

# Journal of Psychopharmacology

<http://jop.sagepub.com/>

---

## Differential contribution of GABA<sub>A</sub> receptor subtypes to the anticonvulsant efficacy of benzodiazepine site ligands

Rosa L. Fradley, Martin R. Guscott, Sharlene Bull, David J. Hallett, Simon C. Goodacre, Keith A. Wafford, Elizabeth M. Garrett, Richard J. Newman, Gillian F. O'Meara, Paul J. Whiting, Thomas W. Rosahl, Gerard R. Dawson, David S. Reynolds and John R. Atack

*J Psychopharmacol* 2007 21: 384 originally published online 8 November 2006

DOI: 10.1177/0269881106067255

The online version of this article can be found at:

<http://jop.sagepub.com/content/21/4/384>

---

Published by:



<http://www.sagepublications.com>

On behalf of:



[British Association for Psychopharmacology](http://www.bappp.org.uk)

Additional services and information for *Journal of Psychopharmacology* can be found at:

**Email Alerts:** <http://jop.sagepub.com/cgi/alerts>

**Subscriptions:** <http://jop.sagepub.com/subscriptions>

**Reprints:** <http://www.sagepub.com/journalsReprints.nav>

**Permissions:** <http://www.sagepub.com/journalsPermissions.nav>

**Citations:** <http://jop.sagepub.com/content/21/4/384.refs.html>

>> [Version of Record](#) - Jul 26, 2007

[Proof](#) - Jan 23, 2007

[What is This?](#)

# Differential contribution of GABA<sub>A</sub> receptor subtypes to the anticonvulsant efficacy of benzodiazepine site ligands

*Journal of Psychopharmacology*  
21(4) (2007) 384–391  
© 2007 British Association  
for Psychopharmacology  
ISSN 0269-8811  
SAGE Publications Ltd,  
Los Angeles, London,  
New Delhi and Singapore  
10.1177/0269881106067255

Rosa L. Fradley *Merck, Sharp and Dohme Research Laboratories, Neuroscience Research Centre, Harlow, Essex, UK.*

Martin R. Guscott *Merck, Sharp and Dohme Research Laboratories, Neuroscience Research Centre, Harlow, Essex, UK.*

Sharlene Bull *Merck, Sharp and Dohme Research Laboratories, Neuroscience Research Centre, Harlow, Essex, UK.*

David J. Hallett *Merck, Sharp and Dohme Research Laboratories, Neuroscience Research Centre, Harlow, Essex, UK.*

Simon C. Goodacre *Merck, Sharp and Dohme Research Laboratories, Neuroscience Research Centre, Harlow, Essex, UK.*

Keith A. Wafford *Merck, Sharp and Dohme Research Laboratories, Neuroscience Research Centre, Harlow, Essex, UK.*

Elizabeth M. Garrett *Merck, Sharp and Dohme Research Laboratories, Neuroscience Research Centre, Harlow, Essex, UK.*

Richard J. Newman *Merck, Sharp and Dohme Research Laboratories, Neuroscience Research Centre, Harlow, Essex, UK.*

Gillian F. O'Meara *Merck, Sharp and Dohme Research Laboratories, Neuroscience Research Centre, Harlow, Essex, UK.*

Paul J. Whiting *Merck, Sharp and Dohme Research Laboratories, Neuroscience Research Centre, Harlow, Essex, UK.*

Thomas W. Rosahl *Merck, Sharp and Dohme Research Laboratories, Neuroscience Research Centre, Harlow, Essex, UK.*

Gerard R. Dawson *Merck, Sharp and Dohme Research Laboratories, Neuroscience Research Centre, Harlow, Essex, UK.*

David S. Reynolds *Merck, Sharp and Dohme Research Laboratories, Neuroscience Research Centre, Harlow, Essex, UK.*

John R. Atack *Merck, Sharp and Dohme Research Laboratories, Neuroscience Research Centre, Harlow, Essex, UK.*

## Abstract

Non-selective benzodiazepines, such as diazepam, interact with equivalent affinity and agonist efficacy at GABA<sub>A</sub> receptors containing either an  $\alpha 1$ ,  $\alpha 2$ ,  $\alpha 3$  or  $\alpha 5$  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 ( $\alpha 1H101R$ ,  $\alpha 2H101R$  or  $\alpha 5H105R$ ) or multiple ( $\alpha 125H \rightarrow R$ )  $\alpha$  subunits that render the resulting GABA<sub>A</sub> receptors diazepam-insensitive. Furthermore, the anticonvulsant properties of diazepam, the  $\alpha 1$ - and  $\alpha 3$ -selective compounds zolpidem and TP003, respectively, and the  $\alpha 2/\alpha 3$  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  $\alpha 3$  nor  $\alpha 5$  subtypes appeared to confer anticonvulsant activity. Moreover, whereas the  $\alpha 1$  and  $\alpha 2$  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  $\alpha 2$ -containing GABA<sub>A</sub> 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<sub>A</sub>, epilepsy, benzodiazepines

## 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<sub>A</sub> 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<sub>A</sub> receptor is comprised of five subunits derived from a family of 16 genes ( $\alpha$ 1–6,  $\beta$ 1–3,  $\gamma$ 1–3,  $\delta$ ,  $\epsilon$ ,  $\pi$  and  $\theta$ ; (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<sub>A</sub> receptors in the brain contain  $\alpha$ ,  $\beta$  and  $\gamma$  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<sub>A</sub> receptor population, containing  $\beta$ ,  $\gamma$ 2 and either  $\alpha$ 1,  $\alpha$ 2,  $\alpha$ 3 or  $\alpha$ 5 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<sub>A</sub> receptor subtypes containing a BZ recognition site (i.e.  $\alpha$ 1-,  $\alpha$ 2-,  $\alpha$ 3- and  $\alpha$ 5-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  $\alpha$  subunit which render the resulting GABA<sub>A</sub> 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  $\alpha$ 1-containing GABA<sub>A</sub> 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<sub>A</sub> 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<sub>A</sub> receptor subunits. Therefore the purpose of the present study was to determine the precise contribution of each GABA<sub>A</sub> 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  $\alpha$ 1,  $\alpha$ 2 or  $\alpha$ 5 subunits ( $\alpha$ 1H101R,  $\alpha$ 2H101R and  $\alpha$ 5H105R, respectively) or a triple mutant

containing all three mutations ( $\alpha$ 125H $\rightarrow$ R). In addition, we examined the anticonvulsant effects of the GABA<sub>A</sub> subtype-selective compounds zolpidem ( $\alpha$ 1-preferring; Jones *et al.*, 1997), TP003 ( $\alpha$ 3-selective; Dias *et al.*, 2005) and TP13 ( $\alpha$ 2/ $\alpha$ 3-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  $21 \pm 2^\circ\text{C}$  and  $55 \pm 10\%$  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<sub>A</sub> receptor  $\alpha$ 1H101R, the  $\alpha$ 2H101R (Dias *et al.*, 2005) and the  $\alpha$ 5H105R 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<sub>A</sub>  $\alpha$ 125H $\rightarrow$ R mouse line was generated through crossing of the GABA<sub>A</sub>  $\alpha$ 1,  $\alpha$ 2 and  $\alpha$ 5 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<sub>A</sub>  $\alpha$ 125H $\rightarrow$ 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
GABA $\alpha$ 1H101R	5'-ATT.AAT.GGA.GAG.TGT.GGT.AAT.CTT.T-3' 5'-TCC.TTC.ATG.GTG.AAC.AAG.ACC.AGG-3'
GABA $\alpha$ 2H101R	5'-CCA.TTA.CAC.TCC.TCA.AAT.TGT.GAA.C-3' 5'-GTG.GTC.TGT.GAA.TTC.TAA.TTT.TCT.AG-3'
GABA $\alpha$ 5H105R	5'-GAG.CGA.ATC.ACG.CAG.GTG.CGA.ACA.GAC-3' 5'-CCC.GAC.CTG.CTA.CCC.AGG.GTA-3'

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 490bp WT and 600bp targeted band for the  $\alpha 1H101R$  and 91bp WT and 200bp targeted band for the  $\alpha 2H101R$  mice. The PCR product for  $\alpha 5H105R$  mouse samples needed to be cut with the restriction endonuclease BstBI, which resulted in 400bp WT and 300 and 100bp targeted DNA fragments.

### Drug preparation

Pentylenetetrazole (PTZ; Sigma-Aldrich, Poole, UK) was dissolved in sterile water and administered subcutaneously (s.c.) at 120mg/kg. Diazepam (0.1–20mg/kg; Sigma-Aldrich, Poole, UK) and zolpidem (3–30mg/kg; Sigma-Aldrich, Poole, UK) were suspended in 0.5% methyl cellulose and administered orally (p.o.). TP003 (0.003–1.0mg/kg; 4,2'-Difluoro-5'-[8-fluoro-7-(1-hydroxy-1-methylethyl)imidazo[1,2-*a*]pyridin-3-yl]biphenyl-2-carbonitrile; (Dias *et al.*, 2005) and TP13 (0.003–1.0mg/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

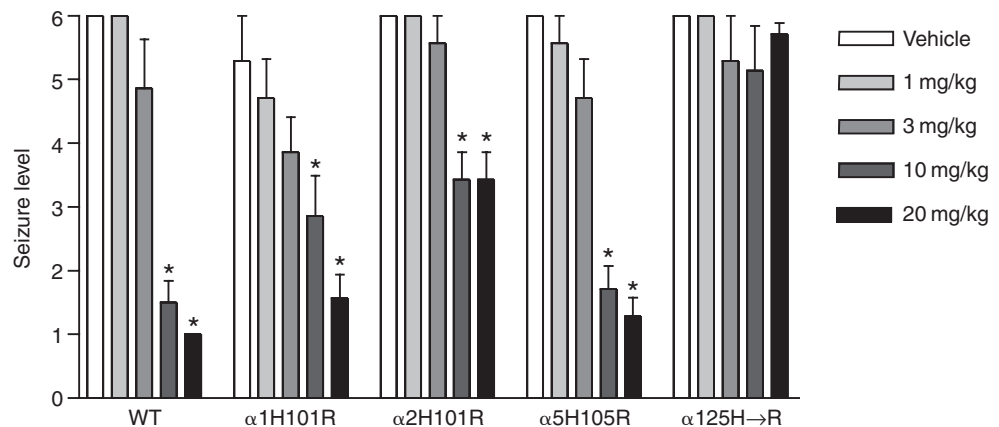
Animals were pretreated with vehicle or test compound. After the appropriate pretreatment time, electrodes dipped in saline were attached to the ears of the animal and an electroshock (1.5 s duration, 0.5 s pulse width, 100 Hz) of 30 mA was given. Animals were observed for a maximum of 2 min following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

### Statistics

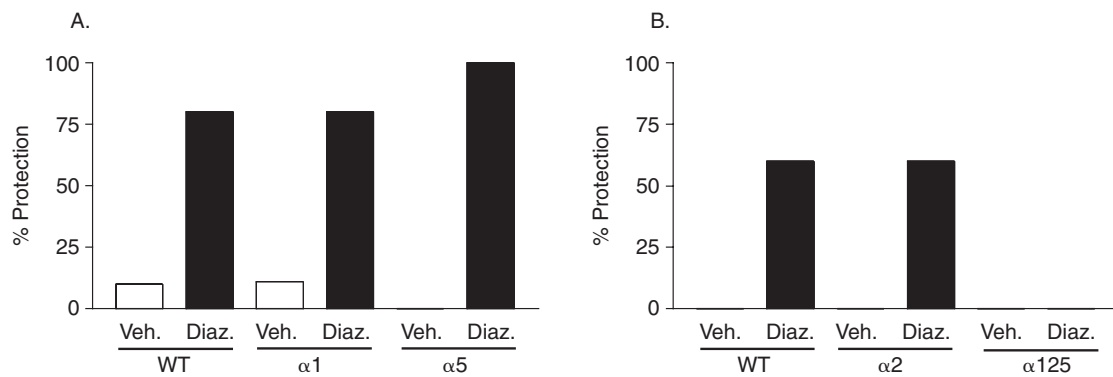
Mean data were analysed by analysis of variance (ANOVA) followed by Newman-Keuls multivariate analysis test using Statistica (StatSoft Inc., Tulsa, USA) statistical software package.  $P < 0.05$  was taken as significant. All data were presented as mean ± SEM unless otherwise stated.

### Results

Wild-type and KI animals with GABA<sub>A</sub> 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  $\alpha 1H101R$ ,  $\alpha 2H101R$  and  $\alpha 5H105R$  mice ( $F_{(4,90)} = 10.74$ ,  $p < 0.001$ ) but animals in which the  $\alpha 1$ -,  $\alpha 2$ - and  $\alpha 5$ -containing GABA<sub>A</sub> receptors were all rendered diazepam insensitive ( $\alpha 125H \rightarrow R$  mice), and in which diazepam only exerts its effects via the  $\alpha 3$  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  $\alpha 1H101R$ ,  $\alpha 2H101R$ ,  $\alpha 5H105R$  or  $\alpha 125H \rightarrow R$  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 2** Protection from MES-induced seizures by diazepam (Diaz., 20 mg/kg) in mice carrying either the  $\alpha 1H101R$ ,  $\alpha 2H101R$ ,  $\alpha 5H105R$  or  $\alpha 125H \rightarrow R$  mutations. Animals were observed for a maximum of 2 min following electroshock and % of animals protected against tonic seizure calculated ( $n=9-10/\text{group}$ ).

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  $\alpha 1$ -,  $\alpha 2$ - and  $\alpha 5$ -containing GABA<sub>A</sub> 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  $\alpha 125H \rightarrow R$  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<sub>A</sub> 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<sub>A</sub> receptors containing either an  $\alpha 1$ ,  $\alpha 2$ ,  $\alpha 3$  or  $\alpha 5$  subunit (Pritchett *et al.*, 1989); zolpidem, which has higher affinity for, and full agonist efficacy at  $\alpha 1$ - compared to  $\alpha 2$ -,  $\alpha 3$ - or  $\alpha 5$ -containing GABA<sub>A</sub> receptors (Jones *et al.*, 1997); TP003, which binds to equivalent affinity to the four subtypes but only possesses efficacy at  $\alpha 3$ -containing GABA<sub>A</sub> receptors (Dias *et al.*, 2005); or TP13, which binds with similar affinity to the four subtypes but has higher efficacy at the  $\alpha 2$  and  $\alpha 3$  compared to  $\alpha 1$

and  $\alpha 5$  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.

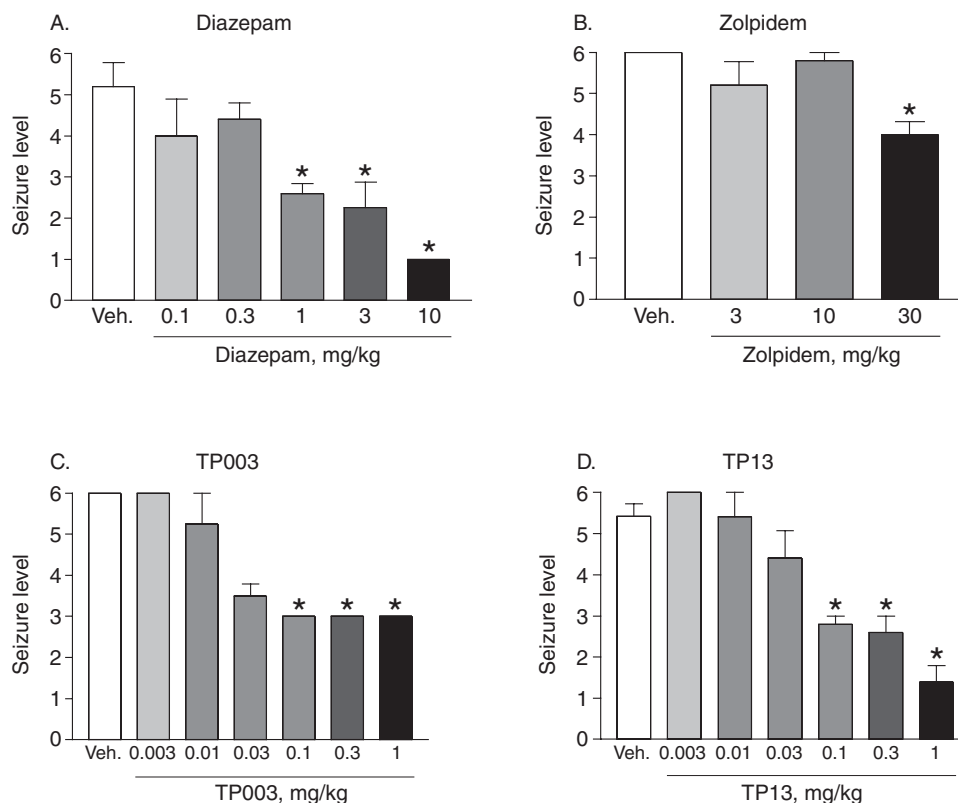
## Discussion

It is widely recognised that GABA<sub>A</sub> receptors are involved in convulsant pathways and previous work has suggested that the  $\alpha 1$  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<sub>A</sub> subtypes to further investigate the roles of the  $\alpha 1$ -,  $\alpha 2$ -,  $\alpha 3$ - and  $\alpha 5$ -containing receptors. The KI mice were grossly phenotypically normal and had normally functioning GABA<sub>A</sub> 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 $\alpha x$ -containing subtype	Diazepam MED, mg/kg	Max. efficacy, Racine seizure level	MES assay, efficacy vs wild type	Contribution to diazepam efficacy
WT	$\alpha 1$ $\alpha 2$ $\alpha 3$ $\alpha 5$	10	$1.0 \pm 0$		
$\alpha 1H101R$	$\alpha 2$ $\alpha 3$ $\alpha 5$	10	$1.6 \pm 0.4$	= WT	$\alpha 1$ = mild
$\alpha 2H101R$	$\alpha 1$ $\alpha 3$ $\alpha 5$	10	$3.4 \pm 0.4$	= WT	$\alpha 2$ = moderate
$\alpha 5H105R$	$\alpha 1$ $\alpha 2$ $\alpha 3$	10	$1.3 \pm 0.3$	$\geq$ WT	$\alpha 5$ = minimal
$\alpha 125H \rightarrow R$	$\alpha 3$	No efficacy	$5.7 \pm 0.2$	No protection	$\alpha 3$ = none

**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  $\pm$  SEM ( $n=6$ /group). \* $p < 0.05$  compared with vehicle-treated animals.



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

Compound		Human recombinant GABA <sub>A</sub> receptors containing $\beta 3$ , $\gamma 2$ plus			
		$\alpha 1$	$\alpha 2$	$\alpha 3$	$\alpha 5$
TP003 <sup>a</sup>	Ki, nM	0.32	0.54	0.50	0.26
	Relative efficacy <sup>b</sup>	0.00	0.12	0.78	0.15
TP13 <sup>a</sup>	Ki, nM	0.20	0.18	0.11	0.09
	Relative efficacy	0.23	0.35	0.43	0.19
Diazepam <sup>c</sup>	Ki, nM	14	20	15	11
	Relative efficacy	0.93	1.02	0.8	0.87
Zolpidem <sup>c</sup>	Ki, nM	27	160	380	>10 000

<sup>a</sup> 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.

<sup>b</sup> Relative efficacy is defined as the extent of the potentiation of GABA EC<sub>20</sub>-equivalent current produced by either TP003 or TP13 compared to that produced by the non-selective full agonist chlordiazepoxide. Data not available for zolpidem.

<sup>c</sup> 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 $\alpha x$ -containing subtype	Max. dose, mg/kg	Max. efficacy, Racine seizure level
Diazepam	$\alpha 1$ $\alpha 2$ $\alpha 3$ $\alpha 5$	10	1.0 $\pm$ 0
Zolpidem	$\alpha 1$ ( $\alpha 2$ $\alpha 3$ )	30	4.0 $\pm$ 0.3
TP003	$\alpha 3$	1	3.0 $\pm$ 0
TP13	( $\alpha 1$ ) $\alpha 2$ $\alpha 3$ ( $\alpha 5$ )	1	1.4 $\pm$ 0.4

The present study demonstrating that the  $\alpha 5$  subtype does not mediate the anticonvulsant effects of diazepam (Figs 1 and 2) is consistent with previous data showing that mice in which the  $\alpha 5$  subunit has been deleted do not undergo spontaneous seizure activity (Collinson *et al.*, 2002) and that a reduction in the expression of  $\alpha 5$ -containing receptors does not alter the sensitivity to PTZ-induced seizures (Crestani *et al.*, 2002). Moreover, an  $\alpha 5$ -selective inverse agonist (which acts to selectively reduce the inhibitory effects of GABA at the  $\alpha 5$  but not  $\alpha 1$ ,  $\alpha 2$  or  $\alpha 3$  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  $\alpha 125H \rightarrow R$  mice when acting solely via the  $\alpha 3$  subtype (Figs 1 and 2) is consistent with the fact that diazepam retains its efficacy in  $\alpha 3H126R$  mice (Low *et al.*, 2000).

Despite the consistency of the present study with previous data suggesting the  $\alpha 3$  and  $\alpha 5$  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  $\alpha 1$  subtype as described previously. Thus, it has previously been reported that the anticonvulsant efficacy of diazepam is reduced in  $\alpha 1H101R$  mice (Rudolph *et al.*, 1999) and that  $\alpha 1$  subunit-containing GABA<sub>A</sub> 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  $\alpha 1$ -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  $\alpha 1$  subunit as at this dose there is also appreciable occupancy of  $\alpha 2$  and  $\alpha 3$ -containing GABA<sub>A</sub> receptors (Atack *et al.*, 1999).

However, even when all data is expressed in the same manner, inconsistencies are still found concerning the  $\alpha 1H101R$  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<sub>A</sub> receptors so preventing the complete protection seen in our data. In contrast, we have found that the GABA<sub>A</sub> $\alpha 2$  subunit plays a larger role in mediating the anticonvulsant properties of diazepam than the  $\alpha 1$ , 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  $\alpha 1$  efficacy but with partial agonist efficacy at the  $\alpha 2$  and  $\alpha 3$  subtypes (TPA023) retains anticonvulsant activity against PTZ-induced seizures (Atack *et al.*, 2006) also suggests that  $\alpha 2$ - and/or  $\alpha 3$ - but not  $\alpha 1$ -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  $\alpha 2$  and  $\alpha 3$  subtypes, then the data with the  $\alpha 125H \rightarrow R$  mice in which the  $\alpha 3$  subtype is not involved, indicates that the  $\alpha 2$  subtype is probably responsible for the anticonvulsant effects of TPA023. However, in the present study the  $\alpha 3$  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<sub>A</sub> 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<sub>A</sub> 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  $\alpha 3$  subtype is not associated with the anticonvulsant effects of diazepam since the GABA<sub>A</sub> $\alpha 125H \rightarrow R$  mice retain only the  $\alpha 3$ -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  $\alpha 2H101R$  and  $\alpha 3H126R$  mice indicate that the  $\alpha 2$  but not  $\alpha 3$  subtype is responsible (Low *et al.*, 2000), whereas the use of subtype-selective compounds implicate the  $\alpha 3$  rather than  $\alpha 2$  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<sub>A</sub> 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<sub>A</sub>  $\alpha$  subunit does not cause a great change in the efficacy of diazepam. However removal of the  $\alpha 1$ ,  $\alpha 2$  and  $\alpha 5$  subunits in the GABA<sub>A</sub> $\alpha 125H \rightarrow R$  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<sub>A</sub> 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  $\alpha 2$  subtype plays a greater role than  $\alpha 1$ -containing GABA<sub>A</sub> 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<sub>A</sub> 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.

## References

- Ashton H (1994) Guidelines for the rational use of benzodiazepines. When and what to use. *Drugs* 48: 25–40
- Atack J R, Smith A J, Emms F, McKernan R M (1999) Regional differences in the inhibition of mouse in vivo [<sup>3</sup>H]Ro 15–1788 binding reflect selectivity for  $\alpha 1$  versus  $\alpha 2$  and  $\alpha 3$  subunit-containing GABA<sub>A</sub> receptors. *Neuropsychopharmacology* 20: 255–262
- Atack J R, Hutson P H, Collinson N, Marshall G, Bentley G, Moyes C, Cook S M, Collins I, Wafford K, McKernan R M, Dawson G R (2005) Anxiogenic properties of an inverse agonist selective for  $\alpha 3$  subunit-containing GABA<sub>A</sub> receptors. *Br J Pharmacol* 144: 357–366
- Atack J R, Wafford K A, Tye S J, Cook S M, Sohal B, Pike A, Sur C, Melillo D, Bristow L, Bromidge F, Ragan I, Kerby J, Street L, Carling R, Castro J L, Whiting P, Dawson G R, McKernan R M (2006) TPA023 [7-(1,1-Dimethylethyl)-6-(2-ethyl-2H-1,2,4-triazol-3-yl-methoxy)-3-(2-fluorophenyl)-1,2,4-triazolo[4,3-b]pyridazine], an agonist selective for  $\alpha 2$ - and  $\alpha 3$ -containing GABA<sub>A</sub> receptors, is a non-sedating anxiolytic in rodents and primates. *J Pharmacol Exp Ther* 316: 410–422
- Carling R W, Madin A, Guiblin A, Russell M G N, Moore K W, Mitchinson A, Sohal B, Pike A, Cook S M, Ragan I C, McKernan R M, Quirk K, Ferris P, Marshall G, Thompson S A, Wafford K A, Dawson G R, Atack J R, Castro J L, Street L J (2005) 7-(1,1-Dimethylethyl)-6-(2-ethyl-2H-1,2,4-triazol-3-ylmethoxy)-3-(2-fluorophenyl)-1,2,4-triazolo[4,3-b]pyridazine: a functionally selective  $\alpha 2/\alpha 3$ -subtype selective agonist that exhibits potent anxiolytic activity but is not sedating in animal models. *J Med Chem* 48: 7089–7092
- Collinson N, Kuenzi F M, Jarolimek W, Maubach K A, Cothliff R, Sur C, Smith A, Otu F M, Howell O, Atack J R, McKernan R M, Seabrook G R, Dawson G R, Whiting P J, Rosahl T W (2002) Enhanced learning and memory and altered GABAergic synaptic transmission in mice lacking the  $\alpha 5$  subunit of the GABA<sub>A</sub> receptor. *J Neurosci* 22: 5572–5580
- Crestani F, Martin J R, Mohler H, Rudolph U (2000) Mechanism of action of the hypnotic zolpidem in vivo. *Br J Pharmacol* 131: 1251–1254
- Crestani F, Assandri R, Tauber M, Martin J R, Rudolph U (2002) Contribution of the  $\alpha 1$ -GABA<sub>A</sub> receptor subtype to the pharmacological actions of benzodiazepine site inverse agonists. *Neuropharmacology* 43: 679–684
- Dawson G R, Maubach K A, Collinson N, Cobain M, Everitt B J, Macleod A M, Choudhury H I, Macdonald L, Pillai G, Rycroft W, Smith A J, Sternfeld F, Tattersall F D, Wafford K A, Reynolds D S, Seabrook G, Atack J R (2006) An inverse agonist selective for  $\alpha 5$  subunit-containing GABA<sub>A</sub> receptors enhances cognition. *J Pharmacol Exp Ther* 316: 1335–1345
- Dias R, Sheppard W F A, Fradley R L, Garrett E M, Stanley J L, Tye S J, Goodacre S, Lincoln R J, Cook S M, Conley R, Hallett D, Humphries A C, Thompson S A, Wafford K A, Street L J, Castro J L, Whiting P J, Rosahl T W, Atack J R, McKernan R M, Dawson G R, Reynolds D S (2005) Evidence for a significant role of  $\alpha 3$ -containing GABA<sub>A</sub> receptors in mediating the anxiolytic effects of benzodiazepines. *J Neuroscience* 25: 10682–10688
- Fritschy J M, Weinmann O, Wenzel A, Benke D (1998) Synapse-specific localization of NMDA and GABA<sub>A</sub> receptor subunits revealed by antigen-retrieval immunohistochemistry. *J Comp Neurol* 390: 194–210
- Haigh J R, Feely M (1988) Tolerance to the anticonvulsant effect of benzodiazepines. *Trends Pharmacol Sci* 9: 361–366
- Jones A, Korpi E R, McKernan R M, Pelz R, Nusser Z, Makela R, Mellor J R, Pollard S, Bahn S, Stephenson F A, Randall A D, Sieghart W, Somogyi P, Smith A J, Wisden W (1997) Ligand-gated ion channel subunit partnerships: GABA<sub>A</sub> receptor  $\alpha 6$  subunit gene inactivation inhibits  $\alpha$  subunit expression. *J Neurosci* 17: 1350–1362
- Jones-Davis D M, Macdonald R L (2003) GABA(A) receptor function and pharmacology in epilepsy and status epilepticus. *Curr Opin Pharmacol* 3: 12–18
- Kralic J E, O'Buckley T K, Khisti R T, Hodge C W, Homanics G E, Morrow A L (2002) GABA<sub>A</sub> receptor alpha-1 subunit deletion alters receptor subtype assembly, pharmacological and behavioral responses to benzodiazepines and zolpidem. *Neuropharmacology* 43: 685–694
- Kuenzi F M, Fitzjohn S M, Morton R A, Collingridge G L, Seabrook G R (2000) Reduced long-term potentiation in hippocampal slices prepared using sucrose-based artificial cerebrospinal fluid. *J Neurosci Methods* 100: 117–122
- Loscher W, Honack D, Fassbender C P, Nolting B (1991) The role of technical, biological and pharmacological factors in the laboratory evaluation of anticonvulsant drugs. III. Pentylentetrazole seizure models. *Epilepsy Res* 8: 171–189
- Loscher W, Schmidt D (1988) Which animal models should be used in the search for new antiepileptic drugs? A proposal based on experimental and clinical considerations. *Epilepsy Res* 2: 145–181
- Low K, Crestani F, Keist R, Benke D, Brunig I, Benson J A, Fritschy J M, Rulicke T, Bluethmann H, Mohler H, Rudolph U (2000) Molecular and neuronal substrate for the selective attenuation of anxiety. *Science* 290: 131–134
- McCabe C, Shaw D, Atack J R, Street L J, Wafford K A, Dawson G R, Reynolds D S, Leslie J C (2004) Subtype-selective GABAergic drugs facilitate extinction of mouse operant behaviour. *Neuropharmacology* 46: 171–178
- McKernan R M, Rosahl T W, Reynolds D S, Sur C, Wafford K A, Atack J R, Farrar S, Myers J, Cook G, Ferris P, Garrett L, Bristow L, Marshall G, Macaulay A, Brown N, Howell O, Moore K W, Carling R W, Street L J, Castro J L, Ragan C I, Dawson G R, Whiting P J (2000) Sedative but not anxiolytic properties of benzodiazepines are mediated by the GABA<sub>A</sub> receptor  $\alpha 1$  subtype. *Nat Neurosci* 3: 587–592
- McKernan R M, Whiting P J (1996) Which GABA<sub>A</sub>-receptor subtypes really occur in the brain? *Trends Neurosci* 19: 139–143
- Meldrum B (2002) Do preclinical seizure models preselect certain adverse effects of antiepileptic drugs. *Epilepsy Res* 50: 33–40
- Pirker S, Schwarzer C, Wieselthaler A, Sieghart W, Sperk G (2000) GABA<sub>A</sub> receptors: immunocytochemical distribution of 13 subunits in the adult rat brain. *Neuroscience* 101: 815–850
- Pritchett D B, Luddens H, Seeburg P H (1989) Type I and type II GABA<sub>A</sub>-benzodiazepine receptors produced in transfected cells. *Science* 245: 1389–1392
- Rudolph U, Crestani F, Benke D, Brunig I, Benson J A, Fritschy J M, Martin J R, Bluethmann H, Mohler H (1999) Benzodiazepine actions mediated by specific gamma-aminobutyric acid<sub>A</sub> receptor subtypes. *Nature* 401: 796–800
- Scheuer M L, Pedley T A (1990) The evaluation and treatment of seizures. *N Engl J Med* 323: 1468–1474

- Schwenk F, Baron U, Rajewsky K (1995) A cre-transgenic mouse strain for the ubiquitous deletion of loxP-flanked gene segments including deletion in germ cells. *Nucleic Acids Res* 23: 5080–5081
- Simon J, Wakimoto H, Fujita N, Lalande M, Barnard E A (2004) Analysis of the set of GABA<sub>A</sub> receptor genes in the human genome. *J Biol Chem* 279: 41422–41435
- Singhi S, Singhi P, Dass R (2003) Status epilepticus: emergency management. *Indian J Pediatr* 70 (suppl. 1): S17–S22
- Sur C, Wafford K A, Reynolds D S, Hadingham K L, Bromidge F, Macaulay A, Collinson N, O'Meara G, Howell O, Newman R, Myers J, Atack J R, Dawson G R, McKernan R M, Whiting P J, Rosahl T W (2001) Loss of the major GABA<sub>A</sub> receptor subtype in the brain is not lethal in mice. *J Neurosci* 21: 3409–3418
- Wafford K A, Macaulay A J, Fradley R, O'Meara G F, Reynolds D S, Rosahl T W (2004) Differentiating the role of  $\gamma$ -aminobutyric acid type<sub>A</sub> (GABA<sub>A</sub>) receptor subtypes. *Biochem Soc Trans* 32: 553–556