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Accessibility
Antihyperalgesia by $\alpha$2-GABA$_A$ Receptors Occurs Via a Genuine Spinal Action and Does Not Involve Supraspinal Sites

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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 GABAAergic 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 $\alpha$-, two $\beta$- and one $\gamma$-2- subunits. Pharmacological properties of the different GABA$_A$R subtypes are best characterized by the type of $\alpha$-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 $\alpha$2-type benzodiazepine pharmacology ($\alpha$2-GABA$_A$Rs) for antihyperalgesia mediated by GABA$_A$ receptors in the spinal cord.
by spinaly 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 z1-GABAARs (Rudolph et al, 1999). More recent experiments using novel benzodiazepine site ligands with improved subunit specificity (ie, reduced or absent activity at z1-GABAARs) 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 GABAARs to this antihyperalgesia is likely, although still not formally proven, the relevance of GABAAR subtypes in supraspinal circuits is unclear. Such supraspinal GABAARs might contribute to antihyperalgesia through a genuine antihyperalgesic effect, eg, through GABAARs 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 z2-GABAARs also contribute to benzodiazepine-mediated anxiolysis (Löw et al, 2000; Morris et al, 2006). In contrast, supraspinal GABAARs might also counteract spinal antihyperalgesia through the silencing antinoceptive 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 GABAARs either facilitates or constrains spinal antihyperalgesia.

To address these questions, we have generated two lines of GABAAR-mutated mice. The first line (hoxb8-z2−/−) carries a tissue-specific deletion of the GABAAR z2 subunit from all spinal neurons, astrocytes, and primary sensory neurons up to the mid cerebral level (approximately C4). This tissue-specific ablation was achieved by crossing mice that carried a GABAAR z2 (Gabra2) allele flanked by two loxP sites (z2fl; 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 z2-GABAAR mouse line (hoxb8-z2R/−), which carries in addition to one z2fl allele, a benzodiazepine-insensitive H101R point-mutated Gabra2 allele (z2R; 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’) z2fl 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[fl]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 z2-GABAARs had been rendered benzodiazepine-insensitive revealed that activation of supraspinal z2-GABAARs neither exerts a positive nor a negative impact on the antihyperalgesic actions of systemically applied HZ166.
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 z2β3/2 or z2(H101R)/β3/2 subunit combination (150–200 μg protein) were incubated with increasing concentrations of HZ166 (10⁻⁸–10⁻⁴ M) and 6.3 nM [³H]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 volume of 200 μl for 90 min on ice. Subsequently, the samples were filtered onto glass fiber filters using a volume of 200 μl. The filters were washed with ice-cold buffer (10 mM Tris-HCl pH 7.4). Non-specific [³H]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 GABAARs were studied in HEK293 cells transiently expressing GABAARs and in spinal cord slices of hoxb8-α2⁻/⁻ mice and wild-type (hoxb8-cre-negative α2 β1/2) littermates. HEK293 cells were transfected with rat z2/z2(H101R), β3 and γ2 GABAAR expression vectors (Benson et al, 1998) using lipofectamine LTX (Invitrogen). The transfection mixture contained (in μg) 1 z2/β3/β, 3 γ2 and 0.5 EGFP (used as a marker of successful transfection). Recordinings 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 CaCl₂, 1.0 MgCl₂, 10 HEPES (pH 7.4), and 10 glucose. Recording electrodes were filled with internal solution containing (in mM) 120 CsCl, 10 EGTA, 4 MgCl₂, 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-α2⁻/⁻ and of hoxb8-cre-negative α2 β1/2 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 = (Rpost-drug - Rpre-drug)/(Rbaseline - Rpre-drug) × 100%, where R is the average response latency or threshold under baseline condition (Rbaseline), after induction of neuropathy or inflammation but before drug injection (Rpre-drug), and 60–90 min after drug injection (Rpost-drug).

Motor coordination 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-α2⁻/⁻ and of hoxb8-cre-negative α2 β1/2 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 $\alpha_2$-GABA$_A$Rs to GABAergic analgesia, three prerequisites were needed: (i) mice that express benzodiazepine-sensitive $\alpha_2$-GABA$_A$R 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 $\alpha_2$-GABA$_A$R was lost in H101R point-mutated receptors.

In order to reach the first prerequisite, we generated two lines of $\alpha_2$-GABA$_A$R-mutated mice using the cre/loxP system. The first line lacked the GABA$_A$R $\alpha_2$ subunit from the spinal cord and from all primary sensory neurons ($hoxb8^{-/-}$), but showed unchanged GABA$_A$R $\alpha_2$ subunit expression in the brain. These mice carried a $hoxb8$-cre transgene and two floxed GABA$_A$R $\alpha_2$ ($\alpha_2^{R}$) alleles. The second line was a conditional point-mutated ($hoxb8^{-/-}$) mouse line, whose spinal $\alpha_2$-GABA$_A$Rs were rendered benzodiazepine insensitive. These mice carried the $hoxb8$-cre transgene together with a $\alpha_2^{B}$ allele and a point-mutated (H101R) GABA$_A$R $\alpha_2$ ($\alpha_2^{R}$) allele. Morphological analyses demonstrated that the supraspinal GABA$_A$R $\alpha_2$ subunit distribution of $hoxb8^{-/-}$ mice was indistinguishable from that of $\alpha_2^{R}$ (wild-type) littermates (Figure 1a). However, transverse sections of the lower lumbar spinal cord of $hoxb8^{-/-}$ mice did not show any GABA$_A$R $\alpha_2$ 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 $\alpha_2$ subunit expression within the upper cervical segments (not shown). The apparent lack of $\alpha_2$-GABA$_A$Rs from the lumbar spinal cord of $hoxb8^{-/-}$ mice indicates that only few, if any, spinal $\alpha_2$-GABA$_A$Rs reside on processes of neurons descending from supraspinal areas. In conditional point-mutated ($hoxb8^{-/-}$) mice and $hoxb8$-cre-negative ($\alpha_2^{R}$) littermates, no differences in $\alpha_2$-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 $\alpha_2$ subunit expressed.

To further corroborate the loss of GABA$_A$R $\alpha_2$ gene expression and to assess possible compensatory changes in the expression of other GABA$_A$R $\alpha$-subunits, we performed qRT-PCR analyses from lumbar DRGs, lumbar spinal cords, and hippocampi of $hoxb8^{-/-}$ mice and $hoxb8$-cre-negative $\alpha_2^{R}$ (wild-type) littermates (Figure 1c). In all three tissues, we detected mRNA encoding for GABA$_A$R $\alpha_1$–$\alpha_5$ subunits, but no $\alpha_6$ subunit mRNA. As expected, the $\alpha_2$ subunit was the most extensively expressed GABA$_A$R $\alpha$-subunit in the lumbar spinal cords and DRGs. Its expression was completely lost in lumbar DRGs and spinal

![Figure 1](image-url)
cords of hoxb8-α2-/- mice, but remained unchanged in the hippocampus. No significant changes were seen in the expression of the other GABA<sub>A</sub>,<sub>R</sub> α subunits in any of the three tissues. For spinal cord and DRG tissue, we also analyzed the expression of ρ1, ρ2, and ρ3 subunit genes, which are able to form functional, benzodiazepine-insensitive GABA<sub>A</sub>,<sub>R</sub> (or sometimes also called GABA<sub>C</sub>,<sub>R</sub>) receptors. Expression of ρ-subunits was unchanged in hoxb8-α2-/- mice (data not shown).

In order to assess the contribution of supraspinal α2-GABA<sub>A</sub>,<sub>R</sub>s 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 α2-GABA<sub>A</sub>,<sub>R</sub>s than for example TPA023 (Atack 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<sub>A</sub>,<sub>R</sub>-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 (Benson et al., 1991), 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 (Figure 2c). To this end, we used lumbar spinal cord slices and recorded GABAergic membrane currents from neurons located in the superficial dorsal horn of hoxb8-α2-/- mice and of wild-type (α2<sup>h/h</sup>) littermates. Superfusion

![Image](481)

Figure 2. HZ166 binding properties to recombinant wild-type and α2(H101R)/β3/2-GABA<sub>A</sub>,<sub>R</sub>s and potentiation by HZ166. (a) Binding affinity of HZ166 to wild-type and α2(H101R)/β3/2 point-mutated GABA<sub>A</sub>,<sub>R</sub>s determined by [3H]Ro45-1513 displacement. K<sub>i</sub> in wild-type GABA<sub>A</sub>,<sub>R</sub>s was 221 ± 22 nM (mean ± SEM, n = 8). (b–e) Electrophysiological analyses (e). (b) Activation of wild-type and α2(H101R)/β3/2 GABA<sub>A</sub>,<sub>R</sub>s exhibited similar dependence on GABA concentration. EC<sub>50</sub> was 27 ± 1 µM (mean ± SEM, n = 8) and 40 ± 2 µM (n = 7) for wild-type and α2(H101R)/β3/2 point-mutated GABA<sub>A</sub>,<sub>R</sub>s, 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<sub>50</sub> = 5 µM) in the presence or absence of HZ166 (1 µM). (d) Concentration–response curves of wild-type and α2(H101R)/β3/2 point-mutated GABA<sub>A</sub>,<sub>R</sub>s for HZ166. EC<sub>50</sub> of HZ166 for wild-type GABA<sub>A</sub>,<sub>R</sub>s: 217 ± 20 nM. (e) Statistical comparison of potentiation by HZ166 (1 µM). ***P < 0.001 (wild-type vs point-mutated receptors), 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-α2−/− mice (153 ± 20 pA, n = 8, P = 0.04, unpaired student t-test) (Figure 3a,b). HZ166 (10 μM) potentiated GABAₐR currents in both genotypes. However, potentiation was significantly smaller in hoxb8-α2−/− mice (52 ± 14%, n = 8) than in wild-type (α2²⁰/M) littermates (151 ± 39%, n = 6, P ≥ 0.02, unpaired student t-test) (Figure 3c). Amplitudes of GABAₐR 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 GABAₐR current components in spinal dorsal horn neurons. The retention of benzodiazepine-sensitive GABAₐR currents in hoxb8-α2−/− mice is in line with the results of our qRT-PCR experiments, which had demonstrated the expression of three benzodiazepine-sensitive GABAₐR α-subunits (x1, x3, and x5) in addition to x2 (Figure 1c). Conversely, unchanged expression of ρ-subunits likely explains the reduced benzodiazepine (HZ166) sensitivity of GABAₐR currents in hoxb8-α2−/− mice.

We then went on to investigate hoxb8-α2−/− and hoxb8-α2¹⁰/M mice in behavioral pain models and addressed their susceptibility to antihyperalgesia by systemically applied HZ166. The analysis of hoxb8-α2¹⁰/M mice was particularly interesting here because the unchanged expression of α2-GABAₐRs in the spinal cord (compare Figure 1b) rendered compensatory and adaptive changes highly unlikely. In addition to hoxb8-α2−/− mice and hoxb8-α2¹⁰/M mice (and their hoxb8-cre-negative littermates), we also included homozygous point-mutated mice (α2²⁰/R). The comparison of HZ166-induced antihyperalgesia in α2²⁰/R mice and in wild-type mice allowed us to determine the total contribution of α2-GABAₐRs to antihyperalgesia by HZ166, independent of their location. Conditional hoxb8-α2−/− knock-out mice and conditional hoxb8-α2¹⁰/M knock-in mice as well as the hoxb8-cre-negative (α2²⁰/M and α2²⁰/R) littermates and global α2²⁰/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-α2−/− and wild-type (α2²⁰/M) 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-α2−/−, hoxb8-α2¹⁰/M and α2²⁰/R mice) and in the hoxb8-cre-negative wild-type (α2²⁰/M) and α2²⁰/R 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 α2²⁰/R) 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/6J mice (Di Lio et al, 2011). Antihyperalgesia by HZ166 was reduced in α2²⁰/R mice to 24.7 ± 3.6% and 26.6 ± 7.0% of the maximum possible effect (thermal and mechanical hyperalgesia, 0.05, unpaired Student’s t-test.)
respectively, \( n = 6 \), each), indicating that about 60% of the antihyperalgesic actions of HZ166 were mediated by \( \alpha_2 \)-GABAARs. The degrees of antihyperalgesia observed in both \( \text{hoxb8-} \alpha_2^{-/-} \) mice (24.0 \pm 5.1% and 25.0 \pm 5.1%, \( n = 6 \) each, for thermal and mechanical hyperalgesia, respectively) and \( \text{hoxb8-} \alpha_2^{H101R} \) mice (27.4 \pm 5.1% and 26.4 \pm 5.2%, \( n = 6 \) each) were virtually indistinguishable from that in global \( \alpha_2^{-/-} \) point-mutated mice, indicating that supraspinal \( \alpha_2 \)-GABAARs were not required for antihyperalgesia by systemic HZ166. We also investigated antihyperalgesic properties of HZ166 against thermal inflammatory hyperalgesia (Figure 5h). In wild-type (\( \text{hoxb8-} \alpha_2^{fl/fl} \)) mice, HZ166 reduced thermal hyperalgesia by 58.7 \pm 11.5% (\( n = 7 \)). This antihyperalgesia was almost lost in \( \text{hoxb8-} \alpha_2^{-/-} \) mice (9.7 \pm 4.7%, \( n = 7 \)) and in global \( \alpha_2^{-/-} \) point-mutated (\( \alpha_2^{H101R} \)) mice (3.8 \pm 0.2%, \( n = 7 \)). Antihyperalgesia against mechanical stimuli was relatively small in wild-type mice (26.1 \pm 9.4%, \( n = 7 \)) and almost completely lost in \( \text{hoxb8-} \alpha_2^{-/-} \) mice (4.7 \pm 12.5%, \( n = 8 \)). However, the difference between wild-type and \( \text{hoxb8-} \alpha_2^{-/-} \) mice did not reach statistical significance (\( P = 0.19 \)) and effects of conditional deletion or mutation of the \( \alpha_2 \) subunit were therefore not further investigated.

The results of our experiments not only exclude that analgesia or antihyperalgesia by HZ166 requires supraspinal \( \alpha_2 \)-GABAARs, but also render significant indirect effects (such as reversal of anxiety or stress induced hyperalgesia) through supraspinal \( \alpha_2 \)-GABAARs 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 (Möller et al, 2002). In the course of these studies, it was shown that the sedative actions of benzodiazepines depend on \( \alpha_1 \)-GABA\(_A\)Rs, (McKernan et al,
2000; Rudolph et al., 1999), whereas the anxiolytic actions were caused by a facilitation of α2-GABAA receptors (Loew 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, i.e., 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 GABAAR 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-GABAA receptors 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

Figure 5  Locomotor activity, motor coordination and antihyperalgesia by HZ166 in α2-GABAA receptor-mutant mice. (a, b) Effects of HZ166 (16 mg/kg body weight i.p.) on locomotor activity in hoxb8-α2−/−, α2Y1−2KO 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-α22+) 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 vehicle.
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 $2$-GABA$_A$Rs residing in supraspinal circuits. This appears as an important issue because $2$-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 (Löwe et al., 2000). $2$-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 nociceptive 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$_A$R receptors on hyperalgesia. In the present study, the actions of HZ166 on nociceptive withdrawal responses were nearly identical in $2^{R/R}$ hoxb8-$2^{-/-}$ and hoxb8-$2^{R/-}$ mice, indicating that supraspinal $2$-GABA$_A$Rs did not have a detectable influence on HZ166-mediated antihyperalgesia. The present data therefore unambiguously demonstrate that the major (ie, the $2$-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 $2$-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 $2$-GABA$_A$Rs, which reside on the central terminals of primary nociceptive afferent fibers (Witschi et al., 2011). This subset of $2$-GABA$_A$Rs was specifically ablated through snsc-re-mediated gabra2 gene deletion. The respective $2^{-/-}$ mice were analyzed in the same inflammatory and neuropathic pain models that have been used in this study. These previous experiments had revealed that primary afferent $2$-GABA$_A$Rs make a partial (about 50% of the total $2$ component) contribution to inflammatory antihyperalgesia. The present study shows that the $2$-GABA$_A$R-mediated component of inflammatory antihyperalgesia was completely lost in hoxb8-$2^{-/-}$ mice and hence entirely of spinal origin. Analysis of the $2^{-/-}$ mice in neuropathic pain models had revealed that $2$-GABA$_A$Rs on primary nociceptors did not make any contribution to neuropathic antihyperalgesia. Again this antihyperalgesic action was completely lost in hoxb8-$2^{-/-}$ mice and therefore also exclusively of spinal origin. Early in situ hybridization studies had found no $2$-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 $2$-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 $2$ over $1$ subtype selectivity (Rudolph and Knofflach, 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 $2$-GABA$_A$Rs and a high $2$ over $1$ 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 $2$-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 $2$-GABA$_A$Rs (about 90% and 60% for inflammatory and neuropathic hyperalgesia, respectively).

Antihyperalgesia was not completely lost in the different GABA$_A$R $2$-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-$2^{-/-}$, hoxb8-$2^{R/-}$, and $2^{R/-}$ mice. This is consistent with our previous study employing intrathecal diazepam injections, where between 30 and 50% of the antihyperalgesia remained in $2^{R/-}$ mice. At the spinal level, this remaining component was mediated by $3$-GABA$_A$Rs and/or $5$-GABA$_A$Rs (Knabl et al., 2008). It is likely that these spinal receptors also account for the antihyperalgesia retained in HZ166-treated hoxb8-$2^{-/-}$ and hoxb8-$2^{R/-}$ mice. For a given benzodiazepine site...
agonist, the actual contribution of z2-GABAARs vs z3-GABAARs and z5-GABAARs will depend on its potentiating effects at these GABAAR subtypes. Until similar studies as the present one have also been performed for z3- and z5-GABAARs, it cannot be excluded that GABAARs different from z2 (ie, z3-GABAARs and z5-GABAARs) 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 z2-GABAAR 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.

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REFERENCES

Ahmadi S, Lippross S, Neuhuber WL, Zeilhofer HU (2002). PGE2 selectively blocks inhibitory glycnergic neurotransmission onto rat superficial dorsal horn neurons. Nat Neurosci 5: 34–40.

Andrè J, Zeau B, Pohl M, Cesselin F, Benoliel J, Becker C (2005). Involvement of cholecystokininergic systems in stress-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-1,4-triazol-3-ylmethoxy)-3-(2-fluorophenyl)-1,2,4-triazolo[4,3-b]pyridazine], an agonist selective for α2- and α3-containing GABAAR 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 acidA receptors rendered diazepam-insensitive by point-mutated α-subunits. FEBS Lett 431: 400–404.

Bennet GJ, Xie YK (2007). Activation of the extracellular signal-regulated kinase in the amygdala modulates pain perception. J Neurosci 27: 1543–1551.

Bennet GJ, Xie YK (2008). Reversal of pathological pain through specific spinal GABAergic inhibition. Pain 424: 938–942.

Cook 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: 626–632.

Di Lio A, Benke D, Besson M, Desmeules J, Daali Y, Wang ZJ et al (2011). HZ166, a novel GABAAR 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). GABAAR-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, Reinhold H et al (2004). GlyRα3: an essential target for spinal PGE2-mediated inflammatory pain sensitization. Science 304: 884–887.

Hölzl K, Reinold H, Harvey RJ, Müller U, Narumiya S, Zeilhofer HU (2006). Spinal prostaglandin E receptors of the EP2 subtype and the glycine receptor z3 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, Obara PT (2003). Analgesia and hyperalgesia from GABA-mediated modulation of the cerebral cortex. Nature 424: 316–320.

Knabl J, Witschi R, Hössl K, Reinhold H, Zeilhofer UB, Ahmadi S et al (2008). Reversal of pathological pain through specific spinal GABAAR 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 GABAAR receptor point-mutated mice. Pain 143: 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 z1 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). Comparison of the novel subtype-selective GABA_A receptor-positive allosteric modulator: in vitro actions, pharmacokinetic properties and in vivo antinociceptive efficacy. *J Pharmacol Exp Ther* 327: 954–968.

Morris HV, Dawson GR, Reynolds DS, Atack JR, Stephens DN (2006). Both z2 and z3 GABA_A receptor subtypes mediate the antinociceptive 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’-[(1-hydroxy-1-methyl-ethyl)-benzimidazol-1-yl]-biphenyl-2-carbonitrile] with diazepam, zolpidem, zopiclone, 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 z2/z3 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 acidA 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 acidA 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 pharmacokinetinics and receptor occupancy of the GABA_A receptor subtype selective benzodiazepine site ligand L-838417. *Biopharm Drug Dispos* 26: 13–20.

Tatsu 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* 23: 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, Gundlah 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, Morrischer G, Scheurer L, Deschamps J, Zeilhofer HU (2010). Hoxb8-Cre mice: A tool for brain-sparing strategies to restore diminished inhibitory spinal pain control. *Spinal GABAergic analgesia*.