Agonists at the benzodiazepine-binding site of GABA\textsubscript{A} receptors (BDZs) enhance synaptic inhibition through four subtypes (\(\alpha_1\), \(\alpha_2\), \(\alpha_3\) and \(\alpha_5\)) of GABA\textsubscript{A} receptors (GABA\textsubscript{A}R). When applied to the spinal cord, they alleviate pathological pain; however, insufficient efficacy after systemic administration and undesired effects preclude their use in routine pain therapy. Previous work suggested that subtype-selective drugs might allow separating desired antihyperalgesia from unwanted effects, but the lack of selective agents has hitherto prevented systematic analyses. Here we use four lines of triple GABA\textsubscript{A}R point-mutated mice, which express only one benzodiazepine-sensitive GABA\textsubscript{A}R subtype at a time, to show that targeting only \(\alpha_2\)GABA\textsubscript{A}Rs achieves strong antihyperalgesia and reduced side effects (that is, no sedation, motor impairment and tolerance development). Additional pharmacokinetic and pharmacodynamic analyses in these mice explain why clinically relevant antihyperalgesia cannot be achieved with nonselective BDZs. These findings should foster the development of innovative subtype-selective BDZs for novel indications such as chronic pain.
Chronic pain is a severe medical condition affecting hundreds of millions of patients worldwide. It is widely accepted that diminished inhibition in pain-processing circuits of the spinal dorsal horn is a major contributor to different chronic pain forms\(^1\)-\(^4\). We have previously demonstrated that local spinal application of BDZ site ligands that positively modulate GABA\(_R\) function alleviates neuropathic and inflammatory pain in rodents\(^5\). Translation of these results to routine systemic pain treatment, however, requires a separation of desired antihyperalgesia from unwanted side effects. This separation appears potentially feasible based on the existence of different GABA\(_R\) subtypes.

Most GABA\(_R\)s in the brain and spinal cord are heteropentameric ion channels composed of two \(\alpha\), two \(\beta\) and one \(\gamma\) subunit\(^6\). The high-affinity binding site for BDZs is formed by an interface between one \(\alpha\) subunit and the \(\gamma\) subunit. High-affinity binding of BDZs at this site requires the presence of a histidine residue at a conserved site in the N-terminal domain of the \(\alpha\) subunit. This conserved histidine is present in \(\alpha1\), \(\alpha2\), \(\alpha3\) or \(\alpha5\) subunits, but not in the \(\alpha4\) and \(\alpha6\) subunits\(^7\). Mutation of the histidine residue into an arginine dramatically reduces the affinity of GABA\(_R\)s to BDZs without changing their responses to GABA\(^8\). The generation of histidine to arginine (H\(\rightarrow\)R) point-mutated mice for each of the four BDZ-sensitive GABA\(_R\) subunits has allowed attributing the different in vivo effects of BDZs to defined GABA\(_R\) subtypes\(^9\).

Most importantly, it was shown that the sedative effects of BDZs were strongly reduced in mice carrying the H\(\rightarrow\)R point mutation in their \(\alpha1\) subunits\(^10\),\(^11\), while point-mutating the \(\alpha2\) subunits led to a loss in the anxiolytic effects of BDZs\(^12\). Using this approach, we could demonstrate that mice whose \(\alpha2\)GABA\(_R\)s had been rendered BDZ-insensitive show drastically reduced antihyperalgesic responses to spinal diazepam (DZP)\(^5\). A general consensus on the question, which GABA\(_R\) subtype should best be targeted to achieve maximal antihyperalgesic responses and to best avoid undesired effects has not been reached. A similarly open question is why classical BDZs are largely devoid of analgesic actions in patients.

Highly selective tool compounds that would allow addressing these questions pharmacologically are still lacking\(^13\). For this reason, we have generated triple GABA\(_R\) point-mutated mice, in which only a single GABA\(_R\) subtype remains BDZ-sensitive. We designate these mice hereafter as HRRR, short for that positively modulate GABA\(_R\) function alleviates neuropathic and inflammatory pain in rodents\(^5\). Using immunocytochemistry, we verified that neither the regional distribution nor the expression levels of \(\alpha1\), \(\alpha2\), \(\alpha3\) and \(\alpha5\) subunits and of the \(\gamma2\) subunit differed between wild-type (wt) mice and the four strains of point-mutated mice (Fig. 1a,b).

Similarly, the total number of BDZ-binding sites (wt receptors plus H\(\rightarrow\)R point-mutated receptors) quantified through \(^3\)H\(\text{Ro15-4513}\) autoradiography was unchanged (Fig. 1c). Spinal autoradiography with \(^3\)H\(\text{Flumazenil}\) that binds with high affinity only to nonpoint-mutated GABA\(_R\)s allowed a quantitative analysis of each of the four subtypes of BDZ-binding sites in isolation. In wt mice, \(^3\)H\(\text{Flumazenil}\) exhibited specific binding throughout the spinal grey matter with enrichment in the dorsal horn and around the central canal. The density of binding sites in the dorsal horn was highest for the \(\alpha3\)GABA\(_R\) subtype, followed by \(\alpha2\) and \(\alpha1\), and lowest for the \(\alpha5\) subtype (Fig. 1d). The \(\alpha1\) subtype was concentrated around the central canal, \(\alpha2\) was most abundant in the superficial dorsal horn, where nociceptive fibres terminate, and \(\alpha5\) was found throughout the dorsal horn and around the central canal, while \(\alpha3\) was generally weak. The distribution of subtype-specific \(^3\)H\(\text{Flumazenil}\) binding in the four strains of GABA\(_R\) triple point-mutated mice matched the distribution on the subunits determined with immunocytochemistry on a gross scale. However, the sum of the binding sites in the four strains of triple point-mutated mice was about twice as high as the total number of binding sites in wt mice. The results from the immunocytochemistry and \(^3\)H\(\text{Ro15-4513}\) autoradiography largely rule out changes in protein expression as the underlying cause. Instead, the \(^3\)H\(\text{Flumazenil}\) data are consistent with a high prevalence of GABA\(_R\)s containing two different \(\alpha\) subunits\(^14\),\(^15\) and with a model of the GABA\(_R\) assembly in which nonpoint-mutated \(\alpha\) subunits have a higher probability for interaction with the \(\gamma2\) subunit than H\(\rightarrow\)R point-mutated subunits\(^16\). This conclusion is further supported by the only marginal reduction of BDZ binding observed in spinal cords of the four single point-mutated mouse lines (Fig. 1e).

**Antihyperalgesia by single BDZ-sensitive GABA\(_R\) subtypes.** We next used the triple point-mutated mice to predict pharmacological actions of subtype-selective BDZ site agonists and treated them with DZP, a classical nonselective BDZ, whose agonistic activity was restricted to a single GABA\(_R\) subtype in triple point-mutated mice. We first focused on the antihyperalgesic potential of such an approach (that is, the potential to reduce neuropathy-induced hyperalgesia) and tested DZP in mice subjected to the chronic constriction injury (CCI; ref. 17) model. Baseline mechanical and heat sensitivity were statistically indistinguishable in all strains of mice analysed and all strains of mice developed similar hyperalgesia after CCI (Table 1). All subsequent experiments with DZP were performed on an \(\alpha1R\) point-mutated background to avoid DZP-induced sedation, which is a potential confounding factor in behavioural pain tests. We first tested different doses of systemic (per os (p.o.)) DZP in \(\alpha1\) point-mutated RHHH mice (Fig. 2a,b). On the basis of these results, we chose a dose of 10 mg kg\(^{-1}\) body weight, which is close to the ED\(_{70}\) for subsequent experiments. We then asked which GABA\(_R\) subtype exerts the strongest antihyperalgesic action and tested the effects of DZP on mechanical (von Frey filament test) and heat (Hargreaves test) hyperalgesia in the four strains of GABA\(_A\) triple point-mutated mice (Fig. 2c–e). For both stimuli, the strongest effect was achieved in RHRH mice in which only \(\alpha2\)GABA\(_A\)s were BDZ-sensitive. Exclusive targeting of \(\alpha3\) and \(\alpha5\)GABA\(_A\)s (in RHRH and RRRH mice) also elicited antihyperalgesia but to a smaller extent. Importantly, mice in which all four BDZ receptors had been point-mutated (RRRR mice) did not show any antihyperalgesic response. We next asked whether targeting of a second or third GABA\(_A\) subtype in addition to \(\alpha2\) would increase antihyperalgesic efficacy and...
compared antihyperalgesic responses in triple point-mutated mice with those in mice carrying only one or two point-mutated receptors. We found only small and statistically insignificant differences indicating that adding activity at a subtype different from α2 did not significantly increase antihyperalgesia (Fig. 2f).

The autoradiography data shown in Fig. 1d suggest a high prevalence of GABA<sub>A</sub>Rs with two different α subunits (‘mixed GABA<sub>A</sub>Rs’) in the spinal cord. Because the sum of the four receptor subtypes detected in the triple point-mutated mice greatly exceeded the total number of [<sup>3</sup>H]flumazenil binding sites in the wt mice, the pharmacological effect of a given GABA<sub>A</sub>R subtype may be overestimated when tested in triple point-mutated mice. To address this issue, we compared the level of antihyperalgesia achieved in the triple point-mutated mice with the loss in antihyperalgesia that occurs after mutation of the same subunit (Fig. 2g). For both types of sensory tests (Hargreaves and von Frey filament) and for all three α subunits under study, we found that the level of antihyperalgesia achieved in the triple point-mutated mice always exceeded the loss in antihyperalgesia by mutation of the same subunit consistent with the high
prevalence of mixed GABA\(_A\)Rs in the spinal cord. It is important to note that the rank order of antihyperalgesic efficacies was the same in experiments with triple point-mutated mice and in loss-of-function experiments in single and double point-mutated mice, and also identical to those found in a pain model employing chemical nociceptor activation instead of neuropathy (Fig. 2h).

Taken together, these results support a major contribution of \(\alpha 2\)GABA\(_A\)Rs to antihyperalgesia in different pain models. However, because all experiments had to be conducted on a \(\alpha 2\) point-mutated background to avoid confounding sedation, the relevance of \(\alpha 2\)GABA\(_A\)Rs might be overestimated if ‘pain-relevant’ \(\alpha 2\)GABA\(_A\)Rs contained also \(\alpha 1\) subunits. To address this potential caveat we used HZ-166 (ref. 18), a novel benzodiazepine site ligand with an improved \(\alpha 2/\alpha 1\) selectivity ratio\(^{19}\) and significant analgesic activity already at non-sedative doses\(^{20}\) (Fig. 3). Antihyperalgesia by HZ-166 was almost completely blocked in single \(\alpha 2\)H101R point-mutated (HRHH) mice, further confirming that the ‘pain-relevant’ GABA\(_A\)Rs exhibit an \(\alpha 2\) pharmacology.

**Potential undesired BDZ effects in triple point-mutated mice.** We next used the same triple point-mutated mouse approach to investigate non-pain-related effects. In these experiments, we focused on diminished locomotor activity (as a surrogate parameter of sedation), muscle strength and motor coordination. No significant differences were observed in the behaviour of drug-naive wt and point-mutated mice (Table 2). The DZP treatment strongly reduced locomotor activity in wt mice and in mice with BDZ-sensitive GABA\(_A\)Rs of only the \(\alpha 1\) subtype (HRRR mice; Fig. 4a). No sedative effects were observed in any of the other triple point-mutated mice. Mice with only \(\alpha 2\) BDZ-sensitive GABA\(_A\)Rs (RRHR mice) even showed a strong increase in locomotor activity, which may originate from the anxiolytic effect of DZP occurring through \(\alpha 2\)GABA\(_A\)Rs (ref. 12). Impairment of muscle strength was assessed in the horizontal wire test. Significant muscle relaxation was detected in wt mice and in mice with BDZ-sensitive GABA\(_A\)Rs of either only the \(\alpha 2\) (RRHR mice) or \(\alpha 3\) subtype (RRRR mice; Fig. 4b). Motor coordination, tested in the rotarod test, was significantly impaired by DZP in mice with only \(\alpha 1\) (HRRR mice) and with only \(\alpha 3\) BDZ-sensitive GABA\(_A\)Rs (RRHR mice; Fig. 4c). Quadruple point-mutated (RRRR) mice were completely protected from DZP-induced muscle relaxation and motor impairment. They did, however, show a trend towards reduced locomotor activity (compare Fig. 4a). We therefore assessed changes in locomotion also after higher DZP doses in the quadruple point-mutated mice and found significant and dose-dependent impairment starting at 30 mg kg\(^{-1}\) (Fig. 4d). Unlike \(\alpha 1\)GABA\(_A\)-mediated sedation, impairment of muscle strength was absent in the quadruple point-mutated mice even at doses \(\geq 30\) mg kg\(^{-1}\). The sedative action of DZP remaining in the quadruple point-mutated mice may be attributed to a low-affinity BDZ-binding site at \(\alpha 1\)GABA\(_A\)Rs described earlier\(^{21,22}\).

Our findings on the contribution of \(\alpha 1\), \(\alpha 2\) and \(\alpha 2\)GABA\(_A\)Rs to sedation, anxiolysis and muscle relaxation confirm previous studies using single GABA\(_A\)R point-mutated mice\(^{11,12,23}\). The results from our experiments with triple point-mutated mice on motor coordination, however, differ from those obtained with single point-mutated mice\(^{11,12}\). This discrepancy may arise from the involvement of mixed GABA\(_A\)Rs containing \(\alpha 1\) and \(\alpha 3\) subunits. We therefore tested whether motor coordination would also be impaired by TP003, an \(\alpha 3\)GABA\(_A\)-selective BDZ site agonist\(^{24}\). Two hours after treatment with TP003 (10 mg kg\(^{-1}\), p.o.), the time to fall off the rod was similarly decreased in wt and RRHR mice (by 43.9 ± 10.2%, paired t-test \(P < 0.05\), \(n = 5\), in wt mice, and by 38.5 ± 6.4, \(n = 5\), \(P = 0.05\)), while HRHH and vehicle-treated wt mice showed no impairment (0.7 ± 12.0%, \(n = 5\), \(P = 0.91\) and −4.7 ± 9.2%, \(n = 5\), \(P = 0.59\)).

**Liability to tolerance development.** Another major limitation of classical BDZs is their liability to tolerance development (that is, the loss of activity during prolonged use). We tested whether this tolerance would also occur for the antihyperalgesic actions of BDZs and whether it could be prevented by selectively targeting \(\alpha 2\)GABA\(_A\)Rs. To this end, we applied again CCI surgery and treated mice of the different strains for nine consecutive days with DZP or vehicle, and mechanical hypersensitivity was measured in wt mice (Fig. 5a, b). Tolerance, here defined as a reduction of the antihyperalgesic response, was generally lower than in the single point-mutated (RHHH) mice. We therefore repeated the first experiment with a lower dose of DZP (3 mg kg\(^{-1}\)) to exclude the possibility that the absence of tolerance in the RHHH mice was

### Table 1 | Baseline nociceptive sensitivities of all GABA\(_A\)R genotypes under study.

| Genotype | BDZ-sensitive \(\alpha\) subunit(s) | Pre-CCI | Post-CCI |
|----------|-----------------------------------|---------|---------|
|          |                                   | von Frey (g) (number of mice) | Hargreaves (s) (number of mice) | von Frey (g) (number of mice) | Hargreaves (s) (number of mice) |
| HHHH (wt) | \(\alpha 1, \alpha 2, \alpha 3, \alpha 5\) | 4.04 ± 0.07 (20) | 22.9 ± 0.72 (17) | 1.48 ± 0.23 (8) | 11.7 ± 1.03 (16) |
| RHHH | \(\alpha 2, \alpha 3, \alpha 5\) | 4.41 ± 0.31 (26) | 22.6 ± 0.63 (15) | 1.52 ± 0.11 (15) | 9.68 ± 0.81 (12) |
| RHRR | \(\alpha 2, \alpha 3\) | 4.21 ± 0.12 (29) | 23.4 ± 0.57 (15) | 1.46 ± 0.09 (20) | 10.4 ± 1.15 (10) |
| RRHH | \(\alpha 2, \alpha 5\) | 4.05 ± 0.23 (29) | 22.9 ± 0.60 (20) | 1.26 ± 0.10 (9) | 12.8 ± 1.70 (7) |
| RRHR | \(\alpha 3, \alpha 5\) | 4.23 ± 0.04 (21) | 21.0 ± 0.43 (20) | 1.52 ± 0.06 (8) | 8.54 ± 0.62 (19) |
| RRRR | \(\alpha 5\) | 4.12 ± 0.06 (32) | 22.2 ± 0.53 (32) | 1.42 ± 0.11 (18) | 10.7 ± 0.72 (19) |
| RRRR | None | 4.32 ± 0.07 (23) | 21.5 ± 0.42 (23) | 1.27 ± 0.08 (13) | 10.3 ± 1.00 (9) |
|          |                                   | 20.8 ± 1.48 (6) | 1.32 ± 0.16 (9) | 1.67 ± 0.08 (11) | 10.2 ± 0.89 (12) |

ANOVA, analysis of variance; BDZ, benzodiazepine; CCI, chronic constriction injury; GABA\(_A\)R, GABA\(_A\) receptor. Values are given as mean ± s.e.m. ANOVA followed by Dunnett’s post hoc test with wt (HHHH) as control.
due to a smaller DZP effect. With this lower dose, complete tolerance still developed in RHHH mice (Fig. 5c,d). These experiments indicate that activity at α3 or α5GABA<sub>A</sub>Rs was necessary to induce antihyperalgesic tolerance. We therefore tested double point-mutated mice in which α3GABA<sub>A</sub>Rs or α5GABA<sub>A</sub>Rs were left BDZ-sensitive in addition to α2GABA<sub>A</sub>Rs (RHHR and RRRH mice). Complete tolerance developed in RHHR mice, but not in RRRH mice, indicating that activity at α3GABA<sub>A</sub>Rs was necessary for induction of antihyperalgesic tolerance in mutant mice (Fig. 5e). In this set of experiments, we
finally tested whether the same subtype dependence would also be found for tolerance against α2GABAAR-R-mediated anxiolysis (Fig. 5f). Unlike antihyperalgesic tolerance, anxiolytic tolerance (measured as increased locomotor activity in the open field) still developed in RHRR mice. This dissociation suggests that tolerance development against antihyperalgesia is not a receptor or cell-autonomous process 

**Why classical BDZs lack clinically relevant analgesic properties.** Our present results and previous pain studies employing subtype-selective BDZ site agonists in rodents30,26–29 contrast with the lack of a clear analgesic or antihyperalgesic action of classical BDZs in human patients30,31. Apart from species differences and differences between disease models and actual disease in human patients, we found one possible explanation particularly worth studying. The doses and the degrees of receptor activation required for a relevant effect might be significantly higher in case of antihyperalgesia than of sedation. As a consequence, antihyperalgesia would occur in patients only at doses already inducing strong sedation. The availability of the triple point-mutated mice allowed us to directly compare the

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**Table 2 | Baseline locomotor activity and performance in the horizontal wire and rotarod tests.**

| Genotype | BDZ-sensitive α subunit (-α) | Activity counts (number of mice) | Horizontal wire performance* (number of mice) | Rotarod performance† (number of mice) |
|----------|-------------------------------|----------------------------------|-----------------------------------------------|--------------------------------------|
| HHHH (wt) | α1, α2, α3 and α5 | 962 ± 114 (14) | 92 ± 2.5 (17) | 92 ± 5.6 (5) |
| HRRR | α1 | 1102 ± 104 (9) | 98 ± 1.2 (9) | 98 ± 8.2 (6) |
| RRHR | α2 | 1132 ± 106 (8) | 96 ± 1.2 (8) | 81 ± 5.3 (5) |
| RRRR | α3 | 1279 ± 114 (10) | 99 ± 1.1 (10) | 85 ± 7.7 (5) |
| RHRR | α5 | 987 ± 90.4 (8) | 100 ± 0.0 (8) | 84 ± 11 (5) |
| RRHR | None | 1037 ± 115 (9) | 99 ± 1.2 (9) | 110 ± 13 (5) |

Statistics‡:

- ANOVA, analysis of variance; BDZ, benzodiazepine; wt, wild type.
- Values are given as mean ± s.e.m.
- Horizontal wire performance, expressed as success rate (%).
- Rotarod performance, expressed as time before fall off (s).
- ANOVA followed by Dunnett’s post hoc test with wt (HHHH) as control.

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**Figure 4 | Sedation, muscle relaxation and motor coordination in GABAAR-mutated mice.** Effects of DZP (10 mg kg⁻¹, p.o.) on locomotor activity in the open field test (a), on muscle relaxation in the horizontal wire test (b), and on motor coordination in the rotarod test (c). ***P<0.001, *P<0.05 significant versus vehicle-treated wt (HHHH) mice (ANOVA followed by Dunnett’s post hoc test). Statistics: locomotor activity F(6,177) = 79.5 (n = 106, 26, 8, 8, 9, 13 and 13 mice, for vehicle and DZP-treated wt mice, and DZP-treated HRRR, RRHR, RHRR and RRHR mice, respectively). Horizontal wire F(6,165) = 44.0 (n = 109, 14, 18, 8, 9, 13 and 10 mice). Rotarod F(6,41) = 11.5 (n = 8, 5, 6, 7, 6, 8 and 8 mice). (d) Effects of DZP on locomotor activity and horizontal wire performance in quadruple GABAAR point-mutated (RRRR) mice. **P<0.001; *P<0.05 significant versus vehicle (ANOVA followed by Dunnett’s post hoc test) F(3,33) = 13.4 (locomotor activity), n = 9, 13, 8 and 7 mice, for vehicle, and 10, 30 and 100 mg kg⁻¹ DZP; F(3,30) = 0.44; P > 0.60 (horizontal wire test), n = 9, 10, 8 and 7 mice, for vehicle, and 10, 30 and 100 mg kg⁻¹ DZP. All data points are mean ± s.e.m.
**Figure 5** | Tolerance liability against the antihyperalgesic effects of DZP. Starting on day 5 after the CCI surgery, mice were treated with DZP or vehicle once daily for nine consecutive days. On day 10, mice were given either DZP or vehicle, and antihyperalgesic effects were measured for 2 h. (a) Complete loss of antihyperalgesic activity was observed after 9-day DZP treatment (10 mg kg\(^{-1}\), p.o.) in RHRR mice. (b) Same as a, but RHRR triple-point-mutated mice. These mice were completely protected from tolerance development against DZP-induced antihyperalgesia. (c) Same as a, but lower dose of DZP (3 mg kg\(^{-1}\), p.o.). Tolerance in RHHH mice still developed at a lower dose of DZP, which induced an antihyperalgesic effect similar to that of 10 mg kg\(^{-1}\) in RHRH mice. (d) Statistical comparison. Two-way ANOVA for the interaction pretreatment \(\times\) acute treatment \((F(3,25) = 32.5, n = 5, 8, 7 and 8 mice for veh/veh, veh/DZP, DZP/veh and DZP/DZP groups, respectively); F(3,25) = 0.014 (n = 7, 8, 7 and 6); F(3,24) = 5.97 (n = 8, 7, 7 and 6), for data shown in a–c). Maximum antihyperalgesic activity was calculated for the interval between 80 and 120 min after DZP administration. (e) Double GABA\(_A\)R point-mutated mice (RHRH and RHHH), in which \(z3GABA_A_R\) and \(z5GABA_A_R\) were left BDZ-sensitive in addition to \(z2GABA_A_R\). Tolerance development required the additional presence of DZP-sensitive \(z3GABA_A_R\). Two-way ANOVA for the interaction pretreatment \(\times\) acute treatment. RHRH mice: \(F(3,22) = 22.1 (n = 6, 6, 7 and 6 mice for veh/veh, veh/DZP, DZP/veh and DZP/DZP groups, respectively); RHHH mice: \(F(3,21) = 0 (n = 6 for all groups). (f) Unlike antihyperalgesic effects, anxiolytic effects of DZP were still susceptible to tolerance development in RHRH mice after 9-day treatment with DZP (10 mg kg\(^{-1}\), p.o.). Two-way ANOVA \(F(3,26) = 28.3 (n = 8, 7, 7 and 7) for the interaction pretreatment \(\times\) acute treatment. ***P = 0.001; *P = 0.05. All data points are mean \(\pm\) s.e.m.

Dosages and levels of receptor occupancy at the relevant GABA\(_A\)R subtypes and sites. For these experiments, we chose midazolam (MDZ) as a second classical BDZ in addition to DZP. First, we determined for both drugs their \(z2/z1\) selectivity profiles in electrophysiological experiments on recombinant GABA\(_A\)Rs. As expected, we found that DZP potentiated \(z1/z2/z2/2\) and \(z2/z3/2\) GABA\(_A\)Rs with similar efficacy and potency (Fig. 6a and Table 3). By contrast, MDZ potentiated \(z1/z2/2/2\) GABA\(_A\)Rs more than twice as much as \(z2/z3/2\) GABA\(_A\)Rs (Fig. 6b and Table 3). We then verified that the H \(\rightarrow\) R point mutation blocked not only DZP effects but also MDZ binding and GABA\(_A\)R potentiation (Figs 6cd; see also ref. 32). Next, we compared the dose dependency of DZP- and MDZ-induced antihyperalgesia in mice with only \(z2\) BDZ-sensitive GABA\(_A\)Rs (RHRR mice) with that of DZP- and MDZ-induced sedation in mice with only \(z1\) BDZ-sensitive GABA\(_A\)Rs (HRRR mice). We found that half maximal sedation occurred already at a doses of 0.33 \(\pm\) 0.05 and 0.52 \(\pm\) 0.11 mg kg\(^{-1}\) (mean \(\pm\) s.d.) for DZP and MDZ, respectively, while half maximal antihyperalgesia required 8 \(\pm\) 6 mg kg\(^{-1}\) (DZP) and 10.4 \(\pm\) 2.0 mg kg\(^{-1}\) (MDZ). A rightward shift of the response curve was also observed when the degrees of receptor occupancy required for antihyperalgesia and for sedation were compared. Half maximal sedation was reached when DZP had bound 47 \(\pm\) 6% brain \(z1GABA_A_R\), while half maximal antihyperalgesia required 71 \(\pm\) 2% occupancy at spinal \(z2GABA_A_R\). In case of MDZ, the required receptor occupancies were even further apart (24 \(\pm\) 2% of brain \(z1GABA_A_R\) and 71 \(\pm\) 2% of spinal \(z2GABA_A_R\)).
consistent with the even less favourable α2/α1 selectivity ratio of MDZ. These data indicate that, when applied to wt mice, the DZP and MDZ doses needed for half maximal antihyperalgesia induce an almost complete (~95%) reduction in locomotor activity, while at non-sedative doses both BDZs would not induce significant antihyperalgesia. Dose-limiting sedation is therefore the most likely reason for the absence of a relevant antihyperalgesic activity of classical, nonselective BDZs in human patients.

**Discussion**

In the present study we have employed triple GABAₐR point-mutated mice to characterize the pharmacological actions expected from yet-to-be-developed subtype-selective BDZ-binding site agonists. Our study can be viewed as a ‘restriction-of-function’ approach (‘what effect of DZP remains when only a single GABAₐR subtype is targeted?’) as opposed to previous loss-of-function studies in single GABAₐR point-mutated mice (‘which effects of DZP are lost or reduced when activity at one GABAₐR subtype is abolished compared with DZP-treated wt mice?’). With respect to sedation, anxiolysis and the muscle-relaxant action, the present study confirms previous results obtained with single point-mutated mice: sedative actions of BDZs occur through α1GABAₐR (refs 10,11), anxiolytic effects through α2GABAₐR (ref. 12) and muscle relaxation through α2 and α3GABAₐR (ref. 23).
Discrepancies between results from restriction-of-function and loss-of-function approaches were observed for impairment of motor coordination. The present study shows that undesired impairment of motor coordination is caused by DZP in HRRR and RRHR mice (that is, it is evoked when either \( \alpha1 \) or \( \alpha3 \)GABA\(_A\)Rs are specifically targeted), while previous reports in the respective single point-mutated mice failed to provide evidence for an involvement of these GABA\(_A\)R subtypes\(^{11,12}\). This discrepancy is consistent with the idea that DZP impairs motor coordination through mixed GABA\(_A\)Rs containing an \( \alpha1 \) and an \( \alpha3 \) subunit (\( \alpha1/\alpha3 \)GABA\(_A\)Rs). Immunohistochemical analyses have shown that co-expression of \( \alpha1 \) and \( \alpha3 \) GABA\(_A\)R subunits occurs in subsets of central neurons\(^{33,34}\), and biochemical data have provided direct evidence for the presence of different \( \alpha \) subunits within the same GABA\(_A\)R complex\(^{15}\). In particular, \( \alpha3 \) containing GABA\(_A\)Rs occur mainly as mixed \( \alpha1/\alpha3 \)GABA\(_A\)Rs (refs 14,15). Furthermore, biochemical data also suggest that in mixed GABA\(_A\)Rs with one point-mutated \( \alpha \) subunit the nonpoint-mutated (wt) subunit has a higher evidence for an involvement of these GABA\(_A\)R subtypes\(^{11,12}\).

| Table 3 | PK/PD parameters* of DZP and MDZ. |
|---------|----------------------------------|
|         | DZP                             | MDZ                             |
|         | \( \alpha1/\beta2/\gamma2 \)     | \( \alpha2/\beta3/\gamma2 \)   |
|         | \( \alpha1/\beta2/\gamma2 \)     | \( \alpha2/\beta3/\gamma2 \)   |
|         | \( \xi1/\eta2/\zeta2 \)          | \( \xi2/\eta3/\zeta2 \)        |
|         | \( \xi1/\eta2/\zeta2 \)          | \( \xi2/\eta3/\zeta2 \)        |
|         | ED\(_{50}\) (EC\(_{50}\))        | ED\(_{50}\) (EC\(_{50}\))       |
|         | 0.081±0.02                      | 1.03±0.04                      |
|         | 91.6±7.7                        | 103±11                         |
|         | 1.50±0.60                       | 1.20±0.35                      |
|         | Sedation                        | Analgesia                      |
|         | 1/2 selectivity \(^{3}\)        | 0.25                            |
|         | 0.33±0.05                       | 0.76±0.6                       |
|         | 93.6±4.5                        | 100 (Fixed)                    |
|         | 2.0±0.64                        | 0.75±0.19                      |
|         | ED\(_{50}\) (sedation/analgesia) | Sedation                        |
|         | 0.043                           | Analgesia                      |
|         | RO\(_{50}\) (%)                 | 46.9±5.5                       |
|         | 70.9±1.7                        | 100 (Fixed)                    |
|         | 14.1±4.1                        | 14.9±2.0                       |
|         | Rate                             | 4.14±2.10                      |
|         | ED\(_{50}\) (sedation/analgesia) | 0.66                            |
|         | RO\(_{50}\) (%)                 | 0.31                            |

\( \xi \), pharmacokinetic; \( \eta \), pharmacodynamic; DZP, diazepam; MDZ, midazolam; %MPE, percent maximal possible effect; RO, receptor occupancy.

For number of mice per group see Fig. 6.

Values are mean±s.d.

\( ^{3} \) Assessed the presence of a low-affinity BDZ-binding site in \( \alpha1/\gamma \) subunit.

remaining BDZ-sensitive would yield full behavioural effects. Results for antihyperalgesia obtained in the present study were consistent with a contribution of mixed GABA\(_A\)Rs to antihyperalgesia. For all three contributing \( \alpha \) subunits, the loss-of-function obtained through point mutation of one \( \alpha \) subunit was smaller than the restriction-of-function in the respective triple point-mutated mouse. The discrepancies were particularly large for \( \alpha3 \) in mechanical sensitization and for \( \alpha5 \) in heat hyperalgesia, suggesting that these effects occur mainly through mixed GABA\(_A\)Rs. Importantly, the rank orders of antihyperalgesic efficacy were the same for the restriction-of-function and loss-of-function approaches with \( \alpha2 > \alpha5 > \alpha3 \) for mechanical sensitization and \( \alpha2 > \alpha3 > \alpha5 \) for heat hyperalgesia and chemical nociception. The same rank order of efficacies has also been reported previously for antihyperalgesia using local spinal injections in single GABA\(_A\)R point-mutated mice\(^{5}\). Our present results therefore corroborate the critical importance of \( \alpha2/\alpha3 \)GABA\(_A\)Rs as targets for antihyperalgesia.

Our experiments on the quadruple point-mutated mice indicate that antihyperalgesia, anxiolysis, muscle relaxation and impairment of motor coordination occur through the high-affinity BDZ-binding site formed by the \( \alpha1/\gamma \) interface. Among the DZP actions assessed here, only sedation by high doses of DZP occurred in quadruple point-mutated mice. This result is consistent with previous electrophysiological data, which suggested the presence of a low-affinity BDZ-binding site in \( \alpha1/\gamma \)GABA\(_A\)Rs contributing to the anaesthetic actions of BDZs\(^{22}\).

Most BDZ effects quickly diminish during prolonged treatment (that is, they undergo fast tolerance development). In the present study, we show that this is also the case for antihyperalgesia. Previous studies with the non-sedative BDZ site ligands L-838,417 and HZ-166 showed that these compounds have strongly reduced liabilities to tolerance development\(^{5,20,27}\). It has, however, not been possible to attribute this reduced tolerance to generally reduced agonistic activity or to improved subtype specificity. Our present study shows that tolerance can be avoided when only \( \alpha2/\alpha3 \)GABA\(_A\)Rs are targeted, even with compounds that exert full agonistic activity. Additional experiments in RHRH and RRHR mice exclude \( \alpha5 \)GABA\(_A\)Rs and suggest that activity at \( \alpha3 \)GABA\(_A\)Rs is required for tolerance induction. However, because all these experiments were carried out in mice carrying the H\( \rightarrow \)R point mutation in the \( \alpha1 \) subunit, we cannot exclude...
that tolerance occurs through mixed α1/α3GABA_2A-Rs, which in
the wt situation may exhibit an α1 pharmacology. Such a scenario
would explain why compounds with activity at α2GABA_2A-Rs but
absent or reduced activity at α1GABA_2A-Rs lack liability to
tolerance.24,27 Interestingly, tolerance in the anxiolytic
action of DZP was retained in triple point-mutated RHRR mice with
only α2GABA_2A-Rs remaining BDZ-sensitive. This difference
suggests that tolerance development against the different BDZ
effects involves activity at distinct subunits and possibly different
mechanisms. Cell- or receptor-autonomous processes, which
have been proposed for the desensitization of hippocampal
α2GABA_2A-Rs (ref. 25), are unlikely to be responsible for the
tolerance against antihyperalgesia.

Additive (reinforcing) properties of classical BDZs are another
area of concern that has not been addressed in the present study.
Reduced BDZ-induced reward facilitation has been reported in
mice carrying the H-→R point mutation in either the α1, α2 or
α3GABA_2A-R subunits.35,36 Preference for MDZ in a two-bottle
choice paradigm was absent in α1 (refs 31,35) and α2 point-
mutated mice,35 suggesting that the simultaneous additive modulations of both
the α1 and α2GABA_2A-R are required for reinforcement. Tan et al.37 reported that α1GABA_2A-Rs
on GABAergic neurons in the ventral tegmental area (VTA) are
required for MDZ-induced neuronal plasticity, strengthening
glutamatergic excitation in the VTA. These findings suggest that
activity at more than one GABA_2A-R subtype is necessary for BDZ
reinforcement. α2-selective BDZ site agonists might therefore be
largely devoid of addictive properties.

Previous studies have provided evidence that activation of
supraspinal GABA_2A-Rs might enhance pain and counteract spinal
antihyperalgesia, for example, through the inhibition of descending
nociceptive fibre tracts.27,39 In our study, we did not observe
any pronociceptive actions of systemically administered DZP. If
such pronociceptive actions were relevant in the pain models used
here, they would occur through α1GABA_2A-Rs whose functions in
nociception were not addressed in the present study. Any such
effects would be avoided by α1-sparring BDZ site agonists.

The availability of the triple α1GABA_2A-R point-mutated mice
permits a direct pharmacokinetically/pharmacodynamic compar-
ison of α1-mediated sedation and α2-mediated antihyperalgesia
in the absence of confounding behavioural effects from other
GABA_2A-R subtypes. In case of DZP, ER_50 values for sedation
and antihyperalgesia differed by a factor of more than 20, and a 50%
higher degree of receptor occupancy was needed for α2-mediated
antihyperalgesia compared with α1-mediated sedation. New
subtype-selective agents will therefore have to have a high degree
of α2 over α1 selectivity in order to achieve clinically relevant
antihyperalgesia in the absence of sedation. These data also
indicate that dose-limiting sedation most likely underlies the lack
of clinically relevant antihyperalgesic effects of presently used
(noselective) BDZs. This is consistent with a recent clinical
study in human volunteers showing weak antihyperalgesic effects
at doses that caused only mild sedation.40

Which GABA_2A-R subtype should be targeted for an optimal
benefit-risk ratio in pain treatment? Both the present restriction-
of-function and loss-of-function experiments on systemic BDZ
administration and previous experiments with local spinal
injections and single GABA_2A-R point-mutated mice2 attribute
the highest antihyperalgesic efficacy to α2GABA_2A-Rs. The present
study has shown that adding activity at α3GABA_2A-Rs or
α2GABA_2A-Rs increases antihyperalgesic efficacy only moderately
or not at all. Because it is at present not known how the different
mixed GABA_2A-Rs respond to subtype-selective agents, a
conservative prediction should be made on the basis of the
results obtained from triple point-mutated mice. These
experiments indicate that sedation is exclusively due to
activation α1GABA_2A-Rs, which is also consistent with studies
using the subtype-selective agonist TPA023B that completely
lacks agonistic activity at α1GABA_2A-Rs as well as sedation in
humans.34 The present experiments also showed that impaired
motor coordination involves α1GABA_2A-Rs and/or α3GABA_2A-Rs.
None of the undesired effects investigated in the present study
could be attributed to α2GABA_2A-Rs. However, cognitive
impairment by nonselective BDZ likely originates from activity
at α2GABA_2A-Rs, as α2GABA_2A-R-selective inverse agonists enhance
cognitive capabilities.32 Subtype-selective BDZ agonists targeting
only α2GABA_2A-Rs should therefore have the best benefit-risk
ratio. They will produce pronounced antihyperalgesia in the
absence of sedation and will not interfere with motor
coordination and should not lose antihyperalgesic activity
during prolonged treatment. They will however have anxiolytic
properties (at least during the beginning of the treatment) and
muscle-relaxant effects. Both of these actions may be beneficial in
chronic pain patients. Because the side effects studied here are
relevant for other indications, we expect that our present findings
will benefit not only the development of innovative subtype-
selective BDZs as analgesics but also as drugs for the treatment of
several prevalent psychiatric diseases.43

Methods

Mice. Experiments were performed in wt mice, and in homozygous single, double,
and triple (H→R) GABA_2A-R point-mutated mice, that is, in mice
expressing different combinations of BDZ-sensitive and BDZ-insensitive GABA_2A-R
α subunits. All mice were of the same genetic background (129X1/SvJ). Double,
triple and quadruple point-mutated mice were generated by cross-breding single
point-mutated mice described earlier.11,12,24

Autoradiography. The distribution of BDZ-sensitive GABA_2A-R subtypes in the
lumbar spinal cord was examined in 16-μm-thick horizontal sections, which were
cut from fresh-frozen spinal cords. Sections were incubated with 5 nM [3H]flu-
mazenil (50 Ci mmol⁻¹) or 9 nM [3H]Ro15-4513 (22.7 Ci mmol⁻¹)
diluted in 50 mM Tris pH 7.4 for 120 min on ice. After washing three times for 20 s in
ice-cold buffer, sections were dried and exposed along with [3H]-standards to a tri-
tium-sensitive phosphoimaging screen (Cyclone Storage Phosphor Screen, Perkin
Elmer). Quantification was carried out using the Optiquant software (Perkin
Elmer). Nonspecific binding was assessed by co-incubating 10 μM clonazepam
([1H]fluMexazilin binding) or 10 μM flumazenil ([1H]Ro15–4513 binding).

Immunohistochemistry. The localization of GABA_2B-R subunits was visualized on
40-μm-thick lumbar spinal cord cryosections by DAB immunoperoxidase staining
on sections from artificial cerebrospinal fluid (aCSF)-perfused mice postfixed for
90 min in 4% paraformaldehyde (PFA) (without picric acid).45 Antibodies were
home-made subunit-specific antiserum.44 Final dilutions were 1:20,000 (α1), 1:1,000
(α2), 1:10,000 (α3), 1:5,000 (α5) and 1:10,000 (γ2). Images were taken with a bright
field light microscope connected to a digital camera and processed with the
Axiovision Rel. 4.5 software where an intensity-gradient false-colour filter
was applied.

Behavioural experiments. All behavioural experiments were performed in 7–
to 12-week-old female and male mice. Care was taken to ensure equal numbers of
female and male mice. All behavioural experiments were made by a non-experi-
menter, blinded either to the genotype of the mice or to their treatment with drug
or vehicle. Permission for animal experiments was obtained from the Veterinäramt
des Kantons Zürich (licence numbers 135/2009 and 126/2012).

DZP (suspended in 0.9% saline and 1% Tween® 80) and MDZ (suspended in 0.9%
saline pH 3.0) were applied orally in all experiments. HZ-166 (suspended in 0.5%
cold buffer, sections were dried and exposed along with [3H]-standards to a tri-
tium-sensitive phosphoimaging screen (Cyclone Storage Phosphor Screen, Perkin
Elmer). Quantification was carried out using the Optiquant software (Perkin
Elmer). Nonspecific binding was assessed by co-incubating 10 μM clonazepam
([1H]fluMexazilin binding) or 10 μM flumazenil ([1H]Ro15–4513 binding).

Neuropathic pain was evoked throughCCI1 of the left sciatic nerve proximal
to the trifurcation with three loose (5-0 silk) ligatures. Mice, which showed signs of
paralysis or which did not develop significant hypersensitivity, were excluded from
subsequent experiments. Effects of DZP and MDZ (p.o. in 0.9% saline and 1% Tween® 80) on thermal and mechanical hyperalgesia were assessed 1 week after
surgery. Heat hyperalgesia was quantified as the change in the latency of paw
withdrawal evoked by exposure of the plantar side of one hindpaw to a defined
radiant heat stimulus. Mechanical hyperalgesia was assessed with electronic
force transducers (Ugo Basile, Italy). Three to four measurements were
made for each time point and animal for both heat and mechanical
hyperalgesia. Percent maximal possible effect (%MPE) was calculated as follows:
of MDZ and 6.3 nM [3H]Ro15-4513 (22.7 Ci mmol). HEK293 cells were harvested in PBS. HEK293 cells were homogenized in 10 mM Tris. Locomotor activity was measured in an open field arena (10 cm diameter) equipped with four pairs of light beams and photosensors. Mice were placed into the arena 75 min before DZP application. Locomotor activity was analysed for the time interval between 20 and 165 min after DZP administration. Motor control was measured with a rotarod accelerating from 4 to 40 r.p.m. within 5 min. Sixty minutes after DZP administration mice were placed on the rotarod. Five measurements were taken per mouse.

To assess muscle relaxation, mice were placed with their forepaws on a metal horizontal wire placed 20 cm above ground. Successes and failures to grab the wire with at least one hindpaw were recorded between 60 and 180 min after DZP administration.

Electrophysiology. The effects of DZP and MDZ on currents through recombinant GABA<sub>A</sub>Rs were studied in HEK293 cells (ATCC) transiently expressing GABA<sub>A</sub>Rs. HEK293 cells were transfected using lipofectamine LTX<sup>®</sup>. To express the γ2 subunit (required for modulation of GABA<sub>A</sub>Rs by BDZs) in all recorded cells, we transfected cells with a plasmid expressing the γ2 subunit plus enhanced yellow fluorescent protein from an internal ribosomal entry site (IRES), and only selected eGFP-positive cells for recordings. The transfection mixture containing (in µg): 1 µg, 1 µg and 3 µg [3H]Ro15-4513 (GFP, 100% receptor occupancy<sup>47</sup>).</p>
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Author contributions

W.T.R. performed all experiments, except the electrophysiological measurement, and analysed the data. D.B. performed and analysed the radio ligand-binding experiments. M.A.A. performed and analysed the electrophysiology experiments. U.R. helped design the experiments. H.U.Z. designed experiments, analysed data and wrote the manuscript. All authors made comments on the manuscript.

Additional information

Competing financial interests: The authors declare no competing financial interests.

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