Itch suppression in mice and dogs by modulation of spinal α2 and α3GABA_A receptors

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Chronic itch is a highly debilitating condition affecting about 10% of the general population. The relay of itch signals is under tight control by inhibitory circuits of the spinal dorsal horn, which may offer a hitherto unexploited therapeutic opportunity. Here, we found that specific pharmacological targeting of inhibitory α2 and α3GABA_A receptors reduces acute histaminergic and non-histaminergic itch in mice. Systemic treatment with an α2/α3GABA_A receptor selective modulator alleviates also chronic itch in a mouse model of atopic dermatitis and in dogs sensitized to house dust mites, without inducing sedation, motor dysfunction, or loss of antipruritic activity after prolonged treatment. Transsynaptic circuit tracing, immunofluorescence, and electrophysiological experiments identify spinal α2 and α3GABA_A receptors as likely molecular targets underlying the antipruritic effect. Our results indicate that drugs targeting α2 and α3GABA_A receptors are well-suited to alleviate itch, including non-histaminergic chronic itch for which currently no approved treatment exists.
Chronic itch affects between 4–17% of the general population. Most drugs currently used to treat itch are histamine H1 and H4 receptor blockers that work well against acute itch. By contrast, chronic itch is mostly histamine-independent and largely unresponsive to these medications. Frequent causes of histamine-independent itch include, besides atopic dermatitis, cholestatic liver disease, end stage kidney failure, and opioid-therapy. Drugs used to treat itch in these conditions include immune suppressants and drugs acting at the CNS such as gabapentinoids, antidepressants, and opioid receptor antagonists. In the majority of cases, these treatments do not provide adequate relief or cause severe side effects.

Pruritic (itch) stimuli are detected by sensory neurons (primary pruritoceptors) that innervate the skin and transform these stimuli into electrical signals, i.e., action potentials. These action potentials are then relayed via the peripheral and central axons of primary pruritoceptors to central neurons in the spinal or medullary dorsal horn. Only recently have researchers begun to understand the signaling molecules, receptors, transmitters and neuronal pathways of itch. Several “new” G protein-coupled receptors expressed by primary sensory neurons have been identified that are activated by pruritogens. One such receptor is the mas-related G protein coupled receptor A3 (MrgrpA3 in mouse, or MRGPRX1 in human), which is activated by the antimalarial drug chloroquine. Sensory neurons expressing this receptor become excited by a wide variety of pruritogens involved in acute histaminergic and non-histaminergic itch, as well as in chronic itch. Other work addressed neuronal pathways involved in the spinal relay of itch. These studies identified excitatory interneurons expressing gastrin releasing peptide (GRP) or the GRP receptor (GRPR) as key elements of this process. These itch-relay pathways appear to be under tight control by dorsal horn inhibitory neurons. Lack of a certain subset of these neurons that depend on the transcription factor Bhlhb5 leads to severe chronic itch in mice. Local ablation of inhibitory neurons of the deep dorsal horn induces abnormal grooming and biting behavior, and localized hair loss reminiscent of chronic itch syndromes. Conversely, local activation of these neurons through DREADDs (designer receptors exclusively activated by designer drugs) suppressed histamine-dependent and histamine-independent itch, demonstrating that inhibitory dorsal horn neurons exert a profound control over spinal itch relay.

Inhibitory neurons of the spinal dorsal horn release two fast amino acid transmitters, GABA and glycine, to reduce the excitability of their postsynaptic target neurons. In the present study, we focused on the GABAergic system and investigated whether itch, in particular chronic itch, can be suppressed through pharmacological modulation of specific subtypes of spinal GABA<sub>α</sub> receptors (GABA<sub>α</sub>Rs). GABA<sub>α</sub>Rs are pentameric anion channels built from a repertoire of 19 subunits. Most GABA<sub>α</sub>Rs in the brain and spinal cord are composed of α, β, and γ subunits in a 2:2:1 stoichiometry. The mammalian genome harbors 12 genes encoding for these subunits (α1-6, β1-3, and γ1–3). Spinal GABA<sub>α</sub>Rs mainly contain α1, α2, α3, or α5 subunits together with β2/3 subunits and a γ subunit. α4 and α6 subunits are only sparsely expressed or completely lacking. Differences in the physiological functions and pharmacological properties of these GABA<sub>α</sub>Rs are mainly determined by the γ subunit. In the present study, we first used genetically modified mice to identify α2/α3 containing GABA<sub>α</sub>Rs as key elements of spinal itch control. Building on this result we assessed potential antipruritic actions of an α2/α3GABA<sub>α</sub>R selective compound (TPA023B; ref. 19) and showed that it not only reduced acute histamine-dependent and histamine-independent itch in mice but also chronic itch in mice and dogs without apparent side effects.

### Results

#### Inhibitory input to peripheral and spinal pruritoceptors

We first verified that itch relaying GRP neurons receive input from local inhibitory interneurons. Retrograde mono-/transynaptic rabies virus-based tracing experiments initiated from GRP::cre neurons identified numerous inhibitory and excitatory neurons presynaptic to GRP neurons (Fig. 1). About half of the inhibitory neurons were located in lamina II of the dorsal horn, where most inhibitory neurons are purely GABAergic. The other half resided in deeper layers, where most inhibitory neurons co-express glycine and GABA.

We next investigated the presence of α1, α2, α3, and α5GABA<sub>α</sub>R subunits on spinal axon terminals of primary MrgrpA3 positive pruritoceptors and on spinal GRP neurons (Fig. 2). Both MrgrpA3 fibers and GRP neurons are concentrated in lamina II, which also harbors α2 and/or α3GABA<sub>α</sub>R subunits at high density. To visualize MrgrpA3 axons and terminals and GRP neurons, we used GRP::eGFP and MrgrpA3::cre-eGFP;ROSA26<sup>lsl</sup>-tdTom transgenic mice. Immunostaining of spinal cord sections of these mice confirmed that the region of α2 and α3GABA<sub>α</sub>R subunit expression overlapped with that of MrgrpA3 terminals and GRP neurons (Fig. 2a,c). A similar GABA<sub>α</sub>R subunit expression pattern was found in the cervical spinal cord and the medullary dorsal horn (see Supplementary Fig. 1). By contrast, α1 and α5GABA<sub>α</sub>R subunits were largely missing from lamina II. Confocal analysis at higher magnification further demonstrated that α2 and α3GABA<sub>α</sub>R subunits were located on MrgrpA3 fibers and GRP neurons (Fig. 2b,d). To allow a quantification of α2 and α3GABA<sub>α</sub>R subunit expressing neurons, we performed fluorescent in situ hybridization in sections of lumbar dorsal root ganglia (DRGs) and lumbar spinal cords (Fig. 2e). In these experiments, we also included spinal cord sections from GRPR::eGFP transgenic mice. Only about 20% of MrgrpA3 positive DRG neurons expressed α2 and α3GABA<sub>α</sub>R subunit transcripts. This low expression is consistent with previously published single cell RNAseq data (Supplementary Table 1). By contrast, virtually all GRP and GRPR neurons expressed α3GABA<sub>α</sub>R subunit transcripts, and more than 60% of these neurons also expressed α2GABA<sub>α</sub>R subunits. Co-expression of α2 and α3GABA<sub>α</sub>R subunit transcripts with GRP and GRPR transcripts was also observed in human spinal cord tissue (Supplementary Fig. 2).

#### GABA<sub>α</sub>R subtypes with antipruritic efficacy

We then asked whether pharmacological targeting of GABA<sub>α</sub>Rs containing α2 or α3 subunits (α2/α3GABA<sub>α</sub>Rs) reduces itch. To this end, we administered diazepam, a classical benzodiazepine that non-selectively potentiates the activation of benzodiazepine-sensitive GABA<sub>α</sub>Rs. To restrict diazepam’s action to a single GABA<sub>α</sub>R subtype (α1, α2, α3, or α5), we used triple GABA<sub>α</sub>R point mutated mice, which carry a histidine to arginine (H → R) point mutation in three of the four benzodiazepine sensitive GABA<sub>α</sub>R α subunits (designated as HRRR, RRHR, RRHR, and RRHR mice, for mice in which only α1, α2, α3, or α5GABA<sub>α</sub>Rs remained diazepam sensitive). Because mice of this particular genetic background (129SvJ) have not yet been systematically analyzed in itch experiments, we first assessed their sensitivity to different pruritogens. We found that injection of α-methyl serotonin (α-methyl 5-HT), a metabolically more stable derivative of the pivotal itch messenger serotonin, induced robust dose-dependent scratching behavior (Supplementary Fig. 3). We then tested the effect of systemic diazepam (10 mg kg<sup>−1</sup>, p.o.) on scratching responses evoked by α-methyl 5-HT (20 μg). Selective targeting of α2 or α3GABA<sub>α</sub>Rs (in HRRR or RRHR mice) strongly reduced scratching bouts (Fig. 3a-d). No significant...
of 3 mice.

Antipruritic efficacy of an α2/α3 selective GABA\textsubscript{A}R modulator.

We then tested whether the data obtained in genetically modified mice would translate into therapeutic efficacy of GABA\textsubscript{A}R subtype-selective compounds. To this end, we tested the antipruritic efficacy of the α1-sparing GABA\textsubscript{A}R modulator TPA023B (ref. 19). As a prerequisite, we verified the in vitro pharmacological profile of TPA023B in transiently transfected HEK293 cells. As reported previously19, TPA023B had partial agonistic activity at the benzodiazepine binding site of α2β3γ2 and α3β3γ2 GABA\textsubscript{A}R\textsubscript{s}, but did not potentiate α1β2γ2 GABA\textsubscript{A}R\textsubscript{s} and had very weak potentiating effects on α5β2γ2 GABA\textsubscript{A}R\textsubscript{s} (Fig. 4a). It did not activate GABA\textsubscript{A}R\textsubscript{s} in the absence of GABA. We then asked whether this partial agonistic activity would translate to a facilitation of GABAergic inhibition in MrgprA3 or GRP neurons (Fig. 4b, c). In dissociated MrgprA3::cre-eGFP positive DRG neurons, TPA023B (1 µM) led to a slight statistically insignificant increase in GABA\textsubscript{A}R current amplitudes (P = 0.068, two-sided paired t-test, n = 10) (Fig. 4b). A more pronounced effect was observed in GRP dorsal horn neurons in which TPA023B (1 µM) significantly prolonged the decay of GABAergic inhibitory
α2 and α3 GABA<sub>α</sub>Rs are expressed on key elements of a spinal itch relay circuit. Expression of GABA<sub>α</sub>R α subunits in MrgprA3 positive primary pruritoceptors (a, b) and GRP positive dorsal horn neurons (c, d). a–d show transverse sections of the lumbar spinal cord of two MrgprA3::cre;ROSA26<sup>tdTom</sup> and three GRP::eGFP transgenic mice stained with antibodies against α1, α2, α3, and α5GABA<sub>α</sub>R subunits. td-Tom and eGFP are shown in green, GABA<sub>α</sub>R α subunits in red. Overlapping expression (light green/yellow) of GABA<sub>α</sub>R α subunits with tdTom and eGFP was seen for α2 and α3GABA<sub>α</sub>R subunits, but not for α1 and α5GABA<sub>α</sub>R subunits. b, d Confocal analyses. Orthogonal views (stacks of 17–35 sections (1024 × 1024 pixels) at 0.4 µm intervals) verify co-localization of α2 and α3GABA<sub>α</sub>R subunits with MrgprA3 positive fibers and terminals (b) and GRP positive dorsal horn neurons (e) at higher magnification. Arrowheads indicate examples of co-localization. Scales bars, 50 µm (a, c), 5 µm (b, d). e Fluorescent in situ hybridization signals of α2 (red) and α3 (blue) subunits together with eGFP (to detect MrgprA3 neurons DRG neurons in MrgprA3::cre-eGFP transgenic mice), GRP and GRPR in situ hybridization signals (green, in wild-type mice). DAPI staining (gray) was used to indicate the location of cells. Bar charts: percent GABA<sub>α</sub>R α subunit positive neurons among the marker (MrgprA3, GRP, and GRPR) positive neurons. Each data point represents one mouse. Sections were obtained from 3–5 mice. Scale bar, 20 µm.
**Fig. 3** Antipruritic effects of GABA<sub>AR</sub> modulation in GABA<sub>AR</sub> triple point mutated mice. 

**a-d** Suppression of acute itch by α<sub>2</sub>, α<sub>3</sub>, and α<sub>5</sub>GABA<sub>AR</sub> activation (diazepam 10 mg kg<sup>-1</sup>, p.o., given 60 min prior to pruritogen injection) in three lines of triple GABA<sub>AR</sub> point mutated mice (α<sub>2</sub> [RHRR mice], α<sub>3</sub> [RRHR], or α<sub>5</sub> [RRRH]). 

- **a** Number of scratching bouts over time after diazepam injection.
- **b** Comparisons were made for diazepam (filled circles) versus vehicle (open circles) for the three genotypes. 
  
P values were obtained from unpaired two-sided t-tests corrected for three independent comparisons. 

n = 8 and 10 (α<sub>2</sub>, vehicle, diazepam); n = 8 and 8 (α<sub>3</sub>, vehicle, diazepam); n = 7 and 9 (α<sub>5</sub>, vehicle, diazepam).

- **c** Dose-dependence of the antipruritic effects of diazepam in RHRR mice (only α<sub>2</sub>GABA<sub>AR</sub> sensitive to diazepam). ANOVA followed by Dunnett’s post hoc test F(6,36) = 6.02; *P < 0.05; **P < 0.01, n = 7, 7, 4, 6, 7, 5, for vehicle, and 0.1, 0.3, 1.0, 3.0, 10, and 30 mg kg<sup>-1</sup>. 

- **d** Same as **c** but RRHR mice (only α<sub>3</sub>GABA<sub>AR</sub> sensitive to diazepam). ANOVA followed by Dunnett’s post hoc test F(6,37) = 4.42; *P < 0.05; **P < 0.01, n = 7 (vehicle) and 6 for all other groups.

- **e-h** Suppression of chronic itch. Antipruritic effects of α<sub>2</sub>, α<sub>3</sub>, and α<sub>5</sub>GABA<sub>AR</sub>s in the oxazolone model of atopic-like dermatitis. Mice were sensitized to oxazolone over 17 days and treated with diazepam (10 mg kg<sup>-1</sup>, i.p.) or vehicle on day 18. Scratching bouts were counted for 6 h starting 15 min after drug or vehicle administration. 
  
P values obtained from unpaired two-sided t-tests for the three genotypes, corrected for three independent comparisons. Unpaired two-sided t-tests, n = 7 and 8 (α<sub>2</sub>, vehicle, diazepam); n = 7, 8 (α<sub>3</sub>, vehicle, diazepam); n = 8, 8 (α<sub>5</sub>, vehicle, diazepam). Error bars indicate s.e.m.
postsynaptic currents by $43 \pm 10\%$ ($P < 0.01$, paired two-sided t-test, $n = 7$) (Fig. 4c). Together with the in situ hybridization data, these results suggest that GABA$_A$Rs inhibit the spinal relay of itch signals primarily at the level of dorsal horn interneurons rather than via inhibition of primary pruritoceptors.

Would the favorable in vitro profile of TPA023B translate into reduced propensity to side effects? Consistent with the lack of agonistic activity at α1GABA$_A$Rs, TPA023B did not induce sedation at doses up to $3 \text{ mg kg}^{-1}$ (p.o.), but instead increased locomotor activity at $1$ and $3 \text{ mg kg}^{-1}$ (Fig. 5a). TPA023B did not cause muscle relaxation (Fig. 5b) and did not impair motor coordination (Fig. 5c).

We then continued investigating the efficacy of systemic (p. o.) TPA023B against acute itch evoked by α-methyl 5-HT (20 μg), chloroquine (100 μg), or histamine (100 μg) (Fig. 6a). Chloroquine-induced scratching was reduced by TPA023B in wild-type mice at doses ≥ $0.03 \text{ mg kg}^{-1}$ (p.o.), but instead increased locomotor activity at 1 and 3 mg kg$^{-1}$ (Fig. 5a). TPA023B did not cause muscle relaxation (Fig. 5b) and did not impair motor coordination (Fig. 5c).

Subsequent experiments with intrathecal injection of TPA023B (0.3 mg kg$^{-1}$) at the level of the lumbar spinal cord in wild-type mice (Fig. 7a) confirmed that the antipruritic action of TPA023B originated from the spinal cord. Further support was obtained with hoxB8-GABA$_A$Ra2$^{-/-}$ (Fig. 7b) and sns-GABA$_A$Ra2$^{-/-}$ mice (Fig. 7c). These conditional knock-out mice lack α2GABA$_A$Rs either from all spinal cord and DRG neurons (hoxB8-GABA$_A$Ra2$^{-/-}$) or only from small diameter Nav1.8 positive (nociceptive and pruritoceptive) DRG neurons$^{28}$ or only from smaller diameter Nav1.8 positive (nociceptive and pruritoceptive) DRG neurons$^{29}$. Antipruritic effects of systemic TPA023B (3 mg kg$^{-1}$, p.o.) were strongly reduced in hoxB8-GABA$_A$Ra2$^{-/-}$ mice but remained unaltered in sns-GABA$_A$Ra2$^{-/-}$ mice. In line with an action on intrinsic dorsal horn neurons, TPA023B (3 mg kg$^{-1}$, p.o.) also reduced scratching behavior elicited by intrathecal injection of brain-type natriuretic peptide (BNP; 10 nmol) and GRP (1 nmole) (Fig. 7d, e) consistent with an itch inhibitory effect occurring primarily via intrinsic dorsal horn neurons.

Would TPA023B also alleviate chronic itch? We first employed again the oxazolone model of atopic-like dermatitis. Acute treatment with TPA023B (1 mg kg$^{-1}$, i.p.) caused a highly significant reduction in the number of scratching bouts (determined during the interval 15–210 min after drug injection) (Fig. 8a; for potential effects of i.p. TPA023B on locomotor behavior and motor performance see Supplementary Fig. 5). A similar antipruritic action was observed in the dry skin dermatitis model$^{30}$ (Fig. 8b). Treatment of chronic itch conditions with TPA023B would require that no loss of therapeutic activity occurs during repeated applications. To test whether TPA023B would retain therapeutic activity during chronic treatment, we compared the antipruritic activity of TPA023B in drug naïve mice with that in mice treated with TPA023B (1 mg kg$^{-1}$ i.p.) once daily for ten days (Fig. 8c). No significant loss of antipruritic
activity was observed. Treatment with TPA023B over several days also alleviated skin lesions. After seven days of once daily treatment with TPA023B (1 mg kg\(^{-1}\), i.p.), mice showed a progressive reduction in their dermatitis scores (Fig. 8d). Furthermore, chronic treatment with TPA023B also reduced the infiltration of the affected skin by macrophages (Supplementary Fig. 6). By contrast, topical treatment with TPA023B (100 µl 0.3 µM, once daily) over the same time period neither changed scratching behavior nor dermatitis scores (Fig. 8e).

Additional experiments verified a spinal site of action also for chronic itch. First experiments in global α\(3^{\text{R}}/\text{R}\) point mutated mice revealed that eliminating diazepam sensitivity from α2GABA\(\chi\)Rs was not sufficient to block the antipruritic action of TPA023B (Fig. 9a). Because selective targeting of α3GABA\(\chi\)Rs had an even stronger antipruritic effect than targeting α2GABA\(\chi\)Rs (compare Fig. 3h), we focused our efforts on the α3GABA\(\chi\),R subtype and investigated α3GABA\(\chi\),R subunit knockout mice, in which deletion of the α3GABA\(\chi\),R subunit gene was achieved through the insertion of a duplicated exon (5\(^{\text{R}}\)) flanked by loxP sites\(^{31}\). This design allows a cre-dependent (tissue-specific) expression of GABA\(\chi\),Rs but was fully restored in spinal cord-speciﬁc hoxB8-GABA\(\chi\),Ra\(^{3\text{resc/resc}}\) mice (Fig. 9c).

Antipruritic efficacy of TPA023B in pruritic dogs. Encouraged by its efficacy in mice and the absence of apparent side effects, we tested the antipruritic efficacy of TPA023B in a second, hierarchically higher, species (Fig. 10). We chose dogs because models of atopic dermatitis that closely mimic natural disease are well-established in this species\(^{32}\) and because the tolerability of TPA023B in dogs had already been established\(^{33}\). We performed a pseudo-randomized observer-blind placebo-controlled crossover trial and in twelve beagle dogs sensitized to house dust mites by repeated exposure of their lower abdomen to lyophilized extracts of Dermatophagoides farinae\(^{34}\). After a sensitization period of 8 weeks during which the dogs were exposed to the extracts once a week, three challenges on three consecutive days were made in week 12. Nine of the 12 dogs developed clinical signs of pruritus and were included in subsequent experiments. These nine dogs were again challenged in weeks 15 and 18 on three consecutive days. The challenge on day 2 was used to obtain baseline values. The challenge on day 3 was followed by TPA023B (20 mg in one tablet, equivalent to about 2 mg kg\(^{-1}\), p.o.) or placebo administration. The same procedure was repeated in week 18 with a cross-over design. Both the time spent scratching and the numbers of scratching bouts were counted over 6 h after drug administration and normalized to the values on the day before drug exposure. Compared to placebo, a signiﬁcant reduction was observed for both read-outs. Five of the 9 dogs (56%) responded with a reduction by more than 50% (for responses of the individual dogs see Supplementary Table 2). These results indicate that speciﬁc targeting of α2/α3GABA\(\chi\),Rs alleviates itch not only in mice but also in dogs supporting the potential for translation to more complex species, including possibly, also human patients.

Discussion

Local application of TPA023B to the spinal cord and specific genetic ablation of spinal α2GABA\(\chi\),Rs have shown that most if not all of its antipruritic action originates from the spinal (or medullary) dorsal horn. This is consistent with our finding that virtually all GRP and GRPR neurons in the mouse spinal cord express α3GABA\(\chi\),R transcripts and more than 60% of these neurons also express α2GABA\(\chi\),R. Using post mortem tissue samples, we conﬁrmed a similar coexpression of α2 and α3GABA\(\chi\),R subunits on GRP and GRPR neurons for the human spinal cord, in line with the highly conserved expression pattern of spinal GABA\(\chi\),R subunits in rodents and humans\(^{35,36}\).

MrgrprA3 is primarily activated by chloroquine\(^7\). However, the ﬁbers carrying this receptor respond to many pruritogens and convey signals related to both histaminergic and non-histaminergic itch\(^8\). A critical role of GRP\(^37\) and GRPR\(^38\) neurons in spinal itch circuits is meanwhile also well established. After local spinal ablation of GRP neurons, mice respond less to pruritic stimuli\(^37\). This is also supported by a study that employed BNP-conjugated sapatonin to ablate spinal GRP neurons\(^9\). Besides GRP and GRPR positive interneurons, neurokinin 1 (NK1) receptor expressing neurons of the dorsal horn are also relevant to spinal itch relay\(^9,39\). Previous work from our group has shown that these neurons also express α2 and α3GABA\(\chi\),R\(^{34,40}\).

α2/α3GABA\(\chi\),Rs do not only control itch but also pain\(^{33,40}\) (for a review see ref.\(^{41}\)). The latter function is supported by the expression of α2/α3GABA\(\chi\),R receptors on sensory ﬁbers and dorsal horn nociceptive neurons\(^{16,40}\) and also by the efﬁcacy of spinal GABAergic neuron transplants against pain and itch\(^{42,43}\).
Unlike opioids that reduce pain but cause itch\textsuperscript{44}, drugs targeting α2/α3GABA\textsubscript{A}Rs should alleviate both itch and pain. Activation of α3GABA\textsubscript{A}R had a stronger impact on scratching responses than α2GABA\textsubscript{A}Rs. This ratio is reverse in case of analgesia\textsuperscript{23,40} in line with the view that sensory fibers and dorsal horn neurons processing itch or pain are not identical\textsuperscript{3,45}. There were also differences in the contribution of α2 and α3GABA\textsubscript{A}Rs between models of acute and chronic itch. These may hint at potential neuroplastic changes occurring during the transition from acute to chronic itch.

In the present study, we have assessed unwanted effects that typically occur with classical non-selective benzodiazepine site ligands. TPA023B was devoid of sedative effects, did not impair motor coordination and tolerance development to α1 or α3GABA\textsubscript{A}Rs\textsuperscript{23}. The lack of these side effects in TPA023B treated mice may either come from the absence of activity at α1GABA\textsubscript{A}Rs or from the only partial agonistic activity (relative to the full agonist diazepam) at α3GABA\textsubscript{A}Rs.

In human patients, the most challenging type of pruritus is chronic non-histaminergic pruritus which is often due to liver or kidney failure or treatment with opioids\textsuperscript{46}. While anti-histaminergics provide good itch relief in cases associated with urticaria, other forms respond less well to these drugs or not at all. At present, these conditions are mostly treated off-label with systemically applied anticonvulsants, such as gabapentin and pregabalin, antidepressants, opioid antagonists, or more recently with NK1 receptor blockers (aprepitant), or immunosuppressants\textsuperscript{47}. None of these compounds have been approved for systemic treatment of chronic itch conditions. In our study, TPA023B was similarly effective against non-histaminergic and histamine-induced itch, and, in general, more effective in chronic than in acute itch. If the antipruritic efficacy combined with good tolerability observed in the present study translates to human patients, TPA023B or related compounds should be well-suited for the treatment of chronic itch in humans.

In a broader context, our study adds to a growing body of evidence indicating that subtype selective GABA\textsubscript{A}R modulators promise not only better tolerability but may also open new avenues to the treatment of disorders that have hitherto not been considered indications for benzodiazepines. Such potential new opportunities include, in addition to itch and pain, cognitive enhancement by inverse agonists at α5 GABA\textsubscript{A}Rs\textsuperscript{48,49} depression and autism spectrum disorders (for a review see ref.\textsuperscript{50}).

**Fig. 6** Antipruritic actions of TPA023B. a Antipruritic actions of TPA023B (p.o.) in mouse models of acute itch. Circles are total number of scratching bouts observed in individual mice within 30 min after intracutaneous pruritogen injection. ANOVA, followed Dunnett’s post hoc test with vehicle as control. *P < 0.05, **P < 0.01, ***P < 0.001. Chloroquine (100 μg) F(5,29) = 6.44, n = 6, 7, 6, 6, 5 for vehicle, and 0.01, 0.03, 0.1, 1.0, and 3.0 mg kg\textsuperscript{-1}. α-Me5HT-evoked itch (20 μg). n = 8, 6, 7, 6, 6 for vehicle, and 0.01, 0.03, 0.1, 1.0, and 3.0 mg kg\textsuperscript{-1}. b The antipruritic action of TPA023B occurs via the benzodiazepine bind site of GABA\textsubscript{A}Rs. α-methyl 5-HT (20 μg) was injected intracutaneously into the right cheek. TPA023B (1 mg kg\textsuperscript{-1}, p.o.) exerted strong antipruritic actions in wild-type mice. In HRRR mice, in which all TPA023B sensitive GABA\textsubscript{A}R subtypes had been rendered benzodiazepine-insensitive, TPA023B had completely lost its antipruritic action. Two-way ANOVA F(2,22) = 6.45. P = 0.019 for genotype × treatment. P = 0.005 (**) and 0.60 (ns) for treatment effect in wild-type and HRRR mice, respectively (n = 7, 6 (wild-type, vehicle and TPA023B); n = 6, 7 (HRRR mice, vehicle and TPA023B)). ++P < 0.01 relative to TPA023B-treated wild-type mice. c-e TPA023B (3 mg kg\textsuperscript{-1}, p.o.) did not interfere with responses to somatosensory or acute noxious stimulation. c light mechanical stimulation with a paint brush, d punctate mechanical stimulation with von Frey filaments, e noxious heat stimulation with a radiant heat source. Five mice per group. Error bars indicate s.e.m.
wide-spread, almost ubiquitous, expression of GABAARs in the CNS, such new opportunities should not come as a surprise.

Methods

**Mice.** Homozygous triple and quadruple (H → R) GABAAR subunit point-mutated mice were generated by cross breeding of single point-mutated mice\(^{26,51,52}\) and the corresponding control mice were of the C57BL/6 genetic background. BAC transgenic GRP-eGFP (Tg(Grp-EGFP)DV197Gsat/Mmucd), GRP::cre (Tg(Grp-cre)R2fl/fl)\(^{56}\) expressing the TVA transgene from a ubiquitous promoter in a cre background. Other transgenic mice (including single GABAAR point-mutated mice) were obtained from Jackson Laboratories. Sns-cre mice (Tg(Scn10a-cre)1Rkun)\(^{54}\) were kindly provided by Dr. Rohini Kuner from the University of Pennsylvania. AAV preparation. AAV.flex.mCherry-2A-RabG vector was cloned in house and packaged at Penn Vector Core (Perelman School of Medicine, University of Pennsylvania) using their custom service. AAV.flex.mCherry-2A-RabG vector was cloned by excising the ChR2-mCherry fusion protein from pAAV-E1a-DIO-

**Drugs.** Diazepam was obtained from Sigma, TPA023B (6,2′-difluoro-5′-[3-(1-hydroxy-1-methylethyl)imidazo[1,2-b][1,2]benzo[7-yl]biphenyl-2-carbonitrile) was synthesized by ANAWA, purity was >95%. For oral (p.o.) and intraperitoneal (i.p.) administration to mice, diazepam and TPA023B were suspended in 0.9% saline/1% Tween80. For electrophysiological experiments and radioligand binding, TPA023B was dissolved in DMSO and diluted with extracellular solution to 0.01–1 µM (final DMSO concentration ≤0.12%). For experiments in dogs, TPA023B was packed into tablets containing 20 mg TPA023B, 38 mg Prosolv® SMCC390 (JRS Pharma) and 2 mg Ac-Di-Sol® (FMU). Placebo tablets were of the same weight, size, color and composition but contained 2.4 mg Quinoline Yellow (E104) instead of 20 mg TPA023B. Quinoline Yellow was added to ensure the same color of TPA023B and placebo tablets. GRP and BNP were obtained from Tocris. Drugs were supplied in distilled water and diluted in extracellular solution to 1–10 nM per µL. For topical applications, TPA023B (0.3 µM 100 µl) was suspended in ace tone/olive oil (4:1 v/v) and applied on the shaved nape of the neck once daily.

**Human spinal cord tissue.** came from autopsies done at the Department of Neurophysiology, University of Zurich. Both, tissue samples and data were anonymized. The analysis of the small tissue samples used in this study did not require special permission (confirmed by the Ethics Committee of the Canton of Zurich, BASEC Reg. 2017-01065).

**Intrapranal virus injections.** Animals were anesthetized with 2% isoflurane and lumbar vertebrae L4 and L5 were exposed. The animal was then placed in a motorized stereotaxic frame and the vertebral column was immobilized using a pair of spinal adaptors. The vertebral lamina and dorsal spinous process were removed to expose the L4 lumbar segment. The dura was perforated about 500 µm left of the dorsal blood vessel using a beveled 30 G needle. Viral vectors were injected at a depth of 200–300 µm using a glass micropipette (tip diameter 30–40 µm) attached to a 10 µl Hamilton syringe. The rate of injection (30 nl min\(^{-1}\)) was reduced.
Fig. 8 Antipruritic actions of TPA023B in chronic itch models. a Oxazolone model of chronic atopic-like dermatitis. Scratching bouts after injection of TPA023B (1 mg kg\(^{-1}\), i.p.) over time (left). Total numbers of scratching bouts between 15 and 210 min after TPA023B/vehicle administration (right). Unpaired two-sided t-test, \(n = 10\) mice per group. b Same as a but dry skin model of dermatitis, \(n = 6\) mice per group. c Lack of tolerance development after 10 day treatment with TPA023B (TPA, 1 mg kg\(^{-1}\), i.p. once daily). Two-way ANOVA (pretreatment \(\times\) treatment), \(F(1,1) = 0.96, P = 0.34\). Two-sided t-test indicates similar antipruritic effects in vehicle and TPA023B pretreated mice, \(n = 6\) (TPA/TPA), \(n = 5\) for all other groups. d Dermatitis score. Chronic systemic treatment with TPA023B (1 mg kg\(^{-1}\) i.p., once per day) starting on day 11 of oxazolone exposure. Oxazolone challenges were continued during TPA023B treatment every other day. Only mice with a dermatitis score of 5 or higher on day 11 were included. In TPA023B-treated mice the dermatitis score decreased from day 6 of treatment onwards (\(F(9,54) > 38.7; P < 0.026\) to \(P < 0.001\)) (left), but remained almost constant in vehicle-treated mice (right). Mixed repeated measures ANOVA revealed a significant treatment \(\times\) time interaction \(F(9,117) = 22.6; P < 0.001\) for day 8 and 9 (++++). Differences between both treatment groups were significant at day 8 and 9. +++++ = 0.0009 and +++++ = 0.0001. Photographs show the same mice before treatment (left) and after 9 days of treatment (right). e Chronic topical treatment (0.3 µM TPA023B in 100 µl, once per day). Left: total number of scratching bouts between 15 and 210 min after TPA023B on day 9 after treatment begin. Unpaired two-sided t-test, \(P = 0.97, n = 6\) mice per group. Right: same as left but dermatitis score (mixed repeated measures ANOVA treatment \(\times\) time interaction \(F(9,99) = 0.61; P = 0.78\); \(n = 6\) and 7, for vehicle and TPA023B, respectively). Error bars indicate s.e.m.
controlled using a PHD Ultra syringe pump with a nanomite attachment (Harvard Apparatus, Holliston, MA). The micropipette was left in place for 5 min after the injection. Wounds were sutured and the animals were injected i.p. with 0.03 mg kg$^{-1}$ buprenorphine and allowed to recover on a heat mat. Rabies virus injected mice were subjected to perfusion 3–5 days after rabies virus injection. The tissue was cut into 25 μm thick coronal cryosections, which were mounted onto Superfrost Plus microscope slides (Thermo Scientific, Waltham, MA). The following antibodies were used: rat anti-mCherry (1:1000; Invitrogen, Carlsbad, CA; USA; RRID:AB_2534023), guinea pig anti-Lmx1b (1:10,000; gift from Carmen Birchmeier57) rabbit anti Pax2 (1:400; Invitrogen, Carlsbad, CA; USA; RRID:AB_221570), chicken anti-GFP (1:1000; Thermo Fisher Scientific, Waltham, MA, USA; RRID:AB_2536611), rabbit anti-GFP (1:1000; Molecular Probes; RRID:AB_2534023), guinea pig anti-Lmx1b (1:10,000; gift from Carmen Birchmeier57) rabbit anti Pax2 (1:400; Invitrogen, Carlsbad, CA; USA; RRID:AB_2533990), anti PKCγ (1:1000; Santa Cruz Biotechnology, Dallas, TX, USA; RRID:AB_2533990), anti PKCγ (1:1000; Santa Cruz Biotechnology, Dallas, TX, USA; RRID:AB_2533990), anti PKCγ (1:1000; Santa Cruz Biotechnology, Dallas, TX, USA; RRID:AB_2533990), anti PKCγ (1:1000; Santa Cruz Biotechnology, Dallas, TX, USA; RRID:AB_2533990), anti PKCγ (1:1000; Santa Cruz Biotechnology, Dallas, TX, USA; RRID:AB_2533990), anti PKCγ (1:1000; Santa Cruz Biotechnology, Dallas, TX, USA; RRID:AB_2533990), anti PKCγ (1:1000; Santa Cruz Biotechnology, Dallas, TX, USA; RRID:AB_2533990), anti PKCγ (1:1000; Santa Cruz Biotechnology, Dallas, TX, USA; RRID:AB_2533990), anti PKCγ (1:1000; Santa Cruz Biotechnology, Dallas, TX, USA; RRID:AB_2533990), anti PKCγ (1:1000; Santa Cruz Biotechnology, Dallas, TX, USA; RRID:AB_2533990), anti PKCγ (1:1000; Santa Cruz Biotechnology, Dallas, TX, USA; RRID:AB_2533990), anti PKCγ (1:1000; Santa Cruz Biotechnology, Dallas, TX, USA; RRID:AB_2533990), anti PKCγ (1:1000; Santa Cruz Biotechnology, Dallas, TX, USA; RRID:AB_2533990), anti PKCγ (1:1000; Santa Cruz Biotechnology, Dallas, TX, USA; RRID:AB_2533990), anti PKCγ (1:1000; Santa Cruz Biotechnology, Dallas, TX, USA; RRID:AB_2533990), anti PKCγ (1:1000; Santa Cruz Biotechnology, Dallas, TX, USA; RRID:AB_2533990), anti PKCγ (1:1000; Santa Cruz Biotechnology, Dallas, TX, USA; RRID:AB_2533990), anti PKCγ (1:1000; Santa Cruz Biotechnology, Dallas, TX, USA; RRID:AB_2533990), anti PKCγ (1:1000; Santa Cruz Biotechnology, Dallas, TX, USA; RRID:AB_2533990), anti PKCγ (1:1000; Santa Cruz Biotechnology, Dallas, TX, USA; RRID:AB_2533990), anti PKCγ (1:1000; Santa Cruz Biotechnology, Dallas, TX, USA; RRID:AB_2533990), anti PKCγ (1:1000; Santa Cruz Biotechnology, Dallas, TX, USA; RRID:AB_2533990), anti PKCγ (1:1000; Santa Cruz Biotechnology, Dallas, TX, USA; RRID:AB_2533990), anti PKCγ (1:1000; Santa Cruz Biotechnology, Dallas, TX, USA; RRID:AB_2533990), anti PKCγ (1:1000; Santa Cruz Biotechnology, Dallas, TX, USA; RRID:AB_2533990), anti PKCγ (1:1000; Santa Cruz Biotechnology, Dallas, TX, USA; RRID:AB_2533990), anti PKCγ (1:1000; Santa Cruz Biotechnology, Dallas, TX, USA; RRID:AB_2533990), anti PKCγ (1:1000; Santa Cruz Biotechnology, Dallas, TX, USA; RRID:AB_2533990), anti PKCγ (1:1000; Santa Cruz Biotechnology, Dallas, TX, USA; RRID:AB_2533990), anti PKCγ (1:1000; Santa Cruz Biotechnology, Dallas, TX, USA; RRID:AB_2533990) and 3GABAAR subunits in transverse sections of the lumbar spinal dorsal horn (top) and in sagittal brain sections (bottom).
RRID:AB_632234 and cyanine 3 (Cy3)-conjugated, Alexa Fluor 488-conjugated, DyLight 488-conjugated, 647-conjugated, and 649-conjugated donkey secondary antibodies (1:500; Dianova, Hamburg, Germany). Retrograde tracing experiments were done in 8–9-week-old mice.

**Immunohistochemistry and image analysis of GABAAR subunits.** Colocalization of GABAAR α subunits with MrgrprA3 axons and GRP neurons was visualized on 40 μm thick transverse mouse lumbar spinal cord cryosections. Mice were deeply anaesthetized with pentobarbital (nembutal, 50 mg kg⁻¹, i.p.) and perfused with oxygenated aCSF. Spinal cords were rapidly collected by pressure ejection and embedded in NEC50. Thirty three were formalin-fixed, and paraffin-embedded, and three were fresh frozen tissue samples. All samples were screened with control RNAscope fluorescent in situ hybridization probes provided by the manufacturer of the assay (human Polr2a (C1), PPIB (C2) and UBC (C3)). Tissue samples of one patient were well enough preserved to yield reliable and specific results. Two samples had been formalin-fixed and paraffin-embedded, and three were fresh frozen tissue samples. All samples were screened with control RNAscope fluorescent in situ hybridization probes provided by the manufacturer of the assay (human Polr2a (C1), PPIB (C2) and UBC (C3)). Tissue samples of one patient were well enough preserved to yield reliable and specific results.

**RNAscope fluorescent in situ hybridization.** Multiplex fluorescent in situ hybridization was performed using RNAscope (Advanced Cell Diagnostics; ACD; ref. 59). In brief, dissected tissue was snap frozen in liquid nitrogen and later on embedded in NEOS. Thirty five μm sections were cut on a Hyrax C60 cryostat, mounted on superfrost glass slides and stored at −80°C until use. The in situ hybridization was carried out according to the manufactures protocol. The following RNAscope probes were used: GABAAR α1 (catalog number: 435351); GABAAR α2 (435011-C1); GABAAR α3 (435021-C3); GABAAR α5 (319841; GRP (317861 and 317861-C1); GRPR (317871 and 317871-C2); EGF (400281 and 400281-C1). The latter was used to detect eGFP expressed under the genetic control of the MrgrprA3 gene in Grpr3A::Cre-eGFP transgenic mice.

### Human spinal cord tissue samples.

Tissue has been extracted during routine autopsy from five patients. Two samples had been formalin fixed and paraffin embedded, and three were fresh frozen tissue samples. All samples were screened with control RNAscope fluorescent in situ hybridization probes provided by the manufacturer of the assay [human Polr2a (C1), PPIB (C2) and UBC (C3)]. Tissue samples of one patient were well enough preserved to yield reliable and specific results in situ hybridization signals. These samples were from a 37 years old male patient with a post mortem time of 16 h who had died of septic shock and right heart failure. From this patient, small samples from the cervical, thoracic and lumbar spinal cord had been fixed in 4% formalin for 14 days, and were then paraffin-embedded. Ten micrometer thick slices were mounted on superfrost glass slides. Multiplex fluorescent in situ hybridization on human spinal cord tissue was performed using RNAscope® Multiplex Fluorescent Reagent Kit Version 2 (323100, Advanced Cell Diagnostics; ACD; ref. 59). The in situ hybridization was carried out according to the manufactures protocol with the following modifications.

#### Target
retrieval was carried out at 94–98 °C for 30 min in target retrieval buffer. Protease incubation was carried out for 30 min at 40 °C with the protease plus reagent. The following reagents were used: protease plus reagent (Roche, Basel, Switzerland), air dried, fixed with acetone for 2 min at −20 °C and subsequently rehydrated with 80% methanol for 5 min at 4 °C. Specimens were incubated with 5% donkey serum, 0.1% Triton-X and 1% BSA in PBS for 1 h at room temperature, followed by overnight incubation with rat anti-mouse CD68 (1:200; Abcam, Cambridge, United Kingdom) at 4 °C. Samples were incubated with Alexa Fluor 488-conjugated secondary antibodies and Hoechst 33342 (all from Invitrogen, Life Technologies, Carlsbad, USA) for 30 min at room temperature. CD68-stained sections were examined on an Axioplan 2 mot plus microscope (Carl Zeiss, Feldbach, Switzerland), equipped with an AxioCam MRC camera (Zeiss) and a Plan-Neofluar objective (Zeiss). Images of at least four individual fields of view were acquired per section using Axio-Vision software 4.8. Using ImageJ v1.49, the fluorescent area was determined between the stratum corneum and an outline thereof shifted 300 µm into the tissue. Results are presented as CD68-positive area (µm²) per µm basement membrane.

Electrophysiological recordings in HEK293 cells recordings. The effects of TPA023B on currents through recombinant GABAARs were studied in HEK293 cells (ATCC) transiently expressing GABAARs. HEK293 cells were transfected using lipofectamine LT²8. To ensure expression of the γ2 subunit (required for modulation of GABAARs by BD2Zs) in all recorded cells, we transfected cells with a plasmid expressing the γ2 subunit plus eGFP from an IRES, and only selected eGFP-positive cells for recordings. The transfection mixture contained (in µg): 1, 1 β2, 3 γ2/eGFP (used as a marker of successful transfection) or 1 α1, 1 β3, 3 α3/eGFP. Recordings were made 18–36 h after transfection. Whole-cell patch-clamp recordings of GABAAR-evoked currents were made at room temperature (20–24 °C) and at a holding potential of −60 mV. Recording electrodes were filled with solution containing (in mM): 120 CsCl, 10 EGTA, 10 HEPES (pH 7.40), 4 MgCl₂, 0.5 GTP and 2 ATP. The external solution contained (in mM): 150 NaCl, 10 KCl, 2.0 CaCl₂, 1.0 MgCl₂, 10 HEPES (pH 7.4), and 10 glucose. GABA was applied to the recorded cell using a manually controlled pulse (4–6 s) of a low sub-saturating GABA concentration (EC50). EC50 values of GABA were determined for all subunit combinations analyzed. EC50 values and Hill coefficients (nH) were obtained from fits of normalized concentration-response curves to the equation I_{GABA} = I_{max} [GABA]^{nH}/[GABA]^{nH} + [EC50]^{nH}, I_{max} was determined as the average maximal current elicited by a concentration of 1 mM GABA. TPA023B was dissolved in DMSO and subsequently diluted with recording solution so was co-applied together with GABA without preincubation.

Primary pruritoceptive neuron preparation and recordings. Lumbar dorsal root ganglia (DRGs) were dissected from 6–8 weeks old MrgprA3::cre-eGFP mice as previously described60. After removal of the connective tissue, DRGs were incubated twice in Liberase™ DL Research Grade (0.09 mg ml⁻¹) were washed twice in Liberase™ medium and plated on coverslips coated with poly-L-lysine and laminin (Sigma). The sensory neurons were cultured in supplement TNB™ medium and plated on coverslips coated with poly-L-lysine and laminin (Sigma). The sensory neurons were cultured in supplement TNB™ medium containing nM 2S (Alomone Labs, 10 µg / 100 ml TNB medium) at 37 °C in 5% CO₂. Within 48 medium containing mNGF 2.5 S (Alomone Labs, 10 µg / 100 ml TNB medium) at 37 °C in 5% CO₂. Within 48 h after plating, whole-cell path-clamp recordings were performed. TNB™ medium was replaced with extracellular solution containing (in mM): 150 NaCl, 10 KCl, 2.0 CaCl₂, 1.0 MgCl₂, 10 HEPES (pH 7.4), and 10 glucose. GABA-evoked currents were determined for all subunit combinations analyzed. EC50 values and Hill coefficients (nH) were obtained from fits of normalized concentration-response curves to the equation I_{GABA} = I_{max} [GABA]^{nH}/[GABA]^{nH} + [EC50]^{nH}, I_{max} was determined as the average maximal current elicited by a concentration of 1 mM GABA. TPA023B was dissolved in DMSO and subsequently diluted with recording solution so was co-applied together with GABA without preincubation.

Electrophysiological recordings in spinal cord slices. Transverse spinal cord slices (400 µm thick) were prepared from 20 to 29-day-old Gpr85-eGFP mice of either sex. Slices were cut in an ice-cold solution containing (in mM): 130 K-glutamate, 15 KCl, 0.05 EGTA, 20 HEPES, and 25 glucose, pH 7.4 (adjusted with NaOH). D-2-amino-5-phosphonovaleric acid, 50 µM) was added to prevent glutamate excitotoxicity. Slices were maintained in artificial cerebrospinal fluid (ACSF) (in mM): 125 NaCl, 2.5 KCl, 1.25 NaH₂PO₄, 26 NaHCO₃, 5 glucose, 2 CaCl₂, and 1 MgCl₂ (equilibrated with 95% O₂, 5% CO₂). Whole cell patch-clamp recordings were made at room temperature targeting eGFP positive neurons. During recordings, slices were continuously superfused at the rate of 1–2 ml min⁻¹ with a cut of 0.2% CO₂. During recording, 1 µM TPA023B (4 mM in a total volume of 10 µl) was added to the ACSF in the presence of the respective behavioral test. Mechanical sensitivity was assessed with electronic von Frey filaments (no. 7–11, TC Wood, Millis, MA) and quantified as the change in the paw withdrawal responses upon gentle stimulation of the skin. The effects of TPA023B and vehicle were tested as the change in the paw withdrawal thresholds measured in g. Heat hyperalgesia was evaluated in the Hargreaves test as the change in the latency of paw withdrawal to a defined heat stimulus. Responses to light mechanical stimulation of the hairy skin was tested as the change in the paw withdrawal responses upon gentle stimulation with a brush. GATING of the brain was performed on day 2 after injection of the pruritogen (in a total volume of 10 µl). No anesthesia was used. Correct injection was confirmed by the appearance of a slightly domed bulla. After injection, mice were placed back into the cylindrical enclosure and video-taped for 30 min. Videos were reviewed offline. Scratches, attacks and the hind paw directed to the ipsilateral cheek was counted in bouts, with one bout defined as an instance when the mouse lifted its paw to scratch until it returned the paw to the cage floor. In case of experiments in which the pruritogen was injected into the skin of the thigh, the time spent biting the injected skin area was counted in s min⁻¹ as a measure of itch.

Acute itch. was assessed in mice that received intradermal microinjections of pruritogens or 0.9% saline into the right cheek, which had been shaved at least 1 day before the experiment. In two sets of experiments that addressed the contribution of GABAARs on primary and secondary pruritoceptors, pruritogens were injected into the skin of the left thigh (Fig. 7a–c). Before injection, mice were acclimatized to a 15 cm diameter cylindrical enclosure for more than 30 min with cage bedding on the floor. Background white noise generated by a radio at ambient volume was applied to prevent auditory distraction. A 30 gauge needle was inserted into the skin to allow direct injection into the skin. Observers, who did not see the injection, were blind to the genotype of the mice or to their treatment with drug or vehicle. In experiments involving comparisons between diazepam or TPA023B and vehicle, mice were randomly assigned to the different groups. No formal sample size calculation was made. Group sizes were chosen based on previous experience with the respective behavioral test. Mechanical sensitivity was assessed with electronic von Frey filaments (no. 7–11, TC Wood, Millis, MA) and quantified as the change in the paw withdrawal thresholds measured in g. Heat hyperalgesia was evaluated in the Hargreaves test as the change in the latency of paw withdrawal to a defined heat stimulus. Responses to light mechanical stimulation of the hairy skin was tested as the change in the paw withdrawal responses upon gentle stimulation with a brush. GATING of the brain was performed on day 2 after injection of the pruritogen (in a total volume of 10 µl). No anesthesia was used. Correct injection was confirmed by the appearance of a slightly domed bulla. After injection, mice were placed back into the cylindrical enclosure and video-taped for 30 min. Videos were reviewed offline. Scratches, attacks and the hind paw directed to the ipsilateral cheek was counted in bouts, with one bout defined as an instance when the mouse lifted its paw to scratch until it returned the paw to the cage floor. In case of experiments in which the pruritogen was injected into the skin of the thigh, the time spent biting the injected skin area was counted in s min⁻¹ as a measure of itch.

Chronic itch. was investigated in the contact dermatitis model27 and the dry skin model40. To induce contact dermatitis, mice were treated on day 0 with 10% oxazolone in acetone/olive oil (4:1 v/v) on the shaved nape of the neck (100 µl). After a resting period of 7 days, mice were treated with 1% oxazolone in acetone/olive oil.
olive oil (4:1 v−v−1) on the nape of the neck (100 µl) every other day for 10 days. On the day of the experiment, mice were injected with drug or vehicle i.p. under short light anesthesia. Scratching of the hindpaw directed to the ipsilateral cheek was quantified as the number of scratching bouts. In the dry skin model, mice were treated with a mixture of acetone and diethylther (1:1) on the shaved nape of the neck for 15 s, followed by distilled water for 30 s, twice daily for 10 days. On the day of the experiment, vehicle or drug was administered i.p. under short light isoflurane anesthesia.

To quantify the severity of skin lesions a dermatitis score was determined.44 Hemorrhage/erythema, dryness/scarring, and hyperplasia were scored as 0 (none), 1 (mild), 2 (moderate), or 3 (severe) once per day resulting in a score between 0 and 9. Photographs of atop dermatitis-like skin were taken before treatment (day 11) and 9 days after treatment (day 20).

**Locomotor activity, motor coordination and muscle relaxation.** TPA023B (1 mg kg−1, p.o. or i.p.) or vehicle was administered 60 min before the tests. Locomotor activity was measured in an open field arena (10 cm radius) equipped with four pairs of light beams and photoemitters and analyzed for the time interval between 60 and 120 min after TPA023B administration. Motor coordination was assessed with a rotarod accelerating from 4 r.p.m. to 40 r.p.m. within 5 min. Fifteen measurements were taken per mouse. To assess muscle relaxation, mice were placed with their forepaws onto a metal horizontal wire placed 20 cm above ground. Successes and failures to grab the wire with at least one hindpaw were recorded between 60 and 120 min after TPA023B administration.

**Pruritus study in dogs.** Twelve 6 months-old beagle dogs (6 females, 6 males) weighing between 9.0 and 13.0 kg (see Supplementary Table 1) were included in the study. They were sensitized using lyophilized extracts of the house dust mite Dermatophagoides farinae in mineral oil. In order to expose the living epidermis, the skin of the abdomen was tape-stripped. D. farinae extract was gently applied on the tape-stripped skin once a week for eight weeks. At this time point, dogs were considered D. farinae-sensitized even though most of them (8 out of 12) did not exhibit pruritus or clinical signs of atop dermatitis. Four weeks later, the dogs were challenged using D. farinae extracts after tape stripping of the abdominal skin. This challenge was made on three consecutive days (days 1 to 3). Pruritus and clinical signs were assessed on day 2, 3, and 4. All dogs exhibited pruritus and clinical signs compatible with atop dermatitis on the site of challenge but also in remote areas. After the third challenge all dogs exhibited histological signs of atop dermatitis and nine dogs showed positive reactions in intradermal allergen exposure test. These nine dogs were enrolled to assess the end of the video recording and whole blood concentrations of TPA023B were not corrected our outcome parameters for baseline scratching present in the absence of the pruritogenic challenge. Blood samples were taken from all dogs at the end of the video recording and whole blood concentrations of TPA023B were measured by high performance liquid chromatography/high resolution electrospray time-of-flight mass spectrometry to verify drug exposure.

**Statistics.** For most experiments, results of individual mice or cells are displayed as individual symbols. Normal distribution of data was assumed when t-tests or ANOVAs were applied.

**Data availability.** Excel files including the data that support the findings of this study are available at G-Node.org with the identifier doc:10.12751/g-node.fbd85d [https://doi.org/g-node.org/fbd85d96a1f200edc2d2951e351a1a5]. Additional raw data of this study are from the corresponding author upon reasonable request.

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56. Author contributions W.T.R. and E.N. performed and analyzed mouse behavioral experiments; W.T.R. and H.W. did retrograde tracing, immunofluorescence, and in situ hybridization experiments; M.P. performed electrophysiological recordings in spinal cord slices and primary DRG neurons cultures; M.A.A. did the recordings in HEK 293 cells; W.T.R., N.F., A.R., and C.F. performed and analyzed the study in Beagles; S.S. and M.D. analyzed mouse skin samples; D.B. did the binding tests, and J.L.H. measured blood levels of TP0023B. K.F. and A.A. provided human spinal tissue samples and made helpful suggestions. H.U.Z. designed the project, analyzed data, and wrote the manuscript. All authors commented on the manuscript.

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58. Competing interests: W.T.R. and H.U.Z. have submitted a patent application on the use of TP0023B or related compounds against itch. Neurocycle Therapeutics has licensed this patent. J.L.H. is a founder of this company. The remaining authors declare no competing interests.

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