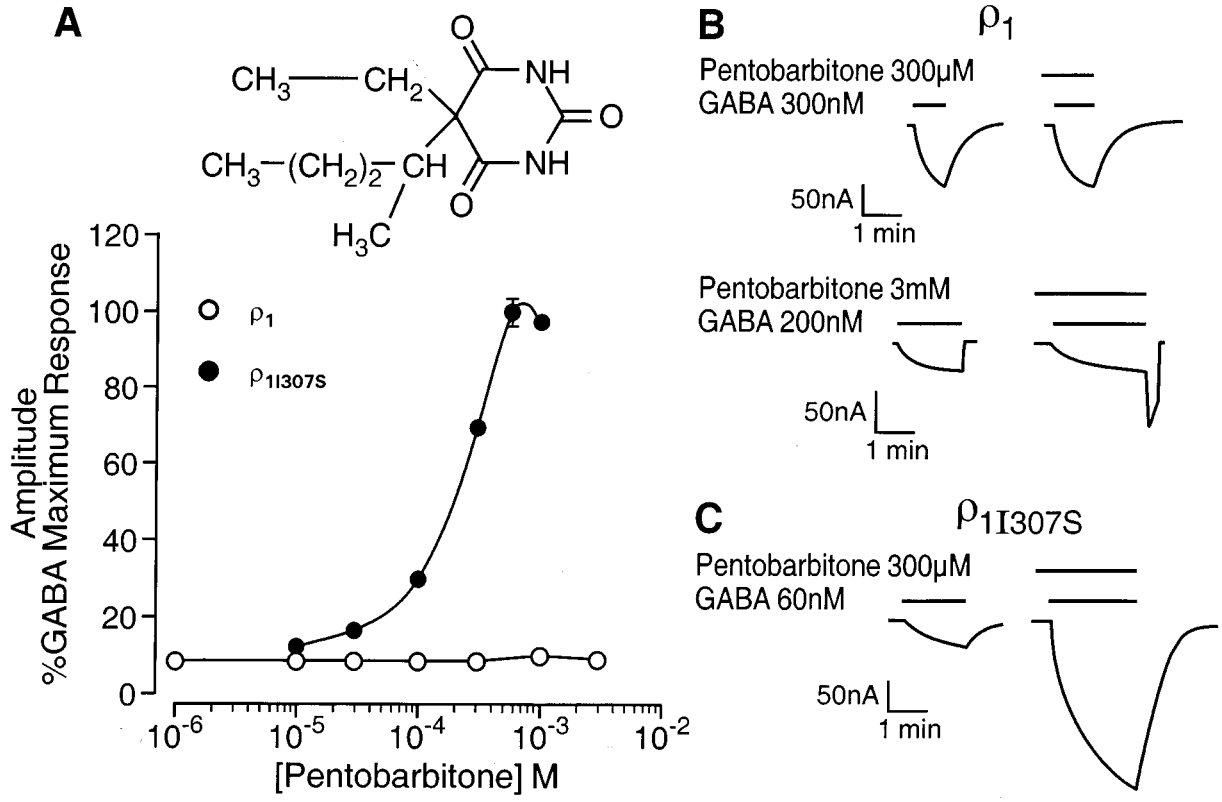


**Figure 1** A single transmembrane amino acid governs pentobarbitone sensitivity of the GABA $\rho_1$ receptor. (A) The graph depicts the relationship between the concentration of pentobarbitone (logarithmic scale) and the peak amplitude of the GABA-evoked current (on a linear scale and expressed as percentage of the response to a maximally effective concentration of GABA) at human $\rho_1$ and $\rho_{1I307S}$ GABA receptors. (B) Pentobarbitone (300 $\mu$M or 3 mM) has no effect on the GABA (300 or 200 nM respectively)-evoked currents recorded from oocytes expressing human $\rho_1$ receptors. However, upon washout of pentobarbitone 3 mM and GABA 200 nM, a transient inward current ('rebound') developed. (C) GABA (60 nM)-evoked currents are greatly enhanced by 300 $\mu$M pentobarbitone for oocytes expressing human $\rho_{1I307S}$ receptors. The insert shows the chemical structure of pentobarbitone.

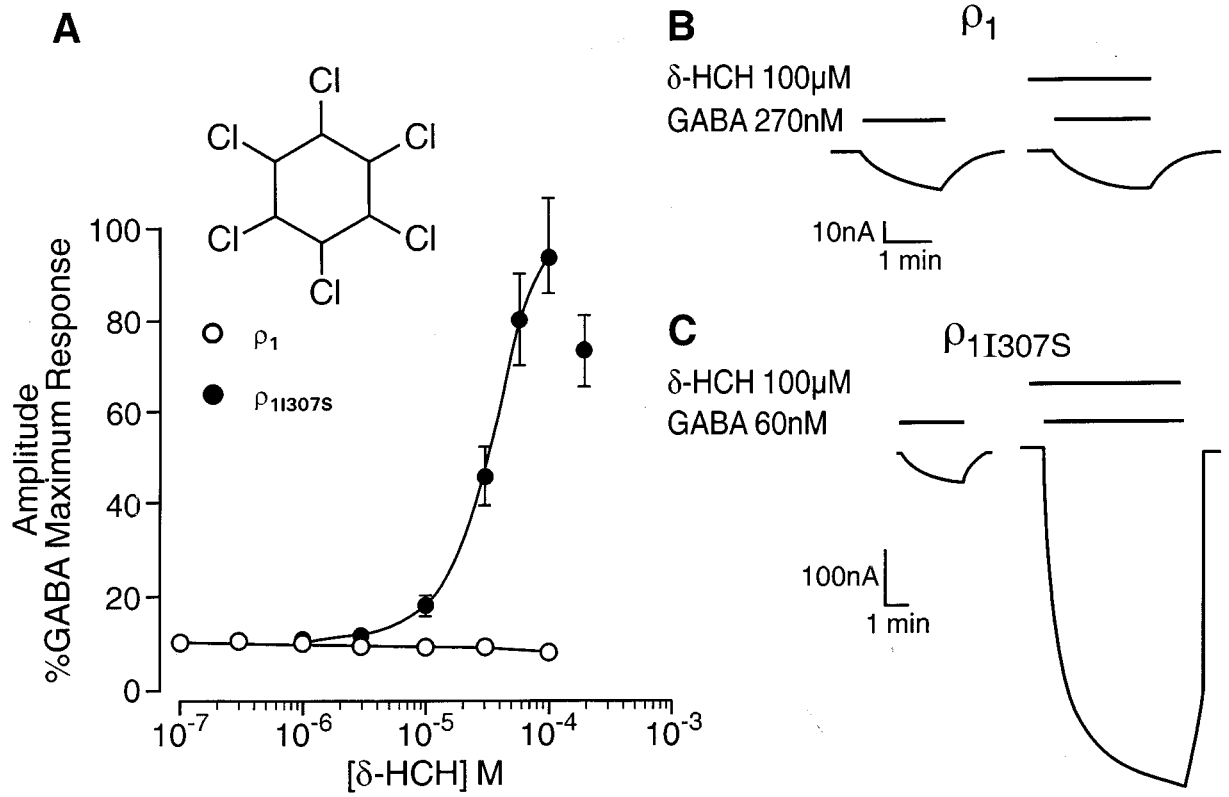


**Figure 2** A single transmembrane amino acid governs $\delta$-HCH sensitivity of the GABA $\rho_1$ receptor. (A) The graph depicts the relationship between the concentration of $\delta$-HCH (logarithmic scale) and the peak amplitude of the GABA-evoked current (on a linear scale and expressed as percentage of the response to a maximally effective concentration of GABA) at human $\rho_1$ and $\rho_{1I307S}$ GABA receptors. (B) $\delta$-HCH (100 $\mu$M) has no effect on the GABA (270 nM)-evoked current recorded from oocytes expressing $\rho_1$ receptors. (C) GABA (60 nM)-evoked currents are greatly enhanced by 100 $\mu$M $\delta$-HCH at oocytes expressing human $\rho_{1I307S}$ receptors. The insert shows the chemical structure of $\delta$-HCH.

the GABA ($EC_{10}$)-evoked current recorded from oocytes expressing the wild-type $\rho$ receptor (Figure 1A and B). However, the washout of high concentrations (600 $\mu$M – 3 mM) of the anaesthetic was associated with the development of a rebound current (Figure 1B). Such currents have also been reported for $GABA_A$ receptors (e.g. Wooltorton *et al.*, 1997). The replacement by serine of isoleucine residue 307, located within the second transmembrane (TM2) domain of the $\rho_1$ GABA receptor, ($\rho_{1I307S}$) produced a 4 fold reduction of the $EC_{50}$ for GABA from $0.8 \pm 0.02$ $\mu$M ($n_H = 2.4 \pm 0.2$; $n = 5$) to $0.2 \pm 0.01$ $\mu$M ($n_H = 3.0 \pm 0.2$; $n = 5$). However, this mutation produced a more fundamental change upon the effect of pentobarbitone. Acting at the $\rho_{1I307S}$ GABA receptor, pentobarbitone produced a concentration-dependent (10 – 600 $\mu$M) enhancement of the GABA ($EC_{10}$)-evoked current with a calculated $EC_{50}$ of $226 \pm 38$ $\mu$M ($n = 4$) and an $E_{max}$ at 600 $\mu$M of $99 \pm 4$% (Figure 1A and C). Hence, concentrations of the anaesthetic that were inert at the wild type receptor produced up to a 10 fold enhancement of the GABA-evoked response of the mutant receptor. At relatively high concentrations, pentobarbitone can directly activate the $GABA_A$ receptor (e.g. Belelli *et al.*, 1996a). However the barbiturate (10 $\mu$M – 3 mM) did not activate either wild type or mutant $\rho_1$ receptors. The $\rho_{1I307S}$ mutation did not influence the actions of other, structurally distinct, anaesthetics.

We have previously demonstrated that relatively low concentrations (3 nM – 1 $\mu$M) of the anaesthetic neurosteroid 5$\beta$3$\alpha$ potentiate the response to GABA mediated by $GABA_A$ receptors (Belelli *et al.*, 1996b). By contrast, 5$\beta$3$\alpha$ (0.1 – 1 $\mu$M) caused a modest but significant inhibition ($P < 0.05$ *vs* control response in the absence of 5$\beta$3$\alpha$) of both wild type and mutant GABA $\rho_1$ receptor, with 1 $\mu$M 5$\beta$3$\alpha$ reducing the GABA ($EC_{50}$)-evoked current to $77 \pm 6$ and $76 \pm 2$% of control ($n = 4$) respectively. Similarly, concentrations of propofol (3 – 30 $\mu$M) known to greatly enhance $GABA_A$ receptor mediated responses (Belelli *et al.*, 1996a), produced a limited but significant depression ($P < 0.05$ *vs* control response in the absence of propofol) of both wild type and mutant GABA $\rho_1$ receptors, with 30 $\mu$M of the anaesthetic reducing the GABA ($EC_{50}$)-evoked current to $86 \pm 5$ and $83 \pm 1$% of control ($n = 3$) respectively. However, neither 5$\beta$3$\alpha$ nor propofol inhibition of the $GABA_A$-evoked response was significantly different ($P > 0.05$) between wild type and mutant $\rho$ receptors. The insecticide lindane ($\gamma$-hexachlorocyclohexane) is a non-competitive antagonist of $GABA_A$ receptors, whereas the $\delta$-isomer ($\delta$-HCH) is a potent positive allosteric modulator (Woodward *et al.*, 1992; Belelli *et al.*, 1996a). $\delta$-HCH (0.1 – 100 $\mu$M) had no effect on GABA ($EC_{10}$)-evoked currents recorded from oocytes expressing the wild type $\rho_1$ receptor ($n = 4$; Figure 2A and B). However, $\delta$-HCH (1 – 100 $\mu$M) greatly enhanced GABA-induced responses mediated by the mutant $\rho_{1I307S}$ receptor ($EC_{50} = 38 \pm 2$ $\mu$M; $E_{max} = 94 \pm 8$%; $n = 4$; see Figure 2C). Hence, as found for pentobarbitone, this single amino acid mutation has revealed a potent, positive allosteric action of $\delta$-HCH.

**Discussion** In contrast to $GABA_A$ receptors, homomeric GABA $\rho$ receptors are insensitive to the positive allosteric actions of benzodiazepines and a number of structurally diverse general anaesthetics including barbiturates, steroids, propofol and isoflurane (Shimada *et al.*, 1992; Mihic & Harris,

1996). The cardinal finding of the present study is that the mutation of a single amino acid from isoleucine (wild type) to serine (the homologous amino acid for $\alpha_{1-6}$, $\gamma_{1-3}$ and $\beta_1$, $GABA_A$ receptor subunits) makes the GABA $\rho_1$ receptor barbiturate-sensitive, such that concentrations of pentobarbitone that had little effect on the wild type recombinant receptor, now produce up to a 10 fold enhancement of the GABA response mediated by the mutant receptor. However, not all general anaesthetics were similarly influenced, as propofol and 5$\beta$3$\alpha$ caused modest inhibition of both the wild type and mutant receptors. This study does not address whether the identified amino acid participates directly in the binding of pentobarbitone to the receptor protein, or alternatively influences the transduction of the allosteric actions of the anaesthetic. However, the effects of the mutation were not exclusive to the barbiturate, as $\delta$-HCH also enhanced GABA function at mutant, but not wild type $\rho_1$ receptors. As pentobarbitone and $\delta$-HCH exhibit little structural similarity, it is difficult to conceive that these compounds bind to a common site. Alternatively, the wild type $\rho_1$ receptor may have distinct binding sites for pentobarbitone and $\delta$-HCH, but only in the mutant receptor does their binding enhance GABA receptor function. The mutation also increased the apparent affinity of GABA for the receptor. The proposed transmembrane location of this amino acid (Wick *et al*., 1998) is difficult to reconcile with the residue contributing to the GABA recognition site directly (Amin & Weiss, 1994), but again favours the mutation modifying the transduction properties of the receptor. Irrespective of whether the identified amino acid participates directly in anaesthetic binding, modifies transduction, or both, it is evident that the anaesthetic pharmacology of $GABA_A$, glycine, invertebrate GABA (RDL) receptors and now $\rho$ GABA receptors is influenced by the nature of the amino acid that occupies the homologous position in these related receptors. Finally, the distinct agonist and antagonist pharmacology of GABA $\rho$ receptors, coupled with their insensitivity to benzodiazepines and a variety of general anaesthetics, has led to them being classified as $GABA_C$ receptors (Shimada *et al*., 1992). However, the present demonstration, that at least part of this distinct pharmacology may be dependent upon a single amino acid warrants caution in such a categorization, and supports the proposal that these ionotropic receptors should, as suggested by sequence homology, be considered as a 'specialized set of $GABA_A$ receptors' (Barnard *et al*., 1998).

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