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The main determinant of furosemide inhibition on $GABA_A$ receptors is located close to the first transmembrane domain

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Abstract

Inhibitory $GABA_A$ receptors are regulated by numerous allosteric modulators, the most receptor-subtype specific of which is furosemide. It recognises receptors of the subunit composition $\alpha 6\beta 2/3\gamma 2$, restricted to cerebellar granule cells. To locate furosemide's site of action we constructed chimeras of the furosemide-sensitive $\alpha 6$ and the furosemide-insensitive $\alpha 1$ subunit, and expressed and studied them together with the $\beta 3$ and $\gamma 2$ subunits in *Xenopus* oocytes by the two-electrode voltage clamp technique. The inhibition of GABA-induced currents by furosemide mainly depended on a short domain proximal to the first transmembrane region of the $\alpha 6$ subunit.

Keywords: $GABA_A$ receptor; α-Subunit; Furosemide; Functional domain

1. Introduction

$GABA_A$ receptors mediate the majority of the inhibitory neurotransmission in the mammalian brain. They have a pentameric structure derived from the assembly of members of the α (1–6), β (1–3), γ (1–3), δ, ε and/or π subunit classes. Each subunit comprises a large extracellular amino-terminal domain containing a conserved 15-amino acid cysteine loop, four transmembrane (TM) regions (TM1-4) and an extracellular carboxy-terminus (Fig. 1). $GABA_A$ receptors possess binding sites for a number of allosteric modulators regulating GABAergic activity (for review see Hevers and Lüddens, 1998). Recombinant receptors containing the $\alpha 6$ subunit, the mRNA of which is restricted to the cerebellar granula cells (Lüddens et al., 1990; Laurie et al., 1992; Bahn et al., 1997) and the more ubiquitous $\alpha 4$ subunit (Wisden et al., 1991) along with $\beta 2$ or $\beta 3$ are highly sensitive to inhibition by the high-ceiling diuretic furosemide but are insensitive to classical benzodiazepine receptor agonists compared to receptors containing the $\alpha 1$ subunit (Korpi et al., 1995; Knoflach et al., 1996; Wafford et al., 1996). However, the domain required for the action of furosemide has been only roughly described to reside amino-terminal to TM1 of the $\alpha 6$ subunit (Fisher et al., 1997). To further pinpoint the main domain we created four chimeras between the $\alpha 1$ and $\alpha 6$ variants covering two adjacent stretches between the cysteine loop and TM2 region. We expressed the α chimeras together with the $\beta 3$ and $\gamma 2$ subunits in *Xenopus* oocytes and measured the effect of furosemide on the GABA-mediated Cl^--current. We identified two furosemide interaction sites, the more prominent of which is located in the carboxy-terminal part of the extracellular domain up to the first half of the first transmembrane region of the $\alpha 6$ subunit.

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2. Materials and methods

2.1. Construction of chimeras

Taq polymerase and *Aoc*I were purchased from Boehringer Mannheim (Mannheim, Germany), *Mlu*I was from New England Biolabs (Bad Schwalbach, Germany). Starting from the previously constructed rat α1 and α6 subunits (α1MA and α6MA) that contained additional unique restriction sites for *Mlu*I (3′ to the cysteine loop) and *Aoc*I (between TM2 and TM3, Wieland et al., 1991), we generated chimeric $GABA_A$ receptors spanning the region between these two sites (Fig. 1). To that end we used the upstream primers α1*Mlu*I and α6*Mlu*I, which hybridised to the sequences surrounding the *Mlu*I recognition sites of α1 and α6, respectively. We named the corresponding downstream primers covering the *Aoc*I sites α1*Aoc*I and α6*Aoc*I. The sequences of these four primers were:

α1 *Mlu*I: 5′ GGGAGCTACGCGTATACAAGAGCAG;
α6 *Mlu*I: 5′ GGGAGCTACGCGTATCCGAAAAGCG;
α1 *Aoc*I: 3′ CATAAGCCACCTTAGGGAGGGAATTTC;
α6 *Aoc*I: 3′ CATAGGACACCTTAGGTAGAGAGTGCC.

We further employed two inner primers with the first half complementary to α1 or α6 and the second half—comprising 15 bases—complementary to α6 or α1, respectively called 5′α1–α6 and 5′α6–α1. Primers exactly reversely complementary to these primers were called 3′α6–α1 and 3′α1–α1. The sequences of primer 5′α1–α6 and 5′α6–α1 were:

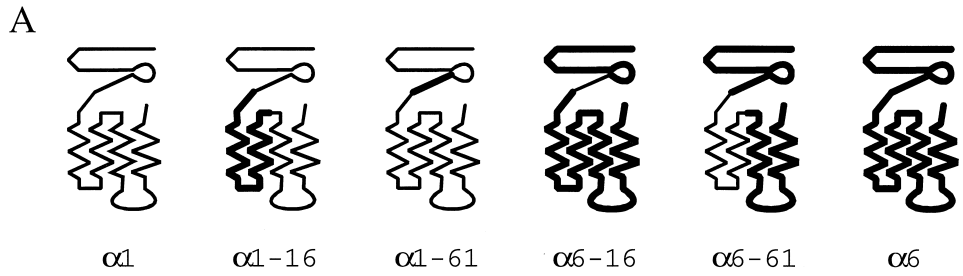
5′ α1–α6: CCAGTATGACCTTCTTGGGCAAACAGTTTCTAG and
5′ α6–α1: CCAGTATGATTTGATTGGGCAAACAGTTGACTC, respectively.

All polymerase chain reactions (PCRs) were performed in the Perkin Elmer instruments 2400 or 9600 in 100 mM Tris–HCl, pH 8.3, 1.5 mM $MgCl_2$, and 500 mM KCl. In a first series of PCRs we used the α1MA as a template with the primer pairs α1MluI/3′α1–α6 and 5′α6–α1/α1*Aoc*I to generate products of 127 bp and 284 bp length, named α1-100 and α1-260, respectively, with the names referring to the length, given in numbers of base pairs, in the final plasmid products. Each initial product contained the 15-base overlap complementary to α6 on the 3′ and 5′ ends plus an extension 5′ to the *Mlu*I or 3′ to the *Aoc*I site, respectively. The corresponding PCR-fragments α6-100 and α6-260 covering the α6-sequence with short α1-extensions were generated using the α6 *Mlu*I/3′ α6–α1 and 5′α1–α6/α6 *Aoc*I primer pairs. All PCRs reactions were run with initial denaturation (94°C) and final elongation steps (72°C) of 5 min each. The two 127 bp and the two 284 bp long fragments were synthesised in 30 cycles of 94°C (30 s), 55°C (30 s) and 72°C (30 s). We then diluted the first four products 1:100 in H_2O. The fragment α6-100, containing the *Mlu*I site of α6, and the adjacent heterologous fragment α1-260, containing the *Aoc*I of α1, were combined for the PCR synthesis of the hybrid α61 fragment, taking advantage of the 15-base overlap of α6-100 with α1-260. The primer pair for this PCR was α6*Mlu*I and α1*Aoc*I. The equivalent construction of α16, extending from the *Mlu*I site of α1 to the *Aoc*I site of α6, employed the primer pair α1*Mlu*I and α6*Aoc*I. The PCR conditions preceding and following the initial denaturation and elongation steps, performed as described above, were: eight cycles with 94°C (15 s); 45°C (3 s); 72°C (30 s) immediately followed by 20 cycles of 94°C (30 s); 55°C (30 s) and 72°C (30 s). The final PCR products called α16 and α61, were purified on a spin column and digested with *Aoc*I and *Mlu*I. After separation on a 2% agarose gel, the appropriate bands of 354 bp length were cut out of the gel and the DNA recovered by a commercial kit (Jet Sorb, Genomed, Research Triangle Park, NC, USA). The α1MA and α6MA plasmids were double digested with *Mlu*I and *Aoc*I. The resulting vectors lacking the 354 bp inserts were recovered from 0.6% agarose gel by the same procedure as described above. The two PCR products α16 and α61 were separately cloned into the two large fragments, resulting in the four chimeras α1-16, α1-61, α6-16 and α6-61, each one containing either a 102 bp or a 258 bp long heterologous insert. The authenticity of the plasmid inserts was verified by sequencing on a Perkin Elmer 470 sequencer. The starting amino acid positions of the exchanged domains in α1 and α6 were for the *Mlu*I site 159 and 158, for the *Aoc*I site 277 and 276 and for the internal overlap 192 and 191, respectively (Fig. 1).

2.2. Electrophysiological methods

Xenopus laevis oocytes were isolated and injected according to standard procedures (Kuner et al., 1993) with mRNA encoding the wild-type or the chimeric α variants in addition to the wild-type rat β3 and rat γ2 short variant. Two-electrode voltage clamp recordings were performed in frog Ringer solution (115 mM NaCl, 1 mM KCl, 1.8 mM $CaCl_2$, 10 mM HEPES, adjusted to pH 7.5 with NaOH). Recording electrodes contained 1 M KCl. Membrane currents were recorded at a holding potential of −70 mV using a Gene Clamp 500 amplifier (Axon Instruments, Foster City, CA, USA). Furosemide (1–3000 μM; Sigma, St. Louis, MO, USA) was co-applied into the bath for 5 s with GABA at the specific EC_{30} concentration which were individually determined in preliminary experiments for all subunit combinations.

The EC_{30} and maximal currents (mean ± S.D.) were for α6β3γ2: 1.5 μM ± 1 and −465 nA ± 156, for α1β3γ2: 71.6 μM ± 18 and −2716 nA ± 413, for α6-16β3γ2:



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                .......160.......170.......180.......190.......200.......210.......220.......230.......240.......250.......260.......270......

rα1             ........TRA.VV.E.TRE.AR..V.A.DG.R.N....L....D.GIVQ.S....VV..TH...K..I...V..T.L..............L.R..........V..............N.....
rα1-16          ........TRA.VV.E.TRE.AR..V.A.DG.R.N....L....S.ETIK.N....VI..VY...Q..M...M..I.T..............I.K..........I..............H.....
rα1-61          ........PKS.II.T.KKG.LY..E.P.ES.S.L....I....D.GIVQ.S....VV..TH...K..I...V..T.L..............L.R..........V..............N.....
rα6-16          ........TRA.VV.E.TRE.AR..V.A.DG.R.N....L....S.ETIK.N....VI..VY...Q..M...M..I.T..............I.K..........I..............H.....
rα6-61          ........PKS.II.T.KKG.LY..E.P.ES.S.L....I....D.GIVQ.S....VV..TH...K..I...V..T.L..............L.R..........V..............N.....
rα6             ........PKS.II.T.KKG.LY..E.P.ES.S.L....I....S.ETIK.N....VI..VY...Q..M...M..I.T..............I.K..........I..............H.....
consensus       LKFGSYAY...E..Y.W...P..SV.V.E..S.L.QYDL.GQTV.S....S.TGEY..MT..FHL.RK.GYF.IQ.Y.PCIMTVILSQVSFW.N.ESVPARTVFG.TTVLTMTTLSISAR.SLPKV
                                                                                                                  TM1                 TM2
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Fig. 1. (A) Chimeric α subunits constructed from α1 and α6. The chimeric nature of the different subunits is indicated by thin (α1) and bold (α6) lines, respectively, illustrating the 102 bp and 258 bp fragments exchanged. The putative four transmembrane regions are depicted schematically, the putative cysteine loop is represented by a half-circle. (B) Amino acid sequences of the wild type and chimeric α subunits in the region exchanged between the α1 and α6 subunits. Amino acids identical in α1 and α6 are only indicated in the consensus sequence. The amino acids of TM1 and TM2 are underlined. The first digit of the amino acid numbers refer to the position in α1.

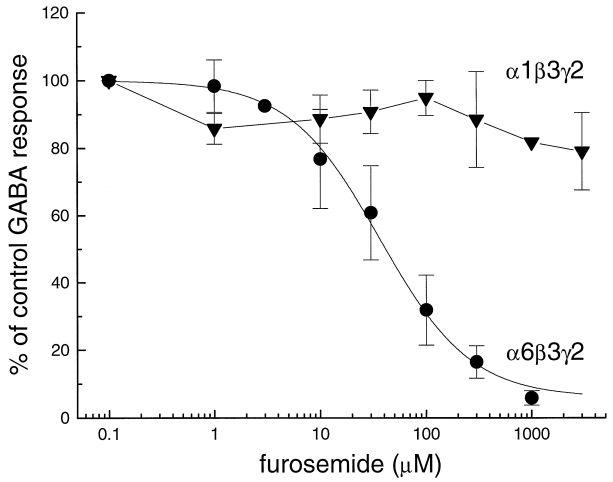

Fig. 2. Inhibition of GABA-mediated currents by furosemide. The dose–response relationships of furosemide were obtained at a GABA concentration yielding 30% of the maximal current. Symbols and bars represent means ± S.D. for α1β3γ2 ($n = 5$) and α6β3γ2 ($n = 6$) and were fit to a four parameter logistic equation. Furosemide blocked the current with an IC_{50} of 37 μM ± 17 in α6β3γ2 receptors but had only a minor effect in α1β3γ2 receptors.

18.3 μM ± 4.6 and −1220 nA ± 535, for α6-61β3γ2: 4.3 μM ± 1.2 and −896 nA ± 178, for α1-16β3γ2: 6.9 μM ± 1.4 and −2756 nA ± 960 and for α1-61β3γ2: 9.1 μM ± 2.3 and −2230 nA ± 484, respectively. The variations of the maximal currents of the various wild-type and chimeric receptors are most likely due to different expression levels as previously observed by binding assays and electrophysiological recordings of recombinant $GABA_A$ receptors (Kleingoor et al., 1993; Korpi and Lüddens, 1997). Applications were separated by 50-s washes. Only oocytes showing stable control responses to GABA before and after each to GABA at the start and the end of an experiment were considered. Acute action of furosemide on the $Na^+/K^+/Cl^-$ cotransporter could be excluded as no effects of furosemide was observed in the absence of GABA. Furthermore, the reversal potentials of the oocytes were identical (between −30 and −20 mV) immediately before and after five consecutive 5-s applications of 50 μM furosemide plus 1 μM GABA to α6β3γ2 receptor expressing oocytes (data not shown). Each experiment was carried out using at least two different donor frogs.

2.3. Data analysis

Currents were recorded and analysed using the pClamp 6.0.3 software (Axon Instruments). Peak currents were normalised and expressed as a percentage of the control current and fit to the equation: $I = I_{min} + (I_{max} - I_{min})/(1 + x/x_0)^n)$, where I represents the normalised current, x the furosemide concentration, x_0 the concentration inducing 50% of maximal inhibition, and n the Hill coefficient. Data were expressed as mean ± S.D.

3. Results

Preliminary experiments obtained with the use of chimera C1 (Wieland et al., 1991) indicated that the amino acid residues permissive for the main inhibitory action of the loop diuretic furosemide are located within a region between the cysteine-loop and the start of TM3 on the α6 subunit (not shown). To define the domain mediating the action of furosemide four α subunit chimeras were constructed (Fig. 1). The chimeras and the wild-type α1 and α6 subunits were co-expressed with β3 and γ2 subunits in *Xenopus* oocytes and their concentration–response relationships determined. Furosemide reversibly inhibited GABA-mediated currents in α6β3γ2 receptors with an IC_{50} of 37 μM ± 17 (Hill coefficient (n_H): 0.8 ± 0.2). The efficacy of furosemide was 94% ± 2 at 1 mM furosemide ($n = 6$; Fig. 2). In α1β3γ2 receptors 3 mM furosemide blocked GABA-induced currents by 21% ± 10 ($n = 5$). Chimera α1-61, which contained the 102 bp long fragment derived from α6, was even less sensitive to furosemide as 3 mM furosemide did not interfere at all with the GABA-elicited current ($n = 12$; Fig. 3). However, α6-16β3γ2 and α1-16β3γ2 receptors were highly sensitive to inhibition by furosemide with IC_{50} values of 31 μM ± 13 (n_H: 0.85 ± 0.22) and 41 μM ± 16 (n_H: 0.89 ± 0.07), respectively, with a maximal inhibition at 1 mM furosemide of 89% ± 3 ($n = 8$) for α6-16β3γ2 and 78% ± 3 ($n = 6$) for α1-16β3γ2 receptors (Fig. 3). With the chimera complementary to the latter, i.e., α6-61, furosemide had an intermediate effect on GABA-induced currents compared

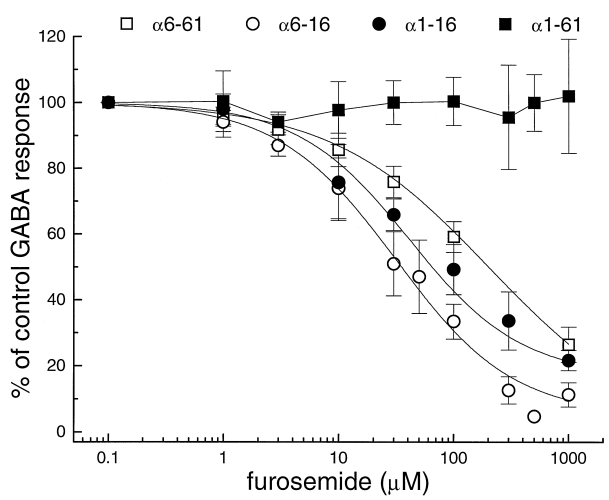

Fig. 3. Inhibition of GABA-mediated currents by furosemide. The dose–response relationships of furosemide were obtained at a GABA concentration yielding 30% of the maximal current. Symbols and bars represent means ± S.D. and were fit to a four parameter logistic equation. All chimeras were expressed together with the β3 and γ2 subunits. The IC_{50} values are 31 μM ± 13 for α6-16β3γ2 ($n = 8$), 41 μM ± 16 for α1-16β3γ2 ($n = 6$) and 201 μM ± 78 for α6-61β3γ2 ($n = 8$) receptors. Furosemide had no effect on α1-61 containing receptors ($n = 12$).

to the other three chimeras, with an IC_{50} value of 201 $\mu M \pm 78$ (n_H: 0.63 ± 0.15) and a maximal inhibition of $73\% \pm 5$ ($n = 8$; Fig. 3).

4. Discussion

Furosemide inhibits GABA-evoked currents at micromolar concentrations in receptors containing an α6 (Korpi et al., 1995) or α4 subunit (Wafford et al., 1996; Knoflach et al., 1996). In contrast, replacing the α4 or α6 subunit by any other α variant leads to a dramatic decrease in the potency and efficacy of furosemide. Previously, it has been demonstrated that furosemide enhances *t*-[^{35}S] butylbicyclophosphorothionate binding to $GABA_A$ receptor channels in the absence and presence of GABA by increasing the affinity of binding, suggesting that it acts non-competitively at an allosteric regulatory site (Korpi et al., 1995). To determine the domains on the α subunits involved in furosemide inhibition we constructed chimeras in which adjacent fragments of 102 bp and 258 bp length of the α1 and α6 subunits were exchanged (Fig. 1). We expressed them together with the natural subunit companions β3 and γ2 (Lüddens and Wisden, 1991).

We found that the 258 bp fragment of the α6 subunit is sufficient for normal furosemide-inhibition of the GABA-induced current in the α6-16β3γ2 and α1-16β3γ2 chimeric receptors. In contrast, α1-61β3γ2 receptors including the 258 bp fragment of the α1 variant were completely insensitive to furosemide. Neither the potency nor the efficacy of furosemide on these chimeras was statistically different from α6 wild-type containing receptors suggesting the main structural domain for furosemide antagonism to reside on this domain of the α6 subunit.

Recently, Fisher et al. (1997) described a high-affinity inhibition by furosemide in a chimeric construct containing the amino-terminal domain of the α6 subunit. This construct included the first half of TM1, thus partly overlapping with the 258 bp fragment. Therefore, together with our present data, the primary determinant for furosemide inhibition appears to be in a short stretch of amino acids amino-terminal to the second half of TM1.

α1β3γ2 and α1-61β3γ2 receptors are insensitive to furosemide, excluding the α1 backbone and the 102 bp fragment of α6 as contributing to the furosemide sensitivity. However, α6-61β3γ2 receptors were also blocked by furosemide but with a potency which was tenfold reduced compared to wild-type α6β3γ2 or α6-16β3γ2 receptors, suggesting an additional recognition site for furosemide on the α6 subunit not detectable in the presence of the high potency site. A similar low potency inhibition by furosemide was previously observed with a chimera containing the amino-terminal extracellular domain of the α1 subunit and the remainder derived from the α6 subunit (Fisher et al., 1997). As the α6-61 chimera originates from the α6 variant with the TM1 and TM2 replaced by the 258 bp fragment of the α1 subunit (Fig. 1), we can conclude that the low-potency site for furosemide is located at the carboxy-terminal end of the α6 starting with TM2, though the possibility remains that this site is part of a single binding pocket.

In addition to α6-containing receptors, furosemide blocks human α4β3γ2 receptors (Wafford et al., 1996), though with the low potency observed by Fisher et al. (1997) for receptors containing the chimeric carboxy-terminal α6 construct (see above). Therefore, they suggested that α4β3γ2 receptors may only contain the lower potency site for furosemide. Our results confirm the existence of a low potency site since we observed it only in α6-61β3γ2 receptors in which the high potency site is absent due to the α1-derived 258 bp fragment. However, Knoflach et al. (1996) reported, that the high-potency furosemide inhibition of GABA-evoked currents of recombinant rat α4β2γ2 and α6β2γ2 receptors is indistinguishable, i.e., in combination with the β2 instead of the β3 subunits. As the amino acid sequences of the human and rat α4 subunits within the exchanged 258 bp region are identical, the different β subunits employed are left as an explanation for the contradictory results. Thus, the α4 but not the α6 containing receptors are postulated to be differentially affected in their furosemide sensitivity by the β2 and β3 variant.

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References

Bahn, S., Jones, A., Wisden, W., 1997. Directing gene expression to cerebellar granule cells using γ-aminobutyric acid type A receptor α6 subunit transgenes. Proc. Natl. Acad. Sci. USA 94, 9417–9421.

Fisher, J.L., Zhang, J., Macdonald, R.L., 1997. The role of α1 and α6 subtype amino-terminal domains in allosteric regulation of γ-aminobutyric $acid_A$ receptors. Mol. Pharmacol. 52, 714–724.

Hevers, W., Lüddens, H., 1998. The diversity of $GABA_A$ receptors: pharmacological and electrophysiological properties of $GABA_A$ channel subtypes. Mol. Neurobiol., in press.

Kleingoor, C., Wieland, H.A., Korpi, E.R., Seeburg, P.H., Kettenmann, H., 1993. Current potentiation by diazepam but not GABA sensitivity is determined by a single histidine residue. Neuroreport 4, 187–190.

Knoflach, F., Benke, D., Wang, Y., Scheurer, L., Lüddens, H., Hamilton, B.J., Carter, D.B., Mohler, H., Benson, J.A., 1996. Pharmacological modulation of the diazepam-insensitive recombinant γ-aminobutyric

acid$_A$ receptors $\alpha 4\beta 2\gamma 2$ and $\alpha 6\beta 2\gamma 2$. Mol. Pharmacol. 50, 1253–1261.

Korpi, E.R., Lüddens, H., 1997. Furosemide interactions with brain GABA$_A$ receptors. Br. J. Pharmacol. 120, 741–748.

Korpi, E.R., Kuner, T., Seeburg, P.H., Lüddens, H., 1995. Selective antagonist for the cerebellar granule cell-specific γ-aminobutyric acid type A receptor. Mol. Pharmacol. 47, 283–289.

Kuner, T., Schoepfer, R., Korpi, E.R., 1993. Ethanol inhibits glutamate-induced currents in heteromeric NMDA receptor subtypes. NeuroReport 5, 297–300.

Laurie, D.J., Seeburg, P.H., Wisden, W., 1992. The distribution of 13 GABA$_A$ receptor subunit mRNAs in the rat brain: II. Olfactory bulb and cerebellum. J. Neurosci. 12, 1063.

Lüddens, H., Wisden, W., 1991. Function and pharmacology of multiple GABA$_A$ receptors. Trends Pharmacol. Sci. 12, 49–51.

Lüddens, H., Pritchett, D.B., Köhler, M., Killisch, I., Keinänen, K., Monyer, H., Sprengel, R., Seeburg, P.H., 1990. Cerebellar GABA$_A$ receptor selective for a behavioural alcohol antagonist. Nature 346, 648.

Wafford, K.A., Thompson, S.A., Thomas, D., Sikela, J., Wilcox, A.S., Whiting, P.J., 1996. Functional characterization of human γ-aminobutyric acid$_A$ receptors containing the $\alpha 4$ subunit. Mol. Pharmacol. 50, 670–678.

Wieland, H.A., Lüddens, H., Seeburg, P.H., 1991. A single histidine in GABA$_A$ receptors is essential for benzodiazepine receptor agonist binding. J. Biol. Chem. 267, 1426–1429.

Wisden, W., Herb, A., Wieland, H., Keinänen, K., Lüddens, H., Seeburg, P.H., 1991. Cloning, pharmacological characteristics and expression pattern of the rat GABA$_A$ receptor $\alpha 4$ subunit. FEBS Lett. 289, 227.