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Affinity of various benzodiazepine site ligands in mice with a point mutation in the $GABA_A$ receptor γ2 subunit

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Abstract

The benzodiazepine binding site of $GABA_A$ receptors is located at the interface of the α and γ subunits. Certain point mutations in these subunits have been demonstrated to dramatically reduce the affinity of benzodiazepine binding site ligands for these receptors. Recently, mice were generated with a phenylalanine (F) to isoleucine (I) substitution at position 77 in the γ2 subunit of $GABA_A$ receptors. Here we tested the potency of 24 benzodiazepine binding site ligands from 16 different structural classes for inhibition of [^{3}H]flunitrazepam binding to brain membranes of these γ2F77I mice. Results indicate that the potency of the classical 1,4-benzodiazepines, of the 1,4-thienodiazepine clotiazepam, the 1,5-benzodiazepine clobazam, or the pyrazoloquinoline CGS 9896 is only 2–7-fold reduced by this γ2F77I point mutation. The potency of the imidazopyrimidines Ru 32698, Ru 33203, and Ru 33356, of the imidazoquinoline Ru 31719, or the pyrazolopyridine CGS 20625 is reduced 10–20-fold, whereas the potency of some imidazobenzodiazepines, β-carbolines, cyclopyrrolones, imidazopyridines, triazolopyridazines, or quinolines is 100–1000-fold reduced. Interestingly, the extent of potency reduction induced by the γ2F77I point mutation varied within the structural classes of compounds. Results support and significantly extend previous observations indicating that the residue γ2F77 is important for high affinity binding of some, but not all benzodiazepine site ligands.

Keywords: $GABA_A$ receptor; γ2 Subunit; Point mutation γ2F77I; Benzodiazepine binding site ligands; Benzodiazepine binding site property; [^{3}H]flunitrazepam

1. Introduction

$GABA_A$ receptors are ligand gated ion channels that can be modulated by a large variety of pharmacologically and clinically important drugs, such as benzodiazepines, barbiturates, neurosteroids, anesthetics and convulsants [1]. A variety of evidence indicates that these compounds exert their action via distinct allosteric binding sites on these receptors. $GABA_A$ receptors are composed of five subunits forming the chloride ion channel that can be opened by GABA [2,3]. So far, at least 19 different subunits belonging to different subunit classes (α, β, γ, δ, ϵ, π, θ, ρ) have been identified in the mammalian brain [4,5]. The majority of $GABA_A$ receptors are composed of 2α, 2β, and 1γ subunit. Using the crystal structure of the acetylcholine binding protein [6] as a template for comparative modeling, homology models of the extracellular domain of $GABA_A$ receptors were recently constructed that for the first time provided insights in the topography of the GABA and benzodiazepine binding site of these receptors [7,8]. Whereas the

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two GABA binding sites of these receptors are located at the β+α-interface, the benzodiazepine binding site is located at the α+γ-interface of $GABA_A$ receptors. Computational docking in models of the benzodiazepine site presently is hampered by model uncertainty leading to unclear side chain positions. In some cases, experimental evidence can possibly discriminate between different ligand positions. For instance, possible dockings of Ro 15-4513, an imidazobenzodiazepine used as photoaffinity label, were discussed recently [9,10].

Various point mutations have been generated in recombinant receptors to identify amino acid residues important for interactions with benzodiazepine binding site ligands [3,11]. The first residue identified to be of importance for the binding of benzodiazepines was the histidine in position 101 of the α1 subunit (α1H101). Mutation of this residue to arginine (α1H101R) dramatically reduced the affinity of classical benzodiazepines, such as diazepam or flunitrazepam, but did not impair the affinity of the imidazobenzodiazepines Ro 15-1788 or Ro 15-4513 [12,13]. In contrast, the affinity of these imidazobenzodiazepines as well as that of flunitrazepam was strongly impaired by substituting tyrosine at the position 209 of the α1 subunit by glutamine (α1Y209Q) [13]. In addition, using [^{3}H]flunitrazepam or [^{3}H]Ro 15-4513 as a photolabel, residues α1H101 [14,15] or α1Y209 [9] were irreversibly labeled,

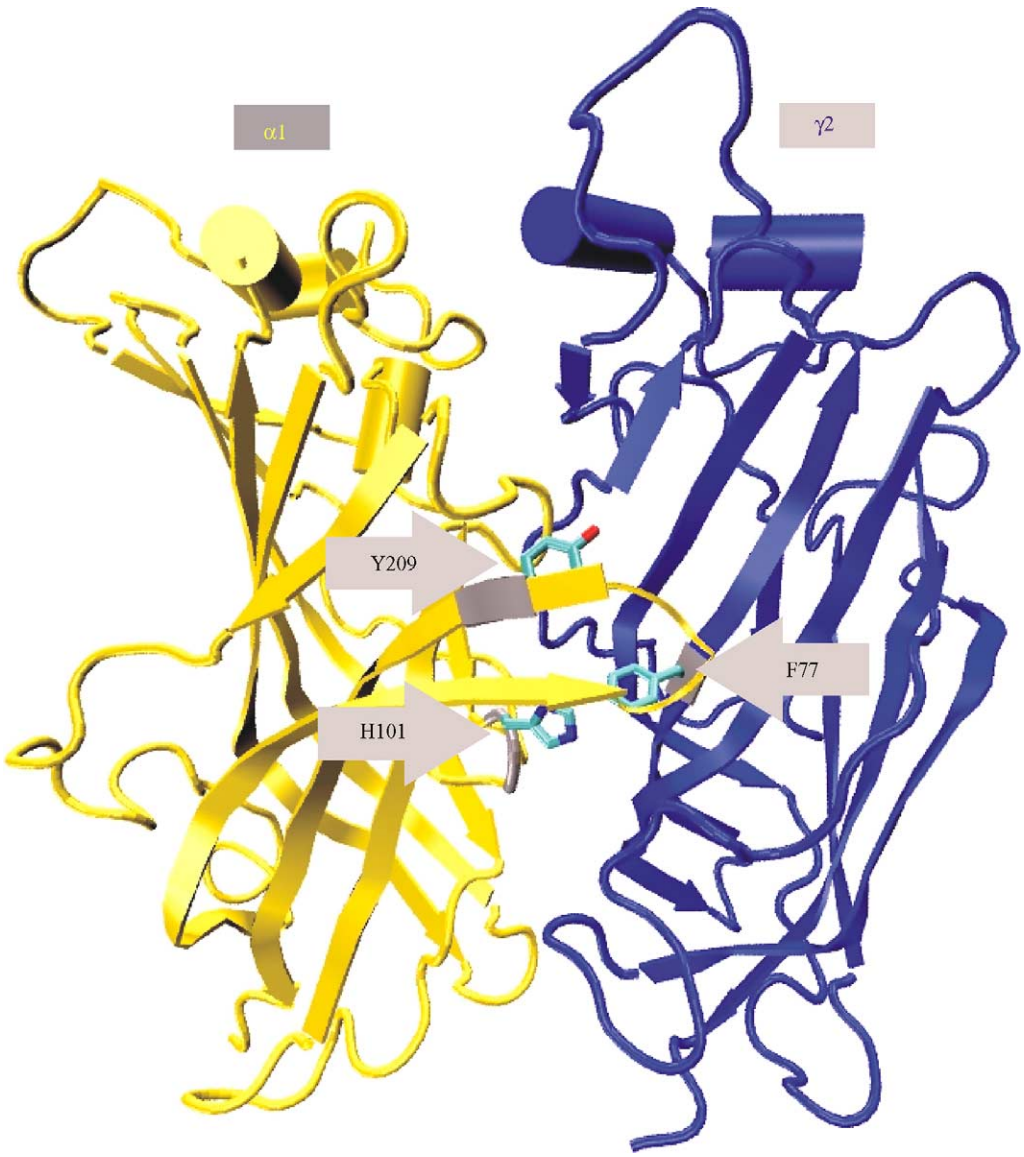
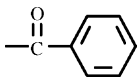
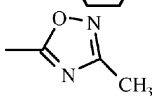
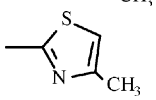
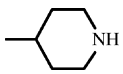
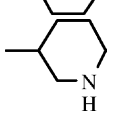


Fig. 1. Homology model of the extracellular domains of the α1 and γ2 subunit, indicating the interface where the benzodiazepine pocket is located. The view is from approximately perpendicular to the pore-mouth with the bottom end of the figure corresponding to the C-terminal end of the extracellular domain. The subunits are shown in ribbon representation, the amino acid side chains of residues α1H101, α1Y209, and γ2F77 are shown in stick representation. The segments of the ribbon representation that correspond to residue α1H101 from the so-called loop A, α1Y209 from the loop C, and γ2F77 from the loop D are coloured gray, the loop C is closest to the viewer, partially obscuring the view of the pocket and of residue α1Y209 that lie behind it. No firm conclusion can be made on the exact position of the amino acid residue side chains. The image was rendered with VMD [27].

Table 1
Structure–affinity relationship of benzodiazepine binding site ligands for $GABA_A$ receptors in C57BL/6J and γ2F77I brain membranes

No.	Compound	Structure	R1	R2	R3	R4	K_i (nM)		
							C57BL/6J	γ2F77I	Ratio
1,4-Benzodiazepines									
1	Flunitrazepam	I	$-CH_3$	$-NO_2$	–F	–	7.2 ± 0.8	18 ± 0.4	2.5
2	Diazepam	I	$-CH_3$	–Cl	–H	–	11 ± 1.3	42 ± 4.4	3.8
3	Flurazepam	I	$-CH_2CH_2N(C_2H_5)_2$	–Cl	–F	–	33 ± 3.3	92 ± 8.3	2.8
4	Midazolam	II	–	–	–	–	3.1 ± 0.2	22 ± 1.8	7.1
1,4-Thienodiazepines									
5	Clotiazepam	III	–	–	–	–	3.2 ± 0.5	4.8 ± 0.8	1.5
1,5-Benzodiazepines									
6	Clobazam	IV	–	–	–	–	374 ± 35	888 ± 124	2.4
Imidazobenzodiazepines									
7	Ro 15-1788	V	–	–	–	–	1.4 ± 0.1	1243 ± 114	888
8	Bretazenil	VI	–H	–Br	–COO-*t*-butyl	–	0.5 ± 0.04	513 ± 58	1026
9	L-655,708	VI	$-OCH_3$	–H	$-COOC_2H_5$	–	63 ± 8.3	>100000	>1000
β-Carbolines									
10	DMCM	VII	$-COOCH_3$	$-C_2H_5$	$-OCH_3$	$-OCH_3$	5.1 ± 0.6	8720 ± 2000	1710
11	β-CCM	VII	$-COOCH_3$	–H	–H	–H	2.3 ± 0.4	689 ± 87	300
12	β-CCP	VII	$-COOC_3H_7$	–H	–H	–H	3.5 ± 0.6	502 ± 80	148
Pyrazoloquinolines									
13	CGS 9896	VIII	–	–	–	–	0.5 ± 0.1	6.9 ± 1.1	6.3
Pyrazolopyridines									
14	CGS 20625	IX	–	–	–	–	0.5 ± 0.1	10 ± 1.4	20
Cyclopyrrolones									
15	Zopiclone	X	–	–	–	–	51 ± 4	15970 ± 2111	313
Imidazopyridines									
16	Zolpidem	XI	–	–	–	–	91 ± 13	30680 ± 5500	337
Triazolopyridazines									
17	Cl 218872	XII	–	–	–	–	358 ± 51	36040 ± 5760	100
Imidazoquinolines									
18	Ru 31719	XIII	–	–	–	–	21 ± 4	210 ± 33	10
Imidazopyrimidines									
19	Ru 32698	XIV		–	–	–	114 ± 18	1578 ± 248	14
20	Ru 33203	XIV		–	–	–	230 ± 60	2500 ± 530	11
21	Ru 33356	XIV		–	–	–	88 ± 11	489 ± 70	5.5
Quinolines									
22	PK 8165	XV		–	–	–	122 ± 16	>100000	>1000
23	PK 9084	XV		–	–	–	574 ± 71	>100000	>1000
Isoquinolines									
24	PK 11195	XVI	–	–	–	–	17000 ± 2050	205400 ± 8620	12

Membranes from C57BL/6J and γ2F77I brains without cerebellum were incubated with 5 nM [^{3}H]flunitrazepam in the absence or presence of 10 μM diazepam and various concentrations of the ligands investigated. The concentrations resulting in half maximal inhibition of specific [^{3}H]flunitrazepam binding (IC50) were converted to K_i values by using the Cheng–Prusoff relationship [19] and the respective K_d values for [^{3}H]flunitrazepam (3.8 ± 0.4 nM and 7.9 ± 0.2 nM for C57BL/6J and γ2F77I, respectively). Data are mean ± S.D. of two to three separate experiments performed in triplicates. 'Structure' refers to compound structures I–XVI shown in Figs. 2 and 3.

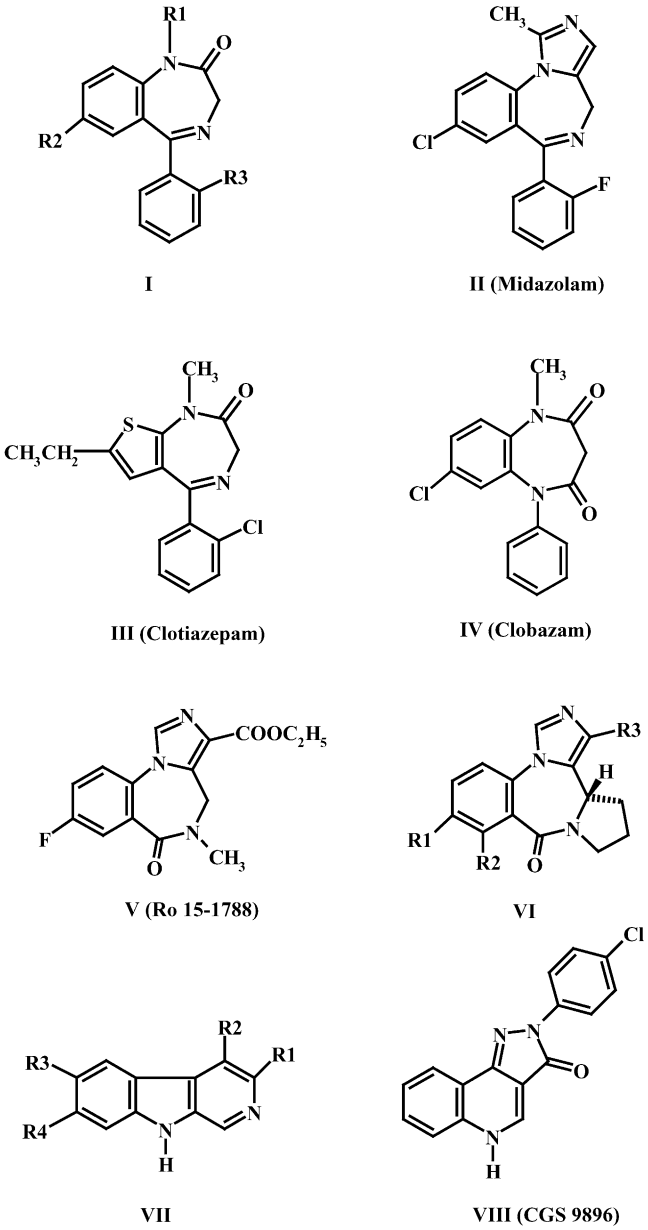


Fig. 2. Chemical structures of compounds used in this study. The roman numerals identify the structures mentioned in Table 1.

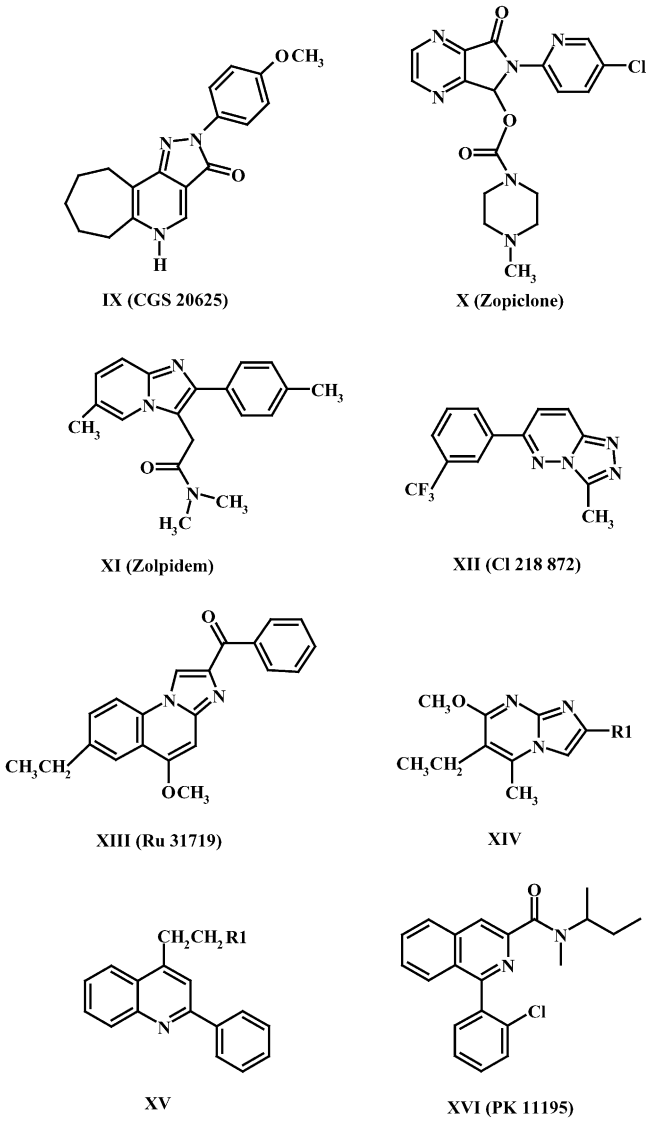


Fig. 3. Chemical structures of compounds used in this study. The roman numerals identify the structures mentioned in Table 1.

respectively, supporting the conclusion that these residues are located in the benzodiazepine binding site of $GABA_A$ receptors and can be contacted by benzodiazepine site ligands with different structure.

In line with the conclusion that the benzodiazepine binding site is formed at the interface of α and γ subunits, several mutations in the γ2 subunit of $GABA_A$ receptors also led to changes in the affinity of various benzodiazepine site ligands [11]. Thus, for instance, the γ2F77I mutation dramatically reduced the affinity of zolpidem, Ro 15-1788, DMCM and Cl 218872 for recombinant α1βγ2F77I receptors, but only weakly reduced the affinity of classical benzodiazepines, such as diazepam or flunitrazepam [16,17]. Interestingly, homology models of $GABA_A$ receptors indicate that residues α1H101 from the so-called loop A, α1Y209 from the loop C, and residue γ2F77 from loop D are situated approximately equidistantly spaced around a solvent-accessible pocket and do indeed line this pocket (Fig. 1, [8]).

Performing binding studies with recombinant receptors, especially when using transiently transfected HEK cells, is rather cumbersome due to the large amounts of cell culture plates needed for dose-response curves. To further investigate the benzodiazepine binding pocket and its interaction with compounds from different structural classes, we here took advantage of the recently generated mice that carry the γ2F77I point mutation [18]. Using brain membranes from γ2F77I or C57BL/6J mice we investigated the potency of 24 benzodiazepine binding site ligands from 16 different structural classes for inhibition of [^{3}H]flunitraze-

pam binding. Results obtained identify compounds from two additional structural classes that are strongly influenced by this mutation.

2. Experimental procedures

2.1. Chemicals

[^{3}H]flunitrazepam (84.5 Ci/mmol) was purchased from Perkin-Elmer Life Sciences. Compounds (see Table 1 and Figs. 2 and 3 for structures) were obtained from the following sources: flunitrazepam (7-nitro-1,3-dihydro-1-methyl-5-o-fluorophenyl-2H-1,4-benzodiazepin-2-one), diazepam (7-chloro-1,3-dihydro-1-methyl-5-phenyl-2H-1,4-benzodiazepin-2-one), flurazepam (7-chloro-1,3-dihydro-1-ethylaminodiethyl-5-o-fluorophenyl-2H-1,4-benzodiazepin-2-one), midazolam (8-chloro-6-(2-fluorophenyl)-1-methyl-4H-imidazo[1,5-a][1,4]benzodiazepine), Ro 15-1788 (ethyl-8-fluoro-5,6-dihydro-5-methyl-6-oxo-4H-imidazo[1,5-a][1,4]benzodiazepine-3-carboxylate), bretazenil (*t*-butyl(s)-8-bromo-11,12,13,13a-tetrahydro-9-oxo-9H-imidazo[1,5-a]pyrrolo[2,1-c][1,4]benzodiazepine-1-carboxylate) (Hoffmann La Roche, Basle, Switzerland); L-655,708 (ethyl-7-methoxy-11,12,13,13a-tetrahydro-9-oxo-9H-imidazo[1,5-a]pyrrolo[2,1-c] [1,4]benzodiazepine-1-carboxylate) was purchased from Tocris Cookson Ltd. UK; clotiazepam (5-(2-chlorophenyl)-7-ethyl-1,3-dihydro-1-methyl-2H-thieno[2,3-e][1,4]diazepin-2-one) (Troponwerke, Köln, Germany); clobazam (7-chloro-1-methyl-5-phenyl-1H-1,5-benzodiazepine-2,4(3H,5H)-dione) (Hoechst, Frankfurt, Germany); methyl-6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate (DMCM), methyl-(β-CCM), propyl-(β-CCP) ester of β-carboline-3-carboxylate (Ferrosan, Soeborg, Denmark); CGS 9896 (2-(4-chlorophenyl)-pyrazolo[4,3-c]quinolin-3-one), CGS 20625 (2-(4-methoxyphenyl)-2,3,5,6,7,8,9,10-octahydrocyclohepta-(b)pyrazolo[4,3-d]pyridin-3-one) (Ciba Geigy, Summit, NJ, USA); zopiclone (4-methyl-1-piperazinecarboxylic acid-6-(5-chloro-2-pyridinyl)-6,7-dihydro-7-oxo-5H-pyrrolo[3,4-b]pyrazin-5-yl ester) (Rhone-Poulenc, Paris, France); zolpidem (*N*,*N*,6-trimethyl-2-(4-methylphenyl)imidazo[1,2-a]-pyridine-3-acetamide) (Synthelabo Recherche, Bagneux, France); Cl 218,872 (3-methyl-6-[3-trifluoromethyl-phenyl]-1,2,4-triazolo[4,3-b]pyridazine) (American Cyanamide Comp., Wayne, NJ, USA); Ru 31719 ((7-ethyl-5-methoxyimidazo[1,2-a]quinolin-2-yl) phenyl methanone), Ru 32698 (6-ethyl-7-methoxy-5-methylimidazo[1,2-a]pyrimidin-2-yl)phenylmethanone, Ru 33203 (5-(6-ethyl-7-methoxy-5-methylimidazo[1,2-a] pyrimidin-2-yl)-3-methyl-[1,2,4]-oxadiazole), Ru 33356 (2-(6-ethyl-7-methoxy-5-methylimidazo[1,2-a]pyrimidin-2-yl)-4-methyl-thiazole) (Roussel Uclaf, Romainville, France); PK 8165 (2-phenyl-4-(4-ethyl-piperidinyl)-quinoline), PK 9084 (2-phenyl-4-(3-ethyl-piperidinyl)-quinoline), PK 11195 (1-(2-chlorophenyl)-*N*-methyl-(1-methyl-propyl)-3-isoquinoline carboxamide) (Pharmuka Laboratories, Gennevilliers, France).

2.2. Mice

Mice containing the point mutation F77I in the $GABA_A$ receptor γ2 subunit gene were generated as described previously [18]. Heterozygous γ2F77/I77 breeding pairs were crossed to give homozygous point mutants and wild-type littermate controls (background: C57BL/6J × 129ola). Since preliminary experiments did not show any differences between EC_{50} values obtained from littermate controls and C57BL/6J mice, in most experiments the potency of compounds for inhibition of [^{3}H]flunitrazepam binding was compared between γ2F77I and C57BL/6J mice.

2.3. Membranes

Brains (with the cerebellum removed) from male and female adult γ2F77I, wild-type littermate controls (γ2F77F) or C57BL/6J mice were homogenized individually in 50 mM Tris/citrate buffer (pH 7.1), containing one complete protease inhibitor cocktail tablet per 50 ml buffer (Roche Diagnostics, Mannheim, Germany). The suspensions were pressed through a set of needles with increasingly small diameters using a syringe. The homogenates were ultracentrifuged at 150,000 × *g*, the pellets washed three times and finally resuspended in 12 ml of a 50 mM Tris/citrate buffer (pH 7.1).

2.4. Receptor binding studies

For receptor binding studies, 300 μl of the suspension were added to a final volume of 1 ml of a solution containing: 50 mM Tris/citrate buffer (pH 7.1); 150 mM NaCl; and various concentrations of [^{3}H]flunitrazepam (84.5 Ci/mmol, Perkin-Elmer Life Sciences) in the absence or presence of 10 μM diazepam. For inhibition studies, membranes were incubated with 5 nM [^{3}H]flunitrazepam in the absence or presence of various concentrations of ligands for the benzodiazepine binding site of $GABA_A$ receptors. After incubation for 90 min at 4 °C, the suspensions were rapidly filtered through Whatman GF/B filters. The filters were washed twice with 5 ml of 50 mM Tris/citrate buffer (pH 7.1) and were transferred to scintillation vials and subjected to liquid scintillation counting (Filter-Count™ Packard; 2100 TR Tri-Carb® Scintillation Analyzer, Packard). Non-specific binding determined in the presence of 10 μM diazepam was subtracted from total [^{3}H]flunitrazepam binding to obtain specific binding. Data were analysed using GraphPad Prism.

3. Results

Brain membranes from γ2F77I mice and littermate controls were incubated with various concentrations of

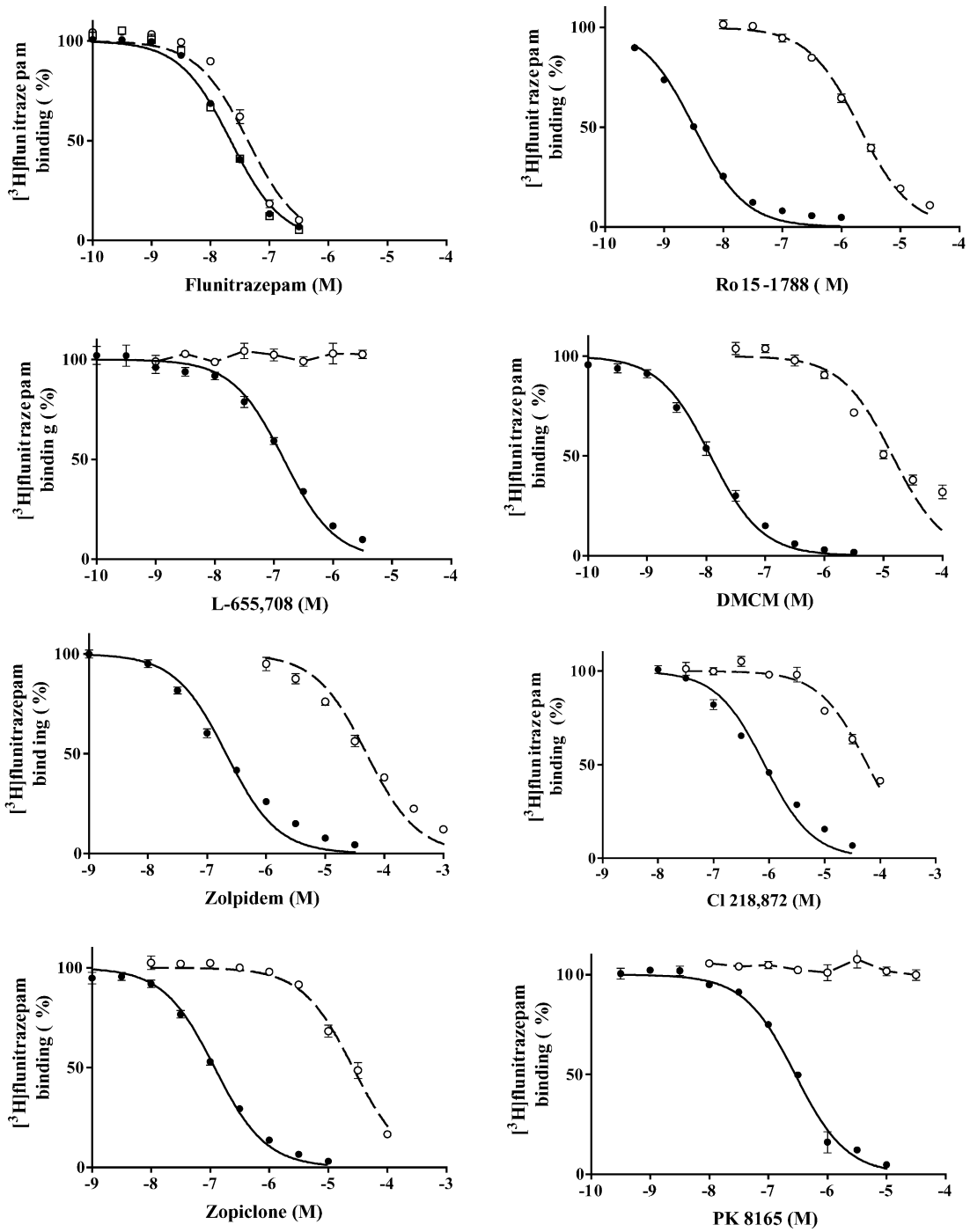


Fig. 4. Potency of various compounds for inhibition of [^{3}H]flunitrazepam binding in brain membranes of C57BL/6J and γ2F77I mice. Brain membranes from C57BL/6J and γ2F77I mice were incubated with 5 nM [^{3}H]flunitrazepam in the absence or presence of 10 μM diazepam or various concentrations of drugs as indicated. Membranes were filtered through Whatman GF/B filters and specifically bound radioactivity was measured. Closed symbols represent data from C57BL/6J and open symbols represent data from γ2F77I brain membranes. Inhibition of [^{3}H]flunitrazepam binding by flunitrazepam was investigated in brain membranes from C57BL/6J, γ2F77I, as well as littermate controls. Data from C57BL/6J, and littermate controls were identical. Data are presented as mean ± S.D. of two to three experiments performed in triplicates and were fitted by GraphPad Prism assuming the presence of a single binding site. No attempt was made to correct for the deviation of this assumption for compounds exhibiting some receptor subtype-selectivity.

[^{3}H]flunitrazepam in the absence or presence of 10 μM diazepam and binding data obtained were subjected to Scatchard analysis. K_d values for [^{3}H]flunitrazepam binding were 7.9 ± 0.2 and 3.8 ± 0.4 for γ2F77I mice and littermate controls, respectively, indicating a 2-fold shift in the affinity of flunitrazepam in γ2F77I mice. Interestingly, the B_{max} values were 4373 ± 290 and 3330 ± 198 (mean ± S.D., n = 3) for γ2F77I mice and littermate controls, respectively, indicating a significant increase (1.3-fold) in the receptor expression in γ2F77I mice. These results are

in contrast to previous results obtained in cerebellum where possible changes in the expression of [^{3}H]flunitrazepam binding sites did not reach significance [18].

In other experiments the potency of a total of 24 benzodiazepine binding site ligands (see Figs. 2 and 3, and Table 1 for structures) for the inhibition of [^{3}H]flunitrazepam binding to brain membranes from γ2F77I and C57BL/6J mice was compared. IC_{50} values were determined and transformed into K_i values using the Cheng–Prusoff relationship [19]. As expected from data of recombinant receptor studies [16,17], the potency for inhibition of [^{3}H]flunitrazepam binding of classical 1,4-benzodiazepines, such as diazepam, flurazepam, or midazolam was reduced 3–7-fold in membranes from γ2F77I mice (Table 1). Investigation of other compounds indicated that the potency of the 1,4-thienodiazepine clotiazepam, the 1,5-benzodiazepine clobazam, or the pyrazoloquinoline CGS 9896 was also only weakly reduced (Table 1). The potency of the imidazopyrimidines Ru 32698, Ru 33203, the imidazoquinoline Ru 31719, the pyrazolopyridine CGS 20625, or the isoquinoline PK 11195 was reduced 10–20-fold in γ2F77I mice (Table 1).

As expected from recombinant receptor studies [16,17], the potency for inhibition of [^{3}H]flunitrazepam binding of the triazolopyridazine Cl 218872 and the imidazopyridine zolpidem was reduced much stronger (100–300-fold), and that of the β-carbolines DMCM, β-CCM, and β-CCP, or the imidazobenzodiazepines Ro 15-1788, Bretazenil, or L-655,708, was reduced up to more than 1000-fold in receptors from γ2F77I mice (Table 1, Fig. 4).

Interestingly, the potency for inhibition of [^{3}H]flunitrazepam binding of the quinolines PK 8165 and PK 9084 and of the cyclopyrrolone zopiclone also was dramatically reduced in membranes from γ2F77I mice (Table 1, Fig. 4).

4. Discussion

In the present study the potency for inhibition of [^{3}H]flunitrazepam binding of 24 benzodiazepine binding site ligands from 16 different structural classes was investigated in mice carrying the point mutation γ2F77I. These mice have been demonstrated previously to behave normally in a range of standard behavioural tests and the overall architecture of the brains also appeared normal [18] as expected from a point mutation that only weakly reduced GABA affinity of the receptor [20]. In contrast to the previous observation that the B_{max} value for [^{3}H]flunitrazepam binding was not significantly different in cerebella of γ2F77I and littermate control mice, in the present study a significant 1.3-fold increase in the B_{max} value was observed in brains without cerebellum of γ2F77I mice. This discrepancy might have been due to the fact that [^{3}H]flunitrazepam in cerebellum nearly exclusively binds to α1βγ2 receptors, whereas in the remaining parts of the brain [^{3}H]flunitrazepam in addition binds to other $GABA_A$ receptor subtypes, such as α2βγ2, α3βγ2, α5βγ2 [5]. Since the effect of the γ2F77I point mutation on GABA potency was investigated in recombinant α1β2γ2 receptors only [20], it is possible that GABA potency is reduced more dramatically in α2βγ2, α3βγ2, α5βγ2 receptor subtypes carrying this point mutation in the forebrain. A more dramatic reduction in the potency of GABA to activate the mutated receptors might have induced an increased compensatory upregulation of $GABA_A$ receptors. Alternatively, this mutation might have impaired an interaction with an endogenous ligand of the benzodiazepine binding site, that in turn could have led to a compensatory upregulation of the number of $GABA_A$ receptors. But it is also possible that the frt-loxP insertion into intron 4 of the γ2 gene [18] could have produced a slight increase in the splicing efficiency or stability and thus in the abundance of the γ2 primary transcript produced from the γ2I77 allele.

As expected from studies with recombinant receptors carrying the point mutation γ2F77I [16,17], the present investigation indicated that the potency for inhibition of [^{3}H]flunitrazepam binding was dramatically reduced for the imidazopyridine zolpidem, the imidazobenzodiazepine Ro 15-1788, the triazolopyridazine Cl 218872, or the β-carboline DMCM, whereas it was only weakly changed for the classical benzodiazepines diazepam, flunitrazepam and midazolam. Whereas the extent of potency reduction was comparable in recombinant receptors and in the brains of γ2F77I mice for flunitrazepam, diazepam, and midazolam, it was dramatically weaker in the brains of γ2F77I mice for Ro 15-1788 (888-fold versus 2020-fold), for zolpidem (337-fold versus >600-fold), for Cl 218872 (100-fold versus >200-fold) and for DMCM (1710-fold versus >5000-fold) [16,17]. Again this discrepancy might have been due to the fact that in recombinant receptor studies only a single type of mutated $GABA_A$ receptor was investigated, whereas in the brains of γ2F77I mice the compounds inhibit binding of [^{3}H]flunitrazepam to a large number of different mutated $GABA_A$ receptor subtypes. The same point mutation could have had different effects on the potency of compounds in different receptor subtypes. In addition, the pharmacological properties of native receptors might be different from those of recombinant receptors due to a different state of phosphorylation, a different extent of clustering or interaction with other proteins or membrane lipids found in neurons but not in HEK cells [21].

Whereas the potency for inhibition of [^{3}H]flunitrazepam binding was strongly reduced for all imidazobenzodiazepines investigated, the potency of the structurally similar midazolam was only weakly reduced in brain membranes from γ2F77I mice. This seems to indicate that the 5-phenyl moiety of midazolam, similar to that of diazepam and the classical benzodiazepines possibly causes a different orientation within the benzodiazepine binding pocket (see also [22–24] for discussion of the orientation of benzodiazepine

site ligands in the binding pocket). This conclusion is supported by the observation that [^{3}H]flunitrazepam irreversibly binds to residue α1H101 [14,15], whereas the imidazobenzodiazepine [^{3}H]Ro 15-4513 irreversibly binds to residue α1Y209 [9].

Similar to the classical benzodiazepines, the 1,4-thienodiazepine clotiazepam, or the 1,5-benzodiazepine clobazam only weakly change their potency for displacing [^{3}H]flunitrazepam in receptors containing the γ2F77I mutation, suggesting an orientation of these compounds in the benzodiazepine binding site similar to that of the classical benzodiazepines. Similarly, the potency of the pyrazoloquinoline CGS 9896, of the pyrazolopyridine CGS 20625, of the imidazoquinoline Ru 31719, of the imidazopyrimidines Ru 32698, Ru 33203, or Ru 33356, or of the isoquinoline PK 11195 was only weakly changed by the γ2F77I mutation. The potency of the cyclopyrrolone zopiclone and of the quinolines PK 8165 or PK 9084, however, was dramatically reduced by this mutation, supporting the conclusion that the amino acid residue γ2F77 is important for the binding of these compounds to the benzodiazepine binding site of $GABA_A$ receptors.

Interestingly, the extent of the potency reduction of compounds belonging to the same structural class was different. Thus, whereas the potency of DMCM was reduced about 1700-fold, that of β-CCM or β-CCP was reduced only 300- and 150-fold in the γ2F77I brains. A similar dependence of the potency reduction on the individual compound structure was observed for the imidazobenzodiazepines. Whereas the potency of the imidazobenzodiazepine antagonist Ro 15-1788 was changed about 900-fold, that of the partial agonist bretazenil or of the partial inverse agonist L-655,708 was shifted >1000-fold. These data seem to indicate that depending on the individual structure of the compounds the presence of the amino acid residue γ2F77 is more or less important for binding to the benzodiazepine binding pocket of $GABA_A$ receptors (Fig. 1).

Interestingly, L-655,708 has been demonstrated to exhibit a 50–100-fold selectivity for receptors containing the α5 subunit over those containing the α1, α2, or α3 subunit [25,26]. On re-introducing the wild-type γ2 subunit into selected brain regions or neuronal populations of γ2F77I mice [18], it should therefore be possible to selectively modulate α5 subunit-containing receptors in these cells and thus, study their function in the brain.

In summary, the present study confirmed and significantly extended previous findings on the consequences of the γ2F77I mutation for binding of various classes of benzodiazepine binding site ligands to $GABA_A$ receptors. Previously, only five classes of compounds were investigated in recombinant receptors containing this point mutation. Here we studied the consequences of the γ2F77I point mutation on the binding pharmacology of 16 different structural classes of compounds. The potency of most of these compounds is only weakly influenced by this mutation. In the course of this study, however, in addition to the four classes of compounds previously identified to be strongly influenced by this mutation, two other classes of compounds were identified that seem to strongly depend on the presence of the γ2F77 for their binding to the benzodiazepine binding pocket. These data should help to develop an integrated pharmacophore model of the benzodiazepine binding pocket of the $GABA_A$ receptors and represent a further step in the direction of a more rational drug design.

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