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Differential contribution of $GABA_A$ receptor subtypes to the anticonvulsant efficacy of benzodiazepine site ligands

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Abstract

Non-selective benzodiazepines, such as diazepam, interact with equivalent affinity and agonist efficacy at $GABA_A$ receptors containing either an α1, α2, α3 or α5 subunit. However, which of these particular subtypes are responsible for the anticonvulsant effects of diazepam remains uncertain. In the present study, we examined the ability of diazepam to reduce pentylenetetrazole (PTZ)-induced and maximal electroshock (MES)-induced seizures in mice containing point mutations in single (α1H101R, α2H101R or α5H105R) or multiple (α125H→R) α subunits that render the resulting $GABA_A$ receptors diazepam-insensitive. Furthermore, the anticonvulsant properties of diazepam, the α1- and α3-selective compounds zolpidem and TP003, respectively, and the α2/α3 preferring compound TP13 were studied against PTZ-induced seizures. In the transgenic mice, no single subtype was responsible for the anticonvulsant effects of diazepam in either the PTZ or MES assay and neither the α3 nor α5 subtypes appeared to confer anticonvulsant activity. Moreover, whereas the α1 and α2 subtypes played a modest role with respect to the PTZ assay, they had a negligible role in the MES assay. With respect to subtype-selective compounds, zolpidem and TP003 had much reduced anticonvulsant efficacy relative to diazepam in both the PTZ and MES assays whereas TP13 had high anticonvulsant efficacy in the PTZ but not the MES assay. Taken together, these data not only indicate a role for α2-containing $GABA_A$ receptors in mediating PTZ and MES anticonvulsant activity but also suggest that efficacy at more than one subtype is required and that these subtypes act synergistically.

Keywords
pentylenetetrazole, maximal electroshock, mouse, knock-in mice, $GABA_A$, epilepsy, benzodiazepines

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Introduction

Epilepsy is one of the most common diseases of the brain, affecting at least 50 million people worldwide. It is a chronic and often progressive disorder characterized by the periodic and unpredictable occurrence of epileptic seizures which are caused by an abnormal discharge of cerebral neurons (Scheuer and Pedley, 1990). Benzodiazepines (BZs), such as diazepam, enhance the inhibitory effects of GABA at the $GABA_A$ receptor and have acute anticonvulsant effects and are therefore commonly used for the treatment of seizures and epilepsy in emergency treatment of the disorder (Singhi *et al*., 2003). However, BZs are not used in the prophylactic treatment of epilepsy due to the development of tolerance and dependence (Haigh and Feely, 1988; Ashton, 1994).

GABA is the primary inhibitory neurotransmitter in the central nervous system and the $GABA_A$ receptor is comprised of five subunits derived from a family of 16 genes (α1–6, β1–3, γ1–3, δ, ϵ, π and θ; (Simon *et al*., 2004) which form a ligand-gated chloride channel, the subunit composition of which determines the sensitivity to a variety of pharmacological agents (Jones-Davis and Macdonald, 2003). The majority of $GABA_A$ receptors in the brain contain α, β and γ subunits in a 2:2:1 stoichiometry, with those that contain a BZ recognition site, and representing around three-quarters of the total brain $GABA_A$ receptor population, containing β, γ2 and either α1, α2, α3 or α5 subunits (McKernan and Whiting, 1996). Individual receptor subunits exhibit not only specific neuronal expression patterns, but also distinct sub-cellular localization (Fritschy *et al*., 1998; Pirker *et al*., 2000) which suggests that different receptor subtypes may have different functions. More specifically, the role of the four $GABA_A$ receptor subtypes containing a BZ recognition site (i.e. α1-, α2-, α3- and α5-containing receptors) in mediating the various effects of diazepam has been investigated using not only subtype selective compounds (McKernan *et al*., 2000) but also transgenic knock-in (KI) mice containing mutations in the α subunit which render the resulting $GABA_A$ receptor insensitive to BZs (McKernan *et al*., 2000; Crestani *et al*., 2000; Low *et al*., 2000; Dias *et al*., 2005). Using this latter approach, it has been shown that α1-containing $GABA_A$ receptors contribute to the anticonvulsant properties of BZs (Rudolph *et al*., 1999; Crestani *et al*., 2000; Kralic *et al*., 2002) raising the possibility that drugs could be developed which target specific $GABA_A$ receptor subtypes and retain anticonvulsant efficacy yet are devoid of the tolerance, dependence, sedation, anterograde amnesia and myorelaxation liabilities associated with non-selective BZs (Meldrum, 2002). However, there has been no systematic investigation of the anticonvulsant properties of other $GABA_A$ receptor subunits. Therefore the purpose of the present study was to determine the precise contribution of each $GABA_A$ receptor subunit to the anticonvulsant activity of non-selective benzodiazepines such as diazepam in two different models of epilepsy: pentylenetetrazole (PTZ)- and maximal electroshock (MES)-induced seizures. More specifically, we studied the anticonvulsant effects of diazepam against PTZ- and MES-induced seizures in mice containing single histidine to arginine point mutations in either the α1, α2 or α5 subunits (α1H101R, α2H101R and α5H105R, respectively) or a triple mutant containing all three mutations (α125H→R). In addition, we examined the anticonvulsant effects of the $GABA_A$ subtype-selective compounds zolpidem (α1-preferring; Jones *et al*., 1997), TP003 (α3-selective; Dias *et al*, 2005) and TP13 (α2/α3-preferring, Compound 15; Carling *et al.*, 2005) in these same assays.

Materials and methods

Animals

All animals were group- or singly-housed (depending on fighting behaviour) in solid-bottomed cages with sawdust bedding and environmental enrichment. Food and water were available ad libitum. Temperature and humidity were maintained at 21 ± 2ºC and 55 ± 10% respectively. Lights were on a 12:12 hour light cycle with lights coming on at 7.00 AM. All procedures were carried out in accordance with the UK Animals (Scientific Procedures) Act (1986) and its associated guidelines. Male Swiss-Webster (SW) mice (20–25 g) were obtained from B&K Universal (Hull, UK).

Generation of knock-in mice

Knock-in (KI) mice were generated as described previously (McKernan *et al*., 2000) for the $GABA_A$ receptor α1H101R, the α2H101R (Dias *et al*., 2005) and the α5H105R subunit. Heterozygous, F1 generation mice were further bred with a cre-transgenic mouse (Schwenk *et al*., 1995) to remove the neomycin resistance gene in their offspring. By additional breeding, wild-type and homozygous mice were generated using a randomized breeding strategy and were kept in a mixed 50% C57BL/6–129SvEv genetic background with both male and female animals used for each experiment and ranging from 3–12 months of age.

The $GABA_A$ α125H→R mouse line was generated through crossing of the $GABA_A$ α1, α2 and α5 KI lines until triple homozygotes had been obtained. Wild-type (WT) animals used in behavioural testing were taken from either single KI lines or from lines generated by the crossing of separate KI lines, e.g. the $GABA_A$ α125H→R mice, depending on availability. Whilst all WT animals were considered to be similar, where animals differed in their response, data has been graphed accordingly. DNA from tail or ear biopsies of the various KI mouse lines were prepared as described (Kuenzi *et al*., 2000) and genotyped using the oligonucleotides shown in Table 1.

Table 1

KI line	Genotyping oligonucleotides
GABAα1H101R	5′-ATT.AAT.GGA.GAG.TGT.GGT.AAT.CTT.T-3′
	5′-TCC.TTC.ATG.GTG.AAC.AAG.ACC.AGG-3′
GABAα2H101R	5′-CCA.TTA.CAC.TCC.TCA.AAT.TGT.GAA.C-3′
	5′-GTG.GTC.TGT.GAA.TTC.TAA.TTT.TCT.AG-3′
GABAα5H105R	5′-GAG.CGA.ATC.ACG.CAG.GTG.CGA.ACA.GAC-3′
	5′-CCC.GAC.CTG.CTA.CCC.AGG.GTA-3′

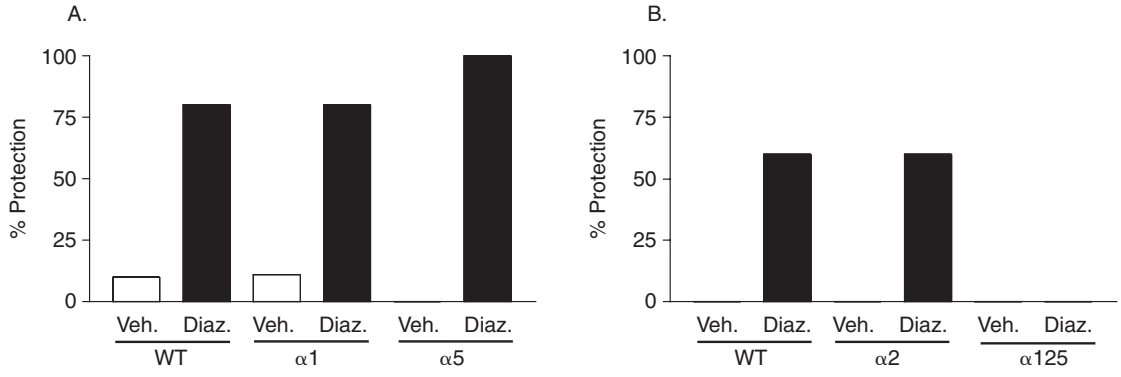

Figure 2 Protection from MES-induced seizures by diazepam (Diaz., 20 mg/kg) in mice carrying either the α1H101R, α2H101R, α5H105R or α125H→R mutations. Animals were observed for a maximum of 2 min following electroshock and % of animals protected against tonic seizure calculated (n=9–10/group).

Fig. 2 shows data from KI animals pretreated with either vehicle or 20 mg/kg diazepam and then subjected to electroshock. Data is shown as % of animals protected from seizure. WT animals from different lines were used as controls and data is presented to show this. Animals lacking binding at α1-, α2- and α5-containing $GABA_A$ receptors were able to be protected from seizure by acute diazepam ($F_{(1,29)}$ =30.94, p<0.001, $F_{(1,36)}$ =35.88, p<0.001 and $F_{(1,35)}$ =72.20, p<0.001 respectively). However, the lack of binding in the α125H→R mice again prevented them being protected against seizure by diazepam. All data is summarized in Table 2.

In the next series of experiments, the anticonvulsant effects of compounds with different $GABA_A$ selectivity profiles were assessed in the PTZ and MES assays in SW mice. Compounds evaluated were diazepam, which has equivalent affinity and full agonist efficacy at $GABA_A$ receptors containing either an α1, α2, α3 or α5 subunit (Pritchett *et al*., 1989); zolpidem, which has higher affinity for, and full agonist efficacy at α1- compared to α2-, α3- or α5-containing $GABA_A$ receptors (Jones *et al*., 1997); TP003, which binds to equivalent affinity to the four subtypes but only possesses efficacy at α3-containing $GABA_A$ receptors (Dias *et al*., 2005); or TP13, which binds with similar affinity to the four subtypes but has higher efficacy at the α2 and α3 compared to α1 and α5 subtypes (Table 2; McCabe *et al*., 2004). Fig. 3 shows that diazepam (Fig. 3A) and TP13 (Fig. 3D) conferred protection against PTZ-induced seizure in a dose-dependant manner ($F_{(6,41)}$ =32.23, p<0.001 and $F_{(6,29)}$ =16.22, p<0.001 respectively). Although zolpidem (Fig. 3B) and TP003 (Fig. 3C) afforded some protection against seizure level ($F_{(3,16)}$ =6.75, p<0.05 and $F_{(6,29)}$ =16.22, p<0.001 respectively) they were less efficacious at the top doses than diazepam and TP13.

Discussion

It is widely recognised that $GABA_A$ receptors are involved in convulsant pathways and previous work has suggested that the α1 subtype plays a role in this (Rudolph *et al*., 1999; Crestani *et al*., 2002). Here we have used both subtype-selective compounds as well as KI animals which lack the BZ binding site at specific $GABA_A$ subtypes to further investigate the roles of the α1-, α2-, α3- and α5-containing receptors. The KI mice were grossly phenotypically normal and had normally functioning $GABA_A$ receptors, other than their inability to bind BZs (Rudolph *et al*., 1999; McKernan *et al*., 2000; Sur *et al*., 2001; Collinson *et al*., 2002; Wafford *et al*., 2004).

Table 2 Summary of effects of different genotypes on efficacy of diazepam in PTZ and MES assays.

Genotype	Diazepam effects mediated via αx-containing subtype	Diazepam MED, mg/kg	Max. efficacy, Racine seizure level	MES assay, efficacy vs wild type	Contribution to diazepam efficacy
WT	α1 α2 α3 α5	10	1.0 ± 0		
α1H101R	α2 α3 α5	10	1.6 ± 0.4	= WT	α1 = mild
α2H101R	α1 α3 α5	10	3.4 ± 0.4	= WT	α2 = moderate
α5H105R	α1 α2 α3	10	1.3 ± 0.3	≥ WT	α5 = minimal
α125H→R	α3	No efficacy	5.7 ± 0.2	No protection	α3 = none

The cycle conditions on the Perkin Elmer thermocycler were 2 min at 93 °C, 2 min at 55 °C, 2 min at 65 °C for 1 cycle, 30 sec at 93 °C, 30 sec at 55 °C, 1 min at 65 °C for 40 cycles, 10 min at 65 °C and 15 °C soak for all mouse lines. PCR amplification resulted in a 490 bp WT and 600 bp targeted band for the α1H101R and 91 bp WT and 200 bp targeted band for the α2H101R mice. The PCR product for α5H105R mouse samples needed to be cut with the restriction endonuclease BstBI, which resulted in 400 bp WT and 300 and 100 bp targeted DNA fragments.

Drug preparation

Pentylenetetrazole (PTZ; Sigma-Aldrich, Poole, UK) was dissolved in sterile water and administered subcutaneously (s.c.) at 120 mg/kg. Diazepam (0.1–20 mg/kg; Sigma-Aldrich, Poole, UK) and zolpidem (3–30 mg/kg; Sigma-Aldrich, Poole, UK) were suspended in 0.5% methyl cellulose and administered orally (p.o.). TP003 (0.003–1.0 mg/kg; 4,2'-Difluoro-5'-[8-fluoro-7-(1-hydroxy-1-methylethyl)imidazo[1,2-á]pyridin-3-yl]biphenyl-2-carbonitrile; (Dias *et al*., 2005) and TP13 (0.003–1.0 mg/kg; 7-Cyclobutyl-6-(2-ethyl-2H-1,2,4-triazol-3-ylmethoxy)-3-phenyl-1,2,4-triazolo[4,3-b] pyridazine) were synthesized at Merck Sharp and Dohme as described elsewhere (Carling *et al*., 2005). Both TP003 and TP13 were suspended in 0.5% methyl cellulose and administered intraperitoneally (i.p.). All drugs were administered in a dosing volume of 10 ml/kg with a pretreatment time of 30 min and are expressed as free base.

PTZ test

Mice were pretreated with either vehicle or test compound 30 min before administration of PTZ (120 mg/kg s.c.) and then placed in a perspex chamber (20 cm × 20 cm) and observed for a further 30 min. The maximum level of seizure reached was rated using a scale adapted from the Racine scale (Loscher *et al*., 1991). In summary:

0 no behavioural response
1 behavioural arrest
2 orofacial movements/chewing/head nodding
3 unilateral/bilateral forelimb clonus without rearing; straub tail, extended body posture
4 all the above plus rearing
5 rearing and falling
6 full tonic seizures

Maximal electroshock test

Animals were pretreated with vehicle or test compound. After the appropriate pretreatment time, electrodes dipped in saline were attached to the ears of the animal and an electroshock (1.5 s duration, 0.5 s pulse width, 100 Hz) of 30 mA was given. Animals were observed for a maximum of 2 min following electroshock and the percentage of animals protected against tonic seizure calculated.

Statistics

Mean data were analysed by analysis of variance (ANOVA) followed by Newman-Keuls multivariate analysis test using Statistica (StatSoft Inc., Tulsa, USA) statistical software package. $P<0.05$ was taken as significant. All data were presented as mean ± SEM unless otherwise stated.

Results

Wild-type and KI animals with $GABA_A$ receptor subunits insensitive to BZs were pretreated with diazepam and then administered PTZ to determine the relative contribution of each subunit to the anticonvulsant activity of diazepam (Fig. 1). Diazepam had a significant effect on seizure protection in a dose-dependent manner in WT animals ($F_{(4,29)} = 40.38$, $p<0.001$). Diazepam retained varying degrees of anticonvulsant efficacy in the α1H101R, α2H101R and α5 H105R mice ($F_{(4,90)} = 10.74$, $p<0.001$) but animals in which the α1-, α2- and α5-containing $GABA_A$ receptors were all rendered diazepam insensitive (α125H→R mice), and in which diazepam only exerts its effects via the α3 subtype, were not protected at all against seizures.

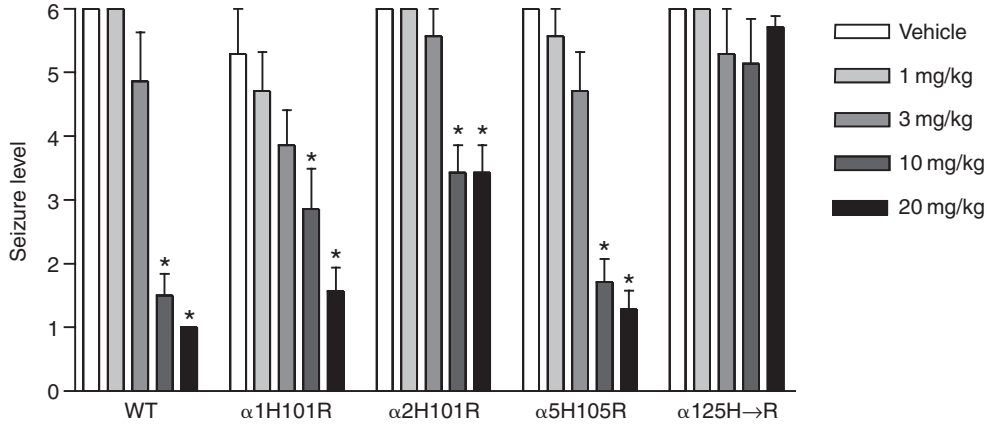

Figure 1 Effects of diazepam on PTZ-induced seizures in WT mice or mice carrying either the α1H101R, α2H101R, α5H105R or α125H→R mutations. Seizure level was measured on the modified Racine Scale (see Methods). Values shown are mean ± SEM. ($n=6$–7/group). *$p<0.05$ compared with vehicle-treated animals

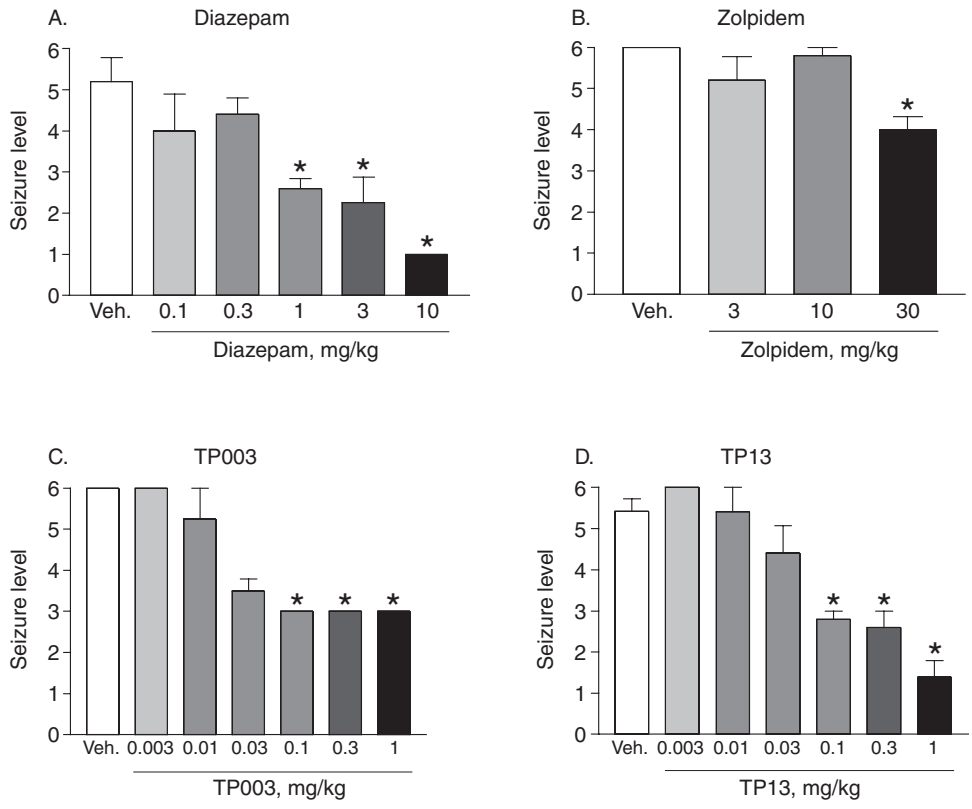

Figure 3 Protection from PTZ-induced seizures by increasing doses of A. diazepam, B. zolpidem, C. TP003 and D. TP13 in SW mice. Seizure level was measured on the Racine Scale. Values shown are mean ± SEM ($n = 6$/group). *$p < 0.05$ compared with vehicle-treated animals.

Table 3 Summary data for compounds used in PTZ and MES.

		Human recombinant $GABA_A$ receptors containing β3, γ2 plus			
Compound		α1	α2	α3	α5
TP003[a]	Ki, nM	0.32	0.54	0.50	0.26
	Relative efficacy[b]	0.00	0.12	0.78	0.15
TP13[a]	Ki, nM	0.20	0.18	0.11	0.09
	Relative efficacy	0.23	0.35	0.43	0.19
Diazepam[c]	Ki, nM	14	20	15	11
	Relative efficacy	0.93	1.02	0.8	0.87
Zolpidem[c]	Ki, nM	27	160	380	>10 000

[a] Data for TP003 and TP13 (also known as Compound 15; Carling *et al*., 2005) taken from Dias *et al.* (2005) and McCabe *et al*. (2004), respectively.
[b] Relative efficacy is defined as the extent of the potentiation of GABA EC20-equivalent current produced by either TP003 or TP13 compared to that produced by the non-selective full agonist chlordiazepoxide. Data not available for zolpidem.
[c] Data for diazepam and zolpidem taken from Atack *et al*. (1999).

Table 4 Summary of efficacy of different subtype-selective compounds in PTZ assays. Brackets indicate lower efficacy.

Compound	Effects mediated via αx-containing subtype	Max. dose, mg/kg	Max. efficacy, Racine seizure level
Diazepam	α1 α2 α3 α5	10	1.0 ± 0
Zolpidem	α1 (α2 α3)	30	4.0 ± 0.3
TP003	α3	1	3.0 ± 0
TP13	(α1) α2 α3 (α5)	1	1.4 ± 0.4

The present study demonstrating that the α5 subtype does not mediate the anticonvulsant effects of diazepam (Figs 1 and 2) is consistent with previous data showing that mice in which the α5 subunit has been deleted do not undergo spontaneous seizure activity (Collinson *et al*., 2002) and that a reduction in the expression of α5-containing receptors does not alter the sensitivity to PTZ-induced seizures (Crestani *et al*., 2002). Moreover, an α5-selective inverse agonist (which acts to selectively reduce the inhibitory effects of GABA at the α5 but not α1, α2 or α3 subtypes) does not alter PTZ sensitivity or induce kindling when dosed chronically (Dawson *et al*., 2006). Similarly, the lack of anticonvulsant efficacy of diazepam in the α125H→R mice when acting solely via the α3 subtype (Figs 1 and 2) is consistent with the fact that diazepam retains its efficacy in α3H126R mice (Low *et al*., 2000).

Despite the consistency of the present study with previous data suggesting the α3 and α5 subtypes play a minimal role in mediating the anticonvulsant effects of diazepam, we were unable to ascribe the anticonvulsant activity of diazepam primarily to the α1 subtype as described previously. Thus, it has previously been reported that the anticonvulsant efficacy of diazepam is reduced in α1H101R mice (Rudolph *et al*., 1999) and that α1 subunit-containing $GABA_A$ receptors play a role in mediating the proconvulsant effects of the inverse agonists DMCM and Ro 15–4513 (Crestani *et al*., 2002). In order to draw parallels between the present study and previous work, it is necessary to express all data in a similar fashion. Previously, the anticonvulsant activity of diazepam against PTZ-induced convulsions in animals was expressed as the percentage of mice entering tonic convulsions or displaying myoclonic jerks (Rudolph *et al*., 1999; Crestani *et al*., 2000). However, in the present study, a more detailed version of the Racine rating scale was employed in which mice scoring a Level 6 are considered to be in tonic convulsions (Loscher *et al*., 1991) and those scoring a Level 2 or 3 would be considered to be displaying myoclonic jerks. It can be seen that in PTZ-induced seizures, 100% of all WT animals score a Level 1 on the Racine Scale at a dose of 20 mg/kg diazepam. This is equivalent to the 0% of animals entering tonic convulsions described in previous work (Rudolph *et al*., 1999; Crestani *et al*., 2000). It should be noted that higher doses of diazepam were not tested to avoid any sedative effects confounding scoring in the Racine Scale. We also concur with previous data presented on the effects of the α1-preferring compound zolpidem (Crestani *et al*., 2000) and have found that at 30 mg/kg, around 80% of WT animals will still display myoclonic jerks but all animals are protected from tonic convulsions, although this may not entirely be due to the α1 subunit as at this dose there is also appreciable occupancy of α2 and α3-containing $GABA_A$ receptors (Atack *et al*., 1999).

However, even when all data is expressed in the same manner, inconsistencies are still found concerning the α1H101R mice. At 20 mg/kg diazepam, 0% and 30% of these mice displayed tonic seizure and myoclonic jerks respectively which was comparable with WT mice, unlike in previous work (Rudolph *et al*., 1999; Crestani *et al*., 2000). It is possible that this may be due to an experimental difference in the route of administration of PTZ and therefore a change in pharmacodynamics leading to a higher exposure in previous work. In turn, this may present a greater insult to the remaining $GABA_A$ receptors so preventing the complete protection seen in our data. In contrast, we have found that the $GABA_A$α2 subunit plays a larger role in mediating the anticonvulsant properties of diazepam than the α1, as 14% and 100% of mice still display tonic seizures and myoclonic jerks respectively at 20 mg/kg diazepam. The fact that a compound lacking α1 efficacy but with partial agonist efficacy at the α2 and α3 subtypes (TPA023) retains anticonvulsant activity against PTZ-induced seizures (Atack *et al*., 2006) also suggests that α2- and/or α3- but not α1-containing receptors play a role in attenuating the effects of PTZ. Assuming that the anticonvulsant effects of TPA023 are mediated by its partial agonist efficacy at the α2 and α3 subtypes, then the data with the α125H→R mice in which the α3 subtype is not involved, indicates that the α2 subtype is probably responsible for the anticonvulsant effects of TPA023. However, in the present study the α3 selective compound TP003 retains a degree of anticonvulsant activity. It may be that protection against tonic or clonic seizure is mediated through efficacy at different $GABA_A$ subtypes. Whilst TP003 has anticonvulsant properties in that it prevents mice from entering a Level 6 (tonic seizure), the data in Fig 3 clearly shows that even at 100% occupancy of $GABA_A$ receptors by TP003 1 mg/kg further protection is not seen. It is the less selective compounds such as diazepam and TP13 that are able to protect against both tonic and clonic seizure (Level 3 – an equivalent to myoclonic jerks shown in previous work (Rudolph *et al*., 1999; Crestani *et al*., 2000). However, and for whatever reason, data from pharmacological and molecular genetic (i.e., transgenic mice) studies are not necessarily comparable. In the present study, experiments with transgenic mice would suggest that the α3 subtype is not associated with the anticonvulsant effects of diazepam since the $GABA_A$α125H→R mice retain only the α3-containing receptors retain diazepam sensitivity, yet these mice are not protected against tonic seizure. Similar inconsistencies have also been found in studies to determine which subtype is responsible for the anxiolytic properties of diazepam. For example, experiments with α2H101R and α3H126R mice indicate that the α2 but not α3 subtype is responsible (Low *et al*., 2000), whereas the use of subtype-selective compounds implicate the α3 rather than α2 subtype (Dias *et al*., 2005; Atack *et al*., 2005).

MES is considered to be a model of generalized seizures of the grand mal (tonic/clonic) type, whereas PTZ is considered to be a model of petit mal (absence or myoclonic) seizures. Due to this, the $GABA_A$ receptor subtypes may have slightly different roles to play in each (Loscher and Schmidt, 1988). From the transgenic data, it can be seen that the removal of any single $GABA_A$ α subunit does not cause a great change in the efficacy of diazepam. However removal of the α1, α2 and α5 subunits in the $GABA_A$α125H→R mice means that diazepam does not retain any anticonvulsant effects. These data when taken together would suggest that only a non-selective agonist such as diazepam is preventative against seizure in this model.

In summary, the present study clearly demonstrates that no single $GABA_A$ receptor subtype is solely responsible for the anticonvulsant effects of GABA and that efficacy at different subtypes may act synergistically. Nevertheless, based on evidence

from the transgenic mice, it would appear that the α2 subtype plays a greater role than α1-containing $GABA_A$ receptors. Subtype selective compounds have been shown to possess a degree of anticonvulsant activity that is not always reconciled with the transgenic data; a discrepancy that has also been observed with respect to anxiolysis. Finally, these data indicate that a compound which selectively targets a single $GABA_A$ receptor subtype is unlikely to possess an anticonvulsant efficacy equivalent to that of a non-selective full agonist such as diazepam, although whether or not such a compound may offer advantages in terms of reduced tolerance remains to be determined.

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