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Furosemide interactions with brain $GABA_A$ receptors

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1 The loop diuretic furosemide is known to antagonize the function of γ-aminobutyric acid type A ($GABA_A$) receptors. The purpose of the present study was to examine the direct interaction of furosemide with the $GABA_A$ receptors by autoradiography and ligand binding studies with native rat and human receptors and with recombinant receptors composed of rat subunits.

2 Autoradiography with [^{35}S]-*t*-butylbicyclophosphorothionate ([^{35}S]-TBPS) as a ligand indicated that furosemide (0.1 – 1 mM) reversed the 5 μM GABA-induced inhibition of binding only in the cerebellar granule cell layer of rat brain sections. In all other regions studied, notably also in the hippocampal and thalamic areas, furosemide failed to antagonize GABA. Furosemide 1 mM decreased [^{35}S]-TBPS binding only in a limited number of brain regions, but facilitation of the GABA-inhibition of the binding was much more widespread.

3 In well-washed rat cerebellar, but not cerebrocortical, membranes, furosemide enhanced the [^{35}S]-TBPS binding over basal level in the absence of added GABA. The $GABA_A$ antagonist, SR 95531, and the convulsant, Ro 5-4864, blocked this furosemide-induced increase. Both interactions with the furosemide enhancement are likely to be allosteric, since furosemide affected the binding of [^{3}H]-SR 95531 and [^{3}H]-Ro 5-4864 identically in the cerebellar and cerebrocortical membranes. Maximal GABA-antagonism induced by furosemide in cerebellar membranes was further increased by SR 95531 but not by Ro 5-4864, indicating additive antagonism only for SR 95531. In human cerebellar receptors, only GABA antagonism by furosemide, but not the enhancement without added GABA, was observed.

4 In recombinant $GABA_A$ receptors, furosemide antagonism of GABA-inhibition of [^{35}S]-TBPS binding depended only on the presence of α6 and β2/3 subunits, irrespective of the presence or absence of γ2 or δ subunits.

5 In α6β3γ2 receptors, clozapine reversed the enhancement of [^{35}S]-TBPS binding by furosemide in the absence of GABA. However, it failed to affect the GABA-antagonism of furosemide, suggesting that the enhancement of basal binding and the GABA antagonism might represent two different allosteric actions of furosemide.

6 In conclusion, the present results indicate that furosemide is a subtype-selective $GABA_A$ antagonist with a mode of action not shared by several other antagonists, which makes furosemide a unique compound for development of potential $GABA_A$ receptor subtype-specific and -selective ligands.

Keywords: $GABA_A$ receptors; furosemide; clozapine; Ro 5-4864; SR 95531; $GABA_A$ antagonism; cerebellar granule cells; $GABA_A$ receptor subunits

Introduction

The main central nervous system inhibitory neurotransmitter receptor, γ-aminobutyric acid type A ($GABA_A$) receptor, mediates, at least partly, behavioral actions of a number of important drugs, including benzodiazepine receptor ligands, barbiturates, anaesthetics and possibly ethanol (Lüddens *et al*., 1995; Sieghart, 1995). This receptor shows enormous molecular heterogeneity, being a pentameric complex of 13 subunits, which belong to α (members 1 – 6), β (1 – 3), γ (1 – 3) or δ (1) classes (Olsen & Tobin, 1990; Wisden & Seeburg, 1992; Stephenson, 1995). It also shows brain regional heterogeneity due to cell-specific expression of different subunits (Laurie *et al*., 1992; Persohn *et al*., 1992; Wisden *et al*., 1992). In spite of this heterogeneity, only a few compounds have been discovered which display some receptor subtype selectivity. All these compounds, such as the hypnotic benzodiazepine receptor ligand, zolpidem (Pritchett & Seeburg, 1990) and the antiepileptics, carbamazepine and phenytoin (Granger *et al*., 1995), show selectivity towards the main receptor subtype containing the α1 subunit.

We have recently discovered that the $Na^+/2\ Cl^-/K^+$ co-transporter blocker furosemide (Greger & Wangemann, 1987) selectively, reversibly, rapidly and noncompetitively antagonizes Cl^- flux in a cerebellar granule cell-specific $GABA_A$ receptor subtype (IC_{50} about 10 μM in α6β2γ2 receptors; Korpi *et al*., 1995a). This antagonism was not due to transporter blockade, since another diuretic with similar specificity for the $Na^+/2\ Cl^-/K^+$ co-transporter, bumetanide, was inactive. The furosemide effect depended on the interplay between α6 and β2 or β3 subunits in $GABA_A$ αβγ receptors. The purpose of the present study was to clarify further the pharmacology of furosemide interaction with the $GABA_A$ receptor by studying both native and recombinant receptors in the presence of known $GABA_A$ antagonists SR 95531 (Heaulme *et al*., 1986), Ro 5-4864 (Weissman *et al*., 1984) and clozapine (Korpi *et al*., 1995b). In addition, we excluded other brain regions as showing similar kind of furosemide antagonism by ligand autoradiography, specifically in hippocampal and thalamic regions.

Methods

Brain samples and membranes

Four-month-old male Wistar rats (Department of Animal Physiology, University of Helsinki, Helsinki, Finland) were decapitated and the cerebral cortex and cerebellum were dis-

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sected and frozen. For autoradiography, whole brains were carefully dissected and frozen on dry ice. Human cerebellar cortical samples were from autopsies at the District of Columbia Medical Examiner's Office from four control subjects used earlier in a benzodiazepine binding study (mean age 47.5 years (range 38–59), 3 males, 1 female, *postmortem* interval 25 h (range 17–31), all with negative *postmortem* toxicology screen, cardiovascular diseases causing their deaths; Korpi *et al*., 1992b). The tissues were homogenized with a Polytron in 50 volumes of ice-cold 50 mM Tris-citrate buffer (pH 7.4) supplemented with 1 mM disodium edetate, centrifuged at 20,000 x *g* for 20 min. The pellets were resuspended in the same buffer and recentrifuged 5 times. The final suspension was prepared in 50 mM Tris-citrate buffer and stored frozen, in aliquots, at −80 °C.

Recombinant receptors

Human embryonic kidney (HEK) 293 cells were transfected (Chen & Okayama, 1987) with rat cDNAs encoding α6, β3, γ2S and δ subunits, subcloned individually into eukaryotic expression vectors (Pritchett *et al*., 1989; Ymer *et al*., 1989; Shivers *et al*., 1989; Lüddens *et al*., 1990). Quantitative ratios of the cDNAs for the α6, β3, γ2S and δ subunits were 5:3:0.5:5. Briefly, cells plated on dishes 15 cm in diameter (Becton Dickinson Labware, Lincoln Park, NJ) were transfected two to three days later. About 20 h after transfection, the medium was changed, and 48 h after transfection, the cells were washed and harvested in phosphate-buffered saline. Cell pellets were homogenized with a Polytron in 50 mM Tris-citrate (pH 7.4) buffer, centrifuged, resuspended, and stored frozen at −80 °C.

Ligand binding

Frozen membranes were thawed, resuspended and centrifuged once, before final resuspension in 50 mM Tris-citrate to give a protein concentration of 20–240 μg ml^{-1} (Bio-Rad Protein Assay kit) in a total volume of 0.5 ml per assay tube (Korpi & Lüddens, 1993). After defined incubation times of duplicate samples, bound and free ligands were separated by rapid filtration of the membranes onto Schleicher & Schuell #52 or Whatman GF/B glass fibre filters. Samples were rinsed twice with 5 ml of ice-cold 10 mM Tris-HCl (pH 7.4). The air-dried filters were immersed in 4 ml of scintillation fluid and their radioactivity was determined.

[^{35}S]-*t*-butylbicyclophosphorothionate ([^{35}S]-TBPS; Du Pont de Nemours, New England Nuclear, Germany) binding at 2 nM concentration was determined after a 90 min incubation at 22°C in 50 mM Tris-citrate buffer supplemented with 200 mM NaCl. Nonspecific binding was defined in the presence of 20 μM picrotoxinin (Sigma, St. Louis, MO). [Butyryl-2,3-^{3}H]-SR 95531 (NEN) binding at 6 nM was determined after a 30 min incubation at 0°C in 50 mM Tris-citrate buffer (pH 7.4), with nonspecific binding being defined in the presence of 100 μM GABA. [N-methyl-^{3}H]-Ro 5-4864 (NEN) binding at 5 nM was determined after a 90 min incubation at 0°C in 50 mM Tris-HCl buffer (pH 7.4), with nonspecific binding being defined in the presence of 10 μM Ro 5-4864. Furosemide (Sigma), 4′-chlorodiazepam (Ro 5-4864; Fluka), clozapine (Sandoz Research Institute, Berne, Switzerland) and the specific $GABA_A$ antagonist 2′-(3′-carboxy-2′,3′-propyl)-3-amino-6-*p*-methoxyphenylpyrazinium bromide (SR 95531; Research Biochemicals, Natick, MA) were used with or without GABA (Serva, Heidelberg, Germany) at 5 μM. Before dilution in assay buffer, furosemide was dissolved at 200 mM concentration in 0.2 M NaOH, Ro 5-4864 at 10 mM concentration in dimethylsulphoxide and clozapine at 100 mM concentration in 0.1 M HCl. SR 95531 and GABA were directly dissolved in the buffer.

Autoradiography

The procedure (Korpi *et al*., 1995b) used was modified from Olsen *et al*. (1990) and Edgar & Schwartz (1990). Briefly, 14 μm frontal sections were cut in a Leitz 1720 cryostat at the following levels (in mm) from the bregma according to Paxinos & Watson (1982): 7, 2.7, 1.5, −0.8, −1.8, −3.3, −5.3, −6.3, −8.5, and −10.3. Sections were preincubated in an ice-water

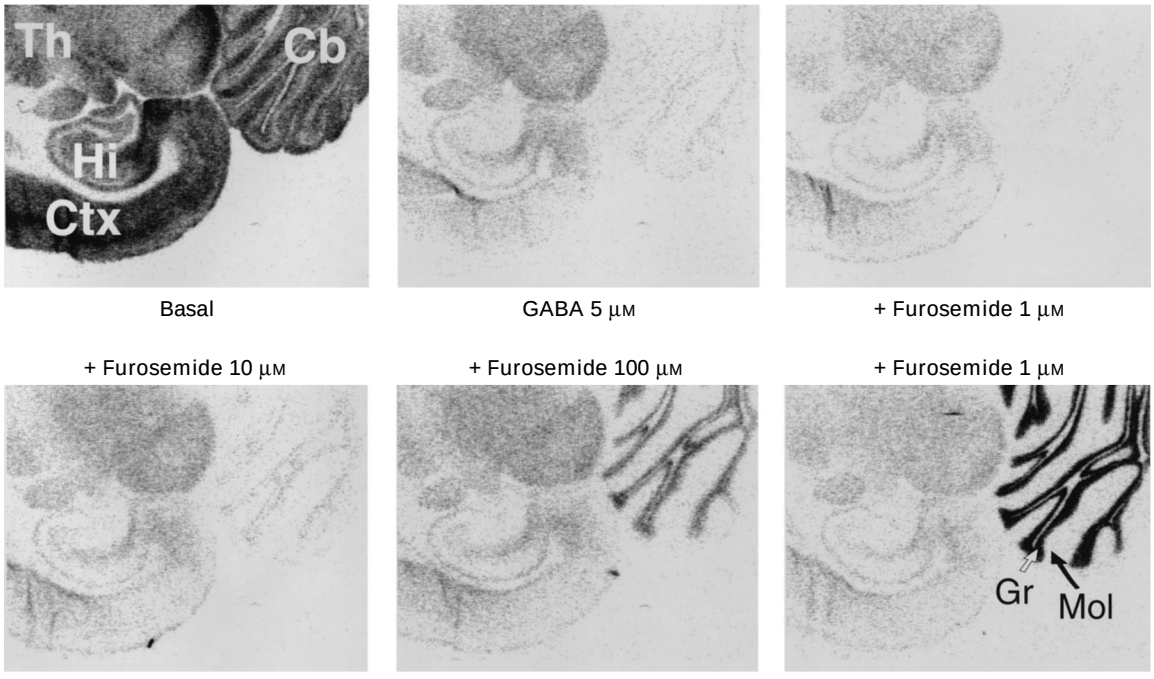

Figure 1 Effect of furosemide on the inhibitory action of GABA on [^{35}S]-TBPS binding in rat hippocampal regions. Representative autoradiographs of serial sections demonstrate basal [^{35}S]-TBPS binding and binding in the presence of 5 μM GABA without and with various concentrations of furosemide. The data indicate that high micromolar concentrations of furosemide attenuate the GABA effects in the cerebellar (Cb) granule cell layer (Gr) but not in cerebellar molecular layer (Mol), hippocampus (Hi), cerebral cortex (Ctx), or thalamus (Th).

bath for 15 min in 50 mM Tris-HCl (pH 7.4) supplemented with 120 mM NaCl. Incubation with [^{35}S]-TBPS (200 d.p.m. μl^{-1}, adjusted to 6 nM with cold TBPS) for 90 min at room temperature (22°C) was performed in the same buffer, by use of 600 μl liquid bubbles over sections on object glasses in a humid chamber. Effects of 5 μM GABA and 1 mM furosemide were tested. After incubation, the sections were washed three times for 15 s in ice-cold incubation buffer, dipped into distilled H_2O, air-dried at room temperature, and exposed to Hyperfilm-ßmax (Amersham, UK) for 3–5 days. Twenty μM picrotoxinin reduced the signal to background level (not shown). Regional labelling intensities were quantitated from

Table 1 Effects of furosemide on regional [^{35}S]-TBPS binding in rat brain sections: quantitative autoradiography

Brain region	*Basal binding*	*Furosemide*	*GABA*	*GABA + furosemide*
Olfactory regions				
Olfactory bulb, external plexiform layer	800±126	79±7[b]	5±1	3±1[a]
glomerular layer	223±47	91±10	9±3	5±1[a]
internal granular layer	107±7	83±10[a]	20±5	12±1[a]
Olfactory tubercle	138±35	85±39	17±5	9±4[a]
Islands of Calleja	681±140	98±12	18±3	11±4[a]
Primary olfactory cortex	193±8	68±5[c]	16±2	10±3[a]
Cerebral cortical regions				
Frontoparietal cortex, somatosensory	293±50	87±11	24±6	12±4[a]
I-III layers	225±28	68±3[c]	15±2	8±3[b]
IV-VI layers	325±58	93±13	28±6	14±4[b]
Frontoparietal cortex, motor	269±31	101±12	29±7	15±5[a]
I-III layers	232±13	83±13	17±4	9±3[a]
IV-VI layers	283±40	107±11	33±8	17±6[a]
Temporal cortex, auditory	258±36	77±12[a]	16±4	8±1[b]
I-III layers	196±43	60±14[a]	14±5	8±3
IV-VI layers	316±61	78±21	19±4	10±3[a]
Limbic regions				
Medial prefrontal cortex	197±19	55±10[b]	15±2	7±1[b]
Anterior cingulate cortex	272±16	70±9[b]	14±3	7±2[b]
Entorhinal cortex	186±28	63±13[a]	14±3	8±1[b]
Subiculum	252±45	88±11	32±6	18±3[b]
Hippocampus, CA1	129±18	68±11	24±6	16±3
CA3	142±20	71±6[b]	23±7	15±3
dentate gyrus	149±16	70±10[b]	22±5	12±2[b]
Bed nucleus stria terminalis	203±16	78±7[a]	24±4	13±4[b]
Nucleus of horizontal limb of diagonal band	438±38	110±13	32±4	17±3[b]
Septohippocampal nucleus/teania tecta	95±22	63±12[a]	21±5	13±5
Lateral septal nuclei	196±39	99±11	39±12	20±8[a]
Triangular septal nucleus	95±33	99±21	52±18	32±10
Bed nucleus of anterior commissura	279±45	81±22	25±6	22±6
Anterior amygdaloid area	213±24	95±12	28±6	18±6
Amygdala	203±21	76±12[a]	21±5	11±4[a]
Posteromedial cortical amygdaloid nucleus	187±34	53±16[b]	14±3	8±1[b]
Basal ganglia				
Nucleus accumbens	185±31	75±14	19±4	10±4[a]
Caudate/Putamen	146±25	103±13	40±9	24±9[a]
Globus pallidus	329±40	106±15	39±7	23±5[b]
Claustrum	311±29	92±6	25±7	13±3[a]
Thalamus				
Paraventricular nucleus	210±27	73±22	20±11	10±6
Anterodorsal nucleus	253±48	110±17	38±13	21±3[a]
Centrolateral/medial nucleus	246±51	104±21	35±9	19±7[a]
Intermediodorsal nucleus	196±27	78±22	21±6	8±4[a]
Ventroposterior nucleus	206±36	117±18	44±7	31±6[a]
Zona incerta/Subthalamic nucleus	235±43	106±17	47±11	25±7[a]
Medial geniculate nucleus	211±46	115±26	45±9	25±4[a]
Hypothalamus				
Lateral preoptic area	216±20	101±11	40±2	22±5[c]
Lateral area	177±18	117±14	46±9	27±7[a]
Anterior area	191±18	95±11	31±10	19±6
Paraventricular nucleus	120±9	75±16	20±8	14±7
Ventromedial nucleus	156±18	74±13[a]	22±7	13±4
Midbrain				
Substantia nigra, pars reticulata	319±58	103±17	32±4	17±5[b]
Interpeduncular nucleus	157±43	100±18	34±8	20±4[a]
Superior colliculus, superior gray layer	264±50	87±15	31±12	15±3[a]
Central gray	222±58	105±16	42±12	20±6[a]
Interior colliculus, central nucleus	320±85	120±19	43±10	20±4[b]
Cerebellum				
Granule cell layer	244±89	255±91[a]	23±6	160±49[b]
Molecular layer	201±45	104±25	12±7	10±2

Basal picrotoxin-sensitive [^{35}S]-TBPS binding at 6 nM is given in $nCi^{-1}g$ (mean±s.d., $n=4$), and values in the presence of furosemide (1 mM), GABA (5 μM) and GABA plus furosemide are expressed as % of the basal binding. Statistical significance of the difference between basal and furosemide and between GABA and GABA + furosemide binding values (Student's t test): [a]$P<0.05$, [b]$P<0.01$, [c]$P<0.001$.

the films by using MCID M4 image analysis device and programme (Imaging Research, St. Catharines, Canada). Locations of various brain areas on exposed films were identified with the aid of the same brain sections stained with thionin. The binding densities for each brain area were averaged from measurements from two to three sections. Plastic ^{14}C-standards (Amersham) exposed simultaneously to the brain sections were used as reference with the resulting binding values given as radioactivity levels estimated for gray matter areas (nCi g^{-1}). Serial horizontal sections from four additional rats were tested for a range of furosemide concentrations (1–1000 μM) in the presence of 5 μM GABA to test whether lower furosemide concentrations would reveal antagonism of GABA-inhibition of [^{35}S]-TBPS binding. A representative set of images from this experiment is given in Figure 1.

Statistics

Statistical significance of the differences from the corresponding control binding and between two population means was assessed by use of two-tailed Student's *t* test with Instat program (GraphPad Software, San Diego, CA).

Results

We used the modulation of the convulsant binding site labelled by [^{35}S]-TBPS as a biochemical test for $GABA_A$ receptor function, since in the presence of GABA this site is allosterically modulated by other ligands in a manner predictive for agonistic and antagonistic efficacy (see Korpi *et al*., 1995b). Quantitative autoradiography of [^{35}S]-TBPS binding in rat brain sections indicated clearly that the cerebellar granule cell layer was the only brain region, where furosemide enhanced the basal binding and abolished the inhibition of the binding by exogenous GABA (Table 1). Since it has been shown that furosemide antagonizes a part of the hippocampal $GABA_A$ response in electrophysiological studies (Pearce, 1993), we looked more carefully at this brain region in the presence of various furosemide concentrations (Figure 1). However, there was no indication of reversal of GABA-inhibition of hippocampal [^{35}S]-TBPS binding, whereas the cerebellar granule cell layer binding was enhanced. Neither was there any furosemide-induced elevation of GABA-inhibited binding in thalamic regions (Table 1, Figure 1), such as medial geniculate nucleus, known to contain α4 subunits which make furosemide-sensitive receptors when expressed recombinantly with β2 and γ2 subunits (Knoflach *et al*., 1996).

Cerebellar membranes from *postmortem* human normal control subjects were used to ascertain whether furosemide antagonism is present also in human cerebellum. Similar to rat cerebellar membranes (Korpi *et al*., 1995a), furosemide reversed the GABA-inhibition of [^{35}S]-TBPS binding, although the binding was not clearly enhanced by it in the absence of GABA (Figure 2).

In addition to specific GABA antagonism in the cerebellar granule cell layer, furosemide at high micromolar and low millimolar concentrations decreased the binding of [^{35}S]-TBPS to cerebellar, hippocampal and cerebrocortical membranes (Korpi *et al*., 1995a). In agreement, 1 mM furosemide significantly decreased the binding in selected brain regions in the absence of added GABA (Table 1), and further enhanced the GABA-inhibition of the binding in most brain regions. Unlike the action of furosemide on the cerebellar granule cell receptors, the 'displacing' action was also shared with another diuretic, bumetanide (Korpi *et al*., 1995a; quantitative data not shown).

Representatives of two different classes of $GABA_A$ antagonists, SR 95531 and Ro 5-4864, were found to reverse the furosemide-induced elevation of [^{35}S]-TBPS binding in cerebellar membranes in the absence of GABA (Figure 3a,c). In the presence of 5 μM GABA, SR 95531 was merely additive to the antagonistic effect of furosemide on the action of GABA,

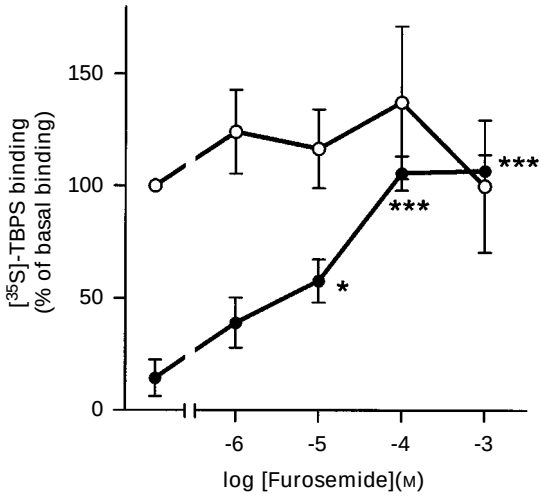

Figure 2 Effects of furosemide in the absence (○) and presence (●) of 5 μM GABA on [^{35}S]-TBPS binding in cerebellar cortical membranes prepared from human *postmortem* samples. Data points, expressed as % of the basal binding, are means ± s.e.mean (vertical lines) for four subjects, with averages of duplicate samples. Significance of the difference from the corresponding values in the absence of furosemide (Student's *t* test): $*P<0.05$, $***P<0.001$.

whereas Ro 5-4864 at 100 μM abolished this effect of furosemide. In cerebrocortical membranes, furosemide did not affect the modulations of SR 95531 and Ro 5-4864 on [^{35}S]-TBPS binding (Figure 3b, d). Ro 5-4864 had a peculiar concentration-dependent action on [^{35}S]-TBPS binding (in agreement with Gee (1987)), which appeared similar in shape, but stronger in cerebrocortical than cerebellar membranes (Figure 4). Furosemide had little effect on cerebellar and cerebrocortical [^{3}H]-SR 95531 binding (Figure 5a), but it affected cerebellar and cerebrocortical binding of [^{3}H]-Ro 5-4864 in concentrations higher than 30 μM (Figure 5b).

Clozapine reversed the elevation of [^{35}S]-TBPS binding by furosemide in cerebellar membranes and slightly decreased the basal binding in the presence of GABA (Figure 6a). It also antagonized GABA-inhibition of the binding irrespective of furosemide. In recombinant $GABA_A$ $\alpha6\beta3\gamma2$ receptors, clozapine still slightly affected the binding in the absence of GABA, but failed to affect the GABA-inhibition of the binding and furosemide antagonism of GABA-inhibition (Figure 6b).

Co-transfection of human embryonic kidney 293 cells with $\alpha6$, $\beta3$, $\gamma2$ and δ subunit combinations revealed that the action of furosemide was independent of the γ variant in the receptor complex (Table 2). The inhibition of [^{35}S]-TBPS binding to $\alpha6\beta3$ as well as $\alpha6\beta3\delta$ receptors by 10 μM GABA was reversed by 300 μM furosemide, indicating that the $\alpha6\beta3$ is sufficient for the expression of the furosemide recognition site on $GABA_A$ receptors. However, furosemide without GABA did not increase the binding above control levels in $\alpha6\beta3$ or $\alpha6\beta3\delta$ receptors in contrast to $\alpha6\beta3\gamma2$ receptors (Table 2; Korpi *et al*., 1995a).

Discussion

The present results indicate several interesting novel features in the mode of the interaction of furosemide with the $GABA_A$ receptor: (1) it shows a selective antagonism at cerebellar granule cell-specific receptors, dependent on the $\alpha6$ and $\beta3$ subunits whether accompanied or not by $\gamma2$ or δ subunits, (2) a similar kind of antagonism by furosemide is undetectable in hippocampal and thalamic regions, in spite of its selective attenuation of a receptor response in hippocampus (Pearce, 1993) and of the sensitivity of $\alpha4\beta2\gamma2$ receptors to furosemide (Knoflach *et al*., 1996), (3) the mechanism of furosemide-

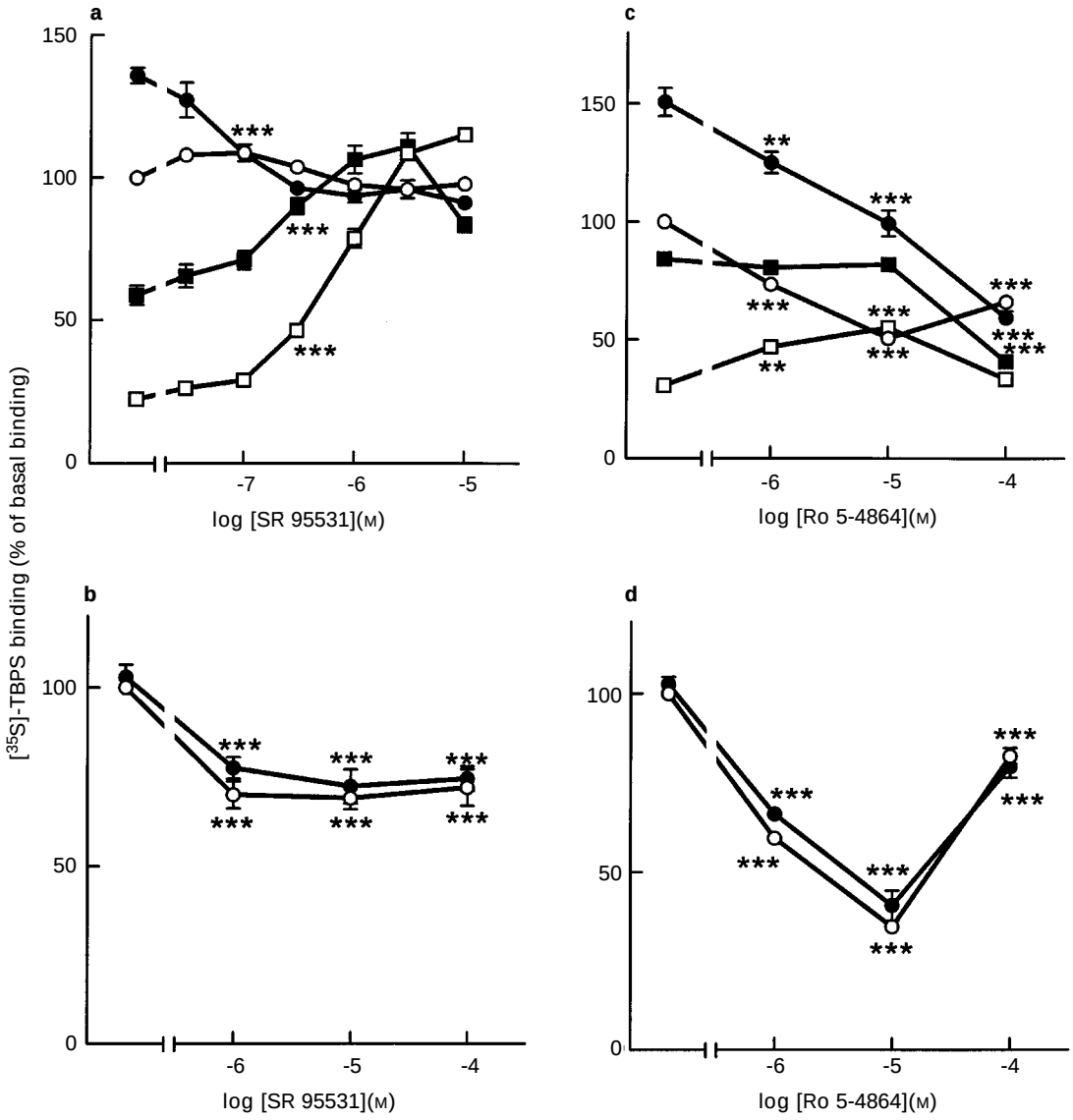

Figure 3 (a, b) Effects of SR 95531 and (c, d) Ro 5-4864 on the [^{35}S]-TBPS binding in cerebellar (a, c) and cerebrocortical (b, d) membranes in basal conditions (○), with 300 μM furosemide (●), with 300 μM furosemide and 5 μM GABA (■) and with 5 μM GABA (□). Data points, expressed as % of the basal binding, are means ± s.e.mean (vertical lines) for three independent experiments on duplicate samples. Significance of the difference from the corresponding values in the absence of SR 95531 or Ro 5-4864 (Student's t test): $^{**}P<0.001$, $^{***}P<0.001$. In (a) only the significances for the lowest SR 95531 concentrations are indicated for clarity.

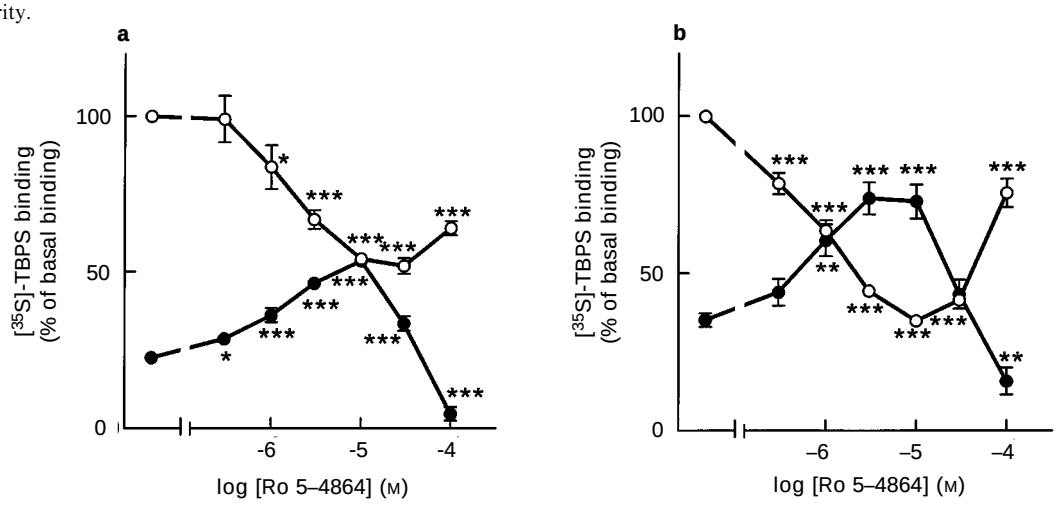

Figure 4 Effects of Ro 5-4864 on the [^{35}S]-TBPS binding in the cerebellar (a) and cerebrocortical (b) membranes in the absence (○) and presence (●) of 5 μM GABA. Data points, expressed as % of the basal binding, are means ± s.e.mean (vertical lines) for three independent experiments on duplicate samples. Significance of the difference from the corresponding values in the absence of Ro 5-4864 (Student's t test): $^{*}P<0.05$, $^{**}P<0.001$, $^{***}P<0.001$.

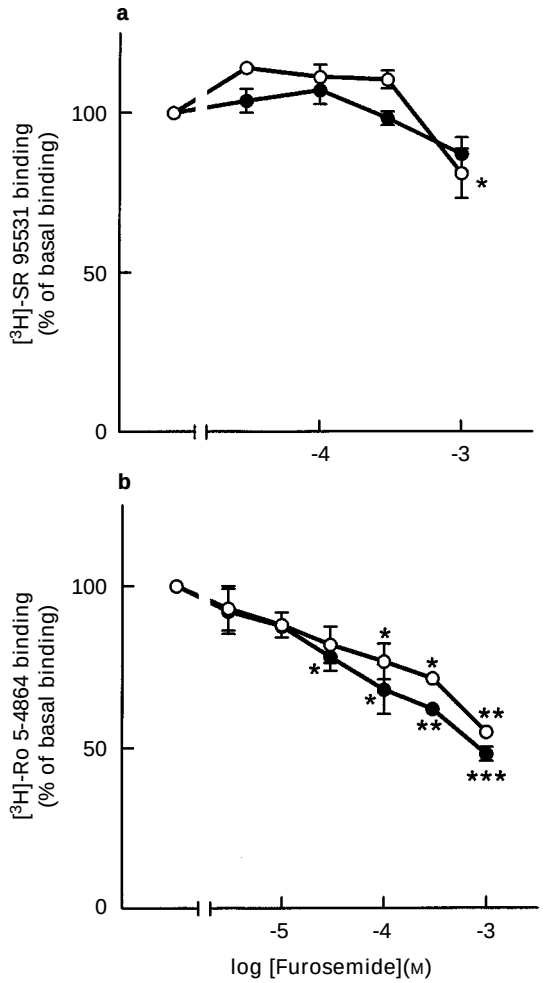

Figure 5 Effects of furosemide on (a) [^{3}H]-SR 95531 and (b) [^{3}H]-Ro 5-4864 binding in the cerebellar (○) and cerebrocortical (●) membranes. Data points, expressed as % of the basal binding, are means ± s.e.mean (vertical lines) for three independent experiments on triplicate samples. Significance of the difference from the corresponding values in the absence of furosemide (Student's t test): $^*P<0.05$, $^{**}P<0.001$, $^{***}P<0.001$.

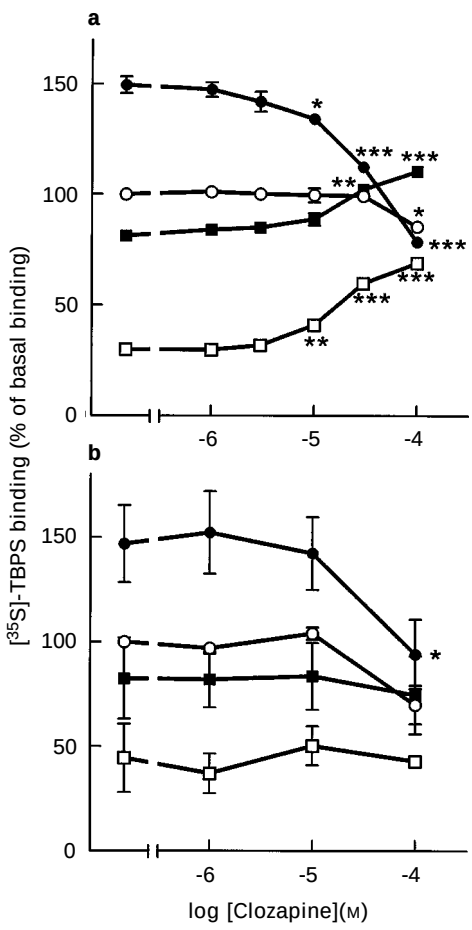

Figure 6 Effects of clozapine on [^{35}S]-TBPS binding in the cerebellar membranes in basal conditions (○), with 300 μM furosemide (●), with 300 μM furosemide and 5 μM GABA (■), and with 5 μM GABA (□). (b) Effects of clozapine on the binding in recombinant $GABA_A$ $\alpha6\beta3\gamma2$ receptors in basal conditions (○), with 100 μM furosemide (●), with 50 μM furosemide and 1 μM GABA (■), and with 1 μM GABA (□). Data points, expressed as % of the basal binding, are means ± s.e.mean (vertical lines) for three independent experiments on duplicate samples. Significance of the difference from the corresponding values in the absence of clozapine (Student's t test): $^*P<0.05$, $^{**}P<0.001$, $^{***}P<0.001$.

induced elevation of [^{35}S]-TBPS binding in the absence of GABA differs from that of its antagonism of GABA-inhibition of the binding, (4) other $GABA_A$ antagonists do not share the molecular interaction of furosemide with the receptor.

Cerebellar granule cells are a unique locus for 'diazepam-insensitivity' of benzodiazepine binding sites (Sieghart *et al.*, 1987; Malminiemi & Korpi, 1989) and for high GABA sensitivity of [^{35}S]-TBPS binding sites (Korpi & Lüddens, 1993), both properties being dependent on the presence of the $GABA_A$ receptor $\alpha6$ subunit in $\alpha\beta\gamma$ receptors (Lüddens *et al.*, 1990; Korpi & Lüddens, 1993). Obviously due to the high GABA sensitivity, the binding of [^{35}S]-TBPS or related ionophore ligands to the granule cell $GABA_A$ receptors increases in the presence of receptor antagonists, such as SR 95531, bicuculline, RU 3156 and Ro 5-4864 (Korpi *et al.*, 1992a; Sapp *et al.*, 1992; Kume & Albin, 1994; Sakurai *et al.*, 1994). However, the action of furosemide on $GABA_A$ receptors seems to be different from that of the other antagonists, because it elevates the binding above basal levels also in the absence, as well as presence, of exogenous GABA in native cerebellar and in recombinant $\alpha6\beta2/3\gamma2$ receptors (Korpi *et al.*, 1995a; Lüddens & Korpi, 1995). The furosemide action in the absence of exogenous GABA, reflecting an increased [^{35}S]-TBPS binding affinity (Korpi *et al.*, 1995a), was readily reversed by SR 95531 and clozapine (Figures 3 and 6), suggesting that it is produced by a rather weak allosteric modification of the $\alpha6$ subunit-containing receptor. Furthermore, SR 95531 and especially clozapine, which in itself is not able to antagonize $\alpha6\beta2/3\gamma2$ receptors (Korpi *et al.*, 1995b), could not block the furosemide antagonism of GABA action in native or recombinant receptors, suggesting a different mechanism of action for furosemide in the absence and presence of GABA.

Table 2 Action of furosemide on GABA-inhibited [^{35}S]-TBPS binding in $\alpha6\beta3$, $\alpha6\beta3\delta$ and $\alpha6\beta3\gamma2$ recombinant receptors

Conditions	$\alpha6\beta3$ *receptors*	$\alpha6\beta3\delta$ *receptors*	$\alpha6\beta3\gamma2$ *receptors*
Basal binding	1065 ± 56	387 ± 52	839 ± 94
GABA 10 μM	55 ± 2	65 ± 7	27 ± 26
Furosemide 300 μM	86 ± 4^a	127 ± 19	154 ± 10^b
GABA + furosemide	98 ± 7^b	133 ± 19^a	160 ± 25^a

Results are means ± s.e.mean for three independent transfections. Basal binding is in fmol mg^{-1} protein, other binding values are as percentages of the basal values. Statistical significance of the difference between basal and furosemide and between GABA and GABA + furosemide binding values (Student's t test): $^aP<0.05$, $^bP<0.01$.

Our present experiments indicate that the GABA antagonism by furosemide only requires the $\alpha 6$ and $\beta 3$ subunits. The latter variant most likely can be replaced by the $\beta 2$, but not by the $\beta 1$ subunit. The interaction of furosemide took place in $\alpha 6\beta 3$ double and in $\alpha 6\beta 3\gamma 2$ and $\alpha 6\beta 3\delta$ triple combinations. However, the elevation of [^{35}S]-TBPS binding without GABA could not be seen in any combination other than $\alpha 6\beta 3\gamma 2$ receptors, again indicating that furosemide produces at least two different alterations in the $GABA_A$ receptor conformation. The enhancement of basal binding by furosemide was not clearly detectable in human cerebellar membranes. One way to interprete these data is that the δ subunit is prominently assembled with $\alpha 6$ and $\beta 2/3$ subunits, even if $\alpha 6\beta 2/3\gamma 2$ receptors must be present as they are responsible for the 'diazepam-insensitive' benzodiazepine binding of the human cerebellum (Turner *et al*., 1991; Korpi *et al*., 1992b).

The picrotoxin-sensitive [^{35}S]-TBPS binding assay used in the present study did not reveal any GABA-antagonism by furosemide in the hippocampus, although electrophysiological studies have clearly demonstrated the presence of a component sensitive to high micromolar concentrations of furosemide in that region (Pearce, 1993). Since furosemide at high concentrations (0.3 – 1 mM) slightly decreases [^{35}S]-TBPS binding in many brain regions (Korpi *et al*., 1995a; Table 1), it is possible that it antagonizes the receptor channel through the picrotoxin site. In this way furosemide could reduce $GABA_A$ responses in non-$\alpha 6$ and non-$\alpha 4$ containing receptors, such as in $\alpha 1\beta 2\gamma 2$ receptors (IC_{50} about 3000 μM; Korpi *et al*., 1995a). Further experiments are needed to establish the action on the picrotoxin-site as an additional mechanism for rapid $GABA_A$ antagonism by high furosemide concentrations. Our autoradiographic data showing no furosemide-induced $GABA_A$ receptor antagonism in the hippocampus and thalamus also indicate that the proportion of $\alpha 4$ subunit-containing receptors is very low, even if its mRNA is abundant in these regions (Wisden *et al*., 1992).

The convulsant benzodiazepine derivative Ro 5-4864, which does not bind to the classical benzodiazepine site of the $GABA_A$ receptors (Weissman *et al*., 1985; Basile *et al*., 1989), had a biphasic interaction on [^{35}S]-TBPS binding in cerebellar and cerebrocortical membranes, being directionally opposite in the presence and absence of exogenous GABA (Gee, 1987; Figure 3). Only high micromolar Ro 5-4864 concentrations were effective in attenuating or blocking the effects of furosemide in cerebellar, but not cerebrocortical membranes. Although furosemide inhibited the high-affinity [^{3}H]-Ro 5-4864 binding in both brain regions, the [^{3}H]-Ro 5-4864 binding measured under these conditions detects mitochondrial peripheral-type benzodiazepine receptors known to be poorly sensitive to furosemide ($IC_{50} = 85$ to > 1000 μM; Lukeman & Fanestil, 1987; Basile *et al*., 1988). Although our experiments cannot fully exclude the involvement of the low-affinity Ro 5-4864 binding sites on the $GABA_A$ receptor in the subtype-selective antagonism by furosemide, it is likely that furosemide acts via (an)other site(s), since the subunit requirements for Ro 5-4864 efficacy are much broader than those for furosemide, and independent of the $\alpha 6$ subunit (Puia *et al*., 1989).

In conclusion, the present data support the idea of selective antagonism of a cerebellar granule cell $GABA_A$ receptor population by furosemide via a novel kind of direct interaction with the receptor subunits. This specific interaction can be used to define the physiological functions of $\alpha 6$ subunit-containing $GABA_A$ receptors (see Zhu *et al*., 1995; Tia *et al*., 1996), since nonselective actions of furosemide on neuronal excitation (Hochman *et al*., 1995) and $GABA_A$ receptor function (Thompson *et al*., 1988; Zhang *et al*., 1991) need either high concentrations, longer periods of action or are nonspecifically shared by other $Na^+/2\ Cl^-/K^+$ co-transporter blockers.

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References

BASILE, A.S., BOLGER, G.T., LUEDDENS, H.W.M. & SKOLNICK, P. (1989). Electrophysiological actions of Ro 5-4864 on cerebellar Purkinje neurons: evidence for 'peripheral' benzodiazepine receptor-mediated depression. *J. Pharmacol. Exp. Ther*., **248,** 463 – 469.

BASILE, A.S., LUEDDENS, H.W.M. & SKOLNICK, P. (1988). Regulation of renal peripheral benzodiazepine receptors by anion transport inhibitors. *Life Sci*., **42,** 715 – 726.

CHEN, C. & OKAYAMA, H. (1987). High-efficiency transformation of mammalian cells by plasmid DNA. *Mol. Cell. Biol*., **7,** 2745 – 2752.

EDGAR, P.P. & SCHWARTZ, R.D. (1990). Localization and characterization of ^{35}S-t-butylbicyclophosphorothionate binding in rat brain: an autoradiographic study. *J. Neurosci*., **10,** 603 – 612.

GEE, K.W. (1987). Phenylquinolines PK 8165 and PK 9084 allosterically modulate [^{35}S]*t*-butylbicyclophosphorothionate binding to a chloride ionophore in rat brain via a novel Ro5 4864 binding site. *J. Pharmacol. Exp. Ther*., **240,** 747 – 753.

GRANGER, P., BITON, B., FAURE, C., VIGE, X., DEPOORTERE, H., GRAHAM, D., LANGER, S.Z., SCATTON, B. & AVENET, P. (1995). Modulation of the γ-aminobutyric acid type A receptor by the antiepileptic drugs carbamazepine and phenytoin. *Mol. Pharmacol*., **47,** 1189 – 1196.

GREGER, R. & WANGEMANN, P. (1987). Loop diuretics. *Renal Physiol*., **10,** 174 – 183.

HEAULME, M., CHAMBON, J.-P., LEYRIS, R., MOLIMARD, J.-C., WERMUTH, C.G. & BIZIERE, K. (1986). Biochemical characterization of the interaction of three pyridazinyl-GABA derivatives with the $GABA_A$ receptor site. *Brain Res*., **384,** 224 – 231.

HOCHMAN, D.W., BARABAN, S.C., OWENS, J.W.M. & SCHWARTZKROIN, P.A. (1995). Dissociation of syncronization and excitability in furosemide blockade of epileptiform activity. *Science*, **270,** 99 – 102.

KNOFLACH, F., BENKE, D., WAND, Y., SCHEURER, L., LÜDDENS, H., HAMILTON, B.J., CARTER, D.B., MOHLER, H. & BENSON, J.A. (1996). Pharmacological modulation of the diazepam-insensitive recombinant $GABA_A$ receptors $\alpha 4\beta 2\gamma 2$ and $\alpha 6\beta 2\gamma 2$. *Mol. Pharmacol*., (in press).

KORPI, E.R., KUNER, T., SEEBURG, P.H. & LÜDDENS, H. (1995a). Selective antagonist for the cerebellar granule cell-specific γ-aminobutyric acid type A receptor. *Mol. Pharmacol*., **47,** 283 – 289.

KORPI, E.R. & LÜDDENS, H. (1993). Regional γ-aminobutyric acid sensitivity of *t*-butylbicyclophosphoro[^{35}S]thionate binding depends on γ-aminobutyric $acid_A$ receptor α subunit. *Mol. Pharmacol*., **44,** 87 – 92.

KORPI, E.R., LÜDDENS, H. & SEEBURG, P.H. (1992a). $GABA_A$ antagonists reveal binding sites for [^{35}S]TBPS in cerebellar granular cell layer. *Eur. J. Pharmacol*., **211,** 427 – 428.

KORPI, E.R., UUSI-OUKARI, M., WEGELIUS, K., CASANOVA, M., ZITO, M. & KLEINMAN, J.E. (1992b). Cerebellar and frontal cortical benzodiazepine receptors in human alcoholics and chronically alcohol-drinking rats. *Biol. Psychiat*., **31,** 774 – 786.

KORPI, E.R., WONG, G. & LÜDDENS, H. (1995b). Subtype specificity of γ-aminobutyric acid type A receptor antagonism by clozapine. *Naunyn-Schmiedeberg's Arch. Pharmacol*., **352,** 365 – 373.

KUME, A. & ALBIN, R.L. (1994). Quantitative autoradiography of 4′-ethynyl-4-*n*-[2,3-$^3H_2$]propylbicycloorthobenzoate binding to the $GABA_A$ receptor complex. *Eur. J. Pharmacol*., **263,** 163 – 173.

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 – 1076.

LÜDDENS, H. & KORPI, E.R. (1995). GABA antagonists differentiate between recombinant $GABA_A$/benzodiazepine receptor subtypes. *J. Neurosci*., **15,** 6957 – 6962.

LÜDDENS, H., KORPI, E.R. & SEEBURG, P.H. (1995). $GABA_A$/benzodiazepine receptor heterogeneity: neurophysiological implications. *Neuropharmacology*, **34,** 245–254.

LÜDDENS, H., PRITCHETT, D.B., KOHLER, 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,** 648–651.

LUKEMAN, D.S. & FANESTIL, D.D. (1987). Interactions of diuretics with the peripheral-type benzodiazepine receptor in rat kidney. *J. Pharmacol. Exp. Ther.*, **241,** 950–955.

MALMINIEMI, O. & KORPI, E.R. (1989). Diazepam-insensitive [^{3}H]Ro 15-4513 binding in intact cultured cerebellar granule cells. *Eur. J. Pharmacol.*, **169,** 53–60.

OLSEN, R.W., MCCABE, R.T. & WAMSLEY, J.K. (1990). $GABA_A$ receptor subtypes: Autoradiographic comparison of GABA, benzodiazepine, and convulsant binding sites in the rat central nervous system. *J. Chem. Neuroanat.*, **3,** 59–76.

OLSEN, R.W. & TOBIN, A.J. (1990). Molecular biology of $GABA_A$ receptors. *FASEB J.*, **4,** 1469–1480.

PAXINOS, G. & WATSON, C. (1982). *The Rat Brain in Stereotaxic Coordinates*. New York: Academic Press.

PEARCE, R.A. (1993). Physiological evidence for two distinct $GABA_A$ responses in rat hippocampus. *Neuron*, **10,** 189–200.

PERSOHN, E., MALHERBE, P. & RICHARDS, J.G. (1992). Comparative molecular neuroanatomy of cloned $GABA_A$ receptor subunits in the rat CNS. *J. Comp. Neurol.*, **326,** 193–216.

PRITCHETT, D.B. & SEEBURG, P.H. (1990). γ-Aminobutyric $acid_A$ receptor α5-subunit creates novel type II benzodiazepine receptor pharmacology. *J. Neurochem.*, **54,** 1802–1804.

PRITCHETT, D.B., SONTHEIMER, H., SHIVERS, B.D., YMER, S., KETTENMANN, H., SCHOFIELD, P.R. & SEEBURG, P.H. (1989). Importance of a novel $GABA_A$ receptor subunit for benzodiazepine pharmacology. *Nature*, **338,** 582–585.

PUIA, G., SANTI, M.R., VICINI, S., PRITCHETT, D.B., SEEBURG, P.H. & COSTA, E. (1989). Differences in the negative allosteric modulation of γ-aminobutyric acid receptors elicited by 4′-chlorodiazepam and by a β-carboline-3-carboxylate ester: a study with natural and reconstituted receptors. *Proc. Natl. Acad. Sci. U.S.A.*, **86,** 7275–7279.

SAKURAI, S.Y., KUME, A., BURDETTE, D.E. & ALBIN, R.L. (1994). Quantitative autoradiography of [^{3}H]*t*-butylbicycloorthobenzoate binding to the γ-aminobutyric acid $receptor_A$ complex. *J. Pharmacol. Exp. Ther.*, **270,** 362–370.

SAPP, D.W., WITTE, U., TURNER, D.M., LONGONI, B., KOKKA, N. & OLSEN, R.W. (1992). Regional variation in steroid anaesthetic modulation of [^{35}S]TBPS binding to γ-aminobutyric $acid_A$ receptors in rat brain. *J. Pharmacol. Exp. Ther.*, **262,** 801–808.

SHIVERS, B.D., KILLISCH, I., SPRENGEL, R., SONTHEIMER, H., KÖHLER, M., SCHOFIELD, P.R. & SEEBURG, P.H. (1989). Two novel $GABA_A$ receptor subunits exist in distinct neuronal subpopulations. *Neuron*, **3,** 327–337.

SIEGHART, W. (1995). Structure and pharmacology of γ-aminobutyric $acid_A$ receptor subtypes. *Pharmacol. Rev.*, **47,** 181–234.

SIEGHART, W., EICHINGER, A., RICHARDS, J.G. & MÖHLER, H. (1987). Photoaffinity labelling of benzodiazepine receptor proteins with the partial inverse agonist [^{3}H]Ro 15-4513: A biochemical and autoradiographic study. *J. Neurochem.*, **48,** 46–52.

STEPHENSON, F.A. (1995). The $GABA_A$ receptors. *Biochem. J.*, **310,** 1–9.

THOMPSON, S.M., DEISZ, R.A. & PRINCE, D.A. (1988). Relative contributions of passive equilibrium and active transport to the distribution of chloride in mammalian cortical neurons. *J. Neurophysiol.*, **60,** 105–124.

TIA, S., WANG, J.F., KOTCHABHAKDI, N. & VICINI, S. (1996). Developmental changes of inhibitory synaptic currents in cerebellar granule neurons: role of $GABA_A$ receptor α6 subunit. *J. Neurosci.*, **16,** 3630–3640.

TURNER, D.M., SAPP, D.W. & OLSEN, R.W. (1991). The benzodiazepine/alcohol antagonist Ro 15-4513: binding to a $GABA_A$ receptor subtype that is insensitive to diazepam. *J. Pharmacol. Exp. Ther.*, **257,** 1236–1242.

WEISSMAN, B.A., COTT, J., HOMMER, D., PAUL, S.M. & SKOLNICK, P. (1984). Electrophysiological and pharmacological actions of the convulsant benzodiazepine Ro 5-4864. *Eur. J. Pharmacol.*, **97,** 257–263.

WEISSMAN, B.A., COTT, J., JACKSON, J.A., BOLGER, G.T., WEBER, K.H., HORST, W.D., PAUL, S.M. & SKOLNICK, P. (1985). 'Peripheral-type' binding sites for benzodiazepines in brain: relationship to the convulsant actions of Ro 5-4864. *J. Neurochem.*, **44,** 1494–1499.

WISDEN, W., LAURIE, D.J., MONYER, H. & SEEBURG, P.H. (1992). The distribution of 13 $GABA_A$ receptor subunit mRNAs in the rat brain. I. Telencephalon, diencephalon, mesencephalon. *J. Neurosci.*, **12,** 1040–1062.

WISDEN, W. & SEEBURG, P.H. (1992). $GABA_A$ receptor channels: from subunits to functional entities. *Curr. Opin. Neurobiol.*, **2,** 263–269.

YMER, S., SCHOFIELD, P.R., DRAGUHN, A., WERNER, P. KÖHLER, M. & SEEBURG, P.H. (1989). $GABA_A$ receptor β subunit heterogeneity: functional expression of cloned cDNAs. *EMBO J.*, **8,** 1665–1670.

ZHANG, L., SPIGELMAN, I. & CARLEN, P.L. (1991). Development of GABA-mediated, chloride-dependent inhibition in CA1 pyramidal neurones of immature rat hippocampal slices. *J. Physiol.*, **444,** 25–49.

ZHU, W.J., VICINI, S., HARRIS, B.T. & GRAYSON, D.R. (1995). NMDA-mediated modulation of γ-aminobutyric acid type A receptor function in cerebellar granule neurons. *J. Neurosci.*, **15,** 7692–7701.

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