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Selective labelling of diazepam-insensitive $GABA_A$ receptors *in vivo* using [^{3}H]Ro 15-4513

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1 Classical benzodiazepines (BZs), such as diazepam, bind to $GABA_A$ receptors containing α1, α2, α3 or α5 subunits that are therefore described as diazepam-sensitive (DS) receptors. However, the corresponding binding site of $GABA_A$ receptors containing either an α4 or α6 subunit do not bind the classical BZs and are therefore diazepam-insensitive (DIS) receptors; a difference attributable to a single amino acid (histidine in α1, α2, α3 and α5 subunits and arginine in α4 and α6).

2 Unlike classical BZs, the imidazobenzodiazepines Ro 15-4513 and bretazenil bind to both DS and DIS populations of $GABA_A$ receptors. In the present study, an *in vivo* assay was developed using lorazepam to fully occupy DS receptors such that [^{3}H]Ro 15-4513 was then only able to bind to DIS receptors.

3 When dosed i.v., [^{3}H]Ro 15-4513 rapidly entered and was cleared from the brain, with approximately 70% of brain radioactivity being membrane-bound. Essentially all membrane binding to DS + DIS receptors could be displaced by unlabelled Ro 15-4513 or bretazenil, with respective ID_{50} values of 0.35 and 1.2 mg kg^{-1}.

4 A dose of 30 mg kg^{-1} lorazepam was used to block all DS receptors in a [^{3}H]Ro 15-1788 *in vivo* binding assay. When predosed in a [^{3}H]Ro 15-4513 binding assay, lorazepam blocked [^{3}H]Ro 15-4513 binding to DS receptors, with the remaining binding to DIS receptors accounting for 5 and 23% of the total (DS plus DIS) receptors in the forebrain and cerebellum, respectively.

5 The *in vivo* binding of [^{3}H]Ro 15-4513 to DIS receptors in the presence of lorazepam was confirmed using α1H101R knock-in mice, in which α1-containing $GABA_A$ receptors are rendered diazepam insensitive by mutation of the histidine that confers diazepam sensitivity to arginine. In these mice, and in the presence of lorazepam, there was an increase of *in vivo* [^{3}H]Ro 15-4513 binding in the forebrain and cerebellum from 4 and 15% to 36 and 59% of the total (i.e. DS plus DIS) [^{3}H]Ro 15-4513 binding observed in the absence of lorazepam.
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Abbreviations: BZ, benzodiazepine; DIS, diazepam-insensitive; DS, diazepam-sensitive

Introduction

The $GABA_A$ receptor is a ligand-gated anion-selective ion channel considered to be a pentamer comprising of a combination of 16 known receptor subunits (α1–6, β1–3, γ1–3, δ, ε, π, θ; Simon *et al*., 2004). It not only possesses a binding site for GABA, the major inhibitory neurotransmitter in the mammalian CNS, but also other recognition sites for a diverse range of modulatory compounds, such as benzodiazepines (BZs), neurosteroids, barbiturates and some anaesthetics (Sieghart, 1995). The clinical utility of BZs as anxiolytics and hypnotics has meant that the binding site for this class of compounds has received much attention.

The majority of $GABA_A$ receptors in the brain possess a BZ binding site and have a stoichiometry of two α, two β and one γ subunits arranged in an αβαβγ sequence as viewed from the synapse (Minier & Sigel, 2004). The BZ binding site occurs at the interface between the α and γ subunits, with both contributing to the binding pocket pharmacology. However, since the γ2 subunit is the predominant γ subunit in the brain, most of the variability in the BZ pharmacology of native $GABA_A$ receptors is due to the α subunit (McKernan & Whiting, 1996). Hence, it has been shown that the classical BZ agonists, such as diazepam and lorazepam, have high affinity for $GABA_A$ receptors containing β and γ2 subunits in conjunction with either an α1, α2, α3 or α5 subunit and these are collectively known as the diazepam-sensitive (DS) receptors (Lüddens *et al*., 1995; Sieghart, 1995). In contrast, $GABA_A$ receptors containing β and γ2 and either an α4 or α6 subunit show essentially no affinity for the classical BZ agonists (Wisden *et al*., 1991; Hadingham *et al*., 1996; Knoflach *et al*., 1996). This suggests that these $GABA_A$ receptors correspond to the diazepam-insensitive (DIS) binding sites previously identified in native tissue using the imidazobenzodiazepine [^{3}H]Ro 15-4513, which binds to both DS and DIS $GABA_A$ receptor populations (Malminiemi & Korpi, 1989; Turner *et al*., 1991; Wong & Skolnick, 1992). The

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diazepam sensitivity of the BZ binding site of α1-, α2-, α3- and α5-containing $GABA_A$ receptors is due to a single histidine residue, which in α4 and α6 subunits is an arginine (Wieland *et al*., 1992; Benson *et al*., 1998). The diazepam sensitivity conferred by this single amino-acid residue has been exploited to generate mice in which this critical α subunit histidine has been mutated to an arginine to generate $GABA_A$ receptor populations that are insensitive to diazepam (Rudolph *et al*., 1999; Löw *et al*., 2000; McKernan *et al*., 2000; Crestani *et al*., 2002). Using this approach, it has been possible to begin to dissect the complex pharmacological actions of diazepam and attribute certain features to particular $GABA_A$ receptor populations, thereby offering the possibility of developing novel drugs that selectively target specific subtypes (Rudolph & Möhler, 2004).

In contrast to DS $GABA_A$ receptors, relatively little is known of the functions of DIS (i.e. α4- and α6-subunit-containing) $GABA_A$ receptors. Thus, although they have unique anatomical localizations (Wisden *et al*., 1992; Pirker *et al*., 2000), their physiological functions remain uncertain, primarily due to the absence of suitable DIS-selective pharmacological tools. In addition to its intrinsic efficacy, a key aspect of the *in vivo* pharmacological effects of any compound acting at either DS or DIS $GABA_A$ receptors is the level of receptor occupancy achieved (Brouillet *et al*., 1991; Facklam *et al*., 1992). [^{3}H]Ro 15-4513 has been used to label DS receptors *in vivo* (Sadzot *et al*., 1989; Mehta & Shank, 1995); however, there are no reports of it being used to specifically label DIS receptors. Therefore, in the present study an *in vivo* binding assay that specifically measures the levels of DIS receptor occupancy was established. Thus, in a manner analogous to the use of [^{3}H]Ro 15-4513 to label DS and DIS receptors *in vitro* (Malminiemi & Korpi, 1989), prior administration of the classical BZ lorazepam was used to block DS receptors, with residual *in vivo* [^{3}H]Ro 15-4513 binding being due to DIS receptors. The pharmacological specificity of this *in vivo* binding of [^{3}H]Ro 15-4513 to DIS was further established in mice in which the histidine residue of the α1 subunit critical for conferring diazepam sensitivity is mutated to an arginine, which results in α1H101R-containing $GABA_A$ receptors being rendered DIS (McKernan *et al*., 2000).

Methods

Chemicals

Both [^{3}H]Ro 15-4513 (853 GBq mmol^{-1}) and [^{3}H]Ro 15-1788 (2812 GBq mmol^{-1}) were purchased from Perkin Elmer LAS (Boston, MA, U.S.A.) Ro 15-4513 was obtained from Sigma-RBI, lorazepam from Sigma-Aldrich (both Gillingham, U.K.) and bretazenil was a gift from Roche Labs (Basel, Switzerland). All other chemicals were purchased from Sigma-Aldrich. The structures of compounds used for the *in vivo* binding studies are shown in Figure 1.

Animals

Male Swiss-Webster mice were obtained commercially (20–25 g; B&K Universal, Hull, U.K.). Transgenic mice (20–40 g in a 75% C57BL6/25% 129SvEv genetic background) containing a histidine to arginine mutation at residue 101 of the α1 subunit (α1H101R) were produced in-house as described previously (McKernan *et al*., 2000). Mice were housed in a temperature- and humidity-controlled environment with a 12 h light/dark cycle, environmental enrichment and free access to food and water. All procedures were carried out in accordance with the Home Office Animals (Scientific Procedures) Act 1986.

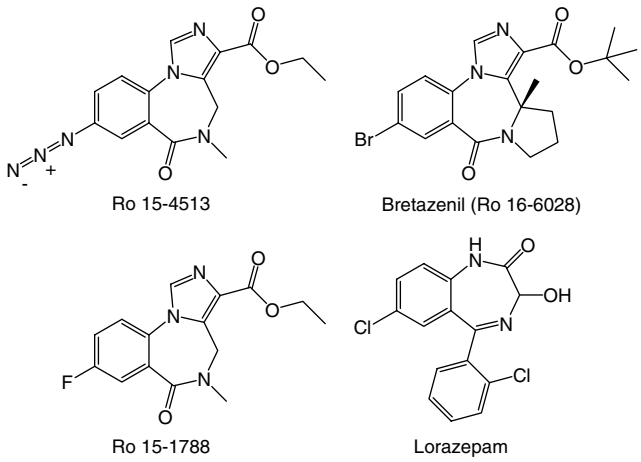

Figure 1 Structures of compounds used in the present study. Lorazepam contains a 1,4-BZ core structure with the 5-phenyl substituent characteristic of other clinically used BZs, such as diazepam, chlordiazepoxide, flunitrazepam and clonazepam. In contrast, Ro 15-4513, bretazenil and Ro 15-1788 all lack the 5-phenyl group, but instead possess an imidazo group at positions 1 and 2 of the diazepine ring.

In vitro *radioligand binding studies*

Human recombinant $GABA_A$ receptors containing β3, γ2 plus α1, α2, α3, α4, α5 or α6 subunits were expressed in L(tk$^-$) cells as described elsewhere (Hadingham *et al*., 1993; 1996; Wafford *et al*., 1996). The affinities of Ro 15-4513, bretazenil, Ro 15-1788 and lorazepam for the BZ binding site of DS (i.e. α1-, α2-, α3- or α5-containing) or DIS (α4- and α6-containing) receptors were determined by measuring the inhibition of [^{3}H]Ro 15-1788 and [^{3}H]Ro 15-4513 binding, respectively (Hadingham *et al*., 1993; 1996). The resulting IC_{50} values were converted to K_i values according to the Cheng–Prusoff equation (Cheng & Prusoff, 1973) using respective K_D values of 0.92, 1.05, 0.58 and 0.45 nM for the binding of [^{3}H]Ro 15-1788 to α1-, α2-, α3- and α5-containing receptors and 5.0 and 6.5 nM for the binding of [^{3}H]Ro 15-4513 to α4- and α6-containing receptors.

Time course of in vivo *[^{3}H]Ro 15-4513 binding*

[^{3}H]Ro 15-4513 (5 μl g^{-1} of a 370 kBq ml^{-1} saline stock solution, equivalent to 37 kBq of [^{3}H]Ro 15-4513 per 20 g mouse) was administered as a bolus *via* the lateral tail vein to Swiss-Webster mice. The animals ($n = 5$ per group) were killed by stunning and decapitation and trunk blood collected into heparinized tubes 0.5, 1, 2, 3, 5, 10, 20 and 30 min after injection. The brains rapidly removed from the skull and the forebrain and cerebellum were separated, weighed and homogenized in 10 volumes of ice-cold phosphate buffer

(10 mM potassium phosphate buffer containing 100 mM KCl, pH 7.4) using a Polytron. The blood was centrifuged for 10 min at $1000 \times g$ and 50 μl aliquots of plasma or 300 μl aliquots of homogenate were added directly to 10 ml liquid scintillation fluid, the latter representing the total brain radioactivity (i.e., unbound + membrane-bound radioactivity). Additional 300 μl aliquots of homogenate were filtered and washed (ice-cold 10 ml phosphate buffer, total wash time $\sim$2 s) through Whatman GF/B 25 mm filter circles and represented membrane-bound radioactivity. The washed filters were then placed in 10 ml of liquid scintillant and shaken for at least 1 h. Radioactivity in each sample was measured using a Beckman LS6500 liquid scintillation counter.

Inhibition of in vivo *[^{3}H]Ro 15-4513 binding by Ro 15-4513 and bretazenil*

Since the membrane binding of [^{3}H]Ro 15-4513 described above comprises both specific binding to $GABA_A$ receptors plus nonspecific binding to non-$GABA_A$ receptor binding sites, prior to differentiating distinct populations of specific binding sites, it was necessary to define the level of nonspecific binding of [^{3}H]Ro 15-4513 *in vivo*. Accordingly, increasing doses of Ro 15-4513 (0.1–100 mg kg^{-1} i.p. in 0.5% carboxymethyl cellulose) or bretazenil (0.03–30 mg kg^{-1} i.p. in 100% polyethylene glycol 300 (PEG 300) vehicle) were administered to male Swiss-Webster mice (n = 4–6 per group), followed 13 min later by an i.v. injection of [^{3}H]Ro 15-4513. After 2 min (a time defined in the time-course experiment described above as giving optimal [^{3}H]Ro 15-4513 binding), mice were killed and the whole brain processed as above.

Defining a dose of lorazepam which blocks DS $GABA_A$ receptors in vivo

In order to establish a dose of lorazepam that blocks essentially all DS receptors *in vivo*, increasing doses of lorazepam (0.1–30 mg kg^{-1} i.p. in 0.5% carboxymethyl cellulose) were administered to male Swiss-Webster mice (n = 3–6 per group), followed 27 min later by i.v. [^{3}H]Ro 15-1788, a radioligand which binds to DS but not, at the low (tracer) concentrations used for an *in vivo* binding assay, DIS $GABA_A$ receptors (Atack *et al*., 1999). After 3 min, mice were killed and the whole brain processed as above. In these experiments, nonspecific binding (c. 80 d.p.m.) was defined using bretazenil (5 mg kg^{-1} i.p. in 100% PEG 300 vehicle).

Measurement of in vivo *binding of [^{3}H]Ro 15-4513 to DIS $GABA_A$ receptors*

Mice (either Swiss-Webster or α1H101R transgenic) were divided into three groups (n = 4–6 per group) which were dosed i.p. either with vehicle (0.5% carboxymethyl cellulose), lorazepam (30 mg kg^{-1}) or bretazenil (20 mg kg^{-1} in 100% PEG 300), followed 28 min later by [^{3}H]Ro 15-4513. After 2 min, mice were killed and processed as outlined above. [^{3}H]Ro 15-4513 binding in bretazenil-treated animals represents nonspecific binding and was generally in the region of 75–100 d.p.m. Specific binding was calculated by subtracting the nonspecific binding from the actual counts in the vehicle- and lorazepam-treated animals, and represents total and DIS binding, respectively.

Results

Ligand binding affinity for human recombinant $GABA_A$ receptors

The *in vitro* binding profiles of compounds used in the present study are presented in Table 1 and confirm previous observations. Hence, the imidazobenzodiazepine Ro 15-4513 has high affinity for $GABA_A$ receptors containing $\beta 3\gamma 2$ plus either α1, α2, α3, α4, α5 or α6. However, α5-containing $GABA_A$ receptors have higher affinity for Ro 15-4513 than the other subtypes and presumably accounts for the super-high-affinity binding site observed in rat brain membranes (Mehta & Shank, 1995). The structurally related imidazobenzodiazepine bretazenil also has relatively high affinity for all subtypes. However, and unlike Ro 15-4513, the affinity of bretazenil for α4- and α6-containing (DIS) $GABA_A$ receptors is lower than its affinity at α1, α2, α3 and α5-containing (DS) receptors (Wong & Skolnick, 1992; Hadingham *et al*., 1993; 1996; Knoflach *et al*., 1996). Similarly, Ro 15-1788 has high affinity for DS receptors, but 50- to 1000-fold lower affinity for DIS, whereas lorazepam has high affinity for DS, but, like other classical BZ agonists, essentially no affinity for DIS (Sieghart, 1995).

Time course of in vivo *[^{3}H]Ro 15-4513 binding*

Figure 2a shows that following i.v. administration of [^{3}H]Ro 15-4513, radioactivity is rapidly cleared from the plasma with a half-life (calculated using a single-phase, exponential

Table 1 Affinities of compounds for different subtypes of human recombinant $GABA_A$ receptors

	Affinity (nM), *at human recombinant $GABA_A$ receptors containing* $\beta 3$, $\gamma 2$ *plus* αx *subunits*					
	(Diazepam-sensitive $GABA_A$ receptors)				*(Diazepam-insensitive $GABA_A$ receptors)*	
	$\alpha 1$	$\alpha 2$	$\alpha 3$	$\alpha 5$	$\alpha 4$	$\alpha 6$
Ro 15-4513	4.8 ± 1.2	7.3 ± 3.2	2.4 ± 1.4	0.13 ± 0.05	5.1 ± 0.9	8.2 ± 1.7
Bretazenil	0.22 ± 0.10	0.37 ± 0.13	0.43 ± 0.14	0.68 ± 0.12	43 ± 9	25 ± 8
Ro 15-1788	1.1 ± 0.3	1.5 ± 0.2	1.0 ± 0.3	0.5 ± 0.1	236 ± 95	410 ± 125
Lorazepam	1.3 ± 0.4	3.5 ± 0.1.1	9.4 ± 2.2	16 ± 4	> 10,000	> 10,000

Human recombinant $GABA_A$ receptors were stably expressed in a fibroblast cell line as described elsewhere (Hadingham *et al*., 1993; 1996; Wafford *et al*., 1996). Binding affinities (K_i) were measured using [^{3}H]Ro 15-1788 and [^{3}H]Ro 15-4513 for α1-, α2-, α3- and α5-containing and α4- and α6-containing receptors, respectively (Hadingham *et al*., 1993; 1996). Values are mean ± s.e.m. of 3–6 separate determinations.

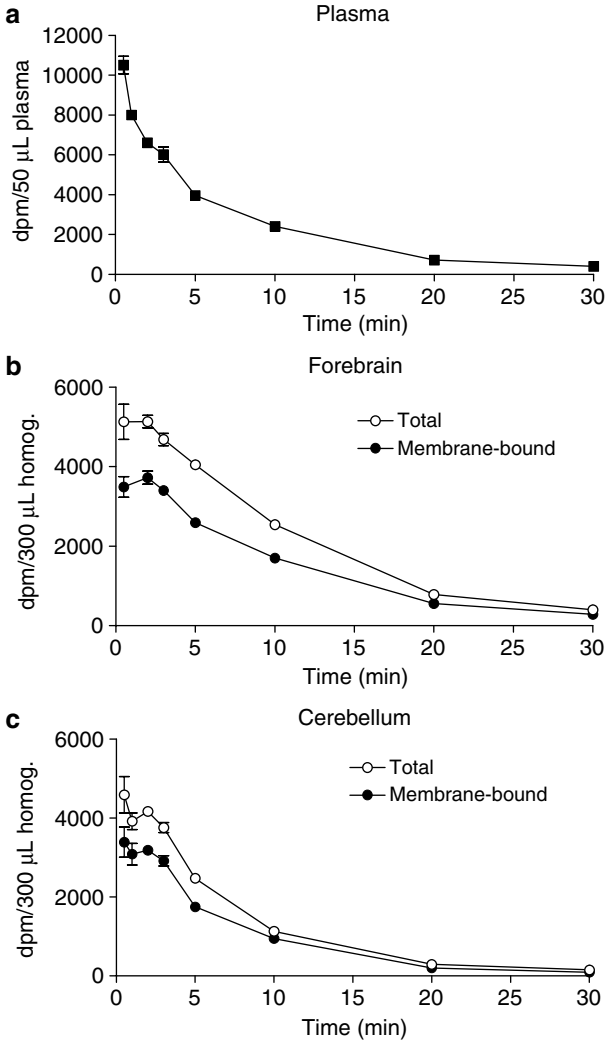

Figure 2 Time–activity curves for the distribution of radioactivity at various times after tail vein injections of [^{3}H]Ro 15-4513 in mouse: (a) 50 μl aliquots of plasma; radioactivity in 300 μl aliquots of (b) mouse forebrain or (c) mouse cerebellar homogenate, which were either unprocessed (total radioactivity) or filtered and washed over Whatman GF/B filters (membrane-bound radioactivity). Values shown are mean $\pm$ s.e.m. ($n = 5$ per group).

decay model) of around 3 min. Uptake into the brain, whether forebrain or cerebellum, was rapid, with a clearance rate that was similar to that of plasma. Moreover, in both brain regions, the time course of membrane-bound radioactivity closely tracked that of the total brain radioactivity, consistent with the forebrain and cerebellum membrane-bound radioactivity constituting the majority (65–70 and 75–80%, respectively) of the total radioactivity. In terms of absolute levels, membrane-bound brain radioactivity levels were comparable in the forebrain and cerebellum at early time points (0.5–3 min), but at later times more radioactivity was retained in the forebrain. For example, 20 min after injection of [^{3}H]Ro 15-4513, levels of radioactivity in the cerebellum had dropped to about one-third of those in the forebrain, suggesting that a component of forebrain membrane binding has a slower rate of dissociation than in the cerebellum.

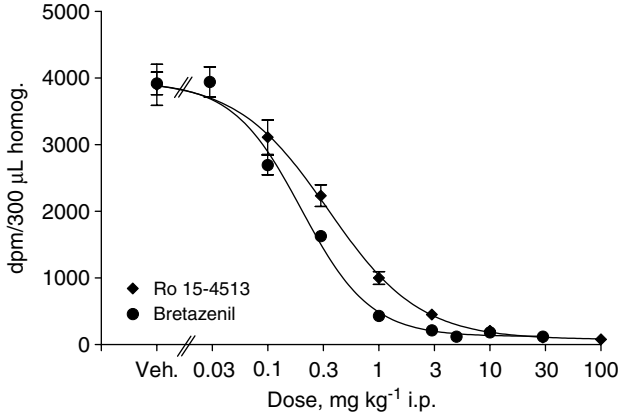

Figure 3 The dose-dependent decrease in brain radioactivity after i.v. injection of [^{3}H]Ro 15-4513 following prior administration of Ro 15-4513 (0.1–100 mg kg^{-1}) or bretazenil (0.03–30 mg kg^{-1}), both administered i.p. with a pretreatment time of 15 min. The respective ID_{50} values for Ro 15-4513 and bretazenil were 0.35 and 0.20 mg kg^{-1}. Values shown are mean $\pm$ s.e.m. ($n = 4$–6 per group).

Inhibition of in vivo *[^{3}H]Ro 15-4513 binding by bretazenil and Ro 15-4513*

Figure 3 shows the dose-dependent decrease in *in vivo* [^{3}H]Ro 15-4513 binding to the combined DS and DIS receptor populations in whole Swiss-Webster mouse brain after pretreatment with either Ro 15-4513 or bretazenil. Both compounds reduced the binding from around 4000 d.p.m./300 μl aliquot of homogenate in the absence of drug to a value in the region of 100–120 d.p.m. at the highest doses of Ro 15-4513 (100 mg kg^{-1}) or bretazenil (30 mg kg^{-1}) tested, demonstrating that the nonspecific *in vivo* binding of this radioligand represents less than 5% of total binding. Using a single-site curve-fitting model, the ID_{50} value for Ro 15-4513 was 0.35 (Hill slope = 1.05) and for bretazenil 0.20 (Hill slope = 1.40). Based on these experiments, a dose of 20 mg kg^{-1} bretazenil was chosen to define nonspecific *in vivo* binding of [^{3}H]Ro 15-4513 in subsequent experiments.

Inhibition of in vivo *[^{3}H]Ro 15-1788 and [^{3}H]Ro 15-4513 binding by lorazepam*

[^{3}H]Ro 15-1788 in vivo *binding* Figure 4a demonstrates the inhibition of mouse forebrain and cerebellum membrane binding of [^{3}H]Ro 15-1788 with increasing concentrations of lorazepam. At the highest dose (30 mg kg^{-1}), lorazepam produced essentially complete inhibition of [^{3}H]Ro 15-1788 binding with levels of radioactivity at this dose, 80–100 d.p.m., corresponding to the level of nonspecific binding defined using bretazenil (5 mg kg^{-1}). The ID_{50}'s for the inhibition of [^{3}H]Ro 15-1788 in mouse forebrain and cerebellum were 0.48 and 0.29 mg kg^{-1}, respectively, the corresponding Hill slopes being 0.94 and 1.07. Thus, a dose of 30 mg kg^{-1} lorazepam completely inhibited the *in vivo* binding of [^{3}H]Ro 15-1788 to DS binding sites.

[^{3}H]Ro 15-4513 in vivo *binding* In contrast to [^{3}H]Ro 15-1788, lorazepam was unable to completely block the specific *in vivo* binding of [^{3}H]Ro 15-4513 (Figure 4b). Thus, 30 mg kg^{-1} lorazepam was able to reduce *in vivo* [^{3}H]Ro

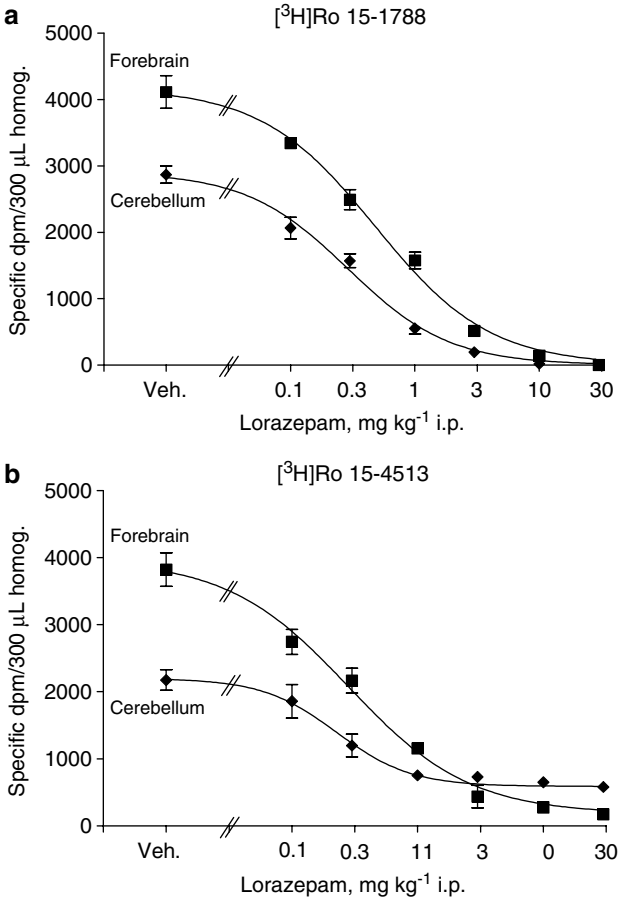

Figure 4 Inhibition of the specific *in vivo* binding of (a). [^{3}H]Ro 15-1788 and (b) [^{3}H]Ro 15-4513 by increasing doses of lorazepam (administered i.p. in 0.5% carboxymethyl cellulose vehicle with pretreatment time of 30 min). Lorazepam was able to reduce the level of binding of [^{3}H]Ro 15-1788 to levels of nonspecific binding (5 mg kg^{-1} bretazenil, c. 80 d.p.m. in both the forebrain and cerebellum). However, even at high doses of lorazepam, there was residual *in vivo* [^{3}H]Ro 15-4513 binding that was appreciably greater than the level of nonspecific binding (110 and 170 d.p.m. in the forebrain and cerebellum, respectively) and which represented 4 and 27% of the specific binding in forebrain and cerebellum, respectively. Values shown are mean ± s.e.m. ($n = 3$–6 per group).

15-4513 binding relative to vehicle-treated mice from around 3820 to 170 d.p.m. in the forebrain and from 2180 to 580 d.p.m. in the cerebellum. This corresponds to a residual radioactivity in the forebrain and cerebellum that represents 4 ± 1 and 27 ± 2% of *in vivo* [^{3}H]Ro 15-4513 binding observed in the absence of lorazepam.

The fact that the lorazepam-displaceable *in vivo* [^{3}H]Ro 15-4513 binding recognizes the same population of binding sites identified using [^{3}H]Ro 15-1788 (i.e. DS binding sites) is demonstrated by the similarity of the potency of lorazepam to inhibit binding to these sites in the forebrain and cerebellum using either radioligand. Hence, the respective ID_{50} values for lorazepam using [^{3}H]Ro 15-1788 or [^{3}H]Ro 15-4513 were 0.48 and 0.33 mg kg^{-1} in the forebrain and 0.29 and 0.24 mg kg^{-1} in the cerebellum.

The reproducibility of the inhibition of *in vivo* [^{3}H]Ro 15-4513 binding by lorazepam was assessed in three separate experiments (Figure 5). In all the three experiments, the proportion of forebrain [^{3}H]Ro 15-4513 binding that could not be inhibited by lorazepam (i.e. DIS binding) was between 4 and 6%, whereas in the cerebellum the corresponding percentage was more variable and ranged from 17 to 26%.

Inhibition of in vivo *[^{3}H]Ro 15-4513 binding by lorazepam in α1H101R transgenic mice*

Figure 6a shows that, in the wild-type (75% C57BL6/25% 129SvEv) mice, lorazepam was unable to block 4 ± 1 and 15 ± 2% of *in vivo* [^{3}H]Ro 15-4513 binding in the forebrain and cerebellum, respectively, which is comparable to the corresponding values (5 and 23%) observed in Swiss-Webster mice (Figure 5). Moreover, the absolute levels of *in vivo* [^{3}H]Ro 15-4513 binding in the wild-type mice, which have a 75% C57B1/6, 25% 129SvEv background, were comparable to those seen in the Swiss-Webster mice (i.e. c. 4000 and 2000 d.p.m./300 µl homogenate in forebrain and cerebellum, respectively; Figure 6b). In α1H101R mice (Figure 6b), the proportion of *in vivo* [^{3}H]Ro 15-4513 binding due to DIS binding sites increased from 4 to 36% in the forebrain and from 15 to 59% in the cerebellum.

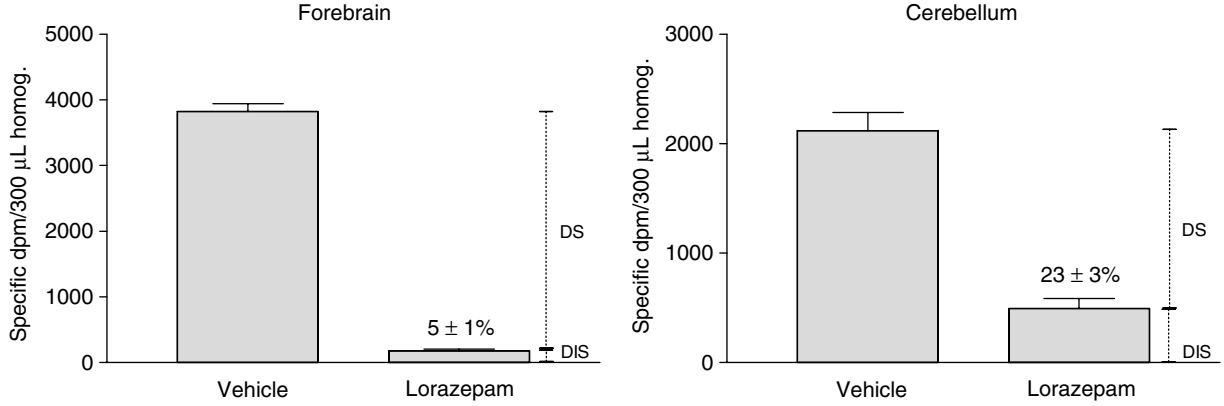

Figure 5 Average of the specific [^{3}H]Ro 15-4513 binding in the forebrain and cerebellum for 30 min in three separate experiments in which Swiss-Webster mice ($n = 4$–6 per group) received i.p. injections of either vehicle (0.5% carboxymethylcellulose) or lorazepam (30 mg kg^{-1}). Specific binding is actual radioactivity minus nonspecific binding (c. 100–125 d.p.m.), defined as radioactivity observed in the forebrain and cerebellum of mice pretreated with bretazenil (20 mg kg^{-1} i.p. in 100% PEG 300). Percentage values show the proportion of *in vivo* separate but identical experiments [^{3}H]Ro 15-4513 binding that cannot be displaced by lorazepam (i.e. DIS binding). Values shown are mean ± s.e.m. ($n = 3$; separate but identical experiments). DS and DIS = diazepam-sensitive and diazepam-insensitive binding, respectively.

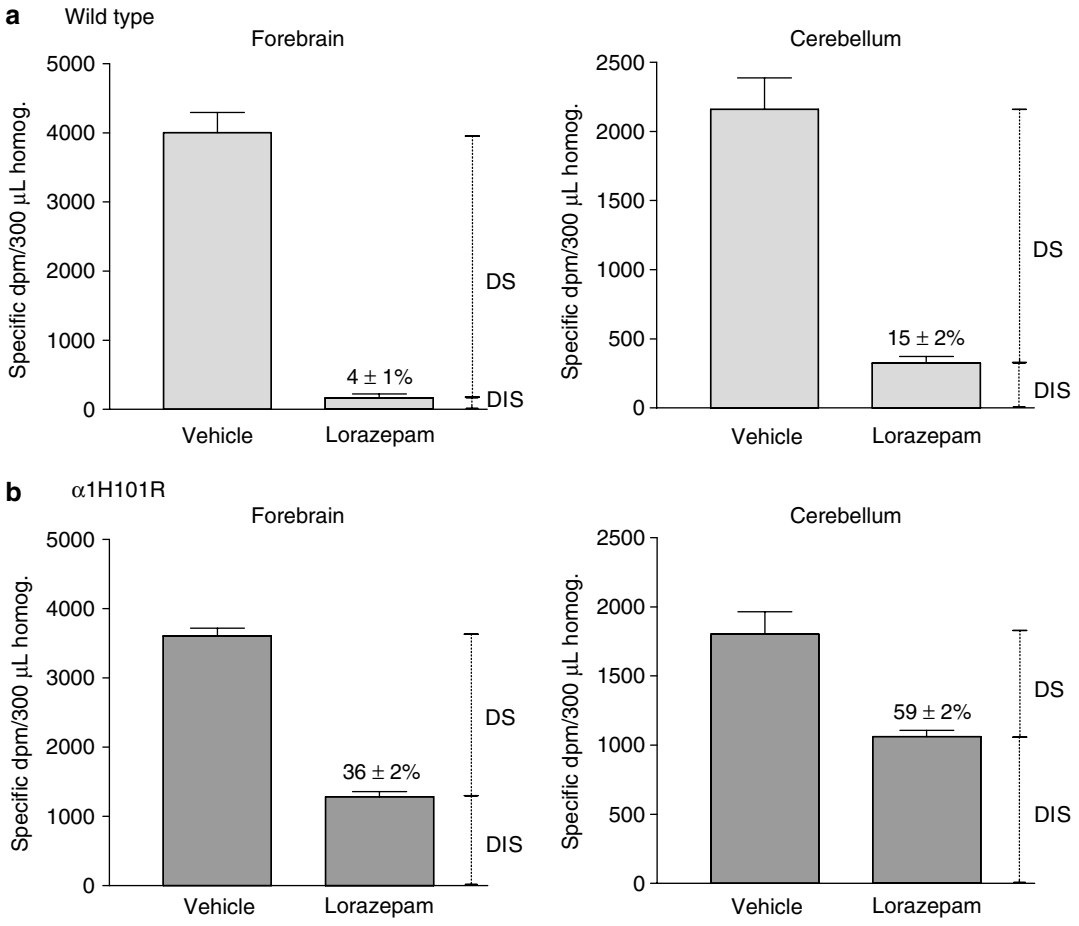

Figure 6 Inhibition of specific *in vivo* [^{3}H]Ro 15-4513 binding in the forebrain and cerebellum of (a) wild-type and (b) α1H101R mice. Mice were pretreated for 30 min with vehicle (0.5% carboxymethyl cellulose) and lorazepam (30 mg kg^{-1} i.p.), with specific binding being defined as actual d.p.m. minus radioactivity in mice pretreated with bretazenil (20 mg kg^{-1} i.p. bretazenil in 100% PEG 300), which gives total receptor blockade and results in forebrain and cerebellum radioactivities of c. 80 and 150 d.p.m., respectively. Percentage values show the proportion of *in vivo* [^{3}H]Ro 15-4513 binding that cannot be displaced by lorazepam. Values shown are mean ± s.e.m. ($n = 5$–6 per group).

Discussion

Properties of [^{3}H]Ro 15-4513 as a radiotracer for in vivo *binding studies*

Following i.v. injection of [^{3}H]Ro 15-4513 to mice, radioactivity is rapidly cleared from plasma, with an estimated half-life in the region of 3 min, consistent with previous data in Cynomolgus monkeys (Halldin *et al*., 1992). Moreover, [^{3}H]Ro 15-4513 was rapidly taken up into and cleared from mouse brain (Figure 2; Sadzot *et al*., 1989), as it is in monkey (Halldin *et al*., 1992) and man (Inoue *et al*., 1992; Suhara *et al*., 1993). The kinetics of [^{3}H]Ro 15-4513 in the mouse are very similar to the structurally related compound [^{3}H]Ro 15-1788, which also has a short plasma half-life with a rapid uptake into and clearance from the brain (Atack *et al*., 1999).

Although brain radioactivity decreased rapidly after i.v. [^{3}H]Ro 15-4513 administration, it was noted, nevertheless, that clearance of radioactivity from the forebrain was slower than from the cerebellum, suggesting that the forebrain contains a receptor population which may have a relatively slow off-rate. More detailed regional analyses suggests that this slow off-rate population is more abundant in the hippocampus than in other cortical and subcortical regions in mouse (Sadzot *et al*., 1989), rhesus monkey (Onoe *et al*., 1996) and man (Inoue *et al*., 1992). This slow off-rate, predominantly hippocampal, high-affinity receptor population has also been identified in rat brain *in vitro* (Mehta & Shank, 1995) and using *in vivo* autoradiography (Nakano *et al*., 1998). It presumably corresponds to binding to the BZ binding site of $GABA_A$ receptors containing an α5 subunit, since such receptors possess around 20–50-fold higher affinity for Ro 15-4513 than α1-, α2-, α3-, α4- and α6-containing subtypes (Table 1; Hadingham *et al*., 1993; 1996). It should be emphasized that this high-affinity binding site is best detected at later time points (20–30 min after i.v. injection of [^{3}H]Ro 15-4513; Sadzot *et al*., 1989), at which time brain [^{3}H]Ro 15-4513 concentrations are low enough to preferentially label this super-high-affinity binding site (Nakano *et al*., 1998). In contrast, in the present study, mice were killed at a much earlier time point (2 min) to maximize the signal seen due to the less-abundant DIS receptor population. Under these conditions, [^{3}H]Ro 15-4513 will not show selectivity for the super-high-affinity site (Nakano *et al*., 1998), but instead will, in the absence of any displacing drug, label the collective DS and DIS $GABA_A$ receptor populations.

From a technical point of view, it should also be noted that in the present study, brain samples were removed, homogenized, filtered and rapidly washed, a process which removes free ligand and low affinity non-specific binding, thereby improving the signal-to-noise ratio. On the other hand, in a study more compatible with the characterization of radioligands for their suitability as PET radiotracers (Sadzot *et al*., 1989), brain regions were dissected and solubilized, a process in which free radioligand and low affinity non-specific binding are included as components of the total radioactivity measured. If this latter methodology was employed for the measurement of DIS binding sites, it is uncertain whether the relatively low levels of DIS binding sites in the cortex could be reliably detected. Indeed, it should be noted that by employing methods similar to those used in the present study (i.e. washing and filtration of homogenates) it was possible to specifically label α5-containing $GABA_A$ receptors (Atack *et al*., 2005) with a radioligand ([^{3}H]L-655708) that was otherwise unsuitable for PET studies (Opacka-Juffry *et al*., 1999).

Pharmacology of in vivo *[^{3}H]Ro 15-4513 binding*

The *in vivo* binding of [^{3}H]Ro 15-4513 could be abolished in a dose-dependent manner by administration of unlabelled Ro 15-4513 with an ID_{50} of 0.35 mg kg^{-1}. This value is appreciably higher than the previously published values, depending on brain region, of 5–13 μg kg^{-1} (Sadzot *et al*., 1989) but this discrepancy is likely due to the use of an i.p. dosing route in the present study compared to an i.v. route in the study of Sadzot *et al*. (1989). Similarly, bretazenil also produced a dose-dependent inhibition of *in vivo* [^{3}H]Ro 15-4513 binding. It might be anticipated that, since bretazenil has higher affinity for DS (affinity = c. 0.4 nM) compared to DIS (c. 20 nM) $GABA_A$ receptors, there would be a biphasic displacement of *in vivo* [^{3}H]Ro 15-4513 binding by bretazenil. However, in the forebrain, the lower-affinity DIS receptor population is too small to reliably detect as a lower-affinity component, whereas in the cerebellum, where DIS are more abundant, the displacement curve did have a shallow Hill slope, but the number of data points was insufficient for an accurate fit of a two-site model.

In vivo *binding of [^{3}H]Ro 15-4513 to DIS using lorazepam to block DS*

Based on its binding to DS rather than DIS receptors (Table 1), lorazepam was chosen to selectively block DS receptors *in vivo*. The binding of lorazepam to DS (as measured using *in vivo* [^{3}H]Ro 15-1788 binding) was dose dependent, with respective ID_{50} values in the forebrain and cerebellum of 0.48 and 0.29 mg kg^{-1}, which are similar to the 0.5–1.6 mg kg^{-1} potency of lorazepam reported previously (Benavides *et al*., 1992). Since 30 mg kg^{-1} lorazepam was able to completely inhibit the *in vivo* binding of [^{3}H]Ro 15-1788, this dose was chosen to selectively block DS $GABA_A$ receptors in subsequent studies.

In the presence of 30 mg kg^{-1} lorazepam, *in vivo* [^{3}H]Ro 15-4513 binding in Swiss-Webster mice was reduced by 94–96% and 74–83% in the forebrain and cerebellum, respectively. Thus, in the forebrain and cerebellum, DIS receptors represented, on average, 5 and 23% of the total *in vivo* binding of [^{3}H]Ro 15-4513 binding observed in the absence of a lorazepam blockade. These *in vivo* data correspond to *in vitro* radioligand saturation analyses or quantitative immunoprecipitation studies, which have demonstrated that DIS sites represent c. 30% of the total binding sites in the cerebellum and around 5% of cortical and thalamic binding sites (Jechlinger *et al*., 1998; Bencsits *et al*., 1999).

In vivo *binding of [^{3}H]Ro 15-4513 to DIS in α1H101R mice*

In order to confirm that in the presence of lorazepam the residual binding of [^{3}H]Ro 15-4513 corresponded to DIS, the *in vivo* binding of [^{3}H]Ro 15-4513 was measured in mice in which the α1 subunit is rendered diazepam (and lorazepam)-insensitive by the mutation of the histidine residue at position 101 to arginine (α1H101R mice; Rudolph *et al*., 1999; McKernan *et al*., 2000). Consequently, the conversion of the α1 subunit from DS to DIS results in α1H101R mice having an increase in DIS binding sites which corresponds to a loss of DS sites (Rudolph *et al*., 1999; McKernan *et al*., 2000).

In vehicle-treated wild-type (75% C57BL6/25% 129SvEv) and α1H101R mice, both genotypes had similar levels of *in vivo* binding of [^{3}H]Ro 15-4513 to the combined DS plus DIS binding site populations (i.e. [^{3}H]Ro 15-4513 binding in the absence of lorazepam), which is consistent with the retention of total [^{3}H]Ro 15-4513 binding sites reported *in vitro* (McKernan *et al*., 2000). Moreover, the increase in DIS receptor population in α1H101R mice from 4 to 36% in the forebrain and 15 to 59% in the cerebellum agrees with *in vitro* data (McKernan *et al*., 2000). This increase in DIS binding occurs due to a reduced DS receptor population caused by loss of lorazepam sensitivity of the α1-containing $GABA_A$ receptors, and is seen not only indirectly in the present study *in vivo* but also *in vitro* (McKernan *et al*., 2000).

Utility of an in vivo *binding assay for DIS*

The ultimate goal of an *in vivo* binding assay should be to relate a pharmacological response to receptor occupancy and intrinsic efficacy (Facklam *et al*., 1992; Jones *et al*., 1994). In this regard, [^{3}H]Ro 15-4513 has proved to be a useful radioligand for use not only in rodent *in vivo* binding assays (Sadzot *et al*., 1989) but also primate (Halldin *et al*., 1992; Onoe *et al*., 1996) and human imaging (Inoue *et al*., 1992; Suhara *et al*., 1993; Lingford-Hughes *et al*., 2002) studies. However, the profound sedative effects produced by the blocking dose of lorazepam used to mask DS receptors in the present study means that this method is unsuitable for studies whereby the behavioural effects of compounds selectively interacting with DIS can be related to receptor occupancy in the same animals (that is, measurement of receptor occupancy immediately following completion of a behavioural task). Moreover, such a method for identifying DIS receptors in mice *in vivo* would be unsuitable for human studies, since although [^{11}C]Ro 15-4513 has proved a successful PET ligand in man (Inoue *et al*., 1992; Suhara *et al*., 1993; Lingford-Hughes *et al*., 2002), the doses of lorazepam required to give complete blockage of DS binding in man are likely to far exceed clinically used doses. For example, it has been estimated that a therapeutically relevant dose of lorazepam corresponds to an occupancy of DS $GABA_A$ receptors of only 3% (Sybirska *et al*., 1993). An alternative approach would be to block DS receptors with an antagonist, which should have

no pharmacological effects. However, the prototypic BZ antagonist Ro 15-1788 (flumazenil) has appreciable affinity for DIS such that at high doses blockade of DS would be accompanied by appreciable occupancy at DIS.

Nevertheless, in the absence of radioligands which directly and specifically label DIS receptors, the use of [^{3}H]Ro 15-4513 in the presence of lorazepam should provide a useful means of evaluating the brain penetrability and receptor occupancy of compounds which interact with DIS (Li *et al*., 2002). Such studies are obviously not suitable for extrapolation to noninvasive clinical PET scanning for the reasons stated above, but nevertheless should facilitate the identification of brain-penetrant compounds which selectively interact with DIS $GABA_A$ receptors and thereby form the basis of an understanding of the roles of this intriguing, anatomically distinct population of $GABA_A$ receptors.

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