Antihyperalgesia by α2-GABAA Receptors Occurs Via a Genuine Spinal Action and Does Not Involve Supraspinal Sites

OPEN

Neuropsychopharmacology (2014) 39, 477–487

www.neuropsychopharmacology.org

Antihyperalgesia by α2-GABA$_A$ Receptors Occurs Via a Genuine Spinal Action and Does Not Involve Supraspinal Sites

Jolly Paul[1,2], Gonzalo E Yévenes[1], Dietmar Benke[1,2], Alessandra Di Lio[1,2], William T Ralvenius[1,2,3], Robert Witschi[1,2], Louis Scheurer[1], James M Cook[4], Uwe Rudolph[5], Jean-Marc Fritschy[1,2] and Hanns Ulrich Zeilhofer*[,1,2,3]

[1]Institute of Pharmacology and Toxicology, University of Zurich, Zurich, Switzerland; [2]Zurich Neuroscience Center (ZNZ), Zurich, Switzerland; [3]Institute of Pharmaceutical Sciences, Swiss Federal Institute of Technology (ETH) Zurich, Zurich, Switzerland; [4]Department of Chemistry and Biochemistry, University of Wisconsin-Milwaukee, USA; [5]Laboratory of Genetic Neuropharmacology, McLean Hospital, and Department of Psychiatry, Harvard Medical School, MA, USA

Drugs that enhance GABAergic inhibition alleviate inflammatory and neuropathic pain after spinal application. This antihyperalgesia occurs mainly through GABA$_A$ receptors (GABA$_A$Rs) containing α2 subunits (α2-GABA$_A$Rs). Previous work indicates that potentiation of these receptors in the spinal cord evokes profound antihyperalgesia also after systemic administration, but possible synergistic or antagonistic actions of supraspinal α2-GABA$_A$Rs on spinal antihyperalgesia have not yet been addressed. Here we generated two lines of GABA$_A$R-mutated mice, which either lack α2-GABA$_A$Rs specifically from the spinal cord, or, which express only benzodiazepine-insensitive α2-GABA$_A$Rs at this site. We analyzed the consequences of these mutations for antihyperalgesia evoked by systemic treatment with the novel non-sedative benzodiazepine site agonist HZ166 in neuropathic and inflammatory pain. Wild-type mice and both types of mutated mice had similar baseline nociceptive sensitivities and developed similar hyperalgesia. However, antihyperalgesia by systemic HZ166 was reduced in both mutated mouse lines by about 60% and was virtually indistinguishable from that of global point-mutated mice, in which all α2-GABA$_A$Rs were benzodiazepine insensitive. The major (α2-dependent) component of GABA$_A$R-mediated antihyperalgesia was therefore exclusively of spinal origin, whereas supraspinal α2-GABA$_A$Rs had neither synergistic nor antagonistic effects on antihyperalgesia. Our results thus indicate that drugs that specifically target α2-GABA$_A$Rs exert their antihyperalgesic effect through enhanced spinal nociceptive control. Such drugs may therefore be well-suited for the systemic treatment of different chronic pain conditions.

Neuropsychopharmacology (2014) **39,** 477–487; doi:10.1038/npp.2013.221; published online 18 September 2013

Keywords: neuropathic pain; analgesia; GABA; benzodiazepine; spinal cord; mouse model

INTRODUCTION

Chronic neuropathic pain syndromes are frequently unresponsive to classical analgesic drugs including cyclooxygenase inhibitors and opioids. Drugs most effective in these pain conditions include anticonvulsant drugs that modulate or block voltage-gated Na^+ or Ca^{2+} channels (Sang and Hayes, 2006). Other anticonvulsive drugs with a different mode of action include the benzodiazepine site agonists, which enhance neuronal inhibition through facilitation of GABA$_A$ receptor-mediated neurotransmission. Diminished GABAergic or glycinergic inhibition in the spinal dorsal horn (ie, in the sensory part of the spinal cord) has been shown to be a major contributor to chronic pain syndromes (Ahmadi *et al*, 2002; Coull *et al*, 2003; Harvey *et al*, 2004), suggesting that drugs that facilitate spinal inhibition might correct a major component of the maladaptive neuroplasticity underlying chronic pain states. In line with this concept, previous work has shown that spinal injection of benzodiazepine site agonists provides pain relief in a number of rodent models of inflammatory and neuropathic pain (Knabl *et al*, 2008; Luger *et al*, 1995; Witschi *et al*, 2011).

Mammalian GABA$_A$Rs form a heterogeneous family of heteropentameric ion channels assembled from a repertoire of 19 subunits. The most prevalent subtypes of GABA$_A$Rs contain two α-, two β- and one γ2- subunits. Pharmacological properties of the different GABA$_A$R subtypes are best characterized by the type of α-subunit present in the individual receptors (Olsen and Sieghart, 2008). Experiments in genetically modified mice demonstrated a particular relevance of GABA$_A$Rs with an α2-type benzodiazepine pharmacology (α2-GABA$_A$Rs) for antihyperalgesia mediated

*Correspondence: Dr HU Zeilhofer, Institute of Pharmacology and Toxicology, University of Zurich, Winterthurerstrasse 190, CH-8057 Zurich, Switzerland, Tel: +41 44 63 55912, Fax: +41 44 63 55988, E-mail: zeilhofer@pharma.uzh.ch
Received 9 May 2013; revised 8 August 2013; accepted 19 August 2013; accepted article preview online 28 August 2013

by spinally applied benzodiazepines (Knabl *et al*, 2008). These and subsequent experiments (Knabl *et al*, 2009) established that the antihyperalgesic actions of benzodiazepine site agonists occur independently from the sedative action, which is mediated by α1-$GABA_A$Rs (Rudolph *et al*, 1999). More recent experiments using novel benzodiazepine site ligands with improved subunit specificity (ie, reduced or absent activity at α1-$GABA_A$Rs) have shown that such novel compounds reduce nerve injury-induced and inflammation-induced hyperalgesia also after systemic administration (Di Lio *et al*, 2011; Knabl *et al*, 2008; Knabl *et al*, 2009; Munro *et al*, 2009; Munro *et al*, 2008; Reichl *et al*, 2012). While the contribution of spinal $GABA_A$Rs to this antihyperalgesia is likely, although still not formally proven, the relevance of $GABA_A$R subtypes in supraspinal circuits is unclear. Such supraspinal $GABA_A$Rs might contribute to antihyperalgesia through a genuine antihyperalgesic effect, eg, through $GABA_A$Rs in the rostral agranular insular cortex (Jasmin *et al*, 2003), or indirectly through the reversal of anxiety-induced or stress-induced hyperalgesia (Andre *et al*, 2005). The latter possibility is particularly relevant as α2-$GABA_A$Rs also contribute to benzodiazepine-mediated anxiolysis (Löw *et al*, 2000; Morris *et al*, 2006). In contrast, supraspinal $GABA_A$Rs might also counteract spinal antihyperalgesia through the silencing antinociceptive tracts descending from the periaqueductal gray or the rostroventromedial medulla (Harris and Westbrook, 1995; Luger *et al*, 1995; Tatsuo *et al*, 1999). It is thus possible that activation of supraspinal $GABA_A$Rs either facilitates or constrains spinal antihyperalgesia.

To address these questions, we have generated two lines of $GABA_A$R-mutated mice. The first line (*hoxb8*-$\alpha2^{-/-}$) carries a tissue-specific deletion of the $GABA_A$R α2 subunit from all spinal neurons, astrocytes, and primary sensory neurons up to the mid cervical level (approximately C4). This tissue-specific ablation was achieved by crossing mice that carried a $GABA_A$R α2 (*Gabra2*) allele flanked by two loxP sites ($\alpha2^{fl}$; Witschi *et al*, 2011) with mice expressing the cre recombinase under the transcriptional control of the *hoxb8* homeobox gene (Witschi *et al*, 2010). The second line can be viewed as a tissue-specific point-mutated α2-$GABA_A$R mouse line (*hoxb8*-$\alpha2^{R/-}$), which carries in addition to one $\alpha2^{fl}$ allele, a benzodiazepine-insensitive H101R point-mutated *Gabra2* allele ($\alpha2^{R}$; Löw *et al*, 2000), and the *hoxb8*-cre transgene. At supraspinal sites, this line expresses the point-mutated allele together with a fully functional ('wild-type') $\alpha2^{fl}$ allele, whereas in primary sensory neurons and in the spinal nervous system only the point-mutated allele is expressed. For pharmacological analyses, we used the novel non-sedative 8-acetyleno-2′-pyridoimidazobenzodiazepine HZ166 (ethyl 8-ethynyl-6-(pyridin-2-yl)-4H-benzo[f]imidazo[1,5-a][1,4]diazepine-3-carboxylate, compound 2 in Rivas *et al*, 2009), which has previously been shown to exhibit antihyperalgesic properties in the absence of sedation in mice (Di Lio *et al*, 2011). Analysis of the antihyperalgesic effects of HZ166 in the two mutated mouse lines and comparison of these effects with those obtained in wild-type mice and in mice in which all α2-$GABA_A$Rs had been rendered benzodiazepine-insensitive revealed that activation of supraspinal α2-$GABA_A$Rs neither exerts a positive nor a negative impact on the antihyperalgesic actions of systemically applied HZ166.

MATERIALS AND METHODS

Generation of $GABA_A$R-Mutated Mice

Three strains of $GABA_A$R α2 (*gabra2*)-mutated mice were used to generate the genotypes needed for the present study: (i) mice carrying $GABA_A$R α2 alleles flanked by loxP sites ($\alpha2^{fl}$; Witschi *et al*, 2011), (ii) mice carrying a H101R point-mutated 'knock-in' allele ($\alpha2^{R}$; Löw *et al*, 2000), and (iii) *hoxb8*-cre transgenic mice (Witschi *et al*, 2010). *Hoxb8*-$\alpha2^{-/-}$ mice and *hoxb8*-cre-negative ($\alpha2^{fl/fl}$) littermates were obtained from crossings of *hoxb8*-cre-positive $\alpha2^{fl/fl}$ mice with $\alpha2^{fl/fl}$ mice. *Hoxb8*-$\alpha2^{R/-}$ mice and *hoxb8*-cre-negative ($\alpha2^{R/fl}$) littermates were generated by crossing *hoxb8*-cre-positive $\alpha2^{fl/fl}$ mice with $\alpha2^{R/R}$. All mouse lines had been backcrossed to a C57BL/6 J background for at least 10 generations and were maintained on this background throughout the study. During the breeding of *hoxb8*-$\alpha2^{-/-}$ mice, a small number of mice were born carrying a non-conditional knockout allele of the *gabra2* gene, which had occurred through undesired recombination events in the germline. To ensure that these mice were excluded from all experiments and breeding, we verified the absence of the knockout allele through PCR analyses performed on DNA extracted from ear biopsies. This tissue was chosen because analysis of *hoxb8*-cre ROSA26-lacZ double transgenic reporter mice had demonstrated that *hoxb8*-cre was not expressed in outer ear tissue at any developmental stage.

Morphology

Distribution of $GABA_A$R α2 protein was studied in brain and spinal cord sections obtained from adult *hoxb8*-$\alpha2^{-/-}$ and *hoxb8*-$\alpha2^{R/-}$ mice and from *hoxb8*-cre^{-} littermates ($\alpha2^{fl/fl}$ and $\alpha2^{R/fl}$ mice). For immunoperoxidase stainings, a polyclonal antibody directed against the N-terminal nine amino acids (NIQEDEAKN) of the $GABA_A$R α2 subunit (Fritschy and Möhler, 1995) was used as the primary antibody. Stainings (triplicates) were made as described previously (Paul *et al*, 2012). Sections from $GABA_A$ receptor-mutated mice and from control littermates were treated in a strictly parallel fashion.

Quantitative reverse Transcriptase PCR

Four to six lumbar spinal cords, lumbar DRGs, and hippocampi were rapidly removed from euthanized adult *hoxb8*-$\alpha2^{-/-}$ mice and $\alpha2^{fl/fl}$ littermates. mRNA was transcribed into cDNA using the QuantiTect Reverse Transcription Kit (Qiagen no.205311). Expression of $GABA_A$R subunits was assessed using *β-actin* as reference gene (for details of the assays see Witschi *et al* (2011)).

[^{3}H]Ro 15-4513 Binding Assay

HEK293 cells maintained in DMEM/10% FBS in poly-lysine-coated culture dishes (10 cm) were transfected with plasmids containing the subunit combination α2β3γ2 or α2(H101R)β3γ2 (7 mg total DNA, ratio 1 : 1 : 2) using jetPEI transfection reagent (Polyplus-transfection). Twenty-four hours after transfection, HEK293 cells were collected in PBS. HEK293 cells were homogenized in 10 vol 10 mM Tris pH 7.5, 0.32 M sucrose, protease inhibitor cocktail (complete

Mini, Roche Applied Science) and centrifuged at 1000 *g* for 10 min. The supernatant was carefully removed and centrifuged at 4 °C again for 20 min at 25 000 *g* at 4 °C. The crude membrane pellet was resuspended in 10 mM Tris-HCl pH 7.4, protease inhibitor cocktail and washed once by centrifugation and re-suspension. Aliquots of the crude membranes prepared from HEK293 cells expressing the $\alpha2\beta3\gamma2$ or $\alpha2$(H101R)$\beta3\gamma2$ subunit combination (150–200 μg protein) were incubated with increasing concentrations of HZ166 (10^{-8}–10^{-4} M) and 6.3 nM [^{3}H]Ro 15-4513 (22.7 Ci/mmol, PerkinElmer) in a total volume of 200 μl for 90 min on ice. Subsequently, the samples were filtered onto glass fiber filters using a 12-channnel semiautomated cell harvester (Scatron) and washed with ice-cold buffer (10 mM Tris-HCl pH 7.4). Non-specific [^{3}H]Ro 15-4513 binding was measured using 10 μM flumazenil. The radioactivity of the filters was determined by liquid scintillation counting using a Tricarb 2500 liquid scintillation analyzer. Binding data were analyzed using the GraphPad Prism software (version 5.02, GraphPad Software, USA).

Electrophysiological Analyses

The effects of HZ166 on $GABA_A$Rs were studied in HEK293 cells transiently expressing $GABA_A$Rs and in spinal cord slices of *hoxb8*-$\alpha2^{-/-}$ mice and wild-type (*hoxb8*-cre-negative $\alpha2^{fl/fl}$) littermates. HEK293 cells were transfected with rat $\alpha2$/$\alpha2$(H101R), $\beta3$ and $\gamma2$ $GABA_A$R expression vectors (Benson *et al*, 1998) using lipofectamine LTX (Invitrogen). The transfection mixture contained (in μg) 1 $\alpha2$/$\beta3$, 3 $\gamma2$ and 0.5 EGFP (used as a marker of successful transfection). Recordings were made 18–36 hours after transfection. Whole-cell patch-clamp recordings of GABA-evoked currents were made at room temperature (20–24 °C) and at a holding potential of −60 mV. The external solution contained (in mM) 150 NaCl, 10 KCl, 2.0 $CaCl_2$, 1.0 $MgCl_2$, 10 HEPES (pH 7.4), and 10 glucose. Recording electrodes were filled with internal solution containing (in mM) 120 CsCl, 10 EGTA, 10 HEPES (pH 7.40), 4 $MgCl_2$, 0.5 GTP, and 2 ATP. CsCl was used instead of KCl to block $GABA_B$ receptor-evoked K^+ currents. GABA was applied to the recorded cell using a manually controlled pulse (4–6 s) of a low subsaturating GABA concentration (EC_5). GABA EC_5 values were determined for wild-type $\alpha2\beta3\gamma2$ and mutant $\alpha2$(H101R)$\beta3\gamma2$ receptors separately. EC_{50} values and Hill coefficients (n_h) were obtained from fits of normalized concentration–response curves to the equation $I_{GABA} = I_{max}[GABA]n_h/([GABA]n_h + [EC_{50}]n_h)$. I_{max} was determined as the current elicited by a saturating concentration of GABA (500 μM). HZ166 was co-applied together with GABA without preincubation.

Transverse spinal cord slices were prepared from 2- to 3-week-old mice of either sex as described previously (Ahmadi *et al*, 2002). Slices were kept in oxygenated (95% O_2/5% CO_2) external solution containing (in mM) 120 NaCl, 5 HEPES, 26 $NaHCO_3$, 1.25 NaH_2PO_4, 2.5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, and 10 glucose (pH 7.35) at 35 °C. Superficial dorsal horn neurons were visually identified using an infrared gradient contrast equipment. Whole-cell patch-clamp recordings were performed at room temperature at a holding potential of −60 mV. Patch pipettes were filled with internal solution containing (in mM) 120 CsCl, 10 EGTA, 4 $MgCl_2$, 0.5 GTP, 2 ATP, and 10 HEPES (pH 7.30 adjusted with CsOH). QX-314 (5 mM) was added to block voltage-activated Na^+ currents in the recorded cell. Slices were continuously superfused with oxygenated external solution at a flow rate of 1.3–1.6 ml/min. After 4–5 min of baseline recording, GABA (50 μM) was bath-applied. Steady-state GABA-evoked currents were achieved usually 3–5 min after application. Subsequently, HZ166 (10 μM) was co-applied with the same GABA concentration for 6 to 7 min. Afterward, GABA and HZ166 were washed-out, or bicuculline (20 μM) was applied. Recordings in which recovery to baseline currents before GABA application was less than 85–90% were excluded from the analysis.

Behavioral Testing

Experiments were performed in 7- to 10-week-old male and female mice. Care was taken to ensure equal numbers of age-matched male and female mice in all experiments. Mechanical and thermal nociceptive sensitivities of *hoxb8*-$\alpha2^{-/-}$ and of *hoxb8*-cre-negative $\alpha2^{fl/fl}$ littermates were determined using electronic von Frey filaments and the plantar test, respectively (for details see Witschi *et al*, 2011).

Antihyperalgesic properties of HZ166 were studied in two models: (i) activity against neuropathic hyperalgesia was assessed in the chronic constriction injury (CCI) model (Bennett and Xie, 1988); unilateral constriction of the left sciatic nerve was performed as described previously (Hösl *et al*, 2006) and (ii) inflammation was evoked through subcutaneous injection of zymosan A (0.06 mg in 20 μl saline (0.9% NaCl)) into one hindpaw. HZ166 was tested 7 days after CCI surgery or 48 h after zymosan A injection, when sensitization had reached a maximum (Witschi *et al*, 2011). Sensitivities of the injured/inflamed paw and of the contralateral control paw were measured alternately and at least five measurements were taken per mouse and time point. Antihyperalgesia was quantified for the time interval of 60–90 min post drug injection, when the drug effect was maximal, and expressed as percent maximum possible analgesia = $(R_{post\text{-}drug} - R_{pre\text{-}drug})/(R_{baseline} - R_{pre\text{-}drug}) \times 100\%$, where R is the average response latency or threshold under baseline condition ($R_{baseline}$), after induction of neuropathy or inflammation but before drug injection ($R_{pre\text{-}drug}$), and 60–90 min after drug injection ($R_{post\text{-}drug}$).

Locomotor activity was assessed as described before (Di Lio *et al*, 2011). HZ166 was administered immediately before placing the mice into the open field arena, and the number of beam crosses per 24 min was determined for a total of 96 min after drug administration.

Motor coordination of *hoxb8*-$\alpha2^{-/-}$ and of *hoxb8*-cre-negative $\alpha2^{fl/fl}$ littermates was investigated in the rotarod test either at two fixed rotational speeds (5 and 10 r.p.m.) for analysis of baseline motor coordination, or with increasing rotational velocity (from 4 r.p.m. to 40 r.p.m. within 5 min) for the analysis of effects of HZ166 on motor coordination. Each mouse was tested three times. HZ166 was given 60 min before testing on the rotarod. Permission for the animal experiments was obtained from the Veterinäramt des Kantons Zürich (ref. no. 135/2009).

RESULTS

To assess a possible influence of supraspinal α2-GABA$_A$Rs to GABAergic analgesia, three prerequisites were needed: (i) mice that express benzodiazepine-sensitive α2-GABA$_A$Rs only in the brain, (ii) a benzodiazepine site agonist that causes significant antihyperalgesia after systemic administration at doses that do not produce confounding sedation or motor impairment, and (iii) we needed to demonstrate that the facilitating action of this agonist on α2-GABA$_A$Rs was lost in H101R point-mutated receptors.

In order to reach the first prerequisite, we generated two lines of α2-GABA$_A$R-mutated mice using the cre/loxP system. The first line lacked the GABA$_A$R α2 subunit from the spinal cord and from all primary sensory neurons (*hoxb8*-α2$^{-/-}$), but showed unchanged GABA$_A$R α2 subunit expression in the brain. These mice carried a *hoxb8*-cre transgene and two floxed GABA$_A$R α2 (α2^{fl}) alleles. The second line was a conditional point-mutated (*hoxb8*-α2$^{R/-}$) mouse line, whose spinal α2-GABA$_A$Rs were rendered benzodiazepine insensitive. These mice carried the *hoxb8*-cre transgene together with a α2^{fl} allele and a point-mutated (H101R) GABA$_A$R α2 (α2^{R}) allele. Morphological analyses demonstrated that the supraspinal GABA$_A$R α2 subunit distribution of *hoxb8*-α2$^{-/-}$ mice was indistinguishable from that of α2$^{fl/fl}$ (wild-type) littermates (Figure 1a). However, transverse sections of the lower lumbar spinal cord of *hoxb8*-α2$^{-/-}$ mice did not show any GABA$_A$R α2 subunit immunoreactivity, indicating highly effective *hoxb8*-cre-mediated gene recombination. Sagittal sections of the cervical spinal cord revealed the expected progressive rostral to caudal loss of GABA$_A$R α2 subunit expression within the upper cervical segments (not shown). The apparent lack of α2-GABA$_A$Rs from the lumbar spinal cord of *hoxb8*-α2$^{-/-}$ mice indicates that only few, if any, spinal α2-GABA$_A$Rs reside on processes of neurons descending from supraspinal areas. In conditional point-mutated (*hoxb8*-α2$^{R/-}$) mice and *hoxb8*-cre-negative (α2$^{R/fl}$) littermates, no differences in α2-subunit immunoreactivity were observed in either the brain or the spinal cord (Figure 1b), suggesting that the loss of one allele had no apparent effect on the amount of GABA$_A$R α2 subunit expressed.

To further corroborate the loss of GABA$_A$R α2 gene expression and to assess possible compensatory changes in the expression of other GABA$_A$R α-subunits, we performed qRT-PCR analyses from lumbar DRGs, lumbar spinal cords, and hippocampi of *hoxb8*-α2$^{-/-}$ mice and *hoxb8*-cre-negative α2$^{fl/fl}$ (wild-type) littermates (Figure 1c). In all three tissues, we detected mRNA encoding for GABA$_A$R α1–α5 subunits, but no α6 subunit mRNA. As expected, the α2 subunit was the most extensively expressed GABA$_A$R α-subunit in the lumbar spinal cords and DRGs. Its expression was completely lost in lumbar DRGs and spinal

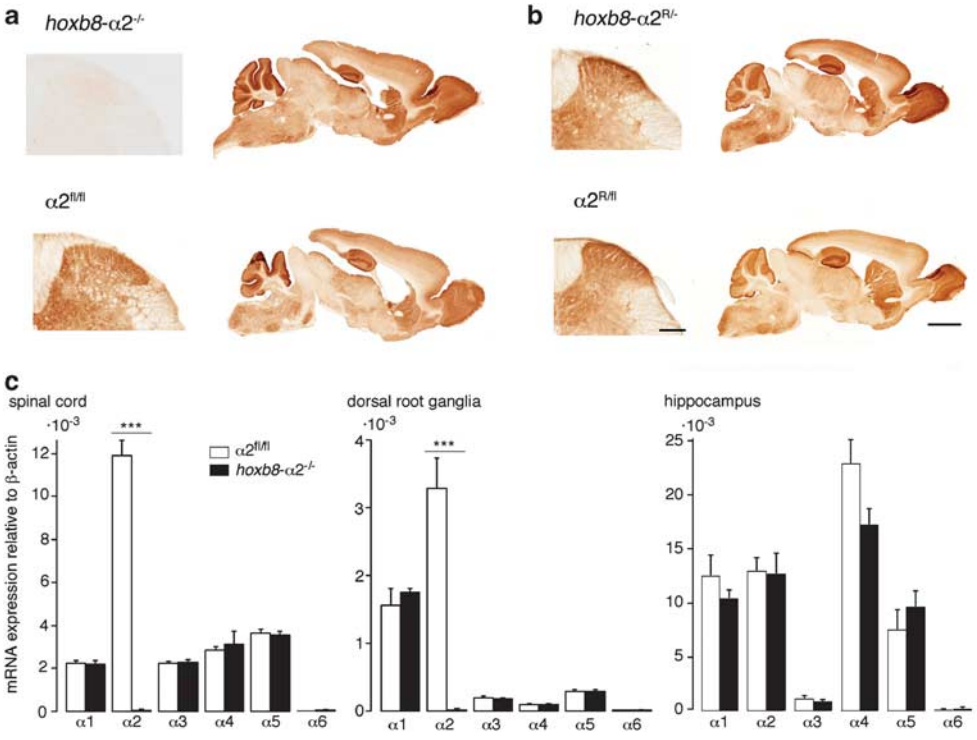

Figure 1 GABA$_A$R α2-subunit distribution in brains and spinal cords of *hoxb8*-α2$^{-/-}$ and *hoxb8*-α2$^{R/fl}$ mice and changes in GABA$_A$R α-subunit expression. (a, b), immunoperoxidase staining of GABA$_A$R α2 subunits in horizontal sections of the lumbar spinal dorsal horn and in sagittal brain sections of spinal cord-specific GABA$_A$R α2-knockout (*hoxb8*-α2$^{-/-}$) and α2(H101R)-knock-in mice (*hoxb8*-α2$^{R/-}$). Bottom row, sections obtained from the respective control littermates (*hoxb8*-cre-negative wild-type and α2$^{R/fl}$). Similar results were obtained from at least two additional mice of each genotype. Scale bars are 200 μm (dorsal horn) and 1 mm (brain), and apply to all sections. (c) Quantitative reverse transcriptase PCR measurements of all six GABA$_A$R α-subunits in lumbar DRGs, lumbar spinal cords, and hippocampi of *hoxb8*-α2$^{-/-}$ and α2$^{fl/fl}$ littermates. mRNA expression relative to β-actin (mean ± SEM, $n = 6$–7 mice per genotype and tissue). mRNA encoding for α6 GABA$_A$R subunit was not detectable in any of the three tissues. ***$P < 0.001$, $P > 0.13$ for all other comparisons, ANOVA followed by Bonferroni *post hoc* correction for 18 independent pair-wise comparisons.

cords of *hoxb8*-$\alpha2^{-/-}$ mice, but remained unchanged in the hippocampus. No significant changes were seen in the expression of the other $GABA_AR$ α subunits in any of the three tissues. For spinal cord and DRG tissue, we also analyzed the expression of $\rho1$, $\rho2$, and $\rho3$ subunit genes, which are able to form functional, benzodiazepine-insensitive $GABA_A$ (or sometimes also called $GABA_C$) receptors. Expression of ρ-subunits was unchanged in *hoxb8*-$\alpha2^{-/-}$ mice (data not shown).

In order to assess the contribution of supraspinal $\alpha2$-$GABA_ARs$ to benzodiazepine-mediated antihyperalgesia, we used the new non-sedative partial benzodiazepine site agonist HZ166 (Rivas *et al*, 2009), which exerts pronounced antihyperalgesic actions in mice in the absence of sedation (Di Lio *et al*, 2011). Among the different available compounds with improved subtype specificity, we chose HZ166 because it possesses higher intrinsic activity at $\alpha2$-$GABA_ARs$ than for example TPA023 (Atack *et al*, 2006) or NS 11394 (Mirza *et al*, 2008), and has better pharmacokinetic properties in mice than L-838,417 (Scott-Stevens *et al*, 2005). Before we analyzed its antihyperalgesic actions in $GABA_AR$-mutated mice, we had to verify that the H101R point mutation abolished modulation by HZ166. Although it has previously been shown that this mutation dramatically reduces the binding of and facilitation by diazepam (Wieland *et al*, 1992), it can have different effects on the action of other benzodiazepine site ligands. The potentiating effect of bretazenil for example is enhanced in the point-mutated receptors, and the action of Ro 45-1513 is converted from negative to positive modulation (Benson *et al*, 1998). We therefore compared the binding affinity of HZ166 with wild-type $\alpha2/\beta3/\gamma2$ and point-mutated $\alpha2(H101R)/\beta3/\gamma2$-$GABA_ARs$ (Figure 2a). In wild-type $\alpha2/\beta3/\gamma2$-$GABA_ARs$, HZ166 displaced [^{3}H] Ro 45-1513 binding with a K_i of 221 ± 22 nM (mean ± SEM, $n=4$). In $\alpha2(H101R)/\beta3/\gamma2$ point-mutated receptors competition was abolished at HZ166 concentration $\leqslant 10\,\mu M$. Only at $100\,\mu M$, a small displacement was detected ($26 \pm 3\%$, mean ± SEM, $n=4$). Subsequent electrophysiological experiments in HEK293 cells transfected with either wild-type or point-mutated $\alpha2(H101R)/\beta3/\gamma2$ $GABA_ARs$ verified that both receptors responded similarly to GABA (Figure 2b). We then investigated the impact of the point mutation on potentiation of $\alpha2/\beta3/\gamma2$ $GABA_ARs$ by HZ166 and showed that HZ166 completely lost its ability for positive allosteric modulation in the point-mutated receptors (Figure 2c–e). Although HZ166 potentiated GABA-evoked currents through wild-type receptors by $232 \pm 20\%$ (HZ166, $5\,\mu M$, $n=8$), potentiation was virtually absent in the point-mutated receptors at HZ166 concentrations $\leqslant 1\,\mu M$ and reached only $17.6 \pm 2.4\%$ at $5\,\mu M$ ($n=7$; $P<0.001$ unpaired student *t*-test, wild-type *vs* point-mutated receptors).

Before proceeding to behavioral experiments, we also investigated the impact of *hoxb8*-cre-mediated ablation of $GABA_AR$ $\alpha2$ subunits on the amplitude of native $GABA_ARs$ in the spinal dorsal horn and on their sensitivity to HZ166 (Figure 3). To this end, we used lumbar spinal cord slices and recorded GABAergic membrane currents from neurons located in the superficial dorsal horn of *hoxb8*-$\alpha2^{-/-}$ mice and of wild-type ($\alpha2^{fl/fl}$) littermates. Superfusion

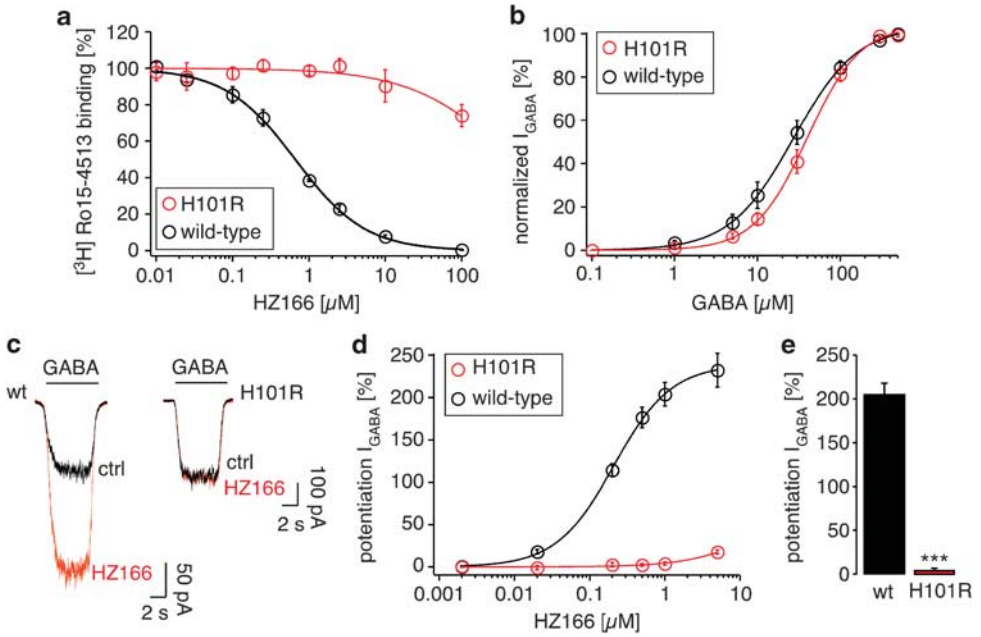

Figure 2 HZ166 binding properties to recombinant wild-type and $\alpha2(H101R)\beta3\gamma2$-$GABA_ARs$ and potentiation by HZ166. (a) Binding affinity of HZ166 to wild-type and $\alpha2(H101R)\beta3\gamma2$ point-mutated $GABA_ARs$ determined by [^{3}H] Ro15-4513 displacement. K_i in wild-type $GABA_ARs$ was 221 ± 22 nM (mean ± SEM, $n=4$). (b–e) Electrophysiological analyses (e). (b) Activation of wild-type and $\alpha2(H101R)\beta3\gamma2$ $GABA_ARs$ exhibited similar dependence on GABA concentration. EC_{50} was $27 \pm 1\,\mu M$ (mean ± SEM, $n=8$) and $40 \pm 2\,\mu M$ ($n=7$) for wild-type and $\alpha2(H101R)\beta3\gamma2$ point-mutated $GABA_ARs$, respectively. Hill coefficients were 1.2 ± 0.03 and 1.4 ± 0.07, and maximum currents were 3.6 ± 0.4 nA and 3.5 ± 0.7 nA. (c–e) Potentiation by HZ166. (c) Example of current traces evoked by GABA ($EC_5 = 5\,\mu M$) in the presence or absence of HZ166 ($1\,\mu M$). (d) Concentration–response curves of wild-type and $\alpha2(H101R)\beta3\gamma2$ point-mutated $GABA_ARs$ for HZ166. EC_{50} of HZ166 for wild-type $GABA_ARs$: 217 ± 20 nM. (e) Statistical comparison of potentiation by HZ166 ($1\,\mu M$). ***$P<0.001$ (wild-type *vs* point-mutated receptors), unpaired Student's *t*-test.

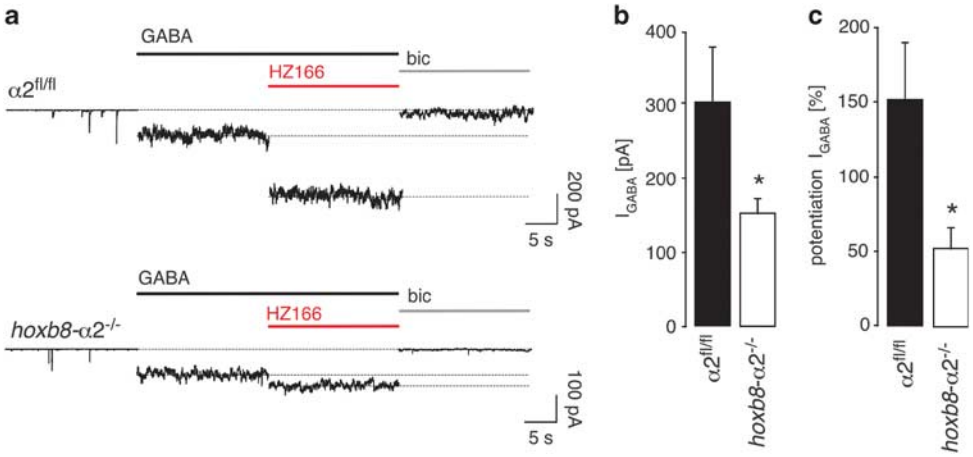

Figure 3 Effects of HZ166 on GABA-evoked membrane currents in *hoxb8*-$\alpha2^{-/-}$ mice and in wild-type ($\alpha2^{fl/fl}$) littermates. (a) Whole-cell voltage-clamp recordings of GABA (50 μM)-evoked membrane currents in superficial dorsal horn neurons of wild-type ($\alpha2^{fl/fl}$) (top) and *hoxb8*-$\alpha2^{-/-}$ mice (bottom). HZ166 (10 μM) was coapplied together with GABA. Bicuculline (bic; 20 μM) was added at the end of the experiment to verify that all currents measured were $GABA_AR$ currents. (b, c) Statistical analysis of $GABA_AR$ current amplitudes (b) and of their potentiation by 10 μM HZ166 (c). $n = 6$ and 8 cells for wild-type ($\alpha2^{fl/fl}$) and *hoxb8*-$\alpha2^{-/-}$ mice, respectively. *$P < 0.05$, unpaired Student's *t*-test.

of the slices with 50 μM GABA elicited average currents of 306 ± 73 pA (mean ± SEM, $n = 6$) in wild-type mice and smaller currents in neurons of *hoxb8*-$\alpha2^{-/-}$ mice (153 ± 20 pA, $n = 8$, $P = 0.04$, unpaired student *t*-test) (Figure 3a,b). HZ166 (10 μM) potentiated $GABA_AR$ currents in both genotypes. However, potentiation was significantly smaller in *hoxb8*-$\alpha2^{-/-}$ mice (52 ± 14%, $n = 8$) than in wild-type ($\alpha2^{fl/fl}$) littermates (151 ± 39%, $n = 6$, $P = 0.02$, unpaired student *t*-test) (Figure 3c). Amplitudes of $GABA_AR$ currents were thus reduced by about half, whereas potentiation by HZ166 was reduced by two-thirds suggesting the presence of both benzodiazepine (HZ166)-sensitive and benzodiazepine (HZ166)-insensitive $GABA_AR$ current components in spinal dorsal horn neurons. The retention of benzodiazepine-sensitive $GABA_AR$ currents in *hoxb8*-$\alpha2^{-/-}$ mice is in line with the results of our qRT-PCR experiments, which had demonstrated the expression of three benzodiazepine-sensitive $GABA_AR$ α-subunits (α1, α3, and α5) in addition to α2 (Figure 1c). Conversely, unchanged expression of ρ-subunits likely explains the reduced benzodiazepine (HZ166) sensitivity of $GABA_AR$ currents in *hoxb8*-$\alpha2^{-/-}$ mice.

We then went on to investigate *hoxb8*-$\alpha2^{-/-}$ and *hoxb8*-$\alpha2^{R/-}$ mice in behavioral pain models and addressed their susceptibility to antihyperalgesia by systemically applied HZ166. The analysis of *hoxb8*-$\alpha2^{R/-}$ mice was particularly interesting here because the unchanged expression of α2-$GABA_ARs$ in the spinal cord (compare Figure 1b) rendered compensatory and adaptive changes highly unlikely. In addition to *hoxb8*-$\alpha2^{-/-}$ mice and *hoxb8*-$\alpha2^{R/-}$ mice (and their *hoxb8*-cre-negative littermates), we also included homozygous point-mutated mice ($\alpha2^{R/R}$). The comparison of HZ166-induced antihyperalgesia in $\alpha2^{R/R}$ mice and in wild-type mice allowed us to determine the total contribution of α2-$GABA_ARs$ to antihyperalgesia by HZ166, independent of their location. Conditional *hoxb8*-$\alpha2^{-/-}$ knock-out mice and conditional *hoxb8*-$\alpha2^{R/-}$ knock-in mice as well as the *hoxb8*-cre-negative ($\alpha2^{fl/fl}$ and $\alpha2^{R/fl}$) littermates and global $\alpha2^{R/R}$ point-mutated mice responded similarly to mechanical stimulation of their hindpaws with electronic von Frey filaments and to thermal stimulation with defined radiant heat (Figure 4a–d). Neuropathic pain sensitization induced through chronic constriction injury (CCI) of the sciatic nerve, and inflammatory hyperalgesia evoked by local subcutaneous zymosan A injection also developed similarly in all genotypes (Figure 4a–d). As a further control experiment, we assessed motor coordination in *hoxb8*-$\alpha2^{-/-}$ and wild-type ($\alpha2^{fl/fl}$) littermates in the rotarod test at two different fixed rotational speeds (5 and 10 r.p.m.). Both lines managed to remain on the rotarod for similar time periods (Figure 4e).

Finally, we addressed the antihyperalgesic action of HZ166 against neuropathic pain in the three types of mutant mice (*hoxb8*-$\alpha2^{-/-}$, *hoxb8*-$\alpha2^{R/-}$ and $\alpha2^{R/R}$ mice) and in the *hoxb8*-cre-negative wild-type ($\alpha2^{fl/fl}$) and $\alpha2^{R/fl}$ littermates. For these experiments, we chose a dose of 16 mg/kg body weight applied intraperitoneally (i.p.), which has previously been shown to produce a saturating antihyperalgesic response in the absence of confounding sedation or motor impairment (Di Lio *et al*, 2011). We first verified that this dose of HZ166 had no effect on motor coordination or locomotor activity in neuropathic mice (Figure 5a–c). We then tested the effects of HZ166 against mechanical and thermal hyperalgesia in neuropathic mice (Figure 5d–g). The antihyperalgesic drug response reached a maximum between 60 and 90 min after drug injection (Figure 5d and f). This time interval was subsequently used to quantify HZ166-induced antihyperalgesia, expressed as percent maximum possible effect (MPE). In wild-type (*hoxb8*-cre-negative $\alpha2^{fl/fl}$) mice, HZ166 reduced thermal and mechanical hyperalgesia by 59.9 ± 1.4% and 61.5 ± 1.5% of pre-drug values (% MPE, mean ± SEM, $n = 6$, each) (Figure 5e and g). Both the time course and the degree of antihyperalgesia were very close to those reported previously by our group for wild-type C57BL/6 J mice (Di Lio *et al*, 2011). Antihyperalgesia by HZ166 was reduced in $\alpha2^{R/R}$ mice to 24.7 ± 3.6% and 26.6 ± 7.0% of the maximum possible effect (thermal and mechanical hyperalgesia,

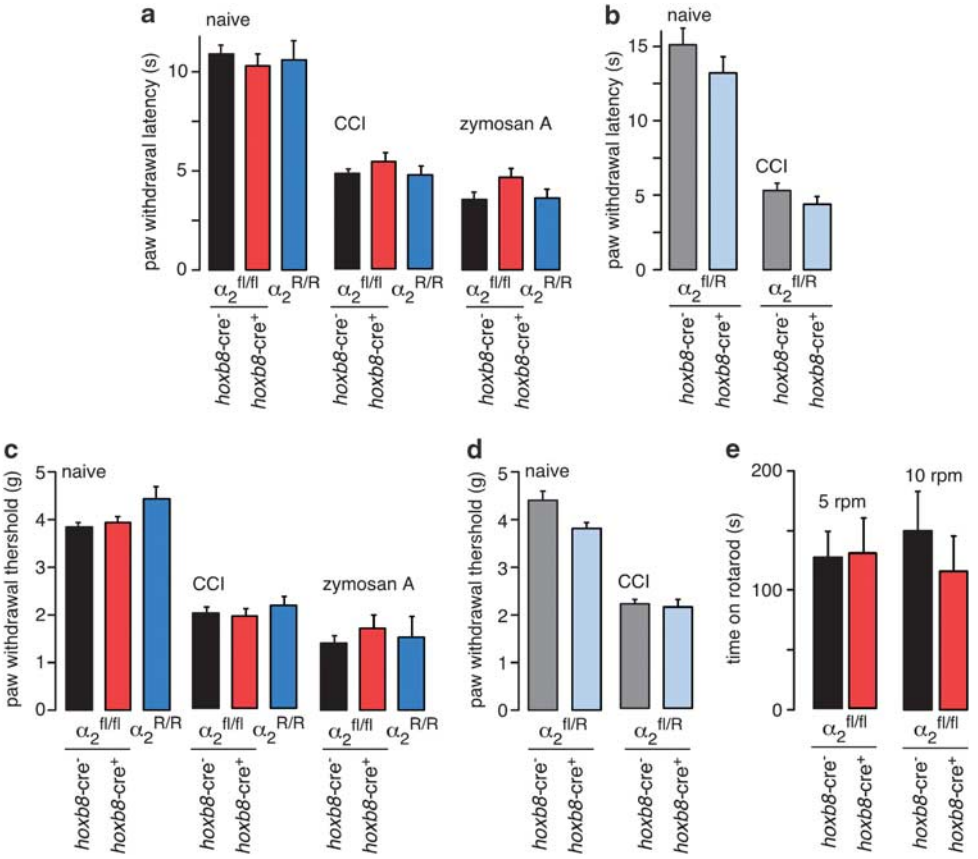

Figure 4 Characterization of baseline nociceptive sensitivity and of neuropathic and inflammatory hyperalgesia and of baseline motor coordination. (a) Baseline heat sensitivity, and neuropathic (CCI) and inflammatory (zymosan A) heat hyperalgesia in *hoxb8*-$\alpha2^{-/-}$ mice ($n=13$, 6, and 7, for naïve, CCI, zymosan A-injected mice), in wild-type ($\alpha2^{fl/fl}$) littermates ($n=27$, 12, and 15), and in separately bred global $\alpha2^{R/R}$ point-mutated mice of the same genetic background ($n=13$, 6, and 7). $P>0.25$, ANOVA followed by Bonferroni *post hoc* test. (b) Same as a, but spinal cord-specific $\alpha2$(H101R) point-mutated (*hoxb8*-$\alpha2^{R/-}$) mice ($n=6$, for both naïve and CCI mice) and *hoxb8*-cre-negative *hoxb8*-$\alpha2^{R/fl}$ littermates ($n=7$, for both naïve and CCI mice). $P>0.26$, unpaired student *t*-test. (c, d) Same as a, b, but mechanical sensitivity and hyperalgesia. $P>0.51$, ANOVA followed by Bonferroni *post hoc* test (c), and $P>0.9$ unpaired Student's *t*-test (d). $n=6$–7 mice, for all genotypes, pain models and tests. (e) Motor coordination assessed in the rotarod test. Time (mean ± SEM) for which the mouse managed to remain on the rod rotating at two different rotational velocities. Unpaired student's *t*-test, $P=0.45$, for *hoxb8*-$\alpha2^{-/-}$ ($n=8$) and wild-type ($\alpha2^{fl/fl}$) littermates ($n=11$), respectively.

respectively, $n=6$, each), indicating that about 60% of the antihyperalgesic actions of HZ166 were mediated by $\alpha2$-GABA$_A$Rs. The degrees of antihyperalgesia observed in both *hoxb8*-$\alpha2^{-/-}$ mice (24.0 ± 5.1% and 25.0 ± 5.1%, $n=6$ each, for thermal and mechanical hyperalgesia, respectively) and *hoxb8*-$\alpha2^{R/-}$ mice (27.4 ± 5.1% and 26.4 ± 5.2%, $n=6$ each) were virtually indistinguishable from that in global $\alpha2^{R/R}$ point-mutated mice, indicating that supraspinal $\alpha2$-GABA$_A$Rs were not required for antihyperalgesia by systemic HZ166. We also investigated antihyperalgesic properties of HZ166 against thermal inflammatory hyperalgesia (Figure 5h). In wild-type (*hoxb8*-cre-negative $\alpha2^{fl/fl}$) mice, HZ166 reduced thermal hyperalgesia by 58.7 ± 11.5% ($n=7$). This antihyperalgesia was almost lost in *hoxb8*-$\alpha2^{-/-}$ mice (9.7 ± 4.7%, $n=7$) and in global $\alpha2$-point-mutated ($\alpha2^{R/R}$) mice (3.8 ± 0.2%, $n=7$). Antihyperalgesia against mechanical stimuli was relatively small in wild-type mice (26.1 ± 9.4%, $n=7$) and almost completely lost in *hoxb8*-$\alpha2^{-/-}$ mice (4.7 ± 12.5%, $n=8$). However, the difference between wild-type and *hoxb8*-$\alpha2^{-/-}$ mice did not reach statistical significance ($P=0.19$) and effects of conditional deletion or mutation of the $\alpha2$ subunit were therefore not further investigated.

The results of our experiments not only exclude that analgesia or antihyperalgesia by HZ166 requires supraspinal $\alpha2$-GABA$_A$Rs, but also render significant indirect effects (such as reversal of anxiety or stress induced hyperalgesia) through supraspinal $\alpha2$-GABA$_A$Rs unlikely. They thus provide firm evidence for a genuine antihyperalgesic action of systemically applied benzodiazepine site agonists through a specific interaction with nociceptive circuits at the spinal level.

DISCUSSION

Studies performed in mice carrying point-mutated benzodiazepine-insensitive GABA$_A$Rs have allowed us to attribute the different *in vivo* actions of benzodiazepines to distinct GABA$_A$R subtypes (Möhler *et al*, 2002). In the course of these studies, it was shown that the sedative actions of benzodiazepines depend on $\alpha1$-GABA$_A$Rs, (McKernan *et al*,

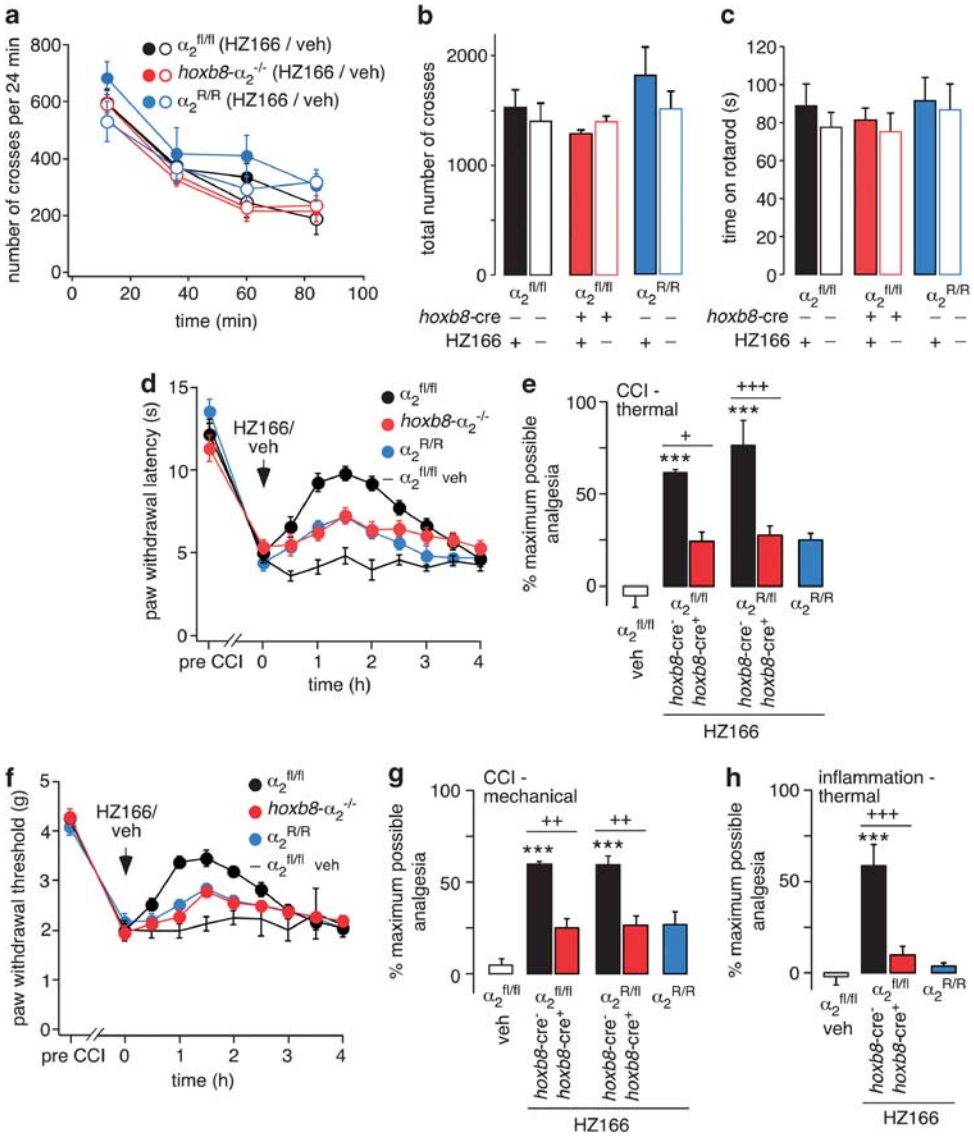

Figure 5 Locomotor activity, motor coordination and antihyperalgesia by HZ166 in α2-GABA$_A$ receptor-mutant mice. (a, b) Effects of HZ166 (16 mg/kg body weight i.p.) on locomotor activity in *hoxb8*-α2$^{-/-}$, α2$^{fl/fl}$, α2$^{R/R}$ mice with neuropathic hyperalgesia (7 days after CCI surgery). (a) Time course. Total number of beam crosses (mean ± SEM) per 24 min over time (0–96 min). (b) Statistical analysis. Two-way ANOVA followed by Bonferroni *post hoc* test. Genotype treatment $F(5,42) = 1.15$, $P = 0.35$. (c) Same genotypes and treatments as in a,b, but effects on motor coordination. Time spent on an accelerating rotarod (rotational velocity increasing from 4 r.p.m. to 40 r.p.m. within 5 min) (s, mean ± SEM). Genotype treatment $F(5,42) = 0.36$, $P = 0.87$. (d–g) Antihyperalgesic actions of the same dose of HZ166 in conditional knock-out (*hoxb8*-α2$^{-/-}$) and conditional knock-in (*hoxb8*-α2$^{R/-}$) mice with neuropathic hyperalgesia (7 days after CCI surgery). (d) Changes in heat hyperalgesia (paw withdrawal latency, s, mean ± SEM) over time after HZ166 (16 mg/kg) or vehicle administration. (e) Statistical analysis. ANOVA followed by Bonferroni *post hoc* test. $F(5,31) = 15.9$. ***$P < 0.001$ significant against vehicle. $^{+}P < 0.05$, and $^{+++}P < 0.001$ significantly different from *hoxb8*-cre-negative littermates. (f, g) Same as d, e, but mechanical hyperalgesia (paw withdrawal thresholds, g). $F(5,39) = 13.57$. $^{++}P < 0.01$ significant against *hoxb8*-cre-negative littermates. (h) Same as e, but antihyperalgesic effects of HZ166 against inflammatory heat hyperalgesia 48 hrs after zymosan A injection. $F(3,25) = 17.4$. $P < 0.001$, significant against *hoxb8*-cre-negative littermates; ***$P < 0.001$ significant against vehicle.

2000; Rudolph *et al*, 1999), whereas the anxiolytic actions were caused by a facilitation of α2-GABA$_A$Rs (Löw *et al*, 2000). Other studies employing the same mouse lines helped to establish additional, less obvious potential indications for subtype-selective benzodiazepine site agonists (Rudolph and Knoflach, 2011). One example of such a previously unforeseen action is antihyperalgesia, ie, the reversal of pathologically exaggerated sensitivity to pain. Although several studies in rodents had suggested pain-modulating actions of benzodiazepine site agonists, it has been notoriously difficult to distinguish apparent analgesia or antihyperalgesia from confounding sedation. Work with GABA$_A$R point-mutated mice provided for the first time compelling evidence for a pain-relieving action of spinal benzodiazepines and demonstrated a critical role of α2-GABA$_A$Rs in this process (Knabl *et al*, 2008). In these experiments, pain relief manifested primarily in a reversal of pathologically increased pain sensitivity rather than in

reduced responses to acute noxious stimuli, indicating that spinal benzodiazepines exerted a hyperalgesic rather than a genuine analgesic effect.

The availability of non-sedative benzodiazepine site agonists suitable for systemic administration prompted the question about a possible role in antihyperalgesia of α2-GABA$_A$Rs residing in supraspinal circuits. This appears as an important issue because α2-GABA$_A$Rs are not only found in the spinal cord but also in supraspinal CNS areas where they mediate for example the anxiolytic effects of classical benzodiazepines (Löw *et al*, 2000). α2-GABA$_A$Rs in the brain might have contributed to benzodiazepine-induced antihyperalgesia through a genuine effect on the supraspinal nociceptive circuits, eg, in the rostral insular cortex (Jasmin *et al*, 2003). Alternatively, their antihyperalgesic effects could also have been secondary, reflecting eg, a reversal of anxiety-induced hyperalgesia (Andre *et al*, 2005; Vidal and Jacob, 1982). Furthermore, supraspinal GABA$_A$Rs in the brainstem, in particular in the rostroventromedial medulla, are known to inhibit antinociceptive fiber tracts descending to the spinal cord and might thereby produce a pronociceptive effect (Harris and Westbrook, 1995; Luger *et al*, 1995; Tatsuo *et al*, 1999). In the present study, we have measured nociceptive withdrawal responses to address these questions. Although these nociceptive responses are primarily mediated by spinal circuits (ie, they remain in spinalized animals, see for example Schouenborg *et al* (1992)), they are highly susceptible to modulation by supraspinal pain control centers, such as the rostral insular cortex (Jasmin *et al*, 2003), the amygdala (Carrasquillo and Gereau, 2007), the rostroventromedial medulla (Tatsuo *et al*, 1999), and the periaqueductal gray (Harris and Westbrook, 1995). As such, they are well-suited for investigating the effects of supraspinal GABA$_A$ receptors on hyperalgesia. In the present study, the actions of HZ166 on nociceptive withdrawal responses were nearly identical in $\alpha2^{R/R}$, *hoxb8*-$\alpha2^{-/-}$ and *hoxb8*-$\alpha2^{R/-}$ mice, indicating that supraspinal α2-GABA$_A$Rs did not have a detectable influence on HZ166-mediated antihyperalgesia. The present data therefore unambiguously demonstrate that the major (ie, the α2-GABA$_A$R-mediated) component of antihyperalgesia by benzodiazepines occurs through a genuine effect on the spinal cord and that this antihyperalgesia is not secondary to effects of benzodiazepines on neuronal circuits in the brain. Our results hence also disprove the possibility that a reversal of anxiety-induced or stress-induced hyperalgesia contributed significantly to the antihyperalgesia measured in our experiments.

Previous studies had used intrathecal injections of diazepam or related benzodiazepines at the lumbar spinal level to demonstrate pain-relieving actions of benzodiazepines or of GABA$_A$R agonists (reviewed in Zeilhofer *et al*, 2009). A critical role of α2-GABA$_A$Rs in neuronal circuits of the spinal cord was therefore likely, yet still not proven, as the compounds injected might have reached supraspinal sites through rostral diffusion. The present work establishes that the spinal cord is the most relevant site for the antihyperalgesic actions of benzodiazepine site agonists.

In a previous study, we have examined the contribution of a subset of spinal α2-GABA$_A$Rs, which reside on the central terminals of primary nociceptive afferent fibers (Witschi *et al*, 2011). This subset of α2-GABA$_A$Rs was specifically ablated through *sns*-cre-mediated *gabra2* gene deletion. The respective *sns*-$\alpha2^{-/-}$ mice were analyzed in the same inflammatory and neuropathic pain models that have been used in the present study. These previous experiments had revealed that primary afferent α2-GABA$_A$Rs make a partial (about 50% of the total α2 component) contribution to inflammatory antihyperalgesia. The present study shows that the α2-GABA$_A$R-mediated component of inflammatory antihyperalgesia was completely lost in *hoxb8*-$\alpha2^{-/-}$ mice and hence entirely of spinal origin. Analysis of the *sns*-$\alpha2^{-/-}$ mice in neuropathic pain models had revealed that α2-GABA$_A$Rs on primary nociceptors did not make any contribution to neuropathic antihyperalgesia. Again this antihyperalgesic action was completely lost in *hoxb8*-$\alpha2^{-/-}$ mice and therefore also exclusively of spinal origin. Early *in situ* hybridization studies had found no α2-GABA$_A$Rs on intrinsic dorsal horn neurons (Persohn *et al*, 1991; Wisden *et al*, 1991), but more recent work provided clear evidence for the expression of these receptors by excitatory and inhibitory neurons in the spinal dorsal horn (Paul *et al*, 2012), which is in line with the data presented here.

Subsequent to the discovery that α2-GABA$_A$Rs are the major target for the anxiolytic actions of benzodiazepines, a significant number of benzodiazepine site agonists have been developed which show reduced sedative properties through improved α2 over α1 subtype selectivity (Rudolph and Knoflach, 2011). These compounds allowed an assessment of the potential analgesic and antihyperalgesic actions of such compounds after systemic administration in wild-type mice without confounding sedation. Studies testing these newly developed compounds revealed significant analgesic or antihyperalgesic properties in rodent pain models (Di Lio *et al*, 2011; Knabl *et al*, 2008; Nickolls *et al*, 2011, for a review see Zeilhofer *et al*, 2012). Comparison of the antihyperalgesic efficacies of different compounds with their pharmacological profiles at different GABA$_A$R subtypes suggests that a rather high intrinsic activity at α2-GABA$_A$Rs and a high α2 over α1 selectivity profile are necessary for significant antihyperalgesia in the absence of sedation (Zeilhofer *et al*, 2012). Although these results were consistent with the findings obtained in the GABA$_A$R point-mutated mice discussed above, final proof that these antihyperalgesic effects indeed originated from α2-GABA$_A$Rs was missing. Here we focused on one such compound, the novel partial benzodiazepine site agonist HZ166. The present study demonstrates that the antihyperalgesic actions of HZ166 were to a large extent mediated by α2-GABA$_A$Rs (about 90% and 60% for inflammatory and neuropathic hyperalgesia, respectively).

Antihyperalgesia was not completely lost in the different GABA$_A$R α2-mutant mice investigated here. Depending on the model used (ie, inflammatory or neuropathic hyperalgesia), between 10 and 40% of the total antihyperalgesia were retained in *hoxb8*-$\alpha2^{-/-}$, *hoxb8*-$\alpha2^{R/-}$, and $\alpha2^{R/R}$ mice. This is consistent with our previous study employing intrathecal diazepam injections, where between 30 and 50% of the antihyperalgesia remained in $\alpha2^{R/R}$ mice. At the spinal level, this remaining component was mediated by α3-GABA$_A$Rs and/or α5-GABA$_A$Rs (Knabl *et al*, 2008). It is likely that these spinal receptors also account for the antihyperalgesia retained in HZ166-treated *hoxb8*-$\alpha2^{-/-}$ and *hoxb8*-$\alpha2^{R/-}$ mice. For a given benzodiazepine site

agonist, the actual contribution of α2-$GABA_A$R *vs* α3-$GABA_A$Rs and α5-$GABA_A$Rs will depend on its potentiating effects at these $GABA_A$R subtypes. Until similar studies as the present one have also been performed for α3- and α5-$GABA_A$Rs, it cannot be excluded that $GABA_A$Rs different from α2 (ie, α3-$GABA_A$Rs and α5-$GABA_A$Rs) also contribute through a supraspinal site.

The present study provides strong evidence for a genuine antihyperalgesic action of systemically applied non-sedative benzodiazepine site agonists and demonstrates the pivotal contribution of spinal cord circuits to this antihyperalgesia. A critical role of inhibitory neurons and neurotransmitter receptors in the spinal dorsal horn has been first proposed in the gate control theory of pain (Melzack and Wall, 1965), but attempts to translate this concept to pain therapy have largely been unsuccessful. The present results show that an enhancement of fast GABAergic inhibition in the spinal dorsal horn is a possible strategy to reverse pathological hyperalgesia. Provided that the results obtained with genetic mouse mutants translate to the action of α2-$GABA_A$R selective drugs in humans, these agents should be devoid of sedation and memory impairment (Rudolph and Knoflach, 2011). Conversely, it is likely that such drugs will exert in addition to their antihyperalgesic actions also anxiolytic and possibly muscle relaxant properties (Rudolph and Knoflach, 2011), both of which should be beneficial to many chronic pain patients. The present finding therefore provide additional impetus for the development of subtype-selective benzodiazepine site agonists as novel antihyperalgesic agents.

FUNDING AND DISCLOSURE

This work was in part supported by a project grant from the Swiss National Science Foundation [131093 to HUZ], by the Special Programme on University Medicine (SPUM) 'Novel approaches to chronic pain' of the Swiss National Science Foundation [124117 to HUZ], by an advanced investigator grant from the European Research Council [DHISP 250128 to HUZ], and by a grant from the National Institutes of Health [NS076517 to JC]. The authors declare that over the past three years, JC has received compensation for professional services from Promentis Pharmaceuticals Inc. and Addiction Therapeutix, UR has received compensation for professional services from Sunovion and from Concert Pharmaceuticals. JC holds a patent on HZ166 and analogs. HUZ has received compensation for professional services from AMT AG Advanced Materials Technology, AstraZeneca Canada, Concert Pharmaceuticals, Geistlich Pharma AG, Grünenthal, Novartis Pharma Schweiz AG, Roche Pharma AG Germany, and Sigma tau Pharma AG. After completion of this study, ADL became an employee of Clinipace Worldwide, JP became an employee of Vifor Pharma, and RW became an employee of Pfizer AG, Switzerland. The remaining authors declare no conflict of interest.

ACKNOWLEDGEMENTS

We thank Mike Poe for the synthesis of HZ166, Isabelle Camenisch for excellent technical assistance, and Dennis Kwame Boadum for animal care.

REFERENCES

Ahmadi S, Lippross S, Neuhuber WL, Zeilhofer HU (2002). PGE_2 selectively blocks inhibitory glycinergic neurotransmission onto rat superficial dorsal horn neurons. *Nat Neurosci* **5**: 34–40.

Andre J, Zeau B, Pohl M, Cesselin F, Benoliel JJ, Becker C (2005). Involvement of cholecystokininergic systems in anxiety-induced hyperalgesia in male rats: behavioral and biochemical studies. *J Neurosci* **25**: 7896–7904.

Atack JR, Wafford KA, Tye SJ, Cook SM, Sohal B, Pike A *et al* (2006). TPA023 [7-(1,1-Dimethylethyl)-6-(2-ethyl-2H-1,2,4-triazol-3-ylmethoxy)-3-(2-fluor ophenyl)-1,2,4-triazolo[4,3-b]pyridazine], an agonist selective for α2- and α3-containing $GABA_A$ receptors, is a nonsedating anxiolytic in rodents and primates. *J Pharmacol Exp Ther* **316**: 410–422.

Bennett GJ, Xie YK (1988). A peripheral mononeuropathy in rat that produces disorders of pain sensation like those seen in man. *Pain* **33**: 87–107.

Benson JA, Löw K, Keist R, Möhler H, Rudolph U (1998). Pharmacology of recombinant γ-aminobutyric $acid_A$ receptors rendered diazepam-insensitive by point-mutated α-subunits. *FEBS Lett* **431**: 400–404.

Carrasquillo Y, Gereau RW (2007). Activation of the extracellular signal-regulated kinase in the amygdala modulates pain perception. *J Neurosci* **27**: 1543–1551.

Coull JA, Boudreau D, Bachand K, Prescott SA, Nault F, Sik A *et al* (2003). Trans-synaptic shift in anion gradient in spinal lamina I neurons as a mechanism of neuropathic pain. *Nature* **424**: 938–942.

Di Lio A, Benke D, Besson M, Desmeules J, Daali Y, Wang ZJ *et al* (2011). HZ166, a novel $GABA_A$ receptor subtype-selective benzodiazepine site ligand, is antihyperalgesic in mouse models of inflammatory and neuropathic pain. *Neuropharmacology* **60**: 626–632.

Fritschy JM, Möhler H (1995). $GABA_A$-receptor heterogeneity in the adult rat brain: differential regional and cellular distribution of seven major subunits. *J Comp Neurol* **359**: 154–194.

Harris JA, Westbrook RF (1995). Effects of benzodiazepine microinjection into the amygdala or periaqueductal gray on the expression of conditioned fear and hypoalgesia in rats. *Behav Neurosci* **109**: 295–304.

Harvey RJ, Depner UB, Wässle H, Ahmadi S, Heindl C, Reinold H *et al* (2004). GlyRα3: an essential target for spinal PGE2-mediated inflammatory pain sensitization. *Science* **304**: 884–887.

Hösl K, Reinold H, Harvey RJ, Müller U, Narumiya S, Zeilhofer HU (2006). Spinal prostaglandin E receptors of the EP2 subtype and the glycine receptor α3 subunit, which mediate central inflammatory hyperalgesia, do not contribute to pain after peripheral nerve injury or formalin injection. *Pain* **126**: 46–53.

Jasmin L, Rabkin SD, Granato A, Boudah A, Ohara PT (2003). Analgesia and hyperalgesia from GABA-mediated modulation of the cerebral cortex. *Nature* **424**: 316–320.

Knabl J, Witschi R, Hösl K, Reinold H, Zeilhofer UB, Ahmadi S *et al* (2008). Reversal of pathological pain through specific spinal $GABA_A$ receptor subtypes. *Nature* **451**: 330–334.

Knabl J, Zeilhofer UB, Crestani F, Rudolph U, Zeilhofer HU (2009). Genuine antihyperalgesia by systemic diazepam revealed by experiments in $GABA_A$ receptor point-mutated mice. *Pain* **1413**: 233–238.

Löw K, Crestani F, Keist R, Benke D, Brünig I, Benson JA *et al* (2000). Molecular and neuronal substrate for the selective attenuation of anxiety. *Science* **290**: 131–134.

Luger TJ, Hayashi T, Weiss CG, Hill HF (1995). The spinal potentiating effect and the supraspinal inhibitory effect of midazolam on opioid-induced analgesia in rats. *Eur J Pharmacol* **275**: 153–162.

McKernan RM, Rosahl TW, Reynolds DS, Sur C, Wafford KA, Atack JR *et al* (2000). Sedative but not anxiolytic properties of

benzodiazepines are mediated by the $GABA_A$ receptor $\alpha1$ subtype. *Nat Neurosci* **3**: 587–592.
Melzack R, Wall PD (1965). Pain mechanisms: a new theory. *Science* **150**: 971–979.
Mirza NR, Larsen JS, Mathiasen C, Jacobsen TA, Munro G, Erichsen HK *et al* (2008). NS11394 [3'-[5-(1-hydroxy-1-methyl-ethyl)-benzoimidazol-1-yl]-biphenyl-2-carbonitr ile], a unique subtype-selective $GABA_A$ receptor positive allosteric modulator: *in vitro* actions, pharmacokinetic properties and *in vivo* anxiolytic efficacy. *J Pharmacol Exp Ther* **327**: 954–968.
Morris HV, Dawson GR, Reynolds DS, Atack JR, Stephens DN (2006). Both $\alpha2$ and $\alpha3$ $GABA_A$ receptor subtypes mediate the anxiolytic properties of benzodiazepine site ligands in the conditioned emotional response paradigm. *Eur J Neurosci* **23**: 2495–2504.
Munro G, Ahring PK, Mirza NR (2009). Developing analgesics by enhancing spinal inhibition after injury: $GABA_A$ receptor subtypes as novel targets. *Trends Pharmacol Sci* **30**: 453–459.
Munro G, Lopez-Garcia JA, Rivera-Arconada I, Erichsen HK, Nielsen EO, Larsen JS *et al* (2008). Comparison of the novel subtype-selective $GABA_A$ receptor-positive allosteric modulator NS11394 [3'-[5-(1-hydroxy-1-methyl-ethyl)-benzoimidazol-1-yl]-biphenyl-2-carbonitr ile] with diazepam, zolpidem, bretazenil, and gaboxadol in rat models of inflammatory and neuropathic pain. *J Pharmacol Exp Ther* **327**: 969–981.
Möhler H, Fritschy JM, Rudolph U (2002). A new benzodiazepine pharmacology. *J Pharmacol Exp Ther* **300**: 2–8.
Nickolls S, Mace H, Fish R, Edye M, Gurrell R, Ivarsson M *et al* (2011). A Comparison of the $\alpha2/3/5$ Selective Positive Allosteric Modulators L-838,417 and TPA023 in Preclinical Models of Inflammatory and Neuropathic Pain. *Adv Pharmacol Sci* **2011**: 608912.
Olsen RW, Sieghart W (2008). International Union of Pharmacology. LXX. Subtypes of γ-aminobutyric $acid_A$ receptors: classification on the basis of subunit composition, pharmacology, and function. Update. *Pharmacol Rev* **60**: 243–260.
Paul J, Zeilhofer HU, Fritschy JM (2012). Selective distribution of $GABA_A$ receptor subtypes in mouse spinal dorsal horn neurons and primary afferents. *J Comp Neurol* **520**: 3895–3911.
Persohn E, Malherbe P, Richards JG (1991). In situ hybridization histochemistry reveals a diversity of $GABA_A$ receptor subunit mRNAs in neurons of the rat spinal cord and dorsal root ganglia. *Neuroscience* **42**: 497–507.
Reichl S, Augustin M, Zahn PK, Pogatzki-Zahn EM (2012). Peripheral and spinal GABAergic regulation of incisional pain in rats. *Pain* **153**: 129–141.
Rivas FM, Stables JP, Murphree L, Edwankar RV, Edwankar CR, Huang S *et al* (2009). Antiseizure activity of novel γ-aminobutyric acidA receptor subtype-selective benzodiazepine analogues in mice and rat models. *J Med Chem* **52**: 1795–1798.
Rudolph U, Crestani F, Benke D, Brünig I, Benson JA, Fritschy JM *et al* (1999). Benzodiazepine actions mediated by specific γ-aminobutyric $acid_A$ receptor subtypes. *Nature* **401**: 796–800.
Rudolph U, Knoflach F (2011). Beyond classical benzodiazepines: novel therapeutic potential of $GABA_A$ receptor subtypes. *Nat Rev Drug Discov* **10**: 685–697.
Sang CS, Hayes KS (2006). Anticonvulsant medications in neuropathic pain. In: McMahon SB, Koltzenburg M (eds). *Wall and Melzack's Textbook of Pain*. 6th edn. Elsevier.
Schouenborg J, Holmberg H, Weng HR (1992). Functional organization of the nociceptive withdrawal reflexes. *II. Changes of excitability and receptive fields after spinalization in the rat. Exp Brain Res* **90**: 469–478.
Scott-Stevens P, Atack JR, Sohal B, Worboys P (2005). Rodent pharmacokinetics and receptor occupancy of the $GABA_A$ receptor subtype selective benzodiazepine site ligand L-838417. *Biopharm Drug Dispos* **26**: 13–20.
Tatsuo MA, Salgado JV, Yokoro CM, Duarte ID, Francischi JN (1999). Midazolam-induced hyperalgesia in rats: modulation via $GABA_A$ receptors at supraspinal level. *Eur J Pharmacol* **370**: 9–15.
Vidal C, Jacob J (1982). Hyperalgesia induced by non-noxious stress in the rat. *Neurosci Lett* **32**: 75–80.
Wieland HA, Lüddens H, Seeburg PH (1992). A single histidine in $GABA_A$ receptors is essential for benzodiazepine agonist binding. *J Biol Chem* **267**: 1426–1429.
Wisden W, Gundlach AL, Barnard EA, Seeburg PH, Hunt SP (1991). Distribution of $GABA_A$ receptor subunit mRNAs in rat lumbar spinal cord. *Brain Res Mol Brain Res* **10**: 179–183.
Witschi R, Johansson T, Morscher G, Scheurer L, Deschamps J, Zeilhofer HU (2010). Hoxb8-Cre mice: A tool for brain-sparing conditional gene deletion. *Genesis* **48**: 596–602.
Witschi R, Punnakkal P, Paul J, Walczak J-S, Cervero F, Fritschy J-M *et al* (2011). Presynaptic $\alpha2$-$GABA_A$ receptors in primary afferent depolarization in spinal pain control. *J Neurosci* **31**: 8134–8142.
Zeilhofer HU, Benke D, Yévenes GE (2012). Chronic pain states: pharmacological strategies to restore diminished inhibitory spinal pain control. *Annu Rev Pharmacol Toxicol* **52**: 111–133.
Zeilhofer HU, Witschi R, Hösl K (2009). Subtype-selective $GABA_A$ receptor mimetics–novel antihyperalgesic agents? *J Mol Med* **87**: 465–469.