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The α2/α3GABA$_A$ receptor modulator TPA023B alleviates not only the sensory but also the tonic affective component of chronic pain in mice

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Abstract

Diminished synaptic inhibition in the spinal dorsal horn is a major contributor to pathological pain syndromes of neuropathic or inflammatory origin. Drugs that enhance the activity of dorsal horn α2/α3GABA$_A$Rs normalize exaggerated nociceptive responses in rodents with neuropathic nerve lesions or peripheral inflammation but lack most of the typical side effects of less specific GABAergic drugs. It is however still unknown whether such drugs also reduce the clinically more relevant conscious perception of pain. Here, we investigated the effects of the α2/α3GABA$_A$R subtype-selective modulator TPA023B on the tonic aversive component of pain in mice with peripheral inflammation or neuropathy. In neuropathic mice with a chronic constriction injury of the sciatic nerve, TPA023B not only reversed hyperalgesia to tactile and heat stimuli but also was highly effective in the conditioned place preference test. In the formalin test, TPA023B not only reduced licking of the injected paw but also reversed facial pain expression scores in the mouse grimace scale assay. Taken together, our results demonstrate that α2/α3GABA$_A$ receptor subtype-selective modulators not only reduce nociceptive withdrawal responses but also alleviate the tonic aversive components of chronic pain.

Keywords: Analgesia, Neuropathic pain, Inflammatory pain, GABA, Benzodiazepine, Subtype-selective, Disinhibition, Conditioned place preference, Paclitaxel, Chronic constriction injury, Mouse grimace scale

1. Introduction

Diminished GABAergic inhibition in the spinal dorsal horn is a major contributor to different chronic pain forms.[16,40,47] Facilitation of GABAergic inhibition in the spinal cord through benzodiazepine (BDZ) site ligands, which positively modulate GABA$_A$ receptor (GABA$_A$R) function, reverses pathologically increased pain sensitivity (hyperalgesia) in rodent models of chronic inflammatory and neuropathic pain.[13] Work in genetically modified (point-mutated "knock-in") mice, in which only 1 GABA$_A$R subtype was left BDZ-sensitive, has demonstrated that targeting α2GABA$_A$R and α3GABA$_A$R provides effective antihyperalgesia in the absence of typical BDZ-mediated side effects.[34] This is in line with previous studies that showed that most undesired effects of classical BDZ agonists, such as sedation, impaired motor coordination, tolerance development, and addiction, can be avoided by sparing modulation of α1GABA$_A$Rs.[34,38,42] These findings have prompted the development of BDZ site ligands with improved subtype selectivity. Several such compounds (L-838,417, HZ-166, SL-651498, and NS11394) have been proven to be efficacious in different preclinical pain models. However, these models used almost exclusively classical withdrawal readout-based pain tests[5,13,14,19,24,31,36] or flexor responses in models using chemically induced nociception.[13,14,25] Such readouts might not properly reflect the multidimensional experience of pain in patients with its complex interplay of sensory, affective, and cognitive components.[2,8] Monitoring these affective aspects of pain in nonverbal animals presents a major challenge. Recently, operant learning paradigms, such as conditioned place preference, and coding of facial expressions of pain have been used to assess affective aspects of pain also in rodents.[10,12,17,33,41]

In this study, we investigated the effects of TPA023B, an α1-sparing GABA$_A$R modulator[44] developed by Merck in the quest for nonsedative anxiolytics.[1,15,39] We assessed effects of TPA023B on the affective and the sensory components of pain in different mouse pain models. Relative to several other α2/α3GABA$_A$R modulators, TPA023B has more favorable pharmacokinetics, and, as shown in a previous study on its antipruritic actions, it is devoid of typical benzodiazepine drug-mediated side effects, including sedation, muscle relaxation, impaired motor coordination, and tolerance development.[35] Furthermore, TPA023B is well tolerated not only in rodents but also in dogs, nonhuman primates, and humans.[1,35,44] Here, we have assessed its effects on evoked nocifensive responses in naive mice and in mice with neuropathic or inflammatory insults and on the affective component of on-going pain in 2 models of mechanical and chemical nerve injury. We show that TPA023B

Sponsorships or competing interests that may be relevant to content are disclosed at the end of this article.

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not only reverses mechanical sensitization in mice with neuropathic pain but also reduces the tonic the aversive component of pain measured in the conditioned place preference test and the mouse grimace scale (MGS) assay. These results indicate that $\alpha2/\alpha3GABA_AR$ modulators are not only antihyperalgesic but also effective against the affective component of chronic pain.

2. Methods

2.1. Animals and animal maintenance

All experiments were performed in 6- to 9-week-old female and male mice at equal numbers, except the use of only male mice in the paclitaxel-induced neuropathic pain model as described by Braz et al.[4] Care was taken to ensure that animals were randomly assigned to each group. Animals were bred in-house, group-housed, and kept on 12/12 light/dark cycle (lights on at 07.00 AM; Zeitgeber time (ZT) = 0; light intensity 50 lux at 1 m above floor) and were provided with food and water at all times. Experiments were performed in wild-type C57BL/6J mice (colony established with breeding pairs obtained from Janvier), in $GABA_AR$ point-mutated mice, in which $\alpha2GABA_ARs$ had been rendered benzodiazepine-insensitive ($\alpha2^{R/R}$; Ref. 18), in mice that lack $\alpha2GABA_ARs$ specifically from the spinal cord (hoxB8-$\alpha2^{-/-}$; Refs. 31, 45) and in corresponding control littermates ($\alpha2^{fl/fl}$). $\alpha2^{R/R}$ mice, which carry a homozygous histidine to arginine (H->R) point mutation at position 101 of the *gabra2* gene, have been described previously by Löw et al.[18] hoxB8-$\alpha2^{-/-}$ mice were bread as described previously.[31,45] All mice were of the C57BL/6J genetic background. Behavioral experiments were conducted by the same female, experienced experimenter, blinded to the genotype of the mice or their treatment with drug or vehicle. All behavioral experiments were performed between 10.00 AM (ZT = 03) and 04.00 PM (ZT = 09). Experimental rooms had a constant temperature (20-21°C), were without daylight, and were illuminated with white light of 50 lux (measured 1 m above the floor). In all tests, mice were allowed to acclimatize to the test apparatus for at least 60 minutes, except for the conditioned place preference test (see below). All procedures were performed in accordance with the Veterinary Office of the Canton of Zürich (licence numbers ZH135/2009, ZH063/2016, and ZH231/2017).

2.2. Drugs and drug administration

TPA023B (6,2′-difluoro-5'-[3-(1-hydroxy-1methylethyl)imidazo[1,2-b][1,2,4]triazin-7-yl]biphenyl-2-carbonitrile) was synthesized by ANAWA (Wangen, Switzerland), and purity was >95%. For per oral (p.o.) administration, TPA023B was suspended in 0.9% saline (sodium chloride, Sigma-Aldrich) and 1% Tween80 (Sigma-Aldrich, St. Louis, MO). For intrathecal (i.t.) administration, TPA023B was suspended in artificial cerebrospinal fluid (ACSF) containing (in mM) 120 NaCl, 5 HEPES, 26 $NaHCO_3$, 1.25 NaH_2PO_4, 2.5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, and 10 glucose (pH 7.35) (all from Sigma-Aldrich). The 4% formalin solution (pH 7.4) was prepared from paraformaldehyde powder dissolved in 0.1-M phosphate buffer containing NaH_2PO_4 and Na_2HPO_4 (all from Sigma-Aldrich). For the p.o. administration of vehicle or drug, a metal (stainless steel) gavage needle (20 G) was used (Thermo Fisher Scientific, Waltham, MA). Mice were not trained for this procedure. To ensure a successful and safe oral gavage performance, the distance from the snout to the caudal point of the sternum (xiphoid process) was measured with the metal needle on the outside of the restrained animal and marked on the needle with a permanent marker. Intrathecal injections were performed under isoflurane (1.5%) anesthesia. A 30-G 0.5 stainless steel needle (Thermo Fisher Scientific) attached to a 25-μL Hamilton syringe was inserted between the groove of L5 and L6 vertebrae. A tail flick indicated a successful insertion of the needle in the intradural space.

2.3. Chronic constriction injury–induced neuropathic pain model

To induce neuropathic pain, chronic constriction injury (CCI) was applied to the left sciatic nerve proximal to the trifurcation with 3 loose (5-0, not absorbable) silk (Ethicon, Somerville, NJ) ligatures in mice anesthetized with isoflurane 1% to 3%.[3] Skin was closed using 5-0 Dermalon suture (Covidien, Minneapolis, MN).

2.4. Paclitaxel-induced neuropathic pain model

We used the paclitaxel model to produce mechanical and heat hypersensitivity that mimics chemotherapy-induced neuropathic pain conditions. In brief, we injected male mice with 250 μL of 1-mg/kg paclitaxel (Sigma-Aldrich), dissolved in 40% DMSO (Sigma-Aldrich), intraperitoneally (i.p.) 4 times, every other day at 10 AM (ZT = 03).[4]

2.5. Formalin test

To chemically induce inflammatory pain, 10 μL of a 4% formalin solution was injected subcutaneously into the left hind paw without anesthesia using a custom-made mouse restrainer. The time spent licking the left hind paw and the facial expression of the mice (MGS, Ref. 17) were evaluated 15 to 60 minutes after injection.

2.6. Assessment of mechanical and thermal sensitivity

Mechanical sensitivity was quantified as the change in the paw withdrawal threshold evoked by an electronic von Frey filament (IITC Inc Life Science, Woodland Hills, CA). Heat sensitivity was determined by the measurement of paw withdrawal latency to a defined radiant heat stimulus applied to the plantar surface of the left hind paw. These experiments were performed using the Plantar Analgesia Meter (IITC Inc Life Science). Heat intensity was set to 14, the plate was prewarmed to 37°C, and the cutoff time was set to 32 seconds to avoid tissue damage.

2.7. Conditioned place preference

The conditioned place preference apparatus consisted of 2 chambers (20 × 20 cm), which were identical in size but could be visually distinguished by their different wall patterns (uniform black vs 3-cm broad black and white horizontal stripes). The chambers were connected through a tunnel (3.8 × 10.0 × 13.7 cm [W × L × H]), the entrances of which were blocked during the conditioning sessions. Mice were recorded with an HD digital video camera, and the time spent in each chamber was analysed manually. Preconditioning started 7 days after CCI surgery. Mice had free access to all chambers for 30 minutes on days 7 and 8 after CCI surgery. On day 9, a preconditioning bias test was performed and the time spent in the 2 chambers was measured for 20 minutes. Mice spending more than 80%, or less than 20% of the total time in 1 chamber, were excluded from the experiment (0, 3, 5, 2, and 0, from the experiments shown in **Figs. 1–5**, respectively). On the conditioning day (day 10), mice were i.t. injected with vehicle (ACSF, total volume of 5 μL) and paired for 45 minutes with a randomly chosen chamber in the morning. Access to the other chamber and tunnel was blocked. Four hours later, mice

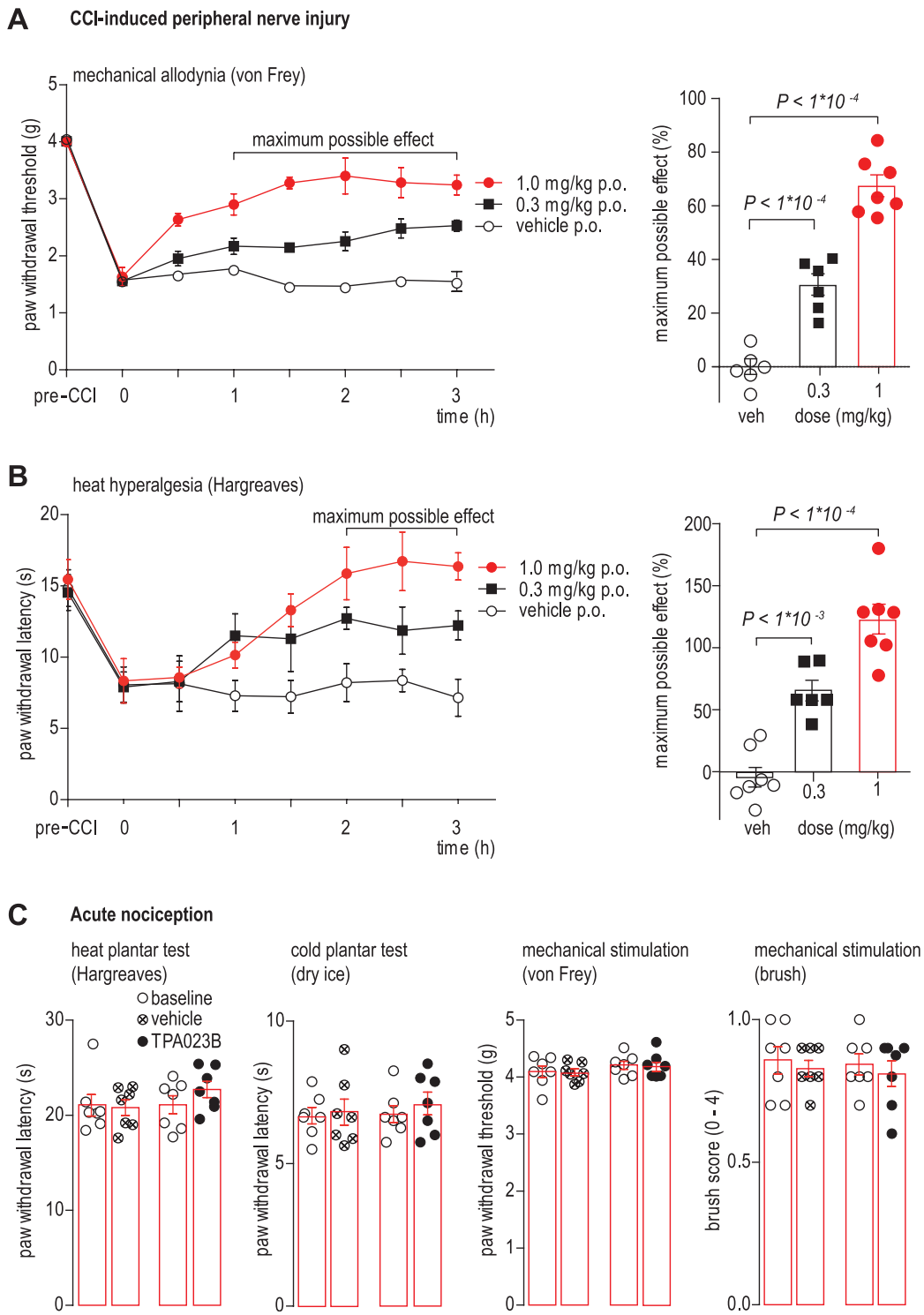

Figure 1. Effects of TPA023B in stimulus-evoked CCI-induced neuropathic and acute nociceptive pain. (A) Reversal of chronic constriction injury (CCI)-induced mechanical hyperalgesia by TPA023B in wild-type (C57BL/6J) mice assessed with von Frey filaments (mean ± SEM, n = 6, 6, and 7 mice, for vehicle, 0.3 mg/kg, and 1 mg/kg TPA023B p.o., respectively). The maximum possible antihyperalgesia (MPE) was calculated from the drug effects during 60 to 180 minutes after TPA023B administration. Statistics, time course of changes in paw withdrawal latencies (left panel): repeated-measures ANOVA followed by the Dunnett post hoc test with predrug baseline (time = 0) as reference. Vehicle: F(5,30) = 0.5; *P* = 0.87, 0.43, 0.81, 0.30, 1.0, and 1.0 for time 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 hours, respectively. TPA023B (0.3 mg/kg): F(5,30) = 2,48; *P* = 0.20, 0.06, 0.0009, 0.043, 0.03, and 0.005, for 0.5, 1.0, 1.5, 2, 2.5, and 3.0 hours, respectively; TPA023B (1 mg/kg): F(6,36) = 4.04; *P* = 0.0001, 0.02, 0.0007, 0.01, 0.01, and 0.005, for 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 hours, respectively. MPE (right panel): one-way ANOVA followed by the Dunnett post hoc test F(2,16) = 87.9. (B) Same as (A) but heat hyperalgesia (n = 7, 6, and 7 mice, for vehicle, 0.3 mg/kg, and 1 mg/kg TPA023B p.o., respectively). Time course (left panel): F(6,36) = 29.18. *P* = 1.0, 0.50, 0.39, 1.0, 1.0, and 0.06, for 0.5, 1.0, 1.5, 2.0, 2.5, and 3 hours, respectively; TPA023B (0.3 mg/kg): F(5,30) = 23.3. *P* = 0.99, 0.04, 0.31, 0.014, 0.03, and 0.01, for 0.5, 1.0, 1.5, 2.0, 2.5, and 3 hours, respectively; TPA023B (1 mg/kg): F(6,36) = 16.3. *P* = 1.0, 0.14, 0.008, 0.004, 0.003, and 0.002, for 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 hours, respectively. MPE (right panel): F(2,17) = 42.9. (C) Effects against acute nociceptive pain in naive C57BL/6J mice. TPA023B (1 mg/kg p.o.) did not alter responses to acute noxious heat (Hargreaves) and cold (dry ice) stimuli nor to light mechanical stimulation with von Frey filaments or a paintbrush (mean ± SEM, n = 7). One-way ANOVA followed by the Bonferroni post hoc test, F(3,24) = 0.9 for the Hargreaves test; F(3,24) = 0.3 for the cold test; F(3,24) = 0.7 for the von Frey test; F(3,24) = 0.2 for the brush test. ANOVA, analysis of variance.

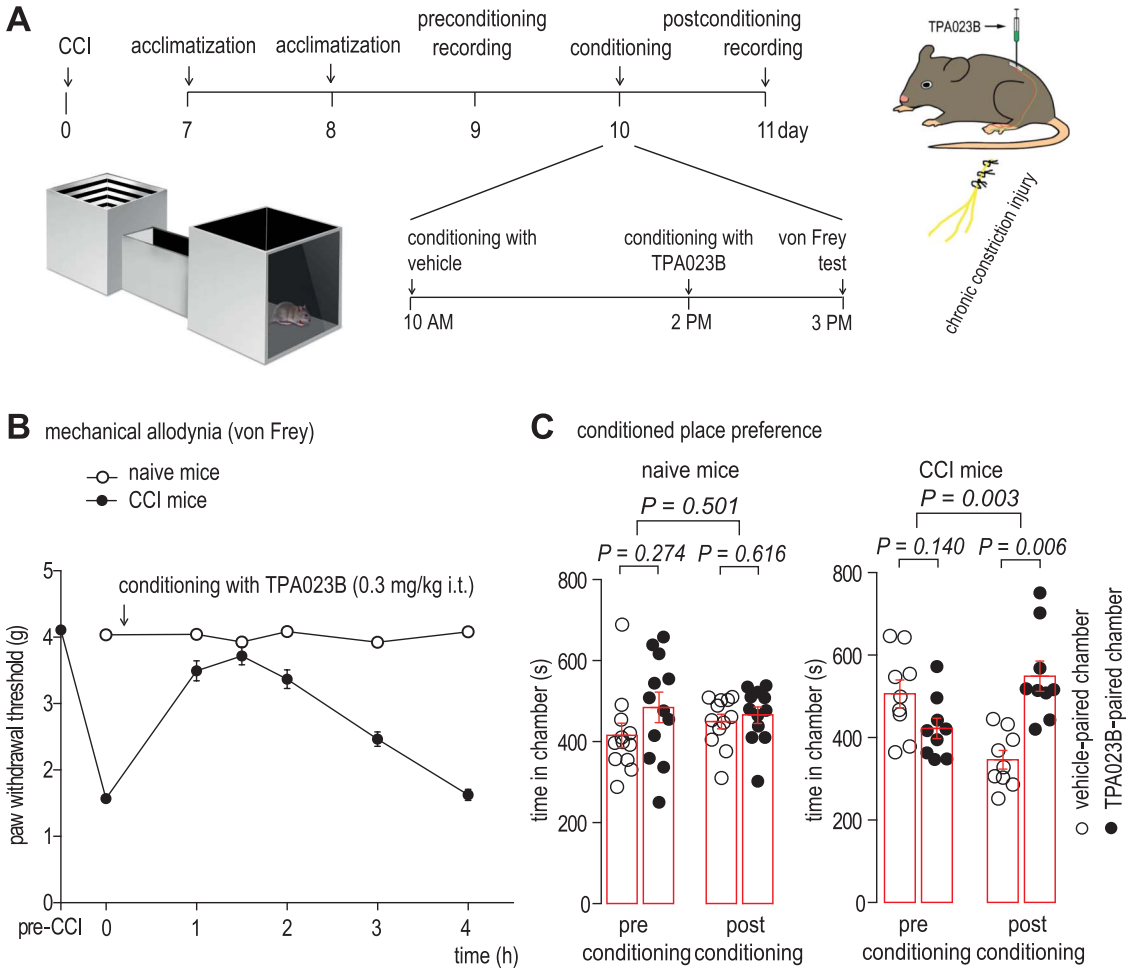

Figure 2. TPA023B reduced the tonic aversive component of on-going pain in the CCI mice. (A) Design and timeline of the conditioned place preference (CPP) experiment. (B) TPA023B (0.3 mg/kg, i.t.) normalized mechanical paw withdrawal thresholds measured on the conditioning day immediately after mice with CCI-induced neuropathy had undergone drug conditioning. Repeated-measures ANOVA followed by the Dunnett post hoc test with paw withdrawal thresholds at predrug baseline (time = 0) as reference. Naive mice (n = 12): $F(11,55) = 0.93$. $P = 1.0$, 0.43, 0.95, 0.62, and 0.97, for 1.0, 1.5, 2.0, 3.0, and 4.0 hours, respectively. CCI mice (n = 9): $F(8,40) = 1.4$. $P < 0.0001$ for 1.0, 1.5, and 2.0 hours, $P = 0.0002$ for 3 hours, and $P = 0.98$ for 4.0 hours. (C) Time (mean ± SEM) spent in vehicle- and TPA023B-paired chambers for naive wild-type (C57BL/6J) mice (n = 12) and wild-type (C57BL/6J) mice that had undergone CCI surgery (n = 9), before and after conditioning. After 2 acclimatization sessions on days 7 and 8, the time spent in the 2 chambers was measured during a 20-minute preconditioning session to ensure the absence of chamber bias. (A) significant chamber x preconditioning/postconditioning interaction was obtained for CCI-operated mice but not for naive mice. Two-way repeated-measures ANOVA: naive mice $F(1,11) = 0.48$; CCI mice $F(1,8) = 18.6$; Subsequent pairwise comparisons demonstrated a preference for the drug-paired chamber only for CCI mice after conditioning. ANOVA, analysis of variance; CCI, chronic constriction injury.

were i.t. injected with TPA023B (0.3 mg/kg in 5 μL) and paired with the other chamber. Chamber pairings were counterbalanced. On the next day (day 10 after CCI surgery), 20 hours after administration of TPA023B and after the afternoon pairing, mice were placed in the tunnel of the conditioned place preference apparatus with access to both chambers and recorded for 20 minutes for analysis of chamber preference. The person analysing the videos was blinded to the genotype and treatment of the animals. The conditioned place preference apparatus was cleaned with water before the next mouse was placed into the apparatus.

2.8. Mouse grimace scale

We used the MGS as a standardized behavioural coding system for facial pain expressions as described by Langford et al.[17] In brief, mice were placed individually in transparent plexiglass cubicles (15 × 15 × 15 cm; L × W × H) for acclimatization. Next, vehicle (0.9% saline and 1% Tween80) or drug (1-mg/kg TPA023B in 250-μL vehicle) was administered orally, and the animals were put back into their cage. One hour later, formalin was injected and mice were placed in cubicles and recorded for 45 minutes with 2 HD digital video cameras positioned on opposite sides. For every condition, 6 images of the face were taken as screenshots from the obtained recordings. A screenshot was extracted on every occasion as long as the face was clearly visible and the mouse was not grooming or sniffing. For analysis, randomly arranged sets of mouse photographs were scored by 2 persons ("coders") blinded to the treatment status of the mice. These coders assigned a value of 0 (not present), 1 (moderately visible), or 2 (severe) for 4 facial features (orbital tightening, nose bulge, cheek bulge, and ear position). When the coder was unsure whether the mouse showed a moderately visible facial expression or not, a score of 1 was given as described in Matsumiya et al.[21] The final MGS score was the average across all criteria of every coder.

2.9. Statistics

Data are expressed as mean ± SEM. Where appropriate, data were analysed using a 1-way or 2-way repeated-measures analysis of variance (ANOVA) and subsequent pairwise

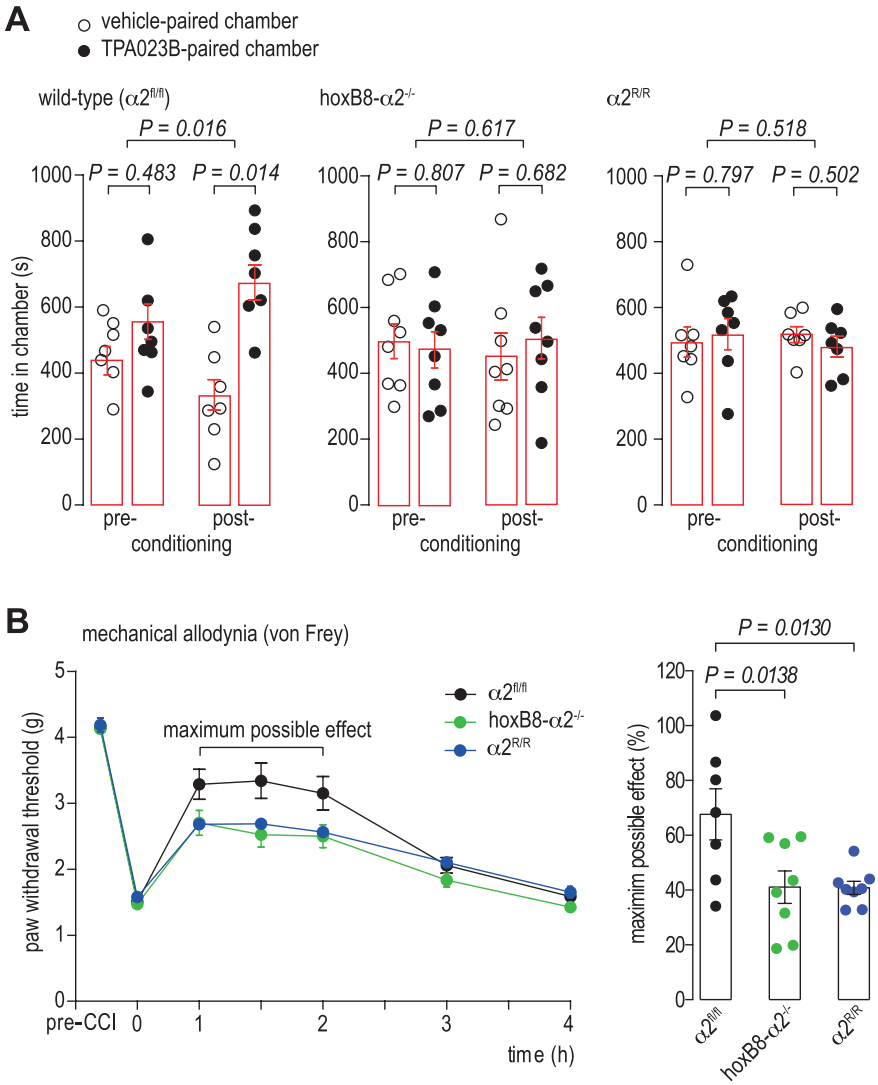

Figure 3. Conditioned place preference in neuropathic mice lacking α2GABA$_A$Rs specifically from the spinal cord. (A) Time (mean ± SEM) spent in vehicle- and TPA023B-paired chambers for $\alpha2^{fl/fl}$ (n = 7), hoxB8-$\alpha2^{-/-}$ (n = 8) and $\alpha2^{R/R}$ mice (n = 7) before and after conditioning. TPA023B (0.3 mg/kg, i.t.) produced place preference in neuropathic $\alpha2^{fl/fl}$ mice for the drug-paired chamber, whereas neuropathic hoxB8-$\alpha2^{-/-}$ mice and $\alpha2^{R/R}$ mice spent similar amounts of time in both chambers. Two-way repeated-measures ANOVA yielded significant chamber x preconditioning/postconditioning interaction for $\alpha2^{fl/fl}$ mice but not for hoxB8-$\alpha2^{-/-}$ and $\alpha2^{R/R}$ mice $\alpha2^{fl/fl}$ (F(1,6) = 10.9, P = 0.016; hoxB8-$\alpha2^{-/-}$: F(1,7) = 0.274; P = 0.617; $\alpha2^{R/R}$: F(1,6) = 0.47; P = 0.518). Pairwise comparisons showed a preference for the TPA023B-paired chamber in $\alpha2^{fl/fl}$ mice after conditioning, but not for any of the other pairs. (B) Partial reversal of mechanical thresholds by TPA023B (0.3 mg/kg, i.t.) measured immediately after neuropathic hoxB8-$\alpha2^{-/-}$ and $\alpha2^{R/R}$ mice underwent drug conditioning. TPA023B almost completely reversed mechanical sensitization in neuropathic $\alpha2^{fl/fl}$ mice (mean ± SEM, n = 7 [$\alpha2^{fl/fl}$], 8 [hoxB8-$\alpha2^{-/-}$], and 7 [$\alpha2^{R/R}$]). Time course (left panel). Repeated-measures ANOVA followed by the Dunnett post hoc test with withdrawal thresholds at predrug baseline (time = 0) as reference. $\alpha2^{fl/fl}$ mice: F(6,30) = 3.67. P = 0.002, 0.003, 0.005, 0.015, and 0.15, for 1.0, 1.5, 2.0, 3.0 and 4.0 hours, respectively; hoxB8-$\alpha2^{-/-}$ mice: F(7,35) = 5.1. P = 0.003, 0.003, 0.001, 0.06 and 1.0, for 1.0, 1.5, 2.0, 3.0, and 4.0 hours, respectively; $\alpha2^{R/R}$ mice: F(6,30) = 2.28. $P < 0.0001$, P = 0.0002, $P < 0.0001$, P = 0.01 and 0.26, for 1.0, 1.5, 2.0, 3.0, and 4.0 hours, respectively. Maximum possible antihyperalgesia (MPE; right panel) was determined from paw withdrawal latencies obtained at 60 to 120 minutes after TPA023B administration. One-way ANOVA followed by the Dunnett post hoc test F(2,20) = 5.9. ANOVA, analysis of variance.

comparisons. Maximum possible analgesic effects were analysed using 1-way ANOVA followed by the Dunnett post hoc tests. Formalin assay data and paclitaxel-induced neuropathy data were analysed using the unpaired t test. In all statistical analyses, results were considered significant when $P < 0.05$.

2.10 Data availability.

Excel files including the data that support the findings of this study are available at G-Node.org with the identifier doi: 10.12751/g-node.ogsof3 [https://doi.gin.g-node.org/10.12751/g-node.ogsof3].

3. Results

3.1. Effects of TPA023B on noxious stimulus-evoked responses in naive and neuropathic mice

TPA023B is one of the most selective nonsedative (α1GABA$_A$R-sparing) benzodiazepine site agonists currently available.[1,44] Although previous work with other α1GABA$_A$R-sparing compounds suggests that it should possess antihyperalgesic efficacy, such actions have not yet been directly demonstrated. We therefore first verified the presence of such antihyperalgesic actions of TPA023B in the CCI model of neuropathic pain. After surgery, all 39 operated mice developed pronounced mechanical and heat hyperalgesia. Seven days after surgery, mice

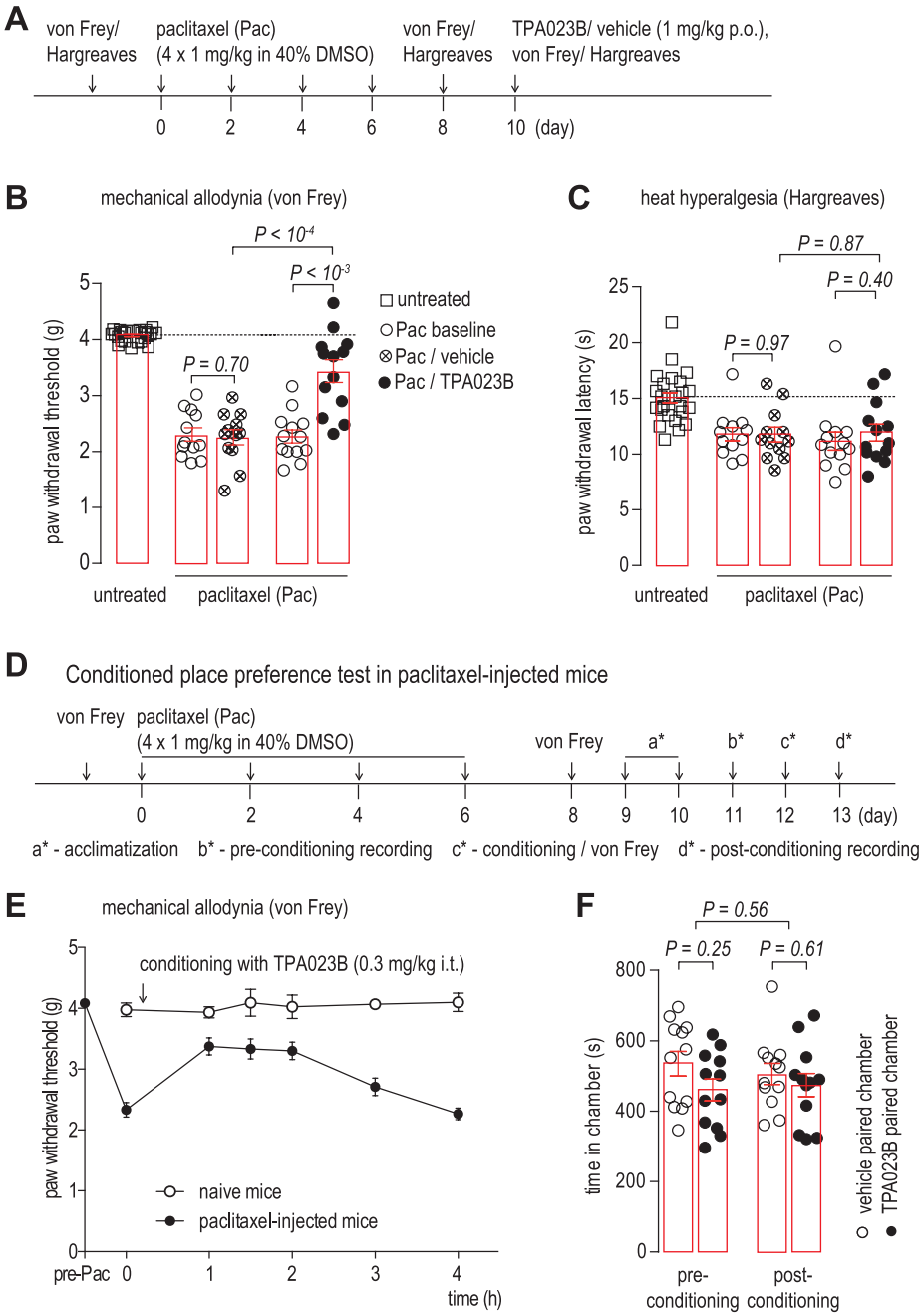

Figure 4. TPA023B reduced mechanical hyperalgesia but not the aversive component of tonic pain in mice with paclitaxel-induced neuropathy. (A) Timeline of paclitaxel injections and stimulus-evoked behavior tests. (B) TPA023B (1 mg/kg p.o.) reversed paclitaxel-induced mechanical hyperalgesia (2-way repeated-measures ANOVA revealed a significant time × drug interaction $F(1,23) = 18.1$, $P < 0.001$; n = 12 and 13, for vehicle- and TPA023B-treated groups). Subsequent pairwise comparisons yield a significant difference for baseline vs TPA023B ($P < 0.0005$). (C) TPA023B (1 mg/kg p.o.) did not alter heat thresholds in the Hargreaves test (2-way repeated-measures ANOVA time x drug interaction $F(1,23) = 0.37$, $P = 0.55$). Same mice as (B). (D) Conditioned place preference protocol in mice with paclitaxel-induced neuropathy. Two-way repeated-measures ANOVA did not yield a significant chamber x preconditioning/postconditioning interaction. $F(1,11) = 0.32$; $P = 0.56$. (E) TPA023B (0.3 mg/kg, i.t.) reduced mechanical sensitization, measured immediately after mice underwent drug conditioning. Repeated-measures ANOVA followed by the Dunnett post hoc test showed significant recovery from paclitaxel-induced mechanical hypersensitivity by TPA023B for all time points ($F(11,55) = 3.22$; $P = 0.0003$, 0.0009, $P < 0.0001$, $P = 0.04$ after 1.0, 1.5, 2.0, and 3.0 hours, respectively) except for 4 hours ($P = 0.99$). No such effect was observed in naive mice: $F(3,15) = 0.94$. $P = 1.0$ after 1.0, 1.5, and 2.0 hours, respectively, $P = 0.75$ after 3 hours, $P = 0.87$ after 4 hours. (F) TPA023B did not produce conditioned place preference for the TPA023B-paired (475 ± 33 seconds) chamber compared with the vehicle-paired (506 ± 30 seconds) chamber in paclitaxel-injected mice. Two-way repeated-measures ANOVA $F(1,44) = 0.5$, followed by pairwise comparisons, n = 12. ANOVA, analysis of variance.

were treated systemically with 2 different doses of TPA023B (0.3 or 1 mg/kg p.o.) or with vehicle. The doses were chosen based on a previous study with well-established dose–response curves.[35] TPA023B exerted a dose-dependent antihyperalgesic action expressed as percent maximum possible effect (mechanical hyperalgesia: 30.4 ± 3.9% and 67.2 ± 4.0%, for 0.3 mg/kg and 1 mg/kg TPA023B, respectively; heat hyperalgesia: 65.1 ± 7.7% and 122.2 ± 12.2%, for 0.3 mg/kg and 1 mg/kg TPA023B,

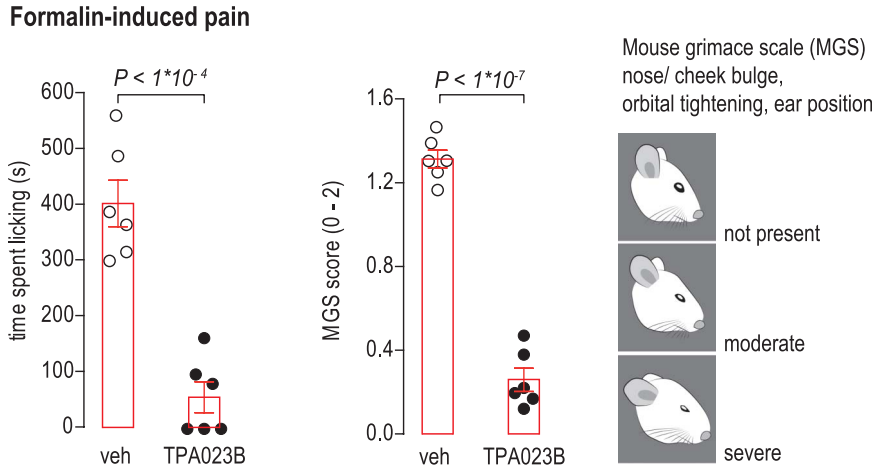

Figure 5. TPA023B alleviated chemically induced inflammatory pain. TPA023B (1 mg/kg p.o.) reduced the time spent licking the injected paw and reversed facial pain expressions in C57BL/6 mice with formalin-induced pain (mean ± SEM, n = 6, unpaired *t* test).

respectively; **Figs. 1A and B**). Separate experiments revealed that significant antihyperalgesia by 1 mg/kg p.o. TPA023B lasts for at least 6 to less than 10 hours (Neumann et al., unpublished). In previous experiments, in which we had used the nonselective prototypical benzodiazepine site agonist diazepam in $GABA_AR$ point-mutated mice, we have found that facilitation of GABAergic inhibition failed to reduce sensitivity to acute nociceptive stimuli in naive (nonsensitized) mice.[35] We therefore tested next whether TPA023B would show a similar lack of acute antinociceptive activity (**Fig. 1C**). Indeed, TPA023B (1 mg/kg p.o.) did not alter response thresholds or latencies upon exposure of naive mice to acute noxious heat (Hargreaves test) or noxious cold exposure (cold plantar test). Furthermore, it did not change responses to light mechanical stimulation with von Frey filaments or a paintbrush.

3.2. TPA023B reduced the tonic aversive component of ongoing pain in neuropathic mice

We next investigated whether the action of TPA023B would be limited to evoked nociceptive responses in sensitized mice or whether it would also reduce the aversive component of tonic pain. To this end, we used the conditioned place preference test (**Fig. 2A**) in CCI mice and applied TPA023B i.t. at a dose of 0.3 mg/kg. The i.t. administration route was chosen for 2 reasons: (1) The faster onset of pain relief (as compared to oral administration) should facilitate the association of drug action with the drug-paired chamber, and (2) potential confounding reward-related or anxiolytic properties, which would originate from supraspinal sites, should be less likely to occur.

On day 7 after CCI surgery, before conditioned place preference conditioning, the presence of mechanical sensitization was confirmed. Animals had paw withdrawal thresholds of 1.57 ± 0.07 g after CCI surgery compared with 4.11 ± 0.05 g before surgery. On days 7, 8, and 9, the preconditioning was performed. Mice were placed into the conditioned place preference apparatus with free access to both chambers. Mice that spent less than 20% or more than 80% in 1 chamber were excluded. On the conditioning day (day 10 after surgery), mice received first a vehicle injection and were then transferred immediately after injection into 1 chamber for 45 minutes without access to the other compartments of the conditioned place preference apparatus ("chamber pairing"). Four hours later, the same mice were injected with TPA023B (0.3 mg/kg, i.t.) and placed into the other chamber for 45 minutes. To verify the presence of an analgesic effect in each of the mice, mechanical pain thresholds were tested with von Frey filaments, immediately after this conditioning. Compared with the values obtained on day 7 (predrug baseline), mice exhibited reduced sensitization (ie, increased paw withdrawal thresholds: 3.49 ± 0.15 g after 1 hour; 3.7 ± 0.13 g after 1.5 hours; 3.37 ± 0.14 g after 2 hours; and 2.46 ± 0.11 g after 3 hours). Within 4 hours after injection, response thresholds returned to pretreatment baseline values (1.63 ± 0.09 g) (**Fig. 2B**). On the next day, animals were placed without further drug treatment into the tunnel connecting the 2 chambers of the conditioned place preference apparatus with free access to both chambers. Mice with CCI-induced hyperalgesia showed a pronounced preference for the drug-paired chamber (549 ± 37 seconds) compared with the vehicle-paired chamber (346 ± 23 seconds) (**Fig. 2C**). Naive mice that had not undergone CCI surgery but the same conditioning procedure spent similar amounts of time in the vehicle (450 ± 18 seconds) and drug-paired (466 ± 20 seconds) chambers, indicating that i.t. administration of TPA023B did not induce place preference in the absence of a tonic pain condition. Naive mice were used as controls instead of sham-operated animals because any post-surgical pain remaining in sham-operated mice might have caused conditioned place preference and would have made it impossible to exclude pain-independent rewarding properties of the test drug.

3.3. Conditioned place preference by TPA023B in neuropathic mice exclusively depends on α2GABA$_A$ receptors located in the spinal cord

Although TPA023B had been i.t. injected in the conditioned place preference experiments described above, we cannot fully exclude that it reached supraspinal sites through rostral diffusion within the cerebrospinal fluid or after absorption into the systemic circulation. To exclude potential effects arising from supraspinal $GABA_ARs$, we took a genetic approach and made use of hoxB8-$\alpha2^{-/-}$ mice that lack $\alpha2GABA_ARs$ specifically from the spinal cord[31] (**Fig. 3A**). We compared the effects of TPA023B in the conditioned place preference test in hoxB8-$\alpha2^{-/-}$ mice with those in global $\alpha2GABA_AR$ point-mutated mice ($\alpha2^{R/R}$), in which all $\alpha2GABA_ARs$ have been rendered insensitive to diazepam[18]

and at the same time also to several other benzodiazepine site agonists including TPA023B.[31,35] $\alpha2^{fl/fl}$ mice were used as wild-type controls. Nine days after CCI surgery, none of the 3 mouse lines showed a pre-existing chamber bias in the conditioned place preference test. After conditioning, $\alpha2^{fl/fl}$ mice showed the expected preference for the TPA023B-paired chamber, similar to what we have previously observed with wild-type C57BL/6 mice. This conditioned place preference was absent in hoxB8-$\alpha2^{-/-}$ mice (and global $\alpha2^{R/R}$ point-mutated mice), indicating that supraspinal $\alpha2GABA_ARs$ did not contribute to the conditioned place preference effect. hoxB8-$\alpha2^{-/-}$ mice spent 499 ± 53 and 472 ± 55 seconds in the vehicle-paired and drug-paired chamber, respectively, and $\alpha2^{R/R}$ point-mutated mice spent 494 ± 46 and 518 ± 47 seconds in the 2 chambers. The same mice were also tested in the von Frey test (**Fig. 3B**). No significant differences were observed between hoxB8-$\alpha2^{-/-}$ mice (41.1 ± 5.9% maximum possible drug effect) and global $\alpha2^{R/R}$ mice (40.9 ± 2.6% maximum possible drug effect), again excluding a contribution of supraspinal $\alpha2GABA_ARs$. However, although the preference for the drug-paired chamber was completely lost in both, hoxB8-$\alpha2^{-/-}$ mice and in global $\alpha2^{R/R}$ mice, both of these mouse lines showed only a partial reduction in TPA023B-induced antihyperalgesia (relative to $\alpha2^{fl/fl}$ mice), suggesting that $GABA_ARs$ contribute differently to the control of the sensory and the aversive components of pain.

3.4. TPA023B effects in mice with paclitaxel-induced neuropathy

We next assessed the antihyperalgesic potential of TPA023B in a second neuropathic pain model. To this end, we chose chronic treatment with the anticancer drug paclitaxel as a model of chemotherapy-induced neuropathic pain. Paclitaxel (1 mg/kg, i.p.) was injected 4 times, every other day (**Fig. 4A**), and led to the development of mechanical and heat hyperalgesia (von Frey test, $P < 0.0001$ and Hargreaves test, $P < 0.0001$, paired t test, n = 25 mice). TPA023B (1 mg/kg p.o.) significantly reduced paclitaxel-induced mechanical sensitization but had no effect on heat hyperalgesia in this model (**Figs. 4B and C**). We then investigated the actions of TPA023B on conditioned place preference in mice with paclitaxel-induced mechanical hyperalgesia using the same conditioning protocol as used previously for the CCI mice (**Fig. 4D**). Paclitaxel reduced the thresholds of paw withdrawal responses upon stimulation with von Frey filaments from 4.08 ± 0.02 g before paclitaxel treatment to 2.33 ± 0.12 g after paclitaxel (n = 12) (**Fig. 4E**). On the conditioning day, mice received first vehicle i.t. treatment and were paired with 1 chamber. Four hours later, mice were treated with TPA023B (0.3 mg/kg, i.t.) paired with the other chamber. After drug conditioning was completed, paw withdrawal thresholds were again assessed with von Frey filaments. To permit blinding of the experimenter, 4 naive animals were included in the von Frey tests. Although TPA023B (0.3 mg/kg, i.t.) was efficacious against paclitaxel-induced mechanical hyperalgesia (3.38 ± 0.14 g after 1 hour, 3.33 ± 0.17 g after 1.5 hours, and 3.3 ± 0.14 g after 2 hours, **Fig. 4E**), it did not induce a place preference (506 ± 30 seconds for the vehicle-paired chamber and 475 ± 33 seconds for TPA023B-paired chamber, **Fig. 4F**).

3.5. TPA023B reverses facial expressions of pain in the formalin test

Finally, we assessed the analgesic actions of TPA023B on formalin-induced spontaneous pain using the MGS assay,[17] which is a standardized facial coding system well-suited for rodent pain models of moderate duration. Systemic treatment with TPA023B (1 mg/kg p.o.) not only significantly reduced the time spent licking the injected paw (401 ± 42 seconds and 57 ± 28 seconds, for vehicle- and TPA023B-treated mice, respectively, n = 6) but also reversed facial pain grimacing (1.31 ± 0.04 MGS score and 0.26 ± 0.06 MGS score for vehicle- and TPA023B-treated mice, respectively, n = 6) caused by subcutaneous formalin injections compared with vehicle-treated mice (**Fig. 5**).

4. Discussion

A large body of evidence indicates that an enhancement of the inhibitory actions of spinal $\alpha2/\alpha3GABA_ARs$ alleviates inflammatory and neuropathic hyperalgesia in mice.[5,13,14,19,24,31,34] Attempts to translate these preclinical concepts into patient therapy have not yet been successful. In fact, a clinical trial testing PF-06372865, an $\alpha2/\alpha3$-selective $GABA_AR$ modulator developed by Pfizer, in patients with chronic low back pain has failed.[9] Possible reasons of this discrepancy include differences between readouts typically used in mouse pain models and in clinical trials. Preclinical testing in rodents largely relies on nocifensive withdrawal responses that measure response thresholds upon exposure to an acute nociceptive stimulus and hence address primarily the sensory component of pain. By contrast, the impairment of patients with chronic pain results mainly from on-going suprathreshold pain and includes a strong affective component.[2,8] Clinical trials on analgesic drugs therefore focus primarily on subjective self-reported pain. Relatively recently developed novel preclinical assays permit a stimulus-independent evaluation of the affective component of on-going and suprathreshold pain also in rodents.[10,12,17,41] In this study, we have used 2 of these assays, namely conditioned place preference in a model of neuropathic pain and the MGS in the formalin test. In both of these assays, we obtained compelling evidence for a beneficial effect of TPA023B on the affective component of tonic pain. We should add here that our experiments on mice exposed to CCI surgery included naive mice as controls but no sham-operated mice. For this reason, the analgesic action observed may include analgesia directed against postoperative pain. However, a previous study[12] found no conditioned place preference with other analgesic drugs (clonidine and lidocaine) in sham-operated mice.

Activation of the brain reward system and anxiolytic drug actions are well-known confounding factors in conditioned place preference paradigms.[43] Rewarding and anxiolytic actions are also known to occur with classical nonselective benzodiazepine site agonists. An activation of the brain reward system in our experiments with TPA023B as an underlying cause of its effects in the conditioned place preference experiments is unlikely because TPA023B did not induce any place preference in naive (pain-free) mice. Furthermore, previous work has demonstrated that $\alpha1$-sparing benzodiazepine site ligands lack rewarding properties.[7,37,42] In this context, it should be noted that relief of pain or, in general, of aversive states activates the brain reward pathway.[27] Such analgesia-induced activation of brain reward circuits likely contributes to the conditioned place preference observed with analgesic treatments. Ruling out a potential anxiolytic action as a confounding factor is more complex, especially since $\alpha2GABA_AR$ modulators, including TPA023B, are anxiolytic[18,28] and because animals with neuropathic pain exhibit elevated levels of anxiety.[22,26] To exclude false-positive effects from an anxiolytic drug action, we verified that the action of

TPA023B in the conditioned place preference paradigm depended exclusively on spinal α2GABA$_A$Rs and did not involve supraspinal receptors.

Our conditioned place preference experiments with i.t. injected TPA023B have yielded another intriguing result. Specific ablation of all α2GABA$_A$Rs from the spinal cord completely prevented TPA023B-induced conditioned place preference in CCI mice. By contrast, the antihyperalgesic action of TPA023B measured in the same mice was only partially reduced, suggesting that the sensory and affective components of pain are differentially sensitive to changes in GABAergic inhibition. Several previous studies have already reported differential effects of adenosine on sensory hyperalgesia and the affective pain component. In particular, King et al.[12] have shown that i.t. administration of adenosine reversed spinal nerve ligation–induced tactile allodynia but did not induce conditioned place preference. Martin et al.[20] have demonstrated that i.t. adenosine reversed mechanical hypersensitivity in rats with spared nerve injury–induced neuropathy but failed to reduce heroin self-administration in these animals. These findings are in line with human data by Eisenach et al.[6] reporting that spinal adenosine blocked secondary hyperalgesia, which was detected with evoked stimuli, in patients with neuropathic pain but had no effect on their overall pain ratings. Despite these examples, it is obvious that the sensory and affective components of pain processing are interrelated because, eg, a complete block of sensory detection would also suppress affective responses.

In this study, we have used 2 different neuropathic pain models, the CCI of the sciatic nerve and paclitaxel-induced neuropathy. In both models, TPA023B was effective against hyperalgesic withdrawal responses, but conditioned place preference by TPA023B was only observed in CCI mice but not in mice with paclitaxel-induced neuropathy. The reason for this discrepancy is currently unknown. Too weak on-going pain in this model might explain the absence of a TPA023B effect in the conditioned place preference model. However, other authors have found effects of other analgesics on conditioned place preference in the same paclitaxel model,[11] putting this explanation into perspective. Alternatively, one might speculate that the impairment of GABAergic inhibition is more severe in CCI-operated mice than in paclitaxel-treated mice, which would render mice with paclitaxel-induced neuropathy less susceptible to analgesia by GABAergic compounds. This explanation would be also supported by the slightly weaker effect on evoked pain responses of TPA023B in paclitaxel-treated mice relative to mice with CCI-induced neuropathy (compare **Figs. 2B and 4E**). Gene expression analyses have indeed found significant reductions in the GABA-producing enzymes GAD-65 and GAD-67 after peripheral mechanical nerve injury,[23] while no significant changes were reported in a study that investigated spinal cord of mice with paclitaxel-induced neuropathy.[4] As GABA$_A$R modulators likely relieve pathological pain associated with a deficit in GABAergic inhibition, this difference may explain why nerve injury–induced hyperalgesia responds better to TPA023B than paclitaxel-induced neuropathic pain.

Similar considerations might also explain the differential efficacy of GABA$_A$R modulators in models of inflammatory or neuropathy-induced hyperalgesia vs acute nociception. Recent work has shown that neuropathic sensitization involves the functionalization of normally silent dorsal horn circuits that connect Aβ fiber input to lamina I projections neurons through multiple interneurons.[32,46] This polysynaptic relay becomes functional in the course of diminished synaptic inhibition and should hence be highly sensitive to drugs strengthening GABAergic inhibition. By contrast, acute nociceptive responses rely more on monosynaptic connections.[19] Such monosynaptic connections are also tuned by GABAergic inhibition but are less likely to be completely silenced by enhancers of GABAergic inhibition. In this context, it might be worth mentioning that TPA023B is effective against acute itch[35] and hence likely in the absence of disinhibition. Interestingly, unlike acute nociceptive transmission, the spinal relay of acute chemical itch signals occurs through at least 2 dorsal horn interneurons and may thus resemble the polysynaptic feature of circuits of hyperalgesia rather than circuits of acute nociception.

The failure of the recent clinical trial on chronic low back pain[9] cannot be ascribed to a lack of effect of α2/α3-selective GABA$_A$R modulators on the tonic aversive component of pain. It is unlikely that differences in the pharmacological properties of TPA023B and PF-6372865, which was tested the failed chronic low back pain trial, explain the failure in the low back pain trial. Both compounds have similar efficacies and affinities for the different GABA$_A$R subtypes, and both penetrate well into the CNS after oral dosing.[1,30] It is hence likely that other factors, such as a too low dose or the selection of a patient population with merely nociceptive rather than neuropathic pain, were responsible for the negative outcome of the chronic low back pain trial.[48]

In summary, our results indicate that a facilitation of GABAergic inhibition in the spinal dorsal horn not only reverses hyperalgesia but also alleviates the tonic aversive component of on-going pain. Therefore, α2/α3-selective GABA$_A$R modulators such as TPA023B hold promising therapeutic potential for the treatment of the sensory as well as affective dimensions of chronic pain.

Conflict of interest statement

The authors have no conflicts of interest to declare.

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