The positive allosteric GABA_B receptor modulator rac-BHFF enhances baclofen-mediated analgesia in neuropathic mice

Khaled Zemoura^a, William T. Ralvenius^a, Pari Malherbe^b and Dietmar Benke^{a,c,d}

^aInstitute of Pharmacology and Toxicology, University of Zurich, Winterthurerstrasse 190, CH-8057 Zurich, Switzerland

^bDiscovery Neuroscience, F. Hoffmann-La Roche AG, pRED, Pharma Research & Early Development, Grenzacherstrasse 124, CH4070, Basel, Switzerland.

^cNeuroscience Center Zurich (ZNZ), Winterthurerstrasse 190, CH-8057 Zurich, Switzerland

^dDrug Discovery Network Zurich (DDNZ), Winterthurerstrasse 190, CH-8057 Zurich

Correspondance to: Dietmar Benke, PhD
Institute of Pharmacology and Toxicology
University of Zurich
Winterthurerstrasse 190
CH-8057 Zurich
Switzerland
e-mail: benke@pharma.uzh.ch
Tel: +41 44 635 5930

Abstract

Neuropathic pain is associated with impaired inhibitory control of spinal dorsal horn neurons, which are involved in processing pain signals. The metabotropic GABA_B receptor is an important component of the inhibitory system and is highly expressed in primary nociceptors and intrinsic dorsal horn neurons to control their excitability. Activation of GABA_B receptors with the orthosteric agonist baclofen effectively relieves neuropathic pain but is associated with severe side effects that prevent its widespread application. The recently developed positive allosteric GABA_B receptor modulators lack most of these side effects and are therefore promising drugs for the treatment of pain. Here we tested the high affinity positive allosteric modulator rac-BHFF for its ability to relieve neuropathic pain induced by chronic constriction of the sciatic nerve in mice. rac-BHFF significantly increased the paw withdrawal threshold to mechanical stimulation in healthy mice, indicating an endogenous GABA_Bergic tone regulating the sensitivity to mechanical stimuli. Surprisingly, rac-BHFF displayed no analgesic activity in neuropathic mice although GABA_B receptor expression was not affected in the dorsal horn as shown by quantitative receptor autoradiography. However, activation of spinal GABA_B receptors by intrathecal injection of baclofen reduced hyperalgesia and its analgesic effect was considerably potentiated by co-application of rac-BHFF. These results indicate that under conditions of neuropathic pain the GABAergic tone is too low to provide a basis for allosteric modulation of GABA_B receptors. However, allosteric modulators would be well suited as an add-on to reduce the dose of baclofen required to achieve analgesia.

Keywords: Allosteric modulation, GABA_B receptor, neuropathic pain, rac-BHFF

1. Introduction

A considerable fraction of the human population worldwide suffers from neuropathic pain, originating from nerve injury or diseases like cancer, diabetes or multiple sclerosis (Bouhassira et al., 2008; Torrance et al., 2013). Because current pharmacological treatments often provide unsatisfactory pain relief, there is a high demand for new drugs addressing neuropathic pain. Impaired inhibitory control of neurons in the dorsal horn of the spinal cord is one main factor that contributes to the expression of neuropathic pain (Zeilhofer et al., 2012b). Hence, pharmacological enhancement of neuronal inhibition has been suggested as a valid strategy to treat neuropathic pain (Zeilhofer et al., 2012a).

The metabotropic GABA_B receptor is one component of the inhibitory system controlling neuronal excitability of spinal cord neurons. Functional GABA_B receptors are heterodimers composed of the two seven membrane spanning domain proteins GABA_{B1} and GABA_{B2} (Bettler et al., 2004). GABA_B receptors are present at high density at sites that are involved in processing pain signals, such as in primary afferent terminals and in neurons of the superficial spinal dorsal horn (Ataka et al., 2000; Charles et al., 2001; Price et al., 1984; Towers et al., 2000; Yang et al., 2002). These characteristics make GABA_B receptors attractive targets for the treatment of neuropathic pain. There is substantial evidence from animal models of neuropathic pain that enhancing GABA_B receptor activity by administration of the agonist baclofen provides analgesia (Hwang and Yaksh, 1997; Malan et al., 2002; Patel et al., 2001; Smith et al., 1994). An analgesic activity of baclofen has also been reported in human patients suffering from intractable neuropathic pain unresponsive to common treatments (Goto et al., 2013; Harmer and Larson, 2002; Hosny et al., 2004; Kopsky et al., 2015; van der Plas et al., 2013; Zuniga et al., 2000). So far, baclofen is the only drug on the market targeting GABA_B receptors primarily for the treatment of severe spasticity (Bowery, 2006). Unfortunately, baclofen poorly crosses the blood-brain barrier and thus requires high concentration when given systemically. This is associated with numerous side effects and prevented widespread applications (Brennan and Whittle, 2008).

Recently, allosteric GABA_B receptor agonists were developed that, in animal models, are associated with less side effects than baclofen (Froestl, 2010; Urwyler, 2011). Here we tested the allosteric GABA_B receptor modulator rac-BHFF for its analgesic activity in the mouse chronic constriction injury (CCI) model of neuropathic pain. rac-

BHFF lack any activity in the absence of orthosteric GABA_B receptor agonists, but potently enhance GABA_B receptor activity (Malherbe et al., 2008). We found that, in contrast to baclofen, rac-BHFF did not relieve neuropathic pain in CCI mice. It, however, significantly increased the analgesic activity of intrathecally administered baclofen. This finding suggests that under conditions of neuropathic pain, the endogenous GABA_Bergic tone is not sufficient to permit an analgesic action via allosteric modulation of GABA_B receptors.

2. Materials and Methods

2.1. Animals

The behavioral experiments were approved by the Veterinäramt des Kantons Zürich (license number 135/2009) and performed with 8 to 10 week old C57BL/6J male mice. The mice were maintained at a 12/12 hour light/dark cycle with free access to food and water. All behavioral experiments were conducted by a male experimenter, blinded to the treatment of the animals.

2.2. Drugs

rac-BHFF was synthesized at F. Hoffmann-La Roche Ltd; (*R*)-baclofen was obtained from Tocris Bioscience; GABA was purchased from Sigma-Aldrich, CGP56999A was kindly provided by Novartis and [³H]CGP 54626 (30 Ci/mmol) was purchased from ANAWA Trading SA.

For behavioral experiments rac-BHFF was prepared immediately prior to use in a mixture containing Cremophor EL, 1,2-propanediol and distilled water (4:1:15) (Malherbe et al., 2008) and administered orally (10 ml/kg). Baclofen was dissolved in physiological saline (0.9% NaCl) and 10 µl were injected intrathecally (5 µg/kg).

2.3. Chronic constriction injury (CCI) model of neuropathic pain

Neuropathic pain was induced in 8 to 10-week-old male mice (C57BL/6J) via unilateral constriction injury of the left sciatic nerve as described (Bennett and Xie, 1988). Anaesthesia was triggered and sustained by 2% isoflurane (Provet AG, Lyssach, Switzerland) in the presence of 30% oxygen. The sciatic nerve was uncovered at the mid-thigh level proximal to the sciatic trifurcation by blunt dissection through the biceps femoris. 5 to 7 mm of nerve was freed of adhering tissue and three chromic gut ties (4/0, Ethicon, New Brunswick, NJ, USA) were loosely knotted round the nerve with about 1 mm gaps. The ties were tightened until they provoked a transitory twitch in the hind limb. In sham-operated animals, the sciatic nerve was uncovered and the connective tissue was freed, but no ties were applied. Seven days after CCI, the mice were evaluated for mechanical sensitization.

2.4. Testing mechanical sensitization

The mice were placed in individual chambers with a mesh floor and allowed to acclimate for 30 min. Then, mechanical sensitization was tested by measuring the paw withdrawal threshold using an electronic von Frey aesthesiometer (IITC, Woodland Hills, CA). Four measurements were done for each time-point and averaged. Measurements of the paw withdrawal thresholds of the injured paw and of the contralateral paw were taken alternatively. Paw withdrawal thresholds were determined before (baseline) and 7 days after surgery.

2.5. Locomotor activity

Locomotor activity was measured in an open field arena (20 cm diameter) equipped with four pairs of light beams and photocells. Locomotor activity, as detected by photocell beam interruptions, was recorded for 6 hours. rac-BHFF was applied immediately before placing the mouse into the arena.

2.6. Receptor autoradiography

Lumbar spinal cord at the level of L4 and L5 from CCI and sham operated mice was rapidly dissected and immediately frozen in dry ice. The spinal cord was cut with a cryostat into 16 μm thick coronal sections and mounted onto microscope slides (Superfrost Plus, Thermo Scientific). For autoradiography, the sections were dried in a stream of cold air and washed two times for 10 min in assay buffer (10 mM Tris pH 7.4, 120 mM NaCl, 4.7 mM KCl, 1.8 mM CaCl_2 , 1.2 mM MgCl_2) and dried again. The sections were then incubated with 6.6 nM [^3H]CGP 54626 diluted in assay buffer for 120 min at room temperature. After washing three times for 20 s in ice-cold assay buffer and a quick dip in distilled water, the sections were thoroughly dried. The dry sections were exposed together with [^3H]-standards to a tritium-sensitive phosphoimaging screen (Cyclone Storage Phosphor Screen, Perkin Elmer). Quantification was done using the Optiquant software (Perkin Elmer). Non-specific binding was determined by including 10 μM CGP 56999A into the incubations.

2.7. Statistical analyses

Percent maximal possible effect (% MPE) was measured as follows:

$$\% \text{ MPE} = (\text{PWT}_t - \text{PWT}_{\text{predrug}}) / (\text{PWT}_{\text{preCCI}} - \text{PWT}_{\text{predrug}}) \times 100$$

PWT_t, paw withdrawal threshold at time point *t*; PWT_{predrug}, paw withdrawal threshold after CCI surgery but before drug application; PWT_{preCCI}, paw withdrawal threshold baseline before CCI surgery.

GraphPad Prism 5 (Graph Pad Software, Inc., San Diego, CA, USA) was used to conduct all statistical analyses. The data are presented as the mean ± SEM. The statistical tests used are indicated in the figure legends.

3. Results

3.1. At high concentration rac-BHFF reduced locomotor activity

Since a potential sedative activity of rac-BHFF would interfere with the intended pain testing, we first evaluated the effect of rac-BHFF on spontaneous locomotor activity during a period of 6 hours. We selected doses of 10 and 50 mg/kg rac-BHFF (p. o.) since a dose as low as 3 mg/kg (p. o.) was previously found to enhance baclofen-induced loss of righting reflex (Malherbe et al., 2008). While 10 mg/kg (p. o.) showed no effect, 50 mg/kg considerably reduced spontaneous activity of the mice (Fig. 1). Therefore, we used a dose of 10 mg/kg rac-BHFF (p. o.) for all further experiments.

3.2. rac-BHFF reduced the sensitivity to mechanical stimulation in healthy mice

Activation of GABA_B receptors by baclofen inhibits A-fibers as well as C-fibers (Ataka et al., 2000; Honsek et al., 2015; Melin et al., 2013) and consequently blocks pinch as well as touch-evoked excitatory postsynaptic currents (EPSCs) (Fukuhara et al., 2013). Therefore, we tested the ability of rac-BHFF to reduce the sensitivity of mice to mechanical stimulation of the hind leg paws using von Frey filaments. As expected, mice displayed a significantly increased paw withdrawal threshold at 60 and 90 min after treatment with rac-BHFF (Fig. 2). At 30 min rac-BHFF did not affect mechanical sensitivity, which is in line with its pharmacokinetics. rac-BHFF reaches maximum plasma levels at 60 min following p. o. administration (Malherbe et al., 2008). This finding suggests that rac-BHFF enhanced an endogenous GABA_B receptor-mediated inhibitory tone, which regulates the sensitivity to mechanical stimuli.

3.3. rac-BHFF failed to relief neuropathic pain

The finding that rac-BHFF decreased the sensitivity to mechanical stimuli in healthy mice favors an antinociceptive activity to mechanical hyperalgesia associated with spinal nerve injury. A potential antihyperalgesic activity of rac-BHFF was assessed in the CCI mouse model of neuropathic pain. Seven days after sciatic nerve ligation, the mice displayed pronounced mechanical hyperalgesia in the affected ipsilateral paw, as indicated by a considerably decreased paw withdrawal threshold (Fig. 3). Surprisingly, administration of rac-BHFF failed to affect the level of hyperalgesia (Fig. 3A). This observation suggests that the normal inhibitory tone provided by GABA_B

receptors is impaired under conditions of neuropathic pain. To test for this, we intrathecally injected neuropathic mice with a low dose of the GABA_B receptor agonist baclofen (5 µg/kg) that provided a submaximal relief to mechanical hyperalgesia (Fig. 3B). The intrathecal route was chosen to avoid a potential sedative activity of baclofen. The additional application of rac-BHFF (10 mg/kg p. o.) considerably potentiated the antihyperalgesic effect of baclofen (Fig. 3B).

These experiments indicate that the endogenous tone of GABA_B receptor-mediated inhibition in neuropathic mice is too low to provide a basis for rac-BHFF-mediated antihyperalgesia.

3.4. GABA_B receptor expression in the dorsal spinal cord is not affected in CCI mice

One additional cause for the lack of antihyperalgesic activity of rac-BHFF might be a potential downregulation of GABA_B receptors during the development of neuropathic pain, as reported for neuropathy in mice induced by diabetes (Wang et al., 2011) or spinal nerve ligation (Wu et al., 2011). Hence, we tested for a downregulation of GABA_B receptors in the dorsal horn of the lumbar spinal cord of CCI mice by quantitative receptor autoradiography using the tritiated high affinity GABA_B receptor antagonist [³H]CGP54626. However, there was no difference in [³H]CGP54626 binding between the affected ipsilateral and unaffected contralateral dorsal horn (Fig. 4). Likewise, no differences in [³H]CGP54626 binding were detected between the dorsal horns of CCI mice and sham operated mice (Fig. 4). Thus, the global expression of GABA_B receptors appears not to be affected in CCI treated mice.

3.5. GABA_B receptor complexes appear not to be affected by CCI

A recent study reported the inactivation of GABA_B receptors in the spinal nerve ligation mouse model by a mechanism that disrupts the receptor heterodimer selectively at the cell surface (Laffray et al., 2012). Thus, we tested whether this mechanism is operative also in the CCI mouse model and therefore might contribute to the ineffectiveness of rac-BHFF. To test for the disruption of GABA_{B1,2} heterodimers, we made use of the fact that GABA_{B1} in the absence of GABA_{B2} displays unchanged affinities for antagonists but dramatically lower affinities for agonists (~100-fold) (Galvez et al., 2001; Galvez et al., 2000; Kaupmann et al., 1998; White et al., 1998). In case of disrupted GABA_B receptor heterodimers, inhibition of [³H]CGP54626 binding to homomeric GABA_{B1} by GABA would require considerably

higher GABA concentrations than the inhibition to GABA_{B1,2} heterodimers. Using quantitative receptor autoradiography we did not observe any differences in the GABA inhibition of [³H]CGP54626 binding in the ipsi- versus contralateral dorsal horn of CCI mice and sham operated mice (Fig. 5). This finding suggests that in the CCI mouse model of neuropathic pain a disruption of GABA_B receptor heterodimers is unlikely a prominent factor contributing to the ineffectiveness of rac-BHFF.

4. Discussion

In the present study we explored the potential analgesic activity of the positive allosteric modulator rac-BHFF in the CCI mouse model of neuropathic pain. Although rac-BHFF significantly increased the paw withdrawal threshold to mechanical stimuli in healthy mice it was inactive in neuropathic mice except when the GABA_B receptors were simultaneously stimulated with the orthosteric agonist baclofen. These results indicate a reduced endogenous GABA_Bergic tone in neuropathic mice that is not sufficient to provide a basis for allosteric modulation of GABA_B receptors.

Positive allosteric modulators of GABA_B receptors bind to a site within the heptahelical domain of GABA_{B2} (Binet et al., 2004), exhibit no or little direct activation of the receptor but strongly increase the affinity and efficacy for orthosteric agonists (for a review see (Urwyler, 2011)). Because positive allosteric modulators enhance GABA_B receptor signaling in a use-dependent manner, i. e. they only act when the receptors are activated by an orthosteric agonist, they are associated with less unwanted side effects than the orthosteric agonist baclofen. Accordingly, positive allosteric modulators of GABA_B receptors are being evaluated for the treatment of a large spectrum of diseases, including anxiety, addiction, gastroesophageal reflux disease and pain (Froestl, 2010). Concerning pain, allosteric modulators of GABA_B receptors have proven to be effective in animal models of acute generalized visceral pain (Brusberg et al., 2009; Kalinichev et al., 2014) as well as in acute and persistent inflammatory pain (Kalinichev et al., 2014). However, it was so far not tested whether positive allosteric modulation of GABA_B receptors would affect neuropathic pain, which is associated with diminished inhibitory neurotransmission. Therefore, we selected the high affinity positive allosteric modulator rac-BHFF to analyze its ability to relief neuropathic pain induced by chronic constriction of the sciatic nerve. rac-BHFF strongly potentiates GABA_B receptor responses, is devoid of any intrinsic activating activity and has a very high oral bioavailability (Malherbe et al., 2008). In addition, rac-BHFF was reported to lack sedative activity in mice and rats (Maccioni et al., 2010; Malherbe et al., 2008), which is a prerequisite for unbiased pain testing. Only at very high concentrations rac-BHFF potentially reduced locomotor activity in mice (300 mg/kg) (Brown et al., 2016) and pigeons (178 mg/kg) (Koek et al., 2013). However, in our setting rac-BHFF severely reduced locomotor activity already at 50 mg/kg. The reason for this discrepancy is currently unclear. We therefore consequently used a dose of 10 mg/kg throughout the tests, which did not affect

locomotor activity. This dose of rac-BHFF was shown to enhance baclofen-induced responses *in vivo* (Gannon and Millan, 2011; Malherbe et al., 2008), indicating that a sufficiently high concentration reached neuronal sites.

We found that treatment of naive mice with rac-BHFF considerably increased the paw withdrawal threshold after mechanical stimulation. This implies the presence of an endogenous tone of GABA_B receptor activity contributing to the regulation of the paw withdrawal threshold. This conclusion is well supported by reports showing that application of the GABA_B receptor agonist baclofen inhibits glutamate release from primary afferent A and C fibers in the superficial dorsal horn (Ataka et al., 2000; Honsek et al., 2015; Melin et al., 2013) and inhibits pinch as well as touch-evoked EPSCs (Fukuhara et al., 2013).

Surprisingly, rac-BHFF was completely inactive in neuropathic mice. Even the withdrawal threshold of the unaffected contralateral paw remained unaffected in contrast to our observation on healthy mice. The failure of rac-BHFF was not due to a general inability of GABA_B receptors to relieve pain in neuropathic mice since activation of the receptors by intrathecal administration of baclofen was analgesic and co-application of rac-BHFF even further increased the paw withdrawal threshold. A reduced GABAergic tone under neuropathic conditions, keeping GABA_B receptors inactive and thus unavailable for allosteric modulation, is the most likely explanation for our findings. In line with this view, GABA levels in the superficial dorsal horn are strongly reduced after inducing neuropathic pain by chronic constriction of the sciatic nerve (Eaton et al., 1998; Vaysse et al., 2011). Interestingly, GABA levels are not only diminished in the ipsilateral but also in the unaffected contralateral dorsal horn. This may explain our observation that rac-BHFF was also inactive at the unaffected paw.

Another mechanism contributing to the inactivity of rac-BHFF might be a potential downregulation of GABA_B receptors. However, there is conflicting data reported on the expression of GABA_B receptors under neuropathic conditions. For instance, in the spinal nerve ligation model of neuropathic pain no change (Engle et al., 2006) or a reduced GABA_B receptor expression (Wu et al., 2011) was observed. This argues against a prominent role of global GABA_B receptor downregulation in neuropathic pain. Accordingly, we detected no change in global GABA_B receptor expression in the dorsal horn of mice after chronic constriction of the sciatic nerve using quantitative receptor autoradiography. We also found no indication for a disruption of

GABA_B receptor complexes that would inactivate the receptors in our neuropathic mice. This intriguing mechanism was reported to contribute to the expression of neuropathic pain induced by spinal nerve ligation (Laffray et al., 2012). Therefore, it is unlikely that alterations at the level of GABA_B receptors significantly contribute to the inactivity of rac-BHFF to relief hyperalgesia in neuropathic mice induced by chronic constriction of the sciatic nerve.

In conclusion, our data suggest that positive allosteric modulators of GABA_B receptors may not be suitable to relief neuropathic pain caused by peripheral nerve injury on their own. This is most likely due to a reduced GABAergic tone, which diminishes GABA_B receptor activity to a level that is not sufficient to provide a basis for allosteric modulation. Therefore, it is likely that allosteric modulation of GABA_B receptors is generally ineffective to relief neuropathic pain caused by injury or diseases associated with a reduced GABAergic tone, such as spinal cord injury (Drew et al., 2004; Gwak and Hulsebosch, 2011; Meisner et al., 2010; Zhang et al., 1994) or HIV-infection (Kanao et al., 2015). However, positive allosteric GABA_B receptor modulators might be useful as add-on to enhance baclofen-mediated analgesia at low baclofen doses, which potentially reduces baclofen-associated side effects.

Acknowledgements

This study was supported by the Swiss National Science Foundation grant 31003A_138382 (DB).

References

- Ataka, T., Kumamoto, E., Shimoji, K., Yoshimura, M., 2000. Baclofen inhibits more effectively C-afferent than Ad-afferent glutamatergic transmission in substantia gelatinosa neurons of adult rat spinal cord slices. *Pain* 86, 273-282.
- Bennett, G. J., Xie, Y. K., 1988. A peripheral mononeuropathy in rat that produces disorders of pain sensation like those seen in man. *Pain* 33, 87-107.
- Bettler, B., Kaupmann, K., Mosbacher, J., Gassmann, M., 2004. Molecular structure and physiological functions of GABA_B receptors. *Physiol. Rev.* 84, 835-867.
- Binet, V., Brajon, C., Le Corre, L., Acher, F., Pin, J. P., Prezeau, L., 2004. The heptahelical domain of GABA_{B2} is activated directly by CGP7930, a positive allosteric modulator of the GABA_B receptor. *J. Biol. Chem.* 279, 29085-29091.
- Bouhassira, D., Lanteri-Minet, M., Attal, N., Laurent, B., Touboul, C., 2008. Prevalence of chronic pain with neuropathic characteristics in the general population. *Pain* 136, 380-387.
- Bowery, N. G., 2006. GABA_B receptor: a site of therapeutic benefit. *Curr. Opin. Pharmacol.* 6, 37-43.
- Brennan, P. M., Whittle, I. R., 2008. Intrathecal baclofen therapy for neurological disorders: a sound knowledge base but many challenges remain. *Br. J. Neurosurg.* 22, 508-519.
- Brown, J. W., Moeller, A., Schmidt, M., Turner, S. C., Nimrich, V., Ma, J., Rueter, L. E., van der Kam, E., Zhang, M., 2016. Anticonvulsant effects of structurally diverse GABA_B positive allosteric modulators in the DBA/2J audiogenic seizure test: Comparison to baclofen and utility as a pharmacodynamic screening model. *Neuropharmacology* 101, 358-369.
- Brusberg, M., Ravnefjord, A., Martinsson, R., Larsson, H., Martinez, V., Lindström, E., 2009. The GABA_B receptor agonist, baclofen, and the positive allosteric modulator, CGP7930, inhibit visceral pain-related responses to colorectal distension in rats. *Neuropharmacology* 56, 362-367.
- Charles, K. J., Evans, M. L., Robbins, M. J., Calver, A. R., Leslie, R. A., Pangalos, M. N., 2001. Comparative immunohistochemical localisation of GABA_{B1a}, GABA_{B1b} and GABA_{B2} subunits in rat brain, spinal cord and dorsal root ganglion. *Neuroscience* 106, 447-467.
- Drew, G. M., Siddall, P. J., Duggan, A. W., 2004. Mechanical allodynia following contusion injury of the rat spinal cord is associated with loss of GABAergic inhibition in the dorsal horn. *Pain* 109, 379-388.
- Eaton, M. J., Plunkett, J. A., Karmally, S., Martinez, M. A., Montanez, K., 1998. Changes in GAD- and GABA- immunoreactivity in the spinal dorsal horn after peripheral nerve injury and promotion of recovery by lumbar transplant of immortalized serotonergic precursors. *J. Chem. Neuroanat.* 16, 57-72.
- Engle, M. P., Gassman, M., Sykes, K. T., Bettler, B., Hammond, D. L., 2006. Spinal nerve ligation does not alter the expression or function of GABA_B receptors in spinal cord and dorsal root ganglia of the rat. *Neuroscience* 138, 1277-1287.
- Froestl, W., 2010. Novel GABA_B receptor positive modulators: a patent survey. *Expert Opin. Ther. Pat.* 20, 1007-1017.
- Fukuhara, K., Katafuchi, T., Yoshimura, M., 2013. Effects of baclofen on mechanical noxious and innocuous transmission in the spinal dorsal horn of the adult rat: in vivo patch-clamp analysis. *Eur. J. Neurosci.* 38, 3398-3407.
- Galvez, T., Duthey, B., Kniazeff, J., Blahos, J., Rovelli, G., Bettler, B., Prezeau, L., Pin, J. P., 2001. Allosteric interactions between GB1 and GB2 subunits are required for optimal GABA_B receptor function. *EMBO J.* 20, 2152-2159.

- Galvez, T., Urwyler, S., Prezeu, L., Mosbacher, J., Joly, C., Malitschek, B., Heid, J., Brabet, I., Froestl, W., Bettler, B., Kaupmann, K., Pin, J. P., 2000. Ca^{2+} requirement for high-affinity g-aminobutyric acid (GABA) binding at GABA_B receptors: Involvement of serine 269 of the GABA_BR1 subunit. *Mol. Pharmacol.* 57, 419-426.
- Gannon, R. L., Millan, M. J., 2011. Positive allosteric modulators at GABA_B receptors exert intrinsic actions and enhance the influence of baclofen on light-induced phase shifts of hamster circadian activity rhythms. *Pharmacol. Biochem. Behav.* 99, 712-717.
- Goto, S., Taira, T., Horisawa, S., Yokote, A., Sasaki, T., Okada, Y., 2013. Spinal cord stimulation and intrathecal baclofen therapy: combined neuromodulation for treatment of advanced complex regional pain syndrome. *Stereotact. Funct. Neurosurg.* 91, 386-391.
- Gwak, Y. S., Hulsebosch, C. E., 2011. GABA and central neuropathic pain following spinal cord injury. *Neuropharmacology* 60, 799-808.
- Harmer, J. P., Larson, B. S., 2002. Pain relief from baclofen analgesia in a neuropathic pain patient who failed opioid and pharmacotherapy: case report. *J. Pain Palliat. Care Pharmacother.* 16, 61-64.
- Honsek, S. D., Seal, R. P., Sandkuhler, J., 2015. Presynaptic inhibition of optogenetically identified VGLuT3+ sensory fibres by opioids and baclofen. *Pain* 156, 243-251.
- Hosny, A., Simopoulos, T., Collins, B., 2004. Response of intractable post herpetic neuralgia to intrathecal baclofen. *Pain Physician* 7, 345-347.
- Hwang, J. H., Yaksh, T. L., 1997. The effect of spinal GABA receptor agonists on tactile allodynia in a surgically-induced neuropathic pain model in the rat. *Pain* 70, 15-22.
- Kalinichev, M., Donovan-Rodriguez, T., Girard, F., Riguet, E., Rouillier, M., Bournique, B., Haddouk, H., Mutel, V., Poli, S., 2014. Evaluation of peripheral versus central effects of GABA_B receptor activation using a novel, positive allosteric modulator of the GABA_B receptor ADX71943, a pharmacological tool compound with a fully peripheral activity profile. *Br. J. Pharmacol.* 171, 4941-4954.
- Kanao, M., Kanda, H., Huang, W., Liu, S., Yi, H., Candiotti, K. A., Lubarsky, D. A., Levitt, R. C., Hao, S., 2015. Gene transfer of glutamic acid decarboxylase 67 by herpes simplex virus vectors suppresses neuropathic pain induced by human immunodeficiency virus gp120 combined with ddC in rats. *Anesth. Analg.* 120, 1394-1404.
- Kaupmann, K., Malitschek, B., Schuler, V., Heid, J., Froestl, W., Beck, P., Mosbacher, J., Bischoff, S., Kulik, A., Shigemoto, R., Karshin, A., Bettler, B., 1998. GABA_B receptor subtypes assemble into functional heteromeric complexes. *Nature* 396, 683-687.
- Koek, W., Cheng, K., Rice, K. C., 2013. Discriminative stimulus effects of the GABA_B receptor-positive modulator rac-BHFF: comparison with GABA_B receptor agonists and drugs of abuse. *J. Pharmacol. Exp. Ther.* 344, 553-560.
- Kopsky, D. J., Keppel Hesselink, J. M., Casale, R., 2015. Walking with neuropathic pain: paradoxical shift from burden to support? *Case Rep. Med.* 2015, 764950.
- Laffray, S., Bouali-Benazzouz, R., Papon, M. A., Favereaux, A., Jiang, Y., Holm, T., Spriet, C., Desbarats, P., Fossat, P., Le Feuvre, Y., Decossas, M., Heliot, L., Langel, U., Nagy, F., Landry, M., 2012. Impairment of GABA_B receptor dimer by endogenous 14-3-3 ζ in chronic pain conditions. *EMBO J.* 31, 3239-3251.

- Maccioni, P., Thomas, A. W., Carai, M. A., Gessa, G. L., Malherbe, P., Colombo, G., 2010. The positive allosteric modulator of the GABA_B receptor, rac-BHFF, suppresses alcohol self-administration. *Drug Alcohol Depend.* 109, 96-103.
- Malan, T. P., Mata, H. P., Porreca, F., 2002. Spinal GABA_A and GABA_B receptor pharmacology in a rat model of neuropathic pain. *Anesthesiology* 96, 1161-1167.
- Malherbe, P., Masciadri, R., Norcross, R. D., Knoflach, F., Kratzeisen, C., Zenner, M. T., Kolb, Y., Marcuz, A., Huwyler, J., Nakagawa, T., Porter, R. H., Thomas, A. W., Wettstein, J. G., Sleight, A. J., Spooren, W., Prinssen, E. P., 2008. Characterization of (R,S)-5,7-di-tert-butyl-3-hydroxy-3-trifluoromethyl-3H-benzofuran-2-one as a positive allosteric modulator of GABA_B receptors. *Br. J. Pharmacol.* 154, 797-811.
- Meisner, J. G., Marsh, A. D., Marsh, D. R., 2010. Loss of GABAergic interneurons in laminae I-III of the spinal cord dorsal horn contributes to reduced GABAergic tone and neuropathic pain after spinal cord injury. *J. Neurotrauma* 27, 729-737.
- Melin, C., Jacquot, F., Dalle, R., Artola, A., 2013. Segmental disinhibition suppresses C-fiber inputs to the rat superficial medullary dorsal horn via the activation of GABA_B receptors. *Eur. J. Neurosci.* 37, 417-428.
- Patel, S., Naeem, S., Kesingland, A., Froestl, W., Capogna, M., Urban, L., Fox, A., 2001. The effects of GABA_B agonists and gabapentin on mechanical hyperalgesia in models of neuropathic and inflammatory pain in the rat. *Pain* 90, 217-226.
- Price, G. W., Wilkin, G. P., Turnbull, M. J., Bowery, N. G., 1984. Are baclofen-sensitive GABA_B receptors present on primary afferent terminals of the spinal cord? *Nature* 307, 71-74.
- Smith, G. D., Harrison, S. M., Birch, P. J., Elliott, P. J., Malcangio, M., Bowery, N. G., 1994. Increased sensitivity to the antinociceptive activity of (+/-)-baclofen in an animal model of chronic neuropathic, but not chronic inflammatory hyperalgesia. *Neuropharmacology* 33, 1103-1108.
- Torrance, N., Ferguson, J. A., Afolabi, E., Bennett, M. I., Serpell, M. G., Dunn, K. M., Smith, B. H., 2013. Neuropathic pain in the community: more under-treated than refractory? *Pain* 154, 690-699.
- Towers, S., Princivalle, A., Billinton, A., Edmunds, M., Bettler, B., Urban, L., Castro-Lopes, J., Bowery, N. G., 2000. GABA_B receptor protein and mRNA distribution in rat spinal cord and dorsal root ganglia. *Eur. J. Neurosci.* 12, 3201-3210.
- Urwyler, S., 2011. Allosteric modulation of family C G-protein-coupled receptors: from molecular insights to therapeutic perspectives. *Pharmacol. Rev.* 63, 59-126.
- van der Plas, A. A., van Rijn, M. A., Marinus, J., Putter, H., van Hilten, J. J., 2013. Efficacy of intrathecal baclofen on different pain qualities in complex regional pain syndrome. *Anesth. Analg.* 116, 211-215.
- Vaysse, L., Sol, J. C., Lazorthes, Y., Courtade-Saidi, M., Eaton, M. J., Jozan, S., 2011. GABAergic pathway in a rat model of chronic neuropathic pain: modulation after intrathecal transplantation of a human neuronal cell line. *Neurosci. Res.* 69, 111-120.
- Wang, X. L., Zhang, Q., Zhang, Y. Z., Liu, Y. T., Dong, R., Wang, Q. J., Guo, Y. X., 2011. Downregulation of GABA_B receptors in the spinal cord dorsal horn in diabetic neuropathy. *Neurosci. Lett.* 490, 112-115.
- White, J. H., Wise, A., Main, M. J., Green, A., Fraser, N. J., Disney, G. H., Barnes, A. A., Emson, P., Foord, S. M., Marshall, F. H., 1998. Heterodimerization is required for the formation of functional GABA_B receptors. *Nature* 396, 679-682.
- Wu, J., Xu, Y., Pu, S., Jiang, W., Du, D., 2011. p38/MAPK inhibitor modulates the expression of dorsal horn GABA_B receptors in the spinal nerve ligation model of neuropathic pain. *Neuroimmunomodulation* 18, 150-155.

- Yang, K., Ma, W. L., Feng, Y. P., Dong, Y. X., Li, Y. Q., 2002. Origins of GABA_B receptor-like immunoreactive terminals in the rat spinal dorsal horn. *Brain Res. Bull.* 58, 499-507.
- Zeilhofer, H. U., Benke, D., Yevenes, G. E., 2012a. Chronic pain states: pharmacological strategies to restore diminished inhibitory spinal pain control. *Annu. Rev. Pharmacol. Toxicol.* 52, 111-133.
- Zeilhofer, H. U., Wildner, H., Yevenes, G. E., 2012b. Fast synaptic inhibition in spinal sensory processing and pain control. *Physiol. Rev.* 92, 193-235.
- Zhang, A. L., Hao, J. X., Seiger, A., Xu, X. J., Wiesenfeld-Hallin, Z., Grant, G., Aldskogius, H., 1994. Decreased GABA immunoreactivity in spinal cord dorsal horn neurons after transient spinal cord ischemia in the rat. *Brain Res.* 656, 187-190.
- Zuniga, R. E., Schlicht, C. R., Abram, S. E., 2000. Intrathecal baclofen is analgesic in patients with chronic pain. *Anesthesiology* 92, 876-880.

Figure legends

Fig. 1. Effect of rac-BHFF on motor activity.

Motor activity of mice was monitored in vehicle and rac-BHFF treated mice. Left, number of beam crosses per 24 min over a period of 6 hours. Right, total number of beam crosses within 6 hours. ***, $P < 0.001$; one-way ANOVA followed by Dunnet's post hoc test; $n = 6$ mice per condition.

Fig. 2. Rac-BHFF increases the paw withdrawal threshold to mechanical stimuli in healthy mice.

Mice were treated with vehicle or rac-BHFF (10 mg/kg p. o.) and tested for paw withdrawal thresholds of the right (upper panel) and left (lower panel) paws using electronic von Frey filaments. ****, $P < 0.0001$; repeated measures two-way ANOVA followed by Bonferroni post hoc test; $n = 4$ mice per condition. Effect of time, $F(3, 36) = 125$ $P < 0.0001$; effect of drug: $F(3, 12) = 200$, $P < 0.0001$.

Fig. 3. Effect of rac-BHFF in the CCI mouse model of neuropathic pain.

A) Administration of rac-BHFF alone is not sufficient to relief neuropathic pain. Seven days after CCI, mice were treated with vehicle or rac-BHFF (10 mg/kg, p. o.) and tested for mechanical sensitization on the ipsilateral (affected) and contralateral (unaffected) paw using electronic von Frey filaments ($n = 6$ mice per condition).

B) rac-BHFF potentiates the analgesic effect of baclofen. Seven days after CCI, mice were treated either with vehicle, baclofen (5 μ g/kg, i. t.) or baclofen (5 μ g/kg, i. t.) plus rac-BHFF (10 mg/kg, p. o.) and tested for mechanical sensitization. Error bars smaller than the symbol are not depicted.

C) Statistical comparison of data shown in B. Maximum antihyperalgesic activity was determined for the interval between 90 and 120 min after drug administration. **, $P < 0.01$, ***, $P < 0.001$; ****, $P < 0.0001$; one-way ANOVA followed by Bonferroni post hoc test; $n = 4$ mice per condition.

Fig. 4. CCI does not downregulate GABA_B receptors.

The expression of GABA_B receptors in sham-operated and CCI mice was determined by quantitative autoradiography on 16 μ m thick cryostat-cut sections from fresh-frozen L4-5 spinal cord using the tritiated GABA_B receptor antagonist [³H]CGP 54626

as radioligand. Top: representative images. Nonspecific binding was determined in the presence of excess CGP 56999A. Bottom: Quantification of specific [³H]CGP 54626 binding in the ipsi and contralateral dorsal horn (13-26 sections from 4 sham and 22-36 section from 4 CCI mice).

Fig. 5. CCI does not appreciably affect GABA_B receptor heterodimers.

The integrity of GABA_B receptor heterodimers was tested using [³H]CGP 54626 binding to CCI and sham L4-5 spinal cord sections in the presence of increasing concentrations of GABA. Because the affinity of GABA to GABA_{B1} in the absence of GABA_{B2} is strongly reduced (~100-fold), GABA is expected to display considerably less potency of inhibiting [³H]CGP 54626 binding in the ipsilateral dorsal horn of CCI mice in case of disrupted GABA_B receptor heterodimers.

A) Representative images. Nonspecific binding was determined in the presence of excess unlabeled CGP 56999A.

B) Quantification of specific [³H]CGP 54626 binding in the ipsi and contralateral dorsal horn of CCI and sham operated mice (8-16 sections per condition from 4 sham and 4 CCI mice).

Fig. 1

Fig. 2

Figure 3

Fig. 3

Figure 4

Fig. 4

Figure 5

Fig. 5