LETTERS

Reversal of pathological pain through specific spinal $GABA_A$ receptor subtypes

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Inflammatory diseases and neuropathic insults are frequently accompanied by severe and debilitating pain, which can become chronic and often unresponsive to conventional analgesic treatment[1,2]. A loss of synaptic inhibition in the spinal dorsal horn is considered to contribute significantly to this pain pathology[3–7]. Facilitation of spinal γ-aminobutyric acid (GABA)ergic neurotransmission through modulation of $GABA_A$ receptors should be able to compensate for this loss[8,9]. With the use of $GABA_A$-receptor point-mutated knock-in mice in which specific $GABA_A$ receptor subtypes have been selectively rendered insensitive to benzodiazepine-site ligands[10–12], we show here that pronounced analgesia can be achieved by specifically targeting spinal $GABA_A$ receptors containing the α2 and/or α3 subunits. We show that their selective activation by the non-sedative ('α1-sparing') benzodiazepine-site ligand L-838,417 (ref. 13) is highly effective against inflammatory and neuropathic pain yet devoid of unwanted sedation, motor impairment and tolerance development. L-838,417 not only diminished the nociceptive input to the brain but also reduced the activity of brain areas related to the associative-emotional components of pain, as shown by functional magnetic resonance imaging in rats. These results provide a rational basis for the development of subtype-selective GABAergic drugs for the treatment of chronic pain, which is often refractory to classical analgesics.

More than 40 years ago, the gate control theory of pain[14] proposed that inhibitory neurons in the superficial dorsal horn of the spinal cord control the relay of nociceptive signals (that is, those evoked by painful stimuli) from the periphery to higher areas of the central nervous system. The pivotal role of inhibitory GABAergic and glycinergic neurons in this process has recently been demonstrated in several reports indicating that a loss of inhibitory neurotransmission underlies several forms of chronic pain[3–7]. Despite this knowledge, inhibitory neurotransmitter receptors have rarely been considered as targets for analgesic treatment. In fact, classical benzodiazepines, which are routinely used for their sedative, anxiolytic and anticonvulsant activity, largely lack clear analgesic efficacy in humans when given systemically[15]. To address this obvious discrepancy we investigated the molecular basis of GABAergic pain control in the spinal cord in an integrative approach based on an electrophysiological and behavioural analysis of genetically modified mice and on functional imaging in rats.

We first tested whether benzodiazepines exert antinociceptive effects at the level of the spinal cord by employing the mouse formalin assay, a model of tonic chemically induced pain. When the classical benzodiazepine diazepam was injected intrathecally into the lumbar spinal canal at doses of 0.01–0.09 mg per kg body weight, an apparent dose-dependent and reversible antinociception was obtained that could be antagonized by systemic treatment with the benzodiazepine antagonist flumazenil (10 mg kg^{-1} intraperitoneally (i.p.)) (Supplementary Fig. 1).

We next sought to identify the $GABA_A$ receptor isoforms responsible for this antinociception. $GABA_A$ receptors are heteropentameric ion channels composed from a repertoire of up to 19 subunits[16]. Benzodiazepine-sensitive isoforms are characterized by the presence of the γ2 subunit and one of four α subunits (α1, α2, α3 or α5)[17]. The generation of four lines of $GABA_A$-receptor point-mutated knock-in mice (α1(H101R), α2(H101R), α3(H126R) and α5(H105R)), in which a conserved histidine residue had been mutated to arginine, rendering the respective subunit insensitive to diazepam, has enabled the attribution of the different actions of diazepam to the individual $GABA_A$ receptor isoforms[10–12]. It also became possible to attribute the sedative effects of diazepam to $GABA_A$ receptors containing an α1 subunit[10] and the anxiolytic effect to those containing an α2 subunit[11] or—at high receptor occupancy—an α3 subunit[18]. We then compared the antinociceptive efficacy of intrathecal diazepam (0.09 mg kg^{-1}) in wild-type mice with that obtained in the four types of $GABA_A$-receptor point-mutated mice in models of inflammatory hyperalgesia induced by subcutaneous injection of zymosan A into one hindpaw and of neuropathic pain evoked by chronic constriction of the left sciatic nerve (chronic constriction injury (CCI) model).

Wild-type mice and all four types of mutant mice developed nearly identical pain sensitization after induction of inflammation or peripheral nerve injury (Fig. 1a, c). In wild-type mice, intrathecal diazepam (0.09 mg kg^{-1}) reversibly reduced inflammatory heat hyperalgesia (Fig. 1b), as well as CCI-induced heat hyperalgesia (Fig. 1d), cold allodynia (Fig. 1e) and mechanical sensitization (Fig. 1f) by 82 ± 13%, 92 ± 6% and 79 ± 9% (means ± s.e.m.), respectively. Responses of the non-inflamed or uninjured side were not significantly changed (Fig. 1a, c), indicating that spinal diazepam acted as an anti-hyperalgesic agent rather than as a general analgesic. Almost identical anti-hyperalgesic effects to those in wild-type mice were seen in mice carrying diazepam-insensitive α1 subunits. By contrast, α2(H101R) mice showed a pronounced reduction in diazepam-induced anti-hyperalgesia, which was consistently observed in all pain models tested. α3(H126R) and α5(H105R) mice showed smaller reductions, which occurred only in a subset of models. Importantly, intrathecal diazepam did not change spontaneous motor activity (Fig. 1g), indicating that the action of diazepam

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remained restricted to the spinal level and did not reach supraspinal sites, where sedation would have been induced.

Anti-hyperalgesic effects of spinal diazepam can in principle originate from the facilitation of $GABA_A$ receptors at different sites. Diazepam might act either on postsynaptic $GABA_A$ receptors located on intrinsic dorsal horn neurons, thereby increasing postsynaptic inhibition, or on $GABA_A$ receptors located on the central terminals of primary afferent nerve fibres to increase primary afferent depolarization and presynaptic inhibition[19]. To identify the benzodiazepine-sensitive $GABA_A$ receptor isoforms expressed at these sites we first employed electrophysiological measurements. GABAergic membrane currents were recorded from superficial dorsal horn neurons in transverse slices of spinal cords and from acutely isolated primary afferent (dorsal root ganglion (DRG)) nociceptive neurons characterized by their sensitivity to capsaicin. In nociceptive DRG neurons obtained from α2(H101R) mice, the facilitation of GABAergic membrane currents by diazepam was completely abolished, whereas no significant alteration was found in neurons from α1(H101R), α3(H126R) and α5(H105R) mice (Fig. 2a). Facilitation of GABAergic membrane currents by diazepam in intrinsic superficial dorsal horn (lamina I/II) neurons was significantly decreased in α2(H101R) and α3(H126R) mice but not in α1(H101R) or α5(H105R) mice (Fig. 2b). We next employed confocal immunofluorescence microscopy of dorsal horn $GABA_A$ receptor α subunits and studied their colocalization with substance P (a marker for primary peptidergic nociceptors) and for neurokinin 1 (NK1) receptors (a marker for intrinsic nociceptive dorsal horn neurons in lamina I). Consistent with our electrophysiological experiments and with previous morphological results in the rat[20] was our observation that α2 and α3 were the most abundant diazepam-sensitive $GABA_A$ receptor α subunits in the mouse spinal dorsal horn (Supplementary Fig. 2). Co-staining experiments with antibodies against substance P or NK1 receptors (Fig. 2c–j and Supplementary Table 1) revealed that α2, but not α1, α3 or α5, were extensively colocalized with substance-P-positive primary afferent terminals in lamina II, whereas colocalization with NK1-receptor-positive lamina I neurons was greatest for the α3 subunit. Staining for α1 and α5 subunits was much less abundant and only occasionally colocalized with either substance P or NK1 receptors. Both sets of experiments indicate that intrinsic dorsal horn neurons express mainly $GABA_A$ receptor isoforms containing α2 and α3 subunits, whereas α2 is the dominant diazepam-sensitive $GABA_A$ receptor α subunit in adult DRG neurons (see also ref. 21).

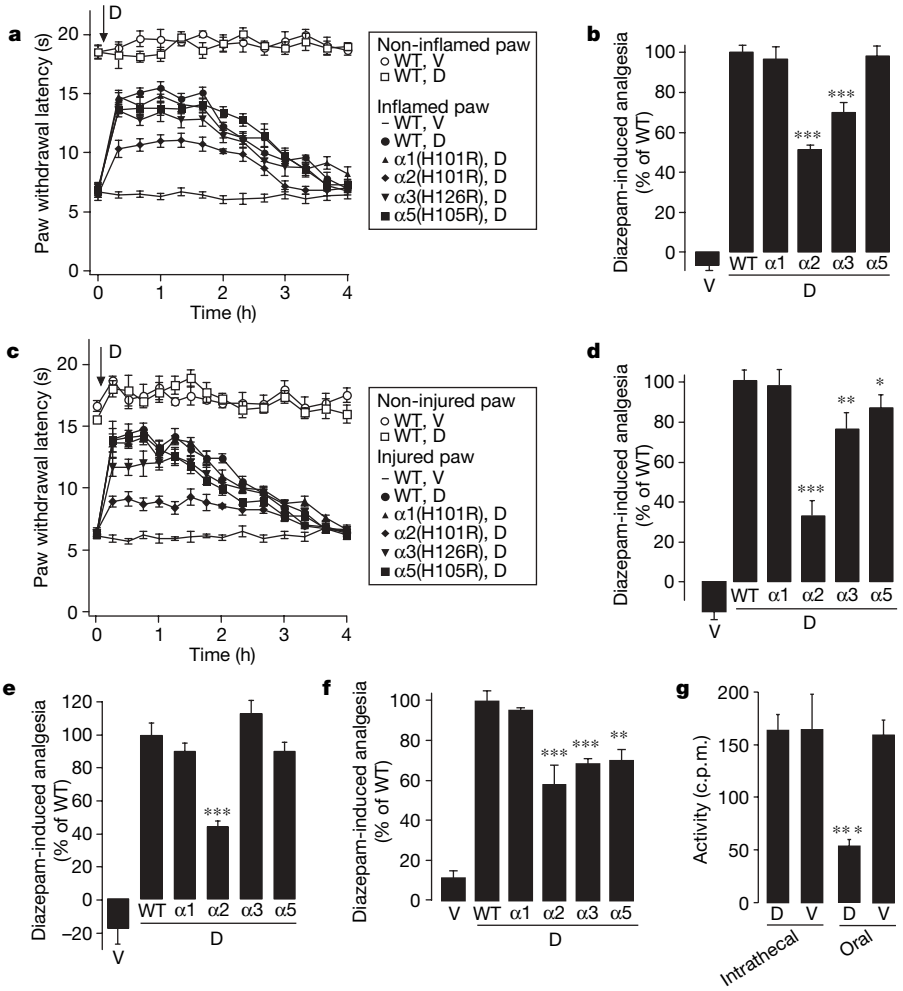

Figure 1 | Antinociceptive effects of spinal diazepam in different mouse pain models. **a**, **b**, Inflammatory pain induced by subcutaneous injection of zymosan A into the left hindpaw in wild-type (WT) mice and $GABA_A$ receptor point-mutated mice (α1(H101R), α2(H101R), α3(H126R), α5(H105R)). **a**, Paw withdrawal latencies (mean ± s.e.m.) in response to a defined radiant heat stimulus versus time after administration of intrathecal diazepam (D; 0.09 mg kg^{-1}; arrowed) 48 h after injection of zymosan A. V, vehicle. **b**, Percentage diazepam-induced analgesia in the different genotypes. **c**, **d**, As in **a** and **b**, but for the CCI model of neuropathic pain. **e**, **f**, Effects of intrathecal diazepam (0.09 mg kg^{-1}) on cold allodynia (**e**) and mechanical sensitivity (**f**) seven days after CCI surgery. Asterisk, $P \leq 0.05$; two asterisks, $P \leq 0.01$; three asterisks, $P \leq 0.001$ (statistically significant against wild type; ANOVA followed by Bonferroni post-hoc test, $n = 6$ or 7 mice per group). **g**, Effects of diazepam (0.09 mg kg^{-1} intrathecally, or 10 mg kg^{-1} orally) on motor activity in the Actiframe test (mean ± s.e.m., $n = 5$ or 6), 10–30 min after intrathecal drug application or 40–80 min after oral drug application. Three asterisks, $P \leq 0.001$ against vehicle (unpaired *t*-test).

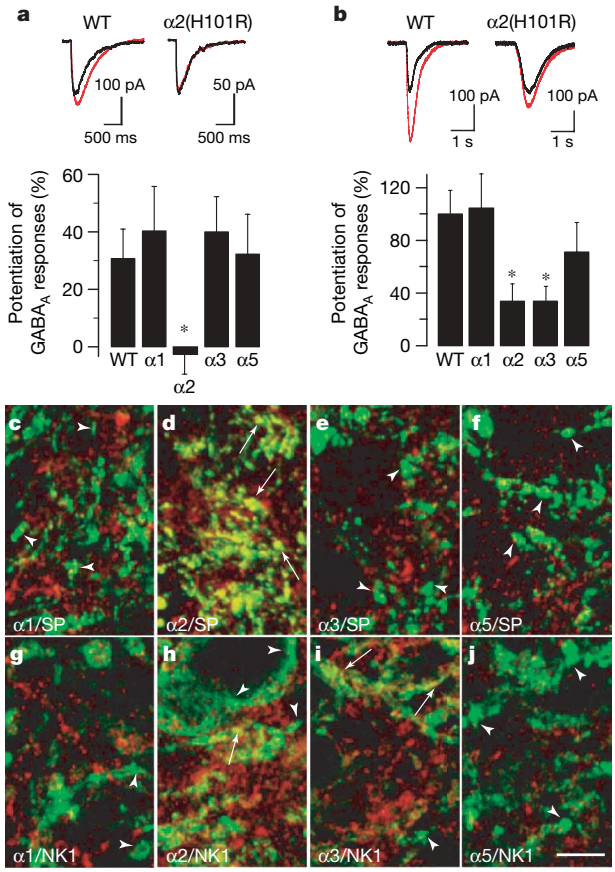

Figure 2 | GABA$_A$ receptor α subunits in capsaicin-sensitive primary afferent DRG neurons and in intrinsic dorsal horn neurons. **a**, **b**, Potentiation of GABAergic membrane currents by diazepam in wild-type (WT) and GABA$_A$ receptor mutant mice. **a**, DRG neurons. Averaged membrane currents evoked by puffer-applied exogenous GABA (1 mM) and percentage potentiation (mean ± s.e.m.) by diazepam (1 μM, $n = 5–9$). Asterisk, $P \leq 0.05$ (significant against all other genotypes; ANOVA followed by Fisher's post-hoc test). **b**, Intrinsic superficial dorsal horn neurons (mean ± s.e.m., $n = 5–10$) Asterisk, $P \leq 0.05$ (significant against wild-type and α1(H101R); ANOVA followed by Fisher's post-hoc test). **c–j**, Double immunofluorescence staining showing differential distribution of GABA$_A$ receptor α subunits (red) relative to substance P (SP)-positive axons and terminals (green) (**c–f**) or NK1 receptor-positive neurons (**g–j**) in laminae I and II. **c**, **g**, α1; **d**, **h**, α2; **e**, **i**, α3; **f**, **j**, α5. Arrows, double-labelled structures. Arrowheads, single-labelled structures devoid of GABA$_A$ receptor labelling. Scale bar, 5 μm (**c–j**).

The decrease in diazepam-induced anti-hyperalgesia in α2(H101R) and α3(H126R) mice corresponds well to the presence of these subunits on primary afferent nerve terminals and/or on intrinsic dorsal horn neurons.

So far, our results indicated that the spinal antinociceptive effect of diazepam is mainly mediated by GABA$_A$ receptor isoforms containing the α2 and α3 subunits, whereas the activation of α1-containing GABA$_A$ receptors is not involved. We therefore tested whether a similar analgesic effect would also be achieved after systemic treatment with subtype-selective benzodiazepine-site agonists, which spare the α1 subunit, by employing the non-sedative benzodiazepine-site ligand L-838,417, which is an antagonist at the α1 subunit and a partial agonist at receptors containing α2, α3 and α5 subunits[13].

Because L-838,417 possesses poor bioavailability and an extremely short half-life in mice[22], it was tested in rats. After systemic treatment, L-838,417 produced dose-dependent and reversible anti-hyperalgesia in both the inflammatory and neuropathic pain models (Fig. 3). As expected, its maximum anti-hyperalgesic effect (Fig. 3a) was less than that of intrathecal diazepam, probably because L-838,417 exerts only partial agonistic activity. Anti-hyperalgesia was again completely reversed by flumazenil (10 mg kg^{-1} i.p.; Fig. 3b), indicating that it was mediated through the benzodiazepine-binding site of GABA$_A$ receptors. It was, however, insensitive to the opioid receptor antagonist naloxone (10 mg kg^{-1} i.p.), demonstrating that opioidergic pathways were not involved (Fig. 3b). L-838,417 did not impair motor coordination (Fig. 3c). We next investigated the effects of L-838,417 against neuropathic pain and compared its analgesic efficacy and its liability to tolerance development (that is, its loss of analgesic activity) with that of morphine. L-838,417 had a maximum analgesic effect comparable to that of morphine (20 mg kg^{-1} i.p.) (Fig. 3d), but unlike morphine it did not lose its efficacy during a chronic (nine-day) treatment period (Fig. 3 d, e).

Finally, functional magnetic resonance imaging (fMRI) was used to assess whether L-838,417 would reduce not only nociceptive behaviour but also the representation of pain in the central nervous system. Changes in blood-oxygenation-level-dependent (BOLD) signals were quantified to measure brain activation evoked by noxious

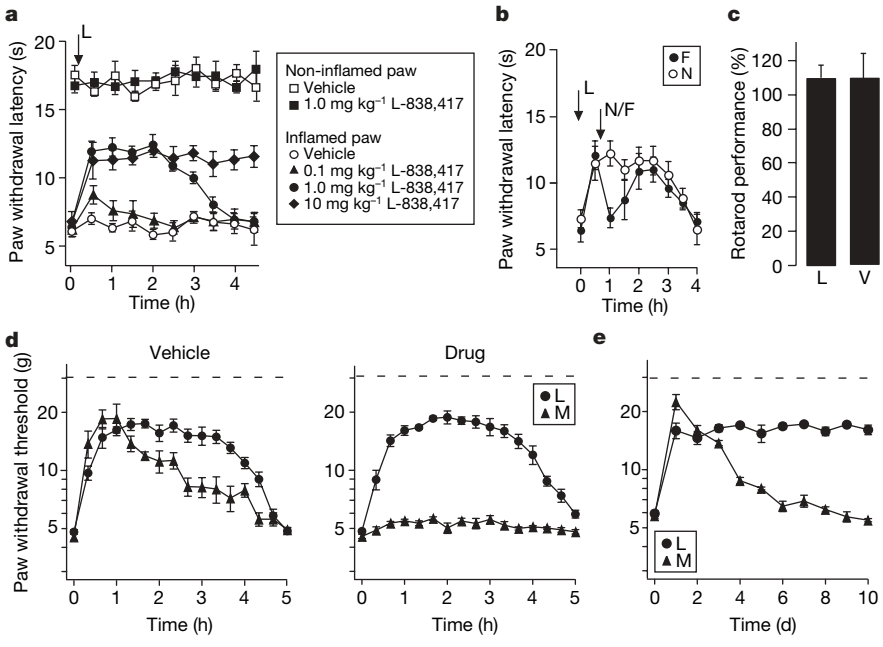

Figure 3 | Anti-hyperalgesic effects of the non-sedative benzodiazepine site ligand L-838,417 in rats. **a**, **b**, Inflammatory hyperalgesia induced by subcutaneous injection of zymosan A (1 mg) into one hindpaw. **a**, Effects of administration of L-838,417 (arrowed) on thermal hyperalgesia 6 h after injection of zymosan A ($n = 4–6$ rats). **b**, Effects of the benzodiazepine site antagonist flumazenil (F; 10 mg kg^{-1} i.p.) and the opioid receptor antagonist naloxone (N; 10 mg kg^{-1} i.p.) on antinociception induced by administration of L-838,417 (L; 1 mg kg^{-1} orally). $n = 3$ rats per group. **c**, Effects of L-838,417 (1 mg kg^{-1} orally) on motor control, shown as percentages of pre-drug rotarod performance ($n = 8$ rats per group). **d**, **e**, Neuropathic pain induced by CCI surgery. **d**, Anti-hyperalgesia by L-838,417 and morphine after chronic treatment (once-daily i.p. injections) for 9 days with either drug (right) or vehicle (left), 16 days after CCI surgery. Dashed lines, thresholds before CCI surgery. **e**, Analgesic efficacy of L-838,417 (L, 1 mg kg^{-1}) and morphine (M, 20 mg kg^{-1}) versus treatment duration. $n = 6$ rats per group. For a comparison with the anti-hyperalgesic activity of intrathecal diazepam in rats see Supplementary Fig. 3. All data are means ± s.e.m.

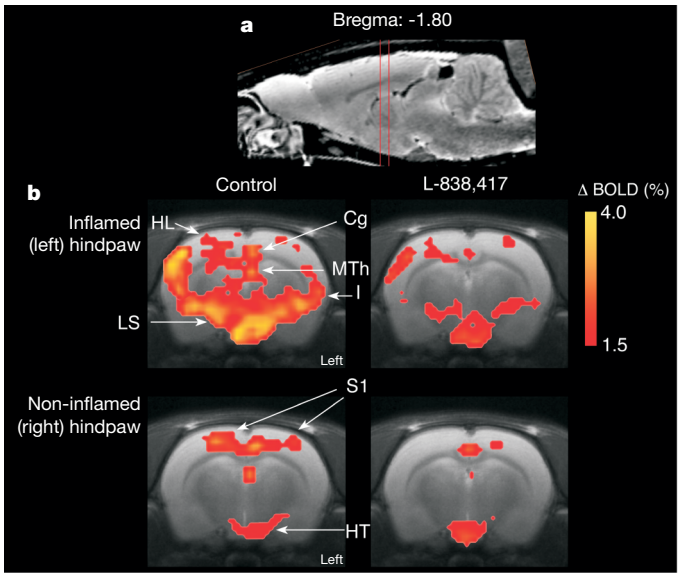

Figure 4 | Effects of L-838,417 (1 mg kg^{-1} i.p.) on the supraspinal representation of pain. **a**, Anatomical slice indicating the position of the functional images. **b**, False-colour images of changes in BOLD signals evoked by stimulation of the left (inflamed) or right (non-inflamed) hindpaw with noxious heat. Images represent group maps across 12 rats averaged from 8 (pre-drug) and 16 (post-drug) stimulations. Experiments started 6 h after subcutaneous zymosan A injection into the left hindpaw. MTh, medial thalamus; S1, primary somatosensory cortex; Cg, cingulate cortex; I, insular cortex; LS, limbic system (including amygdala, entorhinal cortex and hippocampus); HT, hypothalamus; HL, representation of hindlimb in S1. Left, left hemisphere.

heat. Stimulation of the inflamed left or the non-inflamed right hindpaw led to reliable, often bilateral, activation of several brain regions involved in pain processing (Fig. 4). Significantly more brain volume was activated and stronger activation was seen on stimulation of the inflamed paw. L-838,417 (1 mg kg^{-1} i.p.) decreased brain activation induced by noxious heat after stimulation of the inflamed paw. For a quantitative assessment of its analgesic effects, we integrated the stimulus-correlated change in the BOLD signal (F) over all significantly activated voxels of each region of interest and calculated $\Delta F/F$ as $(F_{post} - F_{pre})/F_{pre}$, the relative decrease in F after injection of L-838,417 (Table 1) or vehicle (Supplementary Table 2). Here we focused on brain areas that reflected either the sensory and discriminative component of pain (the medial thalamus and contralateral primary sensory cortex) or its emotional dimension (limbic system and frontal association cortex)[23,24]. After stimulation of the inflamed paw, a pronounced and statistically significant reduction in BOLD signal changes was observed in most brain regions analysed. Smaller changes in brain activation were found when the non-inflamed paw was stimulated, and only negligible effects were seen after innocuous thermal stimulation (Table 1). These results indicate that systemically administered L-838,417 does indeed act as an anti-hyperalgesic agent and reduces BOLD signals in brain areas related to both the sensory and the emotional associative components of pain.

Considerable evidence indicates that a facilitation of GABAergic inhibition can be pro-nociceptive at supraspinal sites, for example the rostral agranular insular cortex[25] or in the periaqueductal grey[26], by reducing the activity of descending antinociceptive neurons. At these sites most $GABA_A$ receptors apparently contain the α1 subunit[27]. Therefore, not only would sparing the α1 subunit avoid unwanted sedation, it would also increase analgesic efficacy. Aside from sedation and tolerance development, addictive properties are of major concern in the development of analgesics. Available evidence indicates that subtype-selective benzodiazepine-site ligands should exhibit at most only modest addictive properties[28] and should not lead to tolerance development[29]. Finally, previous studies have shown that in neuropathic pain after injury to peripheral nerves, GABAergic inhibition can not only be diminished but it can even turn into excitation[6,7]. Our results suggest that sufficient inhibition remains to permit a spinal analgesic effect of drugs that increase GABAergic neurotransmission. Because glycine and GABA are released together at many inhibitory synapses in the dorsal horn[30], a facilitation of GABAergic transmission should also be able to compensate for a selective decrease in glycinergic inhibition[3]. Thus, we have not only identified the $GABA_A$ receptors containing the α2 and α3 subunits as critical components of spinal pain control, but also demonstrated that α1-sparing benzodiazepine-site ligands, which are already in development as anxioselective (non-sedative) agents, might constitute a class of analgesics suitable for the treatment of chronic pain syndromes.

METHODS SUMMARY

Wild-type mice and $GABA_A$ receptor mutant mice (α1(H101R), α2(H101R), α3(H126R) and α5(H105R))[10–12] were maintained on a 129X1/SvJ background. Behavioural experiments were performed on adult mice and Wistar rats. Chemically induced pain was assessed in the formalin test, in which flinches of the injected paw were counted for 60 min after subcutaneous injection of 5% formalin into one hindpaw. Inflammatory pain was induced by subcutaneous injection of zymosan A (0.06 mg in mice; 1 mg in rats) into one hindpaw. Neuropathic pain was studied after chronic constriction of the left sciatic nerve. Heat hyperalgesia was assessed by measuring paw withdrawal latencies on exposure to defined radiant heat. Cold allodynia was measured as the time spent lifting, shaking or licking the paw (seconds per minute) after the application of acetone onto the affected paw. Mechanical sensitivity was assessed with electronic von Frey filaments. Locomotor activity was measured by using microprocessor-controlled activity cages, and motor function was assessed in the rotarod test. In all behavioural tests, the observer was blinded to the genotype or to the drug treatment.

GABAergic membrane currents were recorded from acutely dissociated capsaicin-sensitive DRG neurons (segments L4–L6) and from superficial dorsal horn neurons (layers I and II) in transverse slices of lumbar spinal cord, both obtained from 14–24-day-old mice.

fMRI experiments were performed on adult male Wistar rats slightly anaesthetized with 1–2% isoflurane, using a Bruker 4.7-T Biospec scanner. Heat stimulation was performed by applying temperature ramps to 52 °C and to 42 °C (noxious and innocuous stimulation, respectively) through Peltier elements tightly attached to the hindpaws.

The localization of $GABA_A$ receptor α subunits on primary afferent nerve terminals and on intrinsic dorsal horn neurons was determined by double

Table 1 | Changes in heat-induced brain activation by L-838,417 measured by rat fMRI

Area	Stimulation of inflamed paw with noxious heat		Stimulation of non-inflamed paw with noxious heat		Stimulation of non-inflamed paw with innocuous temperature	
	$\Delta F/F$; incidence*	P†	$\Delta F/F$; incidence*	P†	$\Delta F/F$; incidence*	P†
MTh	−0.35 ± 0.07; 10/12	0.014	−0.29 ± 0.09; 10/12	0.069	−0.10 ± 0.07; 6/12	0.302
S1c	−0.29 ± 0.07; 12/12	0.028	−0.07 ± 0.09; 12/12	0.383	−0.18 ± 0.25; 11/12	0.228
Cg	−0.37 ± 0.07; 11/12	0.034	−0.26 ± 0.08; 12/12	0.078	−0.07 ± 0.08; 10/12	0.152
FAC	−0.55 ± 0.05; 12/12	0.007	−0.30 ± 0.08; 12/12	0.179	−0.09 ± 0.08; 6/12	0.253
LS	−0.36 ± 0.05; 11/12	0.012	−0.06 ± 0.07; 12/12	0.580	−0.04 ± 0.07; 11/12	0.413

Where errors are shown, results are means ± s.e.m. MTh, medial thalamus; S1c, contralateral primary somatosensory cortex; Cg, cingulate cortex; FAC, frontal association cortex; LS, limbic system (including amygdala, entorhinal cortex and hippocampus).
* Number of rats in which a significant noxious heat-induced activation of the respective area occurred/total number of rats studied.
† Significance versus pre-drug (paired Student t-test).

immunofluorescence staining on sections from perfusion-fixed adult mice[27]. Confocal images were processed with Imaris (Bitplane).

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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1. Sandkühler, J. Learning and memory in pain pathways. *Pain* **88,** 113–118 (2000).
2. Woolf, C. J. & Salter, M. W. Neuronal plasticity: increasing the gain in pain. *Science* **288,** 1765–1769 (2000).
3. Ahmadi, S., Lippross, S., Neuhuber, W. L. & Zeilhofer, H. U. PGE_2 selectively blocks inhibitory glycinergic neurotransmission onto rat superficial dorsal horn neurons. *Nature Neurosci.* **5,** 34–40 (2002).
4. Harvey, R. J. *et al.* GlyRα3: an essential target for spinal PGE_2-mediated inflammatory pain sensitization. *Science* **304,** 884–887 (2004).
5. Moore, K. A. *et al.* Partial peripheral nerve injury promotes a selective loss of GABAergic inhibition in the superficial dorsal horn of the spinal cord. *J. Neurosci.* **22,** 6724–6731 (2002).
6. Coull, J. A. *et al.* Trans-synaptic shift in anion gradient in spinal lamina I neurons as a mechanism of neuropathic pain. *Nature* **424,** 938–942 (2003).
7. Coull, J. A. *et al.* BDNF from microglia causes the shift in neuronal anion gradient underlying neuropathic pain. *Nature* **438,** 1017–1021 (2005).
8. Scholz, J. *et al.* Blocking caspase activity prevents transsynaptic neuronal apoptosis and the loss of inhibition in lamina II of the dorsal horn after peripheral nerve injury. *J. Neurosci.* **25,** 7317–7323 (2005).
9. Malan, T. P., Mata, H. P. & Porreca, F. Spinal $GABA_A$ and $GABA_B$ receptor pharmacology in a rat model of neuropathic pain. *Anesthesiology* **96,** 1161–1167 (2002).
10. Rudolph, U. *et al.* Benzodiazepine actions mediated by specific γ-aminobutyric $acid_A$ receptor subtypes. *Nature* **401,** 796–800 (1999).
11. Löw, K. *et al.* Molecular and neuronal substrate for the selective attenuation of anxiety. *Science* **290,** 131–134 (2000).
12. Crestani, F. *et al.* Trace fear conditioning involves hippocampal α5 $GABA_A$ receptors. *Proc. Natl Acad. Sci. USA* **99,** 8980–8985 (2002).
13. McKernan, R. M. *et al.* Sedative but not anxiolytic properties of benzodiazepines are mediated by the $GABA_A$ receptor α1 subtype. *Nature Neurosci.* **3,** 587–592 (2000).
14. Melzack, R. & Wall, P. D. Pain mechanisms: a new theory. *Science* **150,** 971–979 (1965).
15. Enna, S. J. & McCarson, K. E. The role of GABA in the mediation and perception of pain. *Adv. Pharmacol.* **54,** 1–27 (2006).
16. Barnard, E. A. *et al.* International Union of Pharmacology. XV. Subtypes of γ-aminobutyric acid A receptors: classification on the basis of subunit structure and receptor function. *Pharmacol. Rev.* **50,** 291–313 (1998).
17. Wieland, H. A., Lüddens, H. & Seeburg, P. H. A single histidine in $GABA_A$ receptors is essential for benzodiazepine agonist binding. *J. Biol. Chem.* **267,** 1426–1429 (1992).
18. Dias, R. *et al.* Evidence for a significant role of α3-containing $GABA_A$ receptors in mediating the anxiolytic effects of benzodiazepines. *J. Neurosci.* **25,** 10682–10688 (2005).
19. Rudomin, P. & Schmidt, R. F. Presynaptic inhibition in the vertebrate spinal cord revisited. *Exp. Brain Res.* **129,** 1–37 (1999).
20. Bohlhalter, S., Weinmann, O., Möhler, H. & Fritschy, J. M. Laminar compartmentalization of $GABA_A$-receptor subtypes in the spinal cord: an immunohistochemical study. *J. Neurosci.* **16,** 283–297 (1996).
21. Ma, W., Saunders, P. A., Somogyi, R., Poulter, M. O. & Barker, J. L. Ontogeny of $GABA_A$ receptor subunit mRNAs in rat spinal cord and dorsal root ganglia. *J. Comp. Neurol.* **338,** 337–359 (1993).
22. Scott-Stevens, P., Atack, J. R., Sohal, B. & Worboys, P. Rodent pharmacokinetics and receptor occupancy of the $GABA_A$ receptor subtype selective benzodiazepine site ligand L-838417. *Biopharm. Drug Dispos.* **26,** 13–20 (2005).
23. Brooks, J. & Tracey, I. From nociception to pain perception: imaging the spinal and supraspinal pathways. *J. Anat.* **207,** 19–33 (2005).
24. Bushnell, M. C. & Apkarian, A. V. in *Wall and Melzack's Textbook of Pain* (ed. McMahon, S. B. & Koltzenburg, M.) 107–124 (Elsevier Churchill Livingstone, London, 2006).
25. Jasmin, L., Rabkin, S. D., Granato, A., Boudah, A. & Ohara, P. T. Analgesia and hyperalgesia from GABA-mediated modulation of the cerebral cortex. *Nature* **424,** 316–320 (2003).
26. Harris, J. A. & Westbrook, R. F. 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 (1995).
27. Fritschy, J. M. & Möhler, H. $GABA_A$-receptor heterogeneity in the adult rat brain: differential regional and cellular distribution of seven major subunits. *J. Comp. Neurol.* **359,** 154–194 (1995).
28. Ator, N. A. Contributions of $GABA_A$ receptor subtype selectivity to abuse liability and dependence potential of pharmacological treatments for anxiety and sleep disorders. *CNS Spectr.* **10,** 31–39 (2005).
29. van Rijnsoever, C. *et al.* Requirement of α5-$GABA_A$ receptors for the development of tolerance to the sedative action of diazepam in mice. *J. Neurosci.* **24,** 6785–6790 (2004).
30. Keller, A. F., Coull, J. A., Chery, N., Poisbeau, P. & de Koninck, Y. Region-specific developmental specialization of GABA–glycine cosynapses in laminas I–II of the rat spinal dorsal horn. *J. Neurosci.* **21,** 7871–7880 (2001).

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METHODS

Mice and rats. Behavioural experiments were performed in male and female 7–12-week-old mice or in male 7–12-week-old Wistar rats. Permission for the animal experiments was obtained from the Regierung von Mittelfranken (ref. no. 612-2531.31-17/03) and from the Veterinäramt des Kantons Zürich (ref. no. 121/2006 and 34/2007).

Drugs. For intrathecal injection in mice, diazepam was dissolved in 10% dimethyl sulphoxide (DMSO), 90% artificial cerebrospinal fluid (ACSF) (vehicle). Total intrathecal injection volume was 5 µl (for details of the injection procedure see ref. 31). Up to a concentration of 20%, intrathecal DMSO had no effect on pain behaviour in mice. For i.p. injection, diazepam was dissolved in 0.3% Tween 80, 99.7% ACSF. Morphine was dissolved in ACSF. L-838,417 synthesized by Anawa was suspended in 0.5% methylcellulose and 0.9% NaCl and was applied to rats either orally or i.p. in a total volume of 200 µl. Flumazenil (10 mg kg^{-1}) and naloxone (10 mg kg^{-1}) were dissolved in DMSO (1%) and injected i.p. in a total volume of 200 µl.

Formalin test. Formalin (5%, 20 µl) was injected subcutaneously into the dorsal surface of the left hindpaw[32]. Flinches of the injected paw were counted for 60 min starting immediately after formalin injection. Intrathecal drugs (diazepam or vehicle) were injected 10 min before formalin injection. Flumazenil (10 mg kg^{-1}) was injected i.p. 30 min before formalin injection.

Inflammatory pain. Inflammatory pain was assessed in the zymosan A model[33]. In mice, 0.06 mg of zymosan A suspended in 20 µl of 0.9% NaCl was injected subcutaneously into the plantar side of the left hindpaw. The model was also used in rats, but 1 mg of zymosan A was used. Heat hyperalgesia was assessed 24 h and 6 h after induction of inflammation in mice and rats, respectively.

Neuropathic pain. Diazepam, L-838,417 and morphine were analysed in the CCI model[34] in 7–12-week-old mice or rats. Unilateral constriction injury of the left sciatic nerve just proximal to the trifurcation was performed with three loose ligatures. In sham-operated animals the sciatic nerve was exposed and the connective tissue was freed, but no ligatures were applied. In these sham-operated animals only a minor and transient hyperalgesia occurred. Heat hyperalgesia, cold allodynia and mechanical sensitization were assessed 7–9 days after surgery.

Heat hyperalgesia. Paw withdrawal latencies on exposure to a defined radiant heat stimulus were measured with a commercially available apparatus (Plantar Test; Ugo Basile). Four or five measurements were taken in each animal for every time point. Measurements of paw withdrawal latencies of the inflamed or injured paw and of the contralateral paw were made alternately.

Cold allodynia. The time spent lifting, shaking or licking the paw (seconds per minute) was measured for 5 min after application of a drop of acetone onto the affected paw.

Mechanical sensitization. Mechanical sensitivity was assessed with electronic von Frey filaments (IITC). Triple measurements of paw withdrawal thresholds (g) were made for each time point and animal.

Locomotor activity. Locomotor activity was tested with a commercially available microprocessor-controlled activity cage (Actiframe; Gerb Elektronik). Mice were placed in the apparatus 15 min before testing. Motor activity was measured 10–30 min and 40–80 min after intrathecal and oral drug application, respectively.

Motor impairment. A possible impairment of motor function was assessed with the rotarod test[35]. Rats were trained on day zero and the maximum speed tolerated for at least 2 min was determined for each rat. On the following day, rotarod performance was determined again 30 min after treatment with L-838,417 or vehicle (administered orally).

Electrophysiology. DRGs (from segments L4–L6) were removed from 14–24-day-old mice, dissociated and plated on poly-(L-lysine)-coated cover slips (for details see ref. 36). GABA-induced currents were recorded from capsaicin-sensitive DRG neurons 3–30 h after plating. Transverse slices (250 µm thick) of the lumbar spinal cord were prepared from 14–24-day-old mice. GABAergic membrane currents were recorded from superficial dorsal horn neurons (laminae I and II) as described previously[3]. In both preparations, GABA (1 mM) was applied by short (10 ms) puffer applications to the soma of the recorded neuron at a frequency of 0.07 Hz. Diazepam (1 µM) was applied by means of bath perfusion. All recordings were made in the presence of the $GABA_B$ receptor antagonist CGP-55,845 (200 µM).

Immunofluorescence. The localization of $GABA_A$ receptor α subunits on primary afferent nerve terminals and intrinsic dorsal horn neurons was determined by double immunofluorescence staining on sections from perfusion-fixed adult mice[27]. Antibodies were home-made subunit-specific antisera[27] and commercial antibodies against substance P (T1609; Bachem) and NK1 (S8305; Sigma). Sections processed for double immunofluorescence were digitalized by confocal laser scanning microscopy (resolution 90 nm per pixel; two or three images per animal; $n = 3$ mice) and images were processed with Imaris (Bitplane). Double-labelled objects (image profiles) in single confocal sections were identified by a segmentation algorithm (minimal size 0.2 µm^2; minimum intensity 50–90 on a 256-grey-level scale). The numbers of single-labelled and double-labelled profiles were calculated. All values are expressed as percentages of double-labelled profiles relative to the marker indicated.

fMRI methods. fMRI experiments were performed in male Wistar rats weighing 350–400 g. During the measurements, rats were slightly anaesthetized with isoflurane (1–2%) to maintain a respiration rate of about 60 c.p.m. and constant blood *p*CO2 levels. Measurements were made with a Bruker 4.7-T Biospec scanner with a free bore of 40 cm, equipped with an actively radiofrequency-decoupled coil system. A whole-body birdcage resonator enabled homogenous excitation, and a 3-cm quadrature surface coil, which served as a receiver, was located directly above the head of the animal to maximize the signal-to-noise ratio. Constant positioning of the rat's head within the scanner was verified by rapid acquisition of magnetic resonance images at 200-ms intervals. A functional series of 1,470 sets (4 s each, total of 96 min) of 22 axial images (slice thickness 1 mm, field of view 25 × 25 mm^2, 5.20 to −14.60 mm from the bregma[37]) were acquired with the echo planar imaging technique (EPI: matrix 64 × 64, TR = 4,000 ms, TEef = 23.4 ms, two acquisitions). Anatomical scans with a high spatial resolution were obtained with RARE[38] (slice thickness 1 mm, field of view 25 × 25 mm^2, matrix 256 × 256, TR = 400 ms, TE = 18 ms, NEX = 8).

Noxious heat stimulation was performed by applying temperature ramps (34–52 °C (noxious stimulation) or 34–42 °C (innocuous stimulation) with 15-s rise and fall times and a 5-s plateau phase) through two Peltier elements tightly attached to both hindpaws (in awake rats this stimulation method yielded paw withdrawal latencies similar to those obtained in the behavioural tests with radiant heat). Thermal stimuli were applied to the left and right hindpaw alternately at 2-min intervals. After 32 min of recording, L-838,417 (1 mg kg^{-1}) or vehicle was injected through an i.p. catheter without changing the position of the animal in the scanner. After drug injection, recording was continued for 64 min with the same stimulation method.

Data were analysed with Brainvoyager QX after appropriate preprocessing (motion correction, mean intensity adjustment, spatial smoothing 0.6 mm full-width at half-maximum, temporal gaussian smoothing 12 s, and temporal high-pass filtering of nine cycles) with a General Linear Modelling approach with four predictors: inflamed (left)/non-inflamed (right) paw before and after drug injection and Bonferroni correction. *z*-score maps of the individual rats were group analysed with custom-made analysis software (MagnAn[39] running under IDL). Anatomical and functional images were transferred into the register by an affine transformation scheme with only six degrees of freedom derived from the individual brain masks. The registered anatomical data and *z*-score maps were averaged over all animals. Contrast-specific mean *z*-score maps were calculated using a threshold of 3.0. Significantly activated voxels were labelled automatically with a digital standard rat brain atlas[37]. For each rat, brain structure and stimulation condition, we then first calculated the activation intensity as the stimulus-induced relative change in the BOLD signal (F). To quantify the effect of L-838,417 on the stimulus-induced BOLD signal changes we calculated $\Delta F/F$ as $(F_{post} - F_{pre})/F_{pre}$, where F_{post} is the value of F after drug treatment and F_{pre} is the value before drug treatment. Statistical analysis was performed with the paired Student *t*-test. False-colour images of stimulus-induced changes in BOLD signals were obtained by mapping the calculated mean BOLD signal change of each voxel onto all significantly activated voxels. Note that the different colours in Fig. 4 encode F (signal amplitude), not statistical coefficients.

31. Depner, U. B., Reinscheid, R. K., Takeshima, H., Brune, K. & Zeilhofer, H. U. Normal sensitivity to acute pain, but increased inflammatory hyperalgesia in mice lacking the nociceptin precursor polypeptide or the nociceptin receptor. *Eur. J. Neurosci.* **17,** 2381–2387 (2003).
32. Dubuisson, D. & Dennis, S. G. The formalin test: a quantitative study of the analgesic effects of morphine, meperidine, and brain stem stimulation in rats and cats. *Pain* **4,** 161–174 (1977).
33. Hargreaves, K., Dubner, R., Brown, F., Flores, C. & Joris, J. A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia. *Pain* **32,** 77–88 (1988).
34. Bennett, G. J. & Xie, Y. K. A peripheral mononeuropathy in rat that produces disorders of pain sensation like those seen in man. *Pain* **33,** 87–107 (1988).
35. Bonetti, E. P. *et al.* Ro 15–4513: partial inverse agonism at the BZR and interaction with ethanol. *Pharmacol. Biochem. Behav.* **31,** 733–749 (1988).
36. Zeilhofer, H. U., Kress, M. & Swandulla, D. Fractional Ca^{2+} currents through capsaicin- and proton-activated ion channels in rat dorsal root ganglion neurones. *J. Physiol. (Lond.)* **503,** 67–78 (1997).
37. Paxinos, G. & Watson, C. *The Rat Brain in Stereotaxic Coordinates* 4th edn (Academic, San Diego, 1998).
38. Hennig, J., Nauerth, A. & Friedburg, H. RARE imaging: a fast imaging method for clinical MR. *Magn. Reson. Med.* **3,** 823–833 (1986).
39. Hess, A., Sergejeva, M., Budinsky, L., Zeilhofer, H. U. & Brune, K. Imaging of hyperalgesia in rats by functional MRI. *Eur. J. Pain* **11,** 109–119 (2007).