α 6GABA_A Receptor Positive Modulators Alleviate Migraine-like Grimaces in Mice via Compensating GABAergic Deficits in Trigeminal Ganglia

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Abstract

Migraine is caused by hyperactivity of the trigeminovascular system, where trigeminal ganglia (TG) play an important role. This hyperactivity might originate from an underfunctional GABAergic system in TG. To investigate this possibility, we adapted a mouse model of migraine by inducing migraine-like grimaces in male mice via repeated injections of nitroglycerin (NTG, 10 mg/kg, i.p.), once every 2 days, for up to 5 sessions. Migraine-like facial pain scores were measured using the mouse grimace scale. Repeated NTG treatments in mice caused significant increases in migraine-like grimaces that were aborted and prevented by two anti-migraine agents sumatriptan and topiramate, respectively. After 5 sessions of NTG injections, the GABA-synthesizing enzyme, 65-kDa glutamate decarboxylase (GAD65), but not the GABA transporter 1 (GAT1) or the α 6 subunit-containing GABA_A receptors (α 6GABA_ARs), was downregulated in mouse TG tissues. Taking advantage of the unaffected TG α 6GABA_AR expression in NTG-treated mice, we demonstrated that an α 6GABA_AR-selective positive allosteric modulator (PAM), DK-I-56-1, exhibited both abortive and prophylactic effects, comparable to those of sumatriptan and topiramate, respectively, in this migraine-mimicking mouse model. The brain-impermeable furosemide significantly prevented the effects of DK-I-56-1, suggesting its peripheral site of action, likely via preventing α 6GABA_AR modulation in TG. Results suggest that a decreased GABA synthesis caused by the reduced GAD65 expression in TG contributes to the trigeminovascular activation in this repeated NTG-induced migraine-mimicking model and that the unaltered α 6GABA_ARs in TG are potential targets for migraine treatment. Thus, α 6GABA_AR-selective PAMs are potential anti-migraine agents for both abortive and preventive therapies.

Key Words Migraine · α 6 subunit-containing GABA_A receptor · trigeminal ganglia · nitroglycerin · mouse grimace scale · 65-kDa glutamate decarboxylase · GABA transporter 1.

Hung-Ruei Tzeng and Ming Tatt Lee contributed equally to this work.

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Introduction

Migraine is the second leading cause of disability and the third most common disease while its treatment remains an unmet medical need [1]. Hyperactivity of the trigeminovascular system (TGVS) is believed to contribute to the pathogenesis of migraine [2] while the detailed mechanism(s) remain unclear. The TGVS is composed of bilateral trigeminal ganglia (TG) and their peripheral and central axons, as well as their innervated structures. TGVS activation either via peripheral neuroinflammation or central sensitization leads to the release of various vasoactive neuropeptides [3] from peripheral nerve endings in the dura mater [4]. Among these, the calcitonin gene-related peptide (CGRP) is noteworthy. It can trigger neurogenic inflammation in the dura mater, resulting in the prolongation and intensification of migraine via peripheral sensitization [5]. Thus, the elevation of CGRP in the cerebrospinal fluid and plasma has been reported in patients with chronic migraine [6–8] and during migraine attacks [9, 10].

TG are peripheral sensory ganglia that are located outside the blood–brain barrier (BBB). There are abundant GABAergic neurons in TG [11], but the role of the TG GABAergic system in migraine received much less attention than that of other signaling systems, such as CGRP, substance P, TRPV₁ or nitric oxide (NO) [12]. Neurons in TG are surrounded by satellite glial cells (SGCs) [13] that play an important role in the homeostasis of GABA in TG [11]. Although there is no GABAergic innervation in TG, the GABA-synthesizing enzyme, glutamate decarboxylase 65 (GAD65) [14], is expressed in TG neurons but not in SGCs [11]. Nevertheless, SGCs can accumulate GABA from the extracellular space of TG [11]. GABA can thus be released from both GABAergic neurons and SGCs after depolarization and subsequently activate GABA_A receptors (GABA_ARs) on TG neurons, leading to somatic inhibition of neurons within the TG [11]. The involvement of GABA_A receptors is supported by the identification of several GABA_AR subunits, such as $\alpha 1$, $\alpha 3$, $\alpha 5$, $\alpha 6$, $\beta 2/3$, δ and $\gamma 1$ – 3 , in rat TG neurons [11]. The presence of the $\alpha 6$ subunit-containing GABA_AR ($\alpha 6$ GABA_AR) in TG is noteworthy.

A possible importance of the TG GABAergic system in migraine is supported by several lines of evidence. For instance, the anti-migraine agent, valproic acid, is an inhibitor of GABA transaminase, a GABA degradation enzyme, and may enhance the GABA concentration in GABA-containing tissues, probably including TG [15]. Furthermore, Lee et al. [16] have shown that intraperitoneal (i.p.) administration of bicuculline methiodide, a BBB-impermeable GABA_AR antagonist, was able to alleviate the inhibitory effect of valproic acid on migraine-related neuroinflammation induced by electrical stimulation of TG in rats. And recently, we have demonstrated that a pyrazoloquinolinone positive allosteric modulator (PAM) highly selective to $\alpha 6$ GABA_ARs [17],

Compound 6, which was initially coded as PZ-II-029 [18], was able to ameliorate TGVS activation via enhancing GABA actions at TG $\alpha 6$ GABA_ARs in a rat model mimicking migraine induced by intracisternal instillation of capsaicin [19].

In addition to the ophthalmic (V1) branches that innervate the dura mater, maxillary (V2) and mandibular (V3) branches of peripheral trigeminal nerves also originate from TG and are involved in orofacial pain control. For instance, rats that were transfected with the GAD65 gene into TG SGCs had less inflammatory orofacial nociceptive behaviors than the vector-control group, and this effect was reversed by the antagonist of GABA_ARs, but not GABA_BRs [20]. In addition, rats with a 30% reduction of $\alpha 6$ GABA_ARs in TG induced by local siRNA injection displayed more hyperalgesic responses to the temporomandibular joint (TMJ) inflammation than the normal group [21]. Finally, Vasovic et al. [22] also demonstrated that a deuterated analogue of Compound 6, DK-I-56-1 that is also an $\alpha 6$ GABA_AR-selective PAM with a longer half-life [23], was able to alleviate allodynic responses in a rat model of orofacial neuropathic pain. However, to the best of our knowledge, there was no study investigating the association between TG GABAergic transmission and migraine pathogenesis.

To investigate whether an impaired GABAergic system might be associated with the development of migraine, we employed a mouse migraine model induced by repeated nitroglycerin (NTG) injections. This is a preclinical migraine model known to provide clinically relevant histopathological and behavioral readouts [24]. NTG is a well-known trigger of primary headaches [25]. It can increase CGRP levels in migraineurs and is involved in the neuroinflammatory pathway of migraine [26]. A single NTG administration is potent enough to induce headache-like responses in rats and mice [24, 27]. However, it may not adequately represent the pathophysiology of migraine, as acute NTG administration triggers the release of CGRP, but does not affect its gene expression [28], which may explain its short-lived action of 4 h [29]. Interestingly, chronic and intermittent NTG administrations in rats significantly elevates CGRP gene expression [30], suggesting continuous release of CGRP that mimics the clinical manifestation of chronic migraine. Similarly, chronic and intermittent NTG injections in mice was shown to induce migraine-related evoked hyperalgesia [27].

NTG can be metabolized to nitric oxide (NO) through the nitric oxide synthetase (NOS) in neurons and glia, resulting in migraine symptoms through the NO-derived pathways [31]. NO-initiated signaling pathway has been implicated in the pathogenesis of migraine [32, 33]. Elevated NO levels in the peripheral blood have been reported clinically in patients with migraine or with tension-type or cluster headaches [34]. In addition to contributing to vasodilation, NO can also lead to an elevation of CGRP levels in TG [35], dural tissue [36] and

trigeminal nucleus caudalis (TNC) [37], resulting in peripheral and central sensitization, ultimately leading to migraine attacks [38, 39].

In the present study, we adapted a migraine model in mice induced by repeated NTG injections described by Pradhan et al. [27]. However, instead of evoked nociceptive responses, we assessed NTG-induced grimaces, which are migraine-like spontaneous facial nociceptive responses, in mice. Previous immunohistochemical studies in adult rats have shown that the $\alpha 6$ subunit of GABA_ARs is abundantly distributed in both neuronal cells and SGCs of TG [11, 19, 40]. Here, we first measured the expression pattern of $\alpha 6$ GABA_ARs, represented by the immunoreactivity of the $\alpha 6$ subunit, in TG tissues of mice, and then compared possible changes in the expression levels of $\alpha 6$ GABA_ARs after repeated NTG injections. The GABA-synthesizing enzyme, GAD65, and the GABA transporter, GAT1, are key players determining the fate of GABA in TG [11]. We therefore also measured the possible changes in the levels of GAD65 and GAT1 in TG tissues of this migraine model. Finally, we investigated whether $\alpha 6$ GABA_A-selective PAMs, Compound 6 and DK-I-56-1 [23], could inhibit migraine grimaces in mice. Moreover, the addictive potential of the $\alpha 6$ GABA_AR PAM, a general concern for GABA_AR modulators in preclinical safety setting, was also examined via the conditioned place preference (CPP) test.

Materials and Methods

Animals

All animal care and experimental procedures were approved by the Institutional Animal Care and Use Committee of National Taiwan University, College of Medicine. Male Institute of Cancer Research (ICR) mice (6–8 weeks old, 25–35 g) were purchased from BioLASCO (Taiwan Co., Ltd), and were acclimated in a holding room for at least 1 week before the experiments. They were housed 5 per cage in a holding room with 12:12 light-dark cycle, 23 °C room temperature and food and water ad libitum. On the day of *in vivo* experiments, mice in their homecages were moved to a behavioral room and were acclimated there for 1 h before testing. The number of animals used was based on the published literature describing similar experiments [27, 41, 42].

Repeated NTG Administration Protocol

The migraine model induced by chronic intermittent NTG administrations, as described in Fig. 1A, was modified from the ones used in rats reported previously [27, 42]. Briefly, mice were randomly divided into two groups. The NTG group received an NTG (10 mg/kg, i.p.) injection every second day

for five sessions, i.e. on days 1, 3, 5, 7, and 9. The control group received saline injections, instead.

Mouse Grimace Scoring

To study the behavioral readout of NTG-induced migraine-mimicking model, the facial expressions of mice, correlating with migraine-like nociceptive responses, were measured. The NTG/saline-treated mice were placed individually in a custom-built four-cubicle array (each cubicle measuring 7 × 8 × 14 cm high) with one wall and two side walls of stainless steel. The fourth wall was transparent Plexiglas. This arrangement has been reported to be able to encourage the mouse to look towards the transparent wall and hence face the camera, which allows to clearly observe facial expressions [43]. One high-resolution (1920 × 1080) digital video camera was positioned 0.25 m away from the Plexiglas side. The Mouse Grimace Scale (MGS) was utilized to quantify the degree of pain by examining facial features of a mouse during the headache attack state induced by NTG. The MGS scoring procedure is in accordance with a previous report in rats [41] with minor modifications. Briefly, the facial expressions of mice were video-recorded after each NTG injection for 60 min. Images of the mouse facial features were taken every 2 min during 30–60 min after NTG administration. Pictures of each mouse were automatically snapped by a Free Video to JPG Converter®, once every 20 s. A total of 6 pictures were obtained in each 2-min interval, and one picture with the best quality was chosen. Thus, in the end of the 30 min-recording session, 15 qualified photos (one per each 2-min interval) were scored. If the animal was sleeping, actively sniffing or rearing, then the next available picture was used for scoring. Figure 2 demonstrates five representative facial features of MGS, namely orbital tightening (Fig. 2A), ear rearing (Fig. 2B), cheek bulge (Fig. 2C), nose bulge (Fig. 2D), and whisker change (Fig. 2E), in different pain scores. Video-recorded images were displayed on a computer monitor randomly for scoring by an examiner who was blinded to treatment groups. Images were scored individually for each facial feature using a scale of 0–2, as described previously [41]. The sum of all five facial feature scores, termed as “the total grimace score” was used to represent the degree of migraine severity in each mouse.

When establishing the NTG model, the grimace scores were measured on days 1, 3, 5, 7, and 9 (Fig. 1). To investigate the abortive treatment efficacy of the tested drug, the tested drug or its vehicle was given to the mouse 20 min after NTG injection on day 9. For evaluating the preventive treatment efficacy, the tested drug or its vehicle was given to the mouse 30 min before NTG injection in each session, i.e., on days 1, 3, 5, 7, and 9. For both preventive and abortive studies, the mouse grimace scores during 30–60 min after NTG injection on day 9 were collected and analyzed.

Fig. 1 The effect of repeated nitroglycerin (NTG) administration on migraine-like nociceptive responses in ICR mice. (A) The protocol employed to induce migraine in mice by repeated NTG administration. Mice received injections of NTG (10 mg/kg, i.p.) or saline on days 1, 3, 5, 7 and 9. In each session, 30 min after the NTG injection, the facial expressions of each mouse were videotaped for 30 mins and analyzed for the Action Units (AUs) of migraine-like grimacing expressions, termed the Mouse Grimace Scale (MGS) in mice. As detailed in supplementary Fig. 2, these AUs, including orbital tightening (B), ear rearing (C), cheek bulge (D), nose bulge (E) and whisker change (F),

recorded from 30 to 60 mins after saline or NTG administration were averaged (B–F), or summed up (G). Note that either single or repeated NTG administration significantly elevated grimace scores in mice. $^{**}P < 0.01$, $^{***}P < 0.001$ vs saline, when comparing NTG with vehicle group at corresponding time point, two-way ANOVA followed by Sidak's test. In separate groups of mice that underwent the same NTG induction protocol were sacrificed on day 1, 5, or 9 in different batches (red arrows in (A)). Their TG tissues were collected to conduct immunofluorescence (IF) staining, as elaborated in Figs. 3, 4, 5, and S1–S2

Conditioned Place Preference

A biased conditioned place preference (CPP) paradigm, as described in our previous studies [44, 45], was conducted to study if pharmacological activation of $\alpha 6\text{GABA}_A$ Rs would cause potential drug dependence in mice. A three-compartment CPP apparatus was used, which consisted of a white chamber and a black chamber ($13 \times 13 \times 13$ cm) separated by a gray intermediate compartment ($7.2 \times 7.2 \times 13$ cm) with guillotine gates to each chamber. The flooring of the white chamber was made of metal grid while metal bars floor is installed for the black chamber. On day 0, the mouse was allowed to explore freely between black and white chambers for 10 min. The time spent in either the black or white chamber for each mouse was used for grouping mice with an

approximately equal bias for black or white chamber preference. Mice were excluded if the difference spent in either chamber was greater than 100 s, i.e. if they displayed an apparent preference for a specific chamber, or if they spent over 4 min in the intermediate compartment at the preconditioning (PRE) stage. Note that 3 out of 20 mice tested in the present study were excluded, resulting in unequal group sizes in Fig. 7D. During the conditioning training period (days 1, 3, 5, 7, and 9), the mouse was given an intraperitoneal (i.p.) saline injection and confined in its preferred chamber for 30 min in the morning session (A.M.) The same mouse was given DK-I-56-1 (10 mg/kg, i.p.) or its vehicle and confined in its non-preferred chamber for 30 min in the afternoon session (P.M.). The interval between the A.M and P.M session was at least 6 h. On day 10 (POST stage), the mouse

Fig. 2 The Mouse Grimace Scale (MGS) for the Action Units in mice. The example pictures of action units, including orbital tightening (A), ear rearing (B), cheek bulge (C), nose bulge (D) and whisker change (E),

scored as 0, 1, and 2 in mice receiving NTG injection. These migraine-like grimacing expressions were videotaped and scored after receiving NTG injection according to the MGS published previously [41]

was tested for its possible CPP. The CPP score, defined by the difference in time spent between drug-paired and saline-paired chambers, was used to access the degree of drug preference.

Immunofluorescence Staining

Mice were anesthetized with isoflurane (3% for induction) and then sacrificed by intracardiac perfusion with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde (PFA) in PBS. After perfusion, both TG were dissected, postfixed with 4% PFA / PBS solution overnight, and then dehydrated in 30% sucrose for at least 2 days. The trigeminal ganglion was embedded in a Tissue-Tek® Optimal Cutting Temperature (OCT) compound (Sakura Finetek, Flemington, NJ, USA) and cut on a cryostat (Leica CM3050 S) for acquiring sagittal sections of 30 μ m. For each mouse, 6–8 sections were acquired from both TG and 3 alternate sections

containing the whole TG were collected for subsequent immunostaining.

To determine the expression pattern of GAD65 or GAT1 in TG neurons, we performed double-immunohistochemical staining of NeuN (a neuronal marker) and GAD65 or NeuN and GAT1. TG sections were first incubated with the chicken antibody of NeuN overnight at 4 °C (1:400, Cat: AB104224, Lot: GR3227256–2, Abcam, Cambridge, UK, RRID: AB_10711040) followed by the Dylight 405-conjugated Donkey anti-chicken secondary antibody (1:500, Cat: 703–475-155, Lot: 140264, Jackson ImmunoResearch, West Grove, PA, USA, RRID: AB_2340373) for 1.5 h at room temperature. Then, the sections were either incubated with the mouse anti-GAD65 antibody (1:500, Cat: ab26113, Lot: GR3265336–1, Abcam, Cambridge, UK, RRID: AB_448989) or the rabbit anti-GAT1 antibody (1:500, Cat: ab426, Lot: 3220718-10, Abcam, Cambridge, UK, RRID: AB_2189971) overnight at 4 °C followed by their respective secondary antibodies, i.e. the Alexa Fluor 594-conjugated

donkey anti-mouse for GAD65 (1:500, Cat: 715–585-151, Lot: 135835, Jackson ImmunoResearch, West Grove, PA, USA, RRID: AB_2340855) and the Alexa Fluor 594-conjugated donkey anti-rabbit secondary antibody for GAT1 (1:500, Cat: A21207, Lot: 1256153, Life Technology, Carlsbad, CA, USA, RRID: AB_141637) for 1.5 h at room temperature.

Triple-immunohistochemical staining of the $\alpha 6$ subunit, NeuN and glutamine synthetase (GS), a TG satellite glia cell marker [13, 46] was also carried out to examine the distribution of $\alpha 6$ GABA_ARs in neuronal cells and SGCs in TG. TG sections were washed 2 times with PBS for 8 min each, and then incubated in 0.4% TritonX-100 with 10% normal donkey serum blocking in room temperature for 1 h. Then, sections were first incubated with the chicken anti-NeuN antibody (1:400, Cat: AB104224, Lot: GR3227256–2, Abcam, Cambridge, UK, RRID: AB_1071104) overnight at 4 °C, followed by the Dylight Fluor-405 conjugated Donkey anti-chicken secondary antibody (1: 500, Cat: 703–475-155, Lot: 140264, Jackson Immuno Research, West Grove, PA, USA, RRID: AB_2340373) for 1.5 h at room temperature. Thereafter, the sections were incubated with the mouse antibody against GS, overnight at 4 °C (1:500, Cat: MAB302, Lot: 3065028, Millipore, Burlington, CO, USA, RRID: AB_2110656) followed by the Alexa Fluor 594-conjugated donkey anti-mouse secondary antibody (1:500, Cat: 715–585-151, Lot: 135835, Jackson Immuno Research, West Grove, PA, USA, RRID: AB_2340855) for 1.5 h at room temperature. Finally, the sections were incubated with the rabbit anti- $\alpha 6$ antibody overnight at 4 °C (1:200, Cat: NB300-196, Lot: T404R, Novus, Littleton, CO, USA, RRID: AB_350512) followed by the Alexa Fluor 488-conjugated donkey anti-rabbit secondary antibody (1:300, Cat: 406416, Lot: B237604, Biolegend, San Diego, CA, USA, RRID: AB_2563203) for 1.5 h at room temperature. Sections were mounted on glass slides using ProLong Gold Antifade Mountant (Invitrogen, Carlsbad, CA, USA) and sealed with a coverslip.

Similarly, triple-immunohistochemical staining of the $\alpha 6$ subunit, NeuN, CGRP was carried out to examine the expression patterns of $\alpha 6$ GABA_ARs and CGRP in TG neuronal cells. TG sections were washed 2 times with PBS for 8 min each, and then incubated in 0.4% TritonX-100 with 10% normal donkey serum blocking in room temperature for 1 h. The sections were first incubated with the rabbit anti- $\alpha 6$ antibody overnight at 4 °C (1:200, Cat: NB300–196, Lot: T404R, Novus, Littleton, CO, USA, RRID: AB_350512) followed by the Alexa Fluor 488-conjugated donkey anti-rabbit secondary antibody (1:300, Cat: 406416, Lot: B237604, Biolegend, San Diego, CA, RRID: AB_2563306) for 1.5 h at room temperature. Thereafter, the sections were incubated with the chicken anti-NeuN antibody (1:400, Cat: AB134014, Lot: GR3227256–2, Abcam, Cambridge, UK, RRID:

AB_2313589) overnight at 4 °C, followed by the Dylight Fluor-405 conjugated Donkey anti-chicken secondary antibody (1: 500, Cat: 703–475-155, Lot: 140264, Jackson Immuno Research, West Grove, PA, USA, RRID: AB_2340373) for 1.5 h at room temperature. Finally, the sections were incubated with the mouse anti-CGRP antibody overnight at 4 °C (1:200, Cat: ab81887, Lot: GR3283855–2, Abcam, Cambridge, UK, RRID: AB_1658411) followed by the Alexa Fluor 594-conjugated donkey anti-mouse secondary antibody (1:500, Cat: 715–585-151, Lot: 135835, Jackson Immuno Research, West Grove, PA, USA, AB_2340855) for 1.5 h at room temperature. Finally, the sections were mounted on glass slides using ProLong Gold Antifade reagent (Invitrogen, Carlsbad, CA, USA) and sealed with a coverslip.

The double-immunoreactivity of double-IF stained TG sections was quantified by the ZEN Black Software (Carl Zeiss Microscopy GmbH, 2011) according to the guidelines provided (https://www.zeiss.com/content/dam/Microscopy/Downloads/Pdf/FAQs/zen-aim_colocalization.pdf). Briefly, the pixel intensity of the fluorescent stain on each section was employed as the expression intensity of the tagged-protein. The threshold of pixel intensity for each signal in the same section was determined and captured independently. The signal pixel intensity and colocalization pixels were computationally generated in the form of scatter-plot. The weighted colocalization coefficients in percentage were derived from the Pearson correlation coefficient with the scatter-plot threshold set to three times of the standard deviation of the mean value of the background pixels, as reported previously [47, 48].

Chemicals

The clinically used NTG injection solution (Millisrol® inj.) that consists of 0.5 mg/mL NTG in water for injection, was purchased from Nippon Kayaku Co. Ltd., (Tokyo, Japan). The injection volume was 20 mL/kg, i.e. 10 mg/kg of NTG was given to the NTG group of mice; thus, no dilution was required. The control group of mice received 20 mL/kg normal saline injection. Compound 6 [7-methoxy-2-(4-methoxyphenyl)-2,5-dihydro-3H-pyrazolo[4,3-c]quinolin-3-one] and DK-I-56-1 [7-methoxy-2-(4-methoxy-d3-phenyl)-2,5-dihydro-3H-pyrazolo-[4,3-c]quinolin-3-one] were synthesized as previously reported [23]. Furosemide was purchased from Tocris Bioscience (Bristol, UK), sumatriptan and topiramate were from Sigma–Aldrich (St. Louis, MO). Compound 6, DK-I-56-1, topiramate, and furosemide were dissolved in a vehicle consisting of 20% of dimethylsulfoxide (DMSO) and 20% Cremophor®EL (polyoxyethylene castor, Sigma–Aldrich) and 60% Sumatriptan was dissolved in normal saline.

Data Analysis

The experiments were designed to generate groups of equal size, using randomization and blinded analysis. At least two cohorts of mice were used for each dataset and were pooled for final analysis, according to the guidelines published by the National Centre for the Replacement, Refinement and Reduction of Animals in Research, UK [49]. The sample size for each group was estimated using the standard deviation from a pilot study or that obtained in previous literature with an expected power of 80% at the alpha level of 0.05, using an online free G*Power software [50]. The power analysis parameters are listed in Supplementary Table S1. Briefly, for the time-dependent effect of NTG on each AUs (Fig. 1B–F) or on the total grimace score (Fig. 1G), a preliminary study with $n = 3$ indicated a 66% reduction in total grimace score (saline vs NTG groups), with a standard deviation (SD) of 20%. Via G*Power software analysis, the effect size of 1.667, actual power of 97.30%, and total sample size of 6 ($n = 3$ for each group) were obtained (refer to Table S1A for protocol of power analysis). Furthermore, for these data in Fig. 2, there were 6 measurements per mouse. Therefore, the sample size $n = 3$ for each treatment group with 6 repeated measurements per mouse (18 measurements in each group) is sufficient to provide a test result with a large effect size. Thus, for all time-dependent behavioral studies (Fig. 2B–G and Fig. 7D), different cohorts of mice in replicates of $n \geq 3$ (3 or 4) were used and pooled for final ANOVA analysis.

On the other hand, for the protein expression changes measured via IF staining (Figs. 3, 4, and 5), expression changes of 30% were expected to yield significant results, as shown in a previous study indicating that a reduction of 30% in TG α 6GABA_ARs will result in elevated pain response in rats [21]. To the best of our knowledge, no other study reported on the influence of changes in GABAergic signaling in trigeminal-related pain. Thus the 30%-change expectation was applied to IF studies of GAD65, α 6GABA_ARs and GAT1, with an expected 15% SD for each group. The baseline IF data of repeated saline-treated mice were obtained in a preliminary study. Using these assumptions, the power analysis of α 6GABA_ARs expression in TG neurons (Fig. 4A), resulted in an effect size of 2.317, with actual power of 89.24% and total sample size of 10 ($n = 5$ each group) (refer to Table S1B for protocol of power analysis).

For the pharmacological studies, the effect of sumatriptan, the reference drug, was first investigated in a preliminary study, with $n = 3$ for each treatment group (Fig. 6B, control, vehicle, 0.6 mg/kg and 1 mg/kg sumatriptan). The data demonstrated that administration of 1 mg/kg sumatriptan suppressed repeated NTG-induced total grimace score by almost one third, and to a level comparable to that in repeated saline-treated control group, whereas 0.6 mg/kg of sumatriptan exhibited almost half of such effect. As such, power analyses

generated an effect size of 1.658, actual power of 97.18% and suggested a total sample size of 12 ($n = 3$ for each group) (refer to Table S1C for protocol of power analysis). Thus, for all pharmacological studies on total grimace scores (Fig. 6B–D and Fig. 7A), different cohorts of mice in replicates of $n \geq 3$ (3 or 4) were used and were pooled for final ANOVA analysis of the datasets.

The obtained datasets have been evaluated for normal distribution using the Shapiro-Wilk test. However, throughout the experimentation period, mice that exhibited serious signs of injury due to cage mate fighting were excluded in data analyses, causing unequal group sizes (e.g., the NTG-treated groups in Fig. 6C and 7B). The two-way ANOVA followed by Sidak's post hoc test was employed to examine the difference in grimace scores between saline- and NTG-treated groups across time. The one-way ANOVA followed by Sidak's post hoc test was employed to examine the differences among treatment groups. The statistical parameters derived from two-way and one-way ANOVA analyses were tabulated in Supplementary Table S2 and S3, respectively. Student's *t* test was applied when only two groups were compared. Following ANOVA, post hoc tests were run only when *F* values were statistically significant ($p < 0.05$). The Brown-Forsythe test showed no significant variance inhomogeneity for all data sets. The declared group size (n) is the number of mice with each independent treatment and statistical analyses were done using these independent values. No outliers were identified in the data sets of the present study and all data obtained were included in data analysis. The data were expressed as mean $\pm$ SEM with the n number indicating the number of mice tested in the declared group. Statistical significance was set at $P < 0.05$.

Results

Repeated NTG Administrations Induced Migraine-like Grimaces in Mice

Here, we established a migraine-mimicking model induced by an i.p. injection of NTG (10 mg/kg) once every 2 days in up to 5 sessions (Fig. 1A) in ICR mice, similarly as performed in rats [42] and mice [27]. Their facial pain scores were measured by the mouse grimace scale (MGS) as described by Langford [41] for the action units including orbital tightening (Fig. 2A), ear rearing (Fig. 2B), cheek bulge (Fig. 2C), nose bulge (Fig. 2D) and whisker change (Fig. 2E). As shown in Fig. 1, the scores of all action units, except ear rearing, in the NTG-injected group were significantly higher than in the saline control group on the first session (day 1) and remained elevated in the following 4 NTG challenge sessions on days 3, 5, 7, and 9. The total grimace score, calculated by the summation of the 5 action unit scores, in the NTG group was equally

Fig. 3 The effect of repeated nitroglycerin (NTG) administration on the expression level of 65 kDa glutamate decarboxylase (GAD65) in mouse trigeminal ganglion (TG) neurons. Migraine-like grimaces were induced in mice with the same protocol as described in Fig. 1A. Sixty minutes after the 1st (day 1), 3rd (day 5) or final (day 9) session of NTG induction, mice were sacrificed and their TG tissues were collected to conduct immunofluorescence (IF) staining for the GABA-synthesizing enzyme, GAD65 in TG neurons. (A) Representative TG sections prepared from mice sacrificed on day 9 after repeated saline or NTG treatments were double-stained with GAD65 (red, A-a, A-d), NeuN (blue, A-b, A-e) and

costaining of GAD65 and NeuN (A-c, A-f) in saline- and NTG-treated groups, respectively. (B) The percentage of NeuN-immunoreactive cells with the GAD65 immunoreactivity (GAD65/NeuN-ir) in V1 and V2 regions of TG sections prepared from mice on day 9 after repeated saline injections (Saline) or from mice on day 1, 5 or 9 after repeated NTG injections. Note that the percentage of GAD65/NeuN-ir was gradually decreased in repeated NTG-treated group, as compared with the saline-treated group. All data were measured in pixels by the Zen Black software. ** $P < 0.01$, *** $P < 0.001$ vs saline, ### $p < 0.01$ vs day 1, one-way ANOVA followed by Sidak's post hoc test

higher than in the saline-treated group in all 5 sessions measured (Fig. 1G). In the saline group, the total grimace scores on all the saline injection days were not significantly different from the baseline score (day 1) before injection (open bars in Fig. 1G). In the subsequent pharmacological studies, the total grimace score in the last session (day 9) of the NTG challenge was used for the comparison of NTG- and saline-treated mice.

Repeated NTG Administrations in Mice Induced a Robust Reduction of GAD65 in TG Neurons

Next, we performed IF studies on TG tissues isolated from mice after receiving the last (5th) session (on day 9) of the NTG challenge to examine possible histopathological changes in the GABAergic system, including GAD65, GAT-1, and $\alpha 6\text{GABA}_A\text{Rs}$.

Fig. 4 Repeated nitroglycerin (NTG) administration did not affect the expression level of GABA transporter 1 (GAT1) in trigeminal ganglion (TG) neurons and satellite glia cells (SGCs). (A) Representative TG sections were prepared as described in Fig. 1 in repeated saline- or NTG-treated mice sacrificed on day 9, and were double-stained with GAT1 and NeuN. Micrographs are the IF staining of the GAT1 (red, A-a, A-d), NeuN (blue, A-b, A-e) and costaining of GAT1 and NeuN in repeated saline- (A-c) or NTG-treated (A-f) group. (B) The percentage of GAT1/NeuN-ir cells in the V1/V2 region of TG in repeated saline- and NTG-treated groups, respectively. Note that no significant change of GAT1 expression in TG neurons was observed (Student's *t* test, $p =$

0.67 vs saline). (C) Representative TG sections double-stained with GAT1 and glutamine synthetase (GS), an SGC marker, prepared from mice receiving repeated saline injection or mice receiving repeated NTG injection sacrificed on day 9, as described in Fig. 1A. IF staining of the GAT1 (red, C-a, C-d), GS (blue, C-b, C-e) or costaining of GAT1 and GS (C-c, C-f) in repeated saline- and repeated NTG-treated groups, respectively. (D) The percentage of GS-positive cells with GAT1-ir (GAT1/GS-ir) in V1 and V2 regions of TG in both groups. Note that there was no significant change of GAT1 expression in TG SGCs (Student's *t* test, $p = 0.89$ vs saline)

GAD65, but not GAD67, is the GABA-synthesizing enzyme in TG [11]. We therefore compared the levels of GAD65 in TG of NTG- and saline-treated mice. As reported in rats [11], GAD65 (red in Fig. 3A) was abundantly distributed in neuronal cells, which were immunoreactive to NeuN (blue in Fig. 3A), but was not present in SGCs, stained by glutamine synthetase (GS), a glia marker (blue in Supplementary Fig. S1), of mouse TG. The GAD-immunoreactivity (GAD-ir) in TG neurons was significantly decreased in mice having had received 5 NTG challenge sessions (on day 9), as compared with the control group that received 5 saline injections (Fig. 3).

In order to examine whether NTG-induced GAD65 decline in TG neurons is NTG exposure-session dependent, we subsequently analyzed the TG samples from mice treated with 1 (day 1) or 3 repeated sessions (day 5) of NTG (red arrows in Fig. 1A). Interestingly, the downregulation of GAD65 depended on the number of NTG exposures (Fig. 3B). On

day 1, a single NTG injection did not significantly affect the GAD65 expression in TG neurons. However, after 3 and 5 NTG injections, on day 5 and day 9, respectively, the GAD65-ir was significantly and gradually decreased (Fig. 3B).

Repeated NTG Administrations Did Not Alter GAT1 or $\alpha 6$ GABA_AR Levels in TG Neurons or SGCs

GAT1, expressed in both TG neurons and SGCs, is a key transporter for the uptake of extracellular GABA in rat TG tissues [11]. In mice, we also observed abundant expression of GAT1 (red) in neuronal cells (blue in Fig. 4A) and SGCs (blue in Fig. 4C) in mouse TG tissues. Nevertheless, repeated NTG challenge did not result in changes in the expression of GAT1 in either TG neurons (Fig. 4B) or SGCs (Fig. 4D), as compared with the saline injection group.

The $\alpha 6$ GABA_AR_s were investigated in this study as we previously found that they are abundant and highly

Fig. 5 Repeated nitroglycerin (NTG) administration did not affect the expression level of α6 subunit-containing GABA_A receptors (α6GABA_ARs) in trigeminal ganglion (TG) neurons and satellite glia cells (SGCs). (A) Representative TG sections triple-stained with the α6 subunit of GABA_A receptors (green), NeuN (blue, neuronal marker) and glutamine synthetase (GS, red, SGC marker) prepared from repeated saline- or repeated NTG-treated mice sacrificed on day 9, as described in Fig. 1A. Micrographs are the IF staining of the α6 subunit (green, A-a, A-), NeuN (blue, C-b, C-e), or costaining of α6 and NeuN (C-c, C-f) in repeated saline- or NTG-treated group. (D) The percentage of NeuN-positive cells with the α6 immunoreactivity (α6/NeuN-ir) in V1 and

V2 regions of TG in repeated saline- and NTG-injected mice. Note that the percentage of α6/NeuN immunoreactivity between repeated saline and repeated NTG groups was not significantly different. (Student's t test, $p = 0.40$, vs saline). (E) IF staining of the α6 subunit (green, E-a, E-d), GS (red, E-b, E-e) or costaining of α6 and GS in (E-c, E-f) in both groups (F) The percentage of GS-positive cells with the α6 immunoreactivity (α6/GS-ir) in V1 and V2 regions of TG in repeated saline injected and repeated NTG injected mice. Note that the percentage of α6/GS immunoreactivity between repeated saline and repeated NTG groups was not significantly different. (Student's t test, $p = 0.64$, vs saline)

colocalized with CGRP in rat TG neurons and are a potential therapeutic target for migraine [19]. In mouse TG sections, we measured the expression levels of the α6 protein in neurons and in SGCs, respectively, by the colocalization of α6 (green) with NeuN (blue, a neuronal marker), and with GS (red, the glia marker) by triple-IF staining. As shown in Fig. 5, the α6 subunit was abundantly expressed in both neuronal (Fig. 5A, c and h) and glia cells (Fig. 5A, e and j) in adult mice, as reported in adult rats [19]. Between NTG- and saline-treated groups, there was no difference in the expression level of α6 (green) in either NeuN-ir. (blue) cells (blue, Fig. 5A, c vs h) or GS-ir (red) cells (Fig. 5A, e vs j). Interestingly, in line with our previous report in rat TG tissues [19], the α6 subunit of GABA_ARs was expressed not only in CGRP-containing

(yellow arrowheads) but also in CGRP-lacking (open white arrowheads) TG neurons (Supplementary Fig. S2).

Clinical Validity of the Mouse Migraine Model Induced by Repeated NTG Injections

To further examine the clinical validity of this migraine model, we examined whether NTG-induced migraine-like grimaces can be attenuated by an abortive therapeutic agent, sumatriptan. On day 9, 20 min after the final NTG injection, mice were treated with sumatriptan (0.6 and 1 mg/kg, i.p.) and were videotaped 10 min later for their grimace scoring (Fig. 6A). Indeed, sumatriptan significantly and dose-dependently reduced total grimace scores induced by 5 sessions of NTG

Fig. 6 Repeated administration of DK-I-56-1, but not Compound 6, exhibited migraine prophylaxis effect without drug dependence potential. (A) To evaluate the migraine prophylaxis effect of the tested drugs, DK-I-56-1 (3 mg/kg, i.p.), Compound 6 (3 mg/kg, i.p.), topiramate (30 mg/kg, i.p.) or vehicle (i.p.) was administered to the mice 30 mins before nitroglycerin (NTG) injection on days 1, 3, 5, 7, and 9. In the final session, the video was recorded 30 mins after NTG or saline administration for 30 mins, and the total Action Units (AUs) of Mouse Grimace Scale (MGS) were measured. (B) Total grimace scores of the control group that received repeated saline injections and the repeated NTG-treated groups that received vehicle, topiramate (30 mg/kg, i.p.), Compound 6 (3 mg/kg, i.p.), or DK-I-56-1 (3 mg/kg, i.p.) pretreatment

before NTG injection for 5 sessions. Note that pretreatment with DK-I-56-1 and topiramate, but not Compound 6, significantly suppressed migraine-like responses in NTG-treated mice to a level comparable to the control group. ***P* < 0.01, ****P* < 0.001 versus control, ##*P* < 0.01, ###*P* < 0.001 versus vehicle among NTG-treated groups, one-way ANOVA followed by Sidak's post hoc test. (C) The conditioned place preference (CPP) test was conducted in mice with the protocol described in MATERIAL AND METHODS. (D) CPP scores of mice received total 5 injections of vehicle or DK-I-56-1 (10 mg/kg, i.p.) in 9 days. Note that both groups did not develop significant preference towards DK-I-56-1- or vehicle-conditioned chamber

injections in mice (Fig. 6B). At 1 mg/kg, the total grimace score was reduced to the level as in the saline control group.

$\alpha 6$ GABA_ARs PAMs Significantly Aborted NTG-Induced Migraine-like Grimaces in Mice in a Manner Blocked by Furosemide

Two $\alpha 6$ GABA_AR-selective PAMs, Compound 6 and its deuterated analogue, DK-I-56-1 [23], were given to mice 20 min after the last session of NTG injection on day 9, at 1, 3, and 10 mg/kg (i.p.) (Fig. 6A). Both Compound 6 and DK-I-56-1 were effective at 3 and 10 mg/kg (i.p.), and both at 10 mg/kg reduced the total grimace score to the level as in the saline control group (Fig. 6C).

To further substantiate that the "anti-migraine" effects of both Compound 6 and DK-I-56-1 in this model is mediated by $\alpha 6$ GABA_ARs, we examined their interactions with furosemide, an $\alpha 6$ GABA_AR antagonist [51], in this migraine-mimicking model. Coadministration of furosemide (20 mg/kg, i.p.) with Compound 6 (3 mg/kg, i.p.) or DK-I-56-

1 (10 mg/kg, i.p.) significantly prevented the abortive effect of Compound 6 and DK-I-56-1 on NTG-elevated grimace scores (Fig. 6D). Importantly, furosemide per se had no effect on the grimace scores in NTG-treated mice (Fig. 6D).

DK-I-56-1, but Not Compound 6, Provided Prophylactic Anti-migraine Effect in Repeated NTG-Induced Mouse Migraine Model

We further evaluated whether Compound 6 and DK-I-56-1 would also have the potential as a preventive therapy on migraine. Mice were randomly divided into five groups, receiving saline, vehicle, topiramate, a clinically used migraine prophylaxis agent, Compound 6, and DK-I-56-1, respectively, 30 min before each session of NTG injection as described in Fig. 7A. Control groups received repeated saline injections. Interestingly, the results demonstrated that DK-I-56-1 at the dose of 3 mg/kg (i.p.) significantly prevented the elevation of total grimace scores induced by NTG with an efficacy comparable to that of pretreatment with 30 mg/kg (i.p.) topiramate

Fig. 7 Positive allosteric modulators (PAMs) of $\alpha 6$ subunit-containing GABA_A receptors ($\alpha 6$ GABA_ARs) suppressed repeated nitroglycerin (NTG)-induced migraine-like responses in a manner reversed by furosemide, an $\alpha 6$ GABA_AR antagonist. (A) Mice received injections of NTG (10 mg/kg, i.p.) or saline on days 1, 3, 5, 7, and 9. Twenty minutes after the final NTG injection on day 9, mice were treated with the tested drugs including sumatriptan (0.6 or 1.0 mg/kg, i.p.), the $\alpha 6$ GABA_AR-selective PAMs, e.g., Compound 6 (1, 3 or 10 mg/kg, i.p.) or DK-I-56-1 (1, 3 or 10 mg/kg, i.p.). After 10 mins, the facial expressions of each mouse were videotaped for 30 mins and the Action Units (AUs) of Mouse Grimace Scale (MGS) were subsequently measured. The criteria of MGS are detailed in Fig. 2. In separate groups, furosemide (an $\alpha 6$ GABA_AR antagonist, 20 mg/kg, i.p.) was coadministered with Compound 6 (3 mg/kg, i.p.) or DK-I-56-1 (3 mg/kg, i.p.) to discern the involvement of $\alpha 6$ GABA_ARs in migraine-abortive effect of these drugs. (B) Total grimace scores in the control group that received repeated saline injections and in the repeated NTG-injected groups that received vehicle, 0.6 mg/kg or 1 mg/kg of sumatriptan on day 9. Note that sumatriptan significantly reduced total grimace scores in this model. $**P < 0.01$, $***P < 0.001$ versus control, $\#P < 0.05$, $###P < 0.001$ versus vehicle

among NTG-treated groups, one-way ANOVA followed by Sidak's post hoc test. (C) Total grimace scores in the control group that received repeated saline injections and in the repeated NTG-treated groups that received vehicle, Compound 6 (1, 3 or 10 mg/kg, i.p.) or DK-I-56-1 (1, 3, or 10 mg/kg, i.p.) on day 9. Note that 10 mg/kg of Compound 6 and DK-I-56-1, similar to 1 mg/kg or sumatriptan, significantly suppressed the total MGS to the level comparable to the control group. $**P < 0.01$, $***P < 0.001$ versus control, $\#P < 0.05$, $###P < 0.001$ versus vehicle among NTG-treated groups, one-way ANOVA followed by Sidak's post hoc test. (D) Total grimace scores in the control group that received repeated saline injections and in repeated NTG-treated groups that received Compound 6 (3 mg/kg, i.p.) or DK-I-56-1 (10 mg/kg, i.p.) alone or in combination with furosemide (20 mg/kg, i.p.) on day 9. Note that the migraine-abortive effect of Compound 6 or DK-I-56-1 was significantly reversed by furosemide. $***P < 0.001$ versus control, $\#P < 0.05$, $##P < 0.01$ versus vehicle among NTG-treated groups, $\lambda P < 0.05$, $\lambda \lambda P < 0.01$ versus furosemide coadministered group, one-way ANOVA followed by Sidak's post hoc test

(Fig. 7B). However, Compound 6 (3 mg/kg) did not produce a significant preventive effect in a concurrent assay (Fig. 7B).

Absence of Drug Dependence Behavior After Repeated DK-I-56-1 Treatment

We further investigated whether the centrally active $\alpha 6$ GABA_AR PAMs [23, 52] are devoid of drug abuse potentials after repeated uses, which may pose a potential limitation to its pharmacological application. After 5 repeated conditioning sessions with 10 mg/kg (i.p.) DK-I-56-1, as described in

Fig. 7C, DK-I-56-1 did not elicit significant preference towards the DK-I-56-1 conditioned chamber (Fig. 7D).

Discussion

Repeated NTG injections in rats have been reported to be a good animal model of migraine that can provide clinically relevant molecular, histopathological, and behavioral readouts [24]. Here, we established a mouse migraine model induced by repeated NTG injections. The findings that NTG-induced

migraine-like grimaces in mice were dose-dependently attenuated by sumatriptan and prevented by topiramate, comparable to abortive and preventive clinical effects, respectively, of these two anti-migraine agents, supported the clinical validity of this migraine model. The findings that DK-I-56-1 mimicked the abortive and preventive effects of sumatriptan and topiramate, respectively, in this migraine model suggest that positive modulation of TG $\alpha 6$ GABA_ARs by $\alpha 6$ GABA_AR-selective PAMs can be a potential novel therapy for migraine. In addition, we found that the GABA-synthesizing enzyme (GAD65), but not the GABA transporter (GAT-1) or an abundant GABA_A receptor subtype ($\alpha 6$ GABA_AR), were downregulated in TG in this migraine model. These data for the first time point to a possible hypofunction of TG GABAergic transmission in a preclinical model of migraine.

Downregulation of GAD65 but Not GAT-1 in TG Neurons of Repeated NTG-Treated Mice

As the detection of GABA levels is technically challenging, especially in TG, the expression level of GAD, the GABA-synthesizing enzyme, is commonly used as an indirect assessment for GABA production [14]. Here, we demonstrated that the expression of GAD65 in TG neurons was strikingly decreased in the course of repeated NTG administrations (Fig. 3). Downregulation of neuronal GAD65 levels has been shown previously to be associated with chronic pain, such as neuropathic pain [53, 54] and persistent inflammatory pain [55], as observed in the rat dorsal spinal horn and brainstem, respectively. However, so far there were no reports on the association of GAD65 downregulation with chronic pain in TG.

Interestingly, a single administration of NTG did not significantly reduce GAD65 expression in mouse TG, as opposed to repeated (3 and 5 times) NTG administrations (Fig. 3B), although each NTG injection induced an equal degree of grimaces (Fig. 1G). GAD65 downregulation after repeated NTG injections thus represents a chronicity feature of this migraine model. Downregulation could have been caused by enhanced degradation or reduced expression of GAD65. The delayed downregulation of GAD65 suggest an altered gene expression for this effect to occur [24]. A similar chronicity feature of this migraine model has been described previously: Greco et al. (2018) demonstrated that chronic and intermittent, but not single, NTG administrations significantly elevated the CGRP gene expression in rat TG [30]. These two chronicity features might thus be directly or indirectly connected with each other. Downregulation of GAD65 might ensure faithful transmission of CGRP-induced inflammatory pain to the central nervous system by a weakened GABAergic inhibition. In the light of the multiple effects induced by repeated NTG administrations [24], the possible cause–effect relationship behind the upregulation of CGRP and the downregulation of

GAD65 remains to be elucidated. In addition, it also remains to be clarified whether this loss of GAD65 is specifically caused by repeated NTG administrations or whether it is always associated with migraine, irrespective of how migraine is elicited.

On the other hand, repeated NTG administrations did not affect the expression level of GAT1 in TG neurons (Fig. 4A, B) and SGCs (Fig. 4C, D). As GAT1 is known to reaccumulate GABA from the extracellular space [56], this finding suggests that compensatory changes in the GABA uptake system of TG seem not to occur in response to decreased GABA synthesis.

$\alpha 6$ GABA_ARs Are Abundantly Expressed in TG Neurons and SGCs and Were Unaltered in Mice After Repeated NTG Administrations

In the present study, we found that $\alpha 6$ GABA_ARs are highly expressed in TG neurons and SGCs of adult mouse (Fig. 5). Previous studies suggested that the $\alpha 6$ GABA_AR expression in specific brain regions is subjected to up- or downregulation under pathological conditions. For instance, there was a decreased expression of $\alpha 6$ GABA_ARs in the hippocampus of rats that became depressive due to maternal separation [57] and there was an increased expression in the striatum of mice with induced anxiety [58]. Interestingly, the expression level of $\alpha 6$ GABA_ARs in either TG neurons or SGCs did not change in mice that were repeatedly treated by NTG (Fig. 5).

TG $\alpha 6$ GABA_ARs as a Novel Therapeutic Target for Migraine

Recently, we have identified a series of pyrazoloquinolinone compounds, which are PAMs highly selective for $\alpha 6$ GABA_ARs [17, 23], that can alleviate trigeminal neuropathic pain induced by infraorbital nerve ligation [22] and TGVS hypersensitivity induced by intracisternal capsaicin instillation [19] in rats. In the latter study, only pathological changes of capsaicin instillation and their alleviation by the $\alpha 6$ GABA_AR-selective Compound 6 could be investigated, but not its possible migraine-abortive or preventive effect.

Here, we extended our previous studies by employing the migraine-like grimace model induced by repeated NTG-injections in mice to evaluate potential anti-migraine effects of $\alpha 6$ GABA_AR PAMs. In this model, we demonstrated that a migraine-abortive therapeutic agent, sumatriptan (Fig. 6B), a 5-HT_{1B/1D} agonist which can inhibit CGRP release [59] as well as dural stimulation-induced TNC activation [60], significantly abolished NTG-induced grimace elevation. In addition, another anti-migraine agent used for migraine prevention [61], topiramate, also effectively prevented NTG-induced grimace elevation. These positive results of clinically used anti-migraine agents support the predictive validity of this

migraine model for preclinical studies. The negative results of the vehicle in both abortive and preventive experiments further support the reliability of the readouts of this behavioral model of migraine.

Both Compound 6 and its deuterated analogue, DK-I-56-1, given on day 9, the last session of repeated NTG injections, can reduce grimace scores in mice, supporting the effectiveness of $\alpha 6$ GABA_AR PAMs as an abortive treatment for migraine (Fig. 6C). Importantly, this abortive effect is comparable with that of sumatriptan (Fig. 6B). These data further support the conclusion that $\alpha 6$ GABA_AR PAMs can be a potential pharmacotherapy against acute migraine attacks with a comparable efficacy as triptans.

Furosemide, a loop diuretic acting as a luminal Na-K-Cl cotransporter inhibitor [62], has been known to also reversibly antagonize GABA-elicited currents at $\alpha 6\beta 2/3\gamma 2$ -consisting GABA_ARs [63]. Here, we demonstrated that systemic injection of furosemide (20 mg/kg, i.p.) successfully antagonized the anti-migraine effect of both Compound 6 and DK-I-56-1 (Fig. 6D). Since furosemide is BBB-impermeable [62], it could not have acted in the central nervous system. This suggests that the site of action of $\alpha 6$ GABA_ARs PAMs is located in the periphery. TG are a crucial site for mediating migraine pathogenesis [12] and are not enclosed by the BBB. The $\alpha 6$ GABA_ARs expressed in TG may thus be the target of action of these pyrazoloquinolinones.

In addition, we investigated whether $\alpha 6$ GABA_AR PAMs also possess prophylactic effects against migraine development in the current model when repeatedly applied to the mice 30-min before each NTG injection. Interestingly, DK-I-56-1, a deuterated analogue with longer plasma half-life [23], but not Compound 6, prevented grimace score elevation before the last NTG injection (Fig. 7B). The effect of DK-I-56-1 is comparable to the preventive effect of topiramate, a clinically used preventive therapy for migraine.

Proposed Anti-migraine Mechanism of $\alpha 6$ GABA_ARs PAMs in TG

Taken together, we found that the level of GAD65, but not that of $\alpha 6$ GABA_ARs or GAT1, was significantly reduced in TG in the mouse migraine model induced by repeated NTG injections. A proposed mechanism is depicted in Fig. 8. NTG can activate the TGVS at the meningeal neurovascular endings through nitric oxide. A single NTG injection induces headache in man [25] and in rats [24, 64] and increased headache-like grimaces in mice, possibly by the nitric oxide-induced release of CGRP in the meninges. But it does not cause an enhancement of CGRP synthesis by increasing its gene expression [28], or a reduction in the level of GAD65, as demonstrated in this study.

On the other hand, repeated NTG treatments and hence frequent TGVS activation, resembling frequent migraine

attacks, may lead to an impaired GABA- $\alpha 6$ GABA_AR neurotransmission in TG, which is likely attributed to a lower GABA level due to a downregulation of GAD65 in TG neurons. $\alpha 6$ GABA_AR-selective PAMs, such as Compound 6 and DK-I-56-1, may restore the impaired GABAergic transmission in TG, resulting in migraine alleviation.

The subunit composition of $\alpha 6$ GABA_ARs in TG is not known. Since $\alpha 1$, $\alpha 3$, $\alpha 5$, $\alpha 6$, $\beta 2/3$, δ and $\gamma 1$ to $\gamma 3$ subunits of GABA_ARs have been found in TG neurons of rats [11], the subunit composition of $\alpha 6$ GABA_ARs might be similar to that in the cerebellum and comprise $\alpha 6\beta\gamma 2$, $\alpha 1\alpha 6\beta\gamma 2$, $\alpha 6\beta\delta$, and $\alpha 1\alpha 6\beta\delta$ receptors [65]. $\alpha 6\beta\gamma 2$, $\alpha 6\beta\delta$, and $\alpha 1\alpha 6\beta\delta$ receptors are diazepam-insensitive receptors. $\alpha 1\alpha 6\beta\gamma 2$ receptors are diazepam-sensitive when the $\alpha 1$ subunit is neighboring the $\gamma 2$ subunit, as are other receptors possibly formed in TG from the subunits expressed, and a single $\alpha 6$ subunit within a receptor is sufficient to confer a high furosemide sensitivity [66, 67]. In our previous study, diazepam could not block capsaicin-induced TGVS activation, in contrast to Compound 6, topiramate, and other compounds interacting with $\alpha 6$ GABA_ARs that were effective in this migraine-mimicking model [19], suggesting that activation of diazepam-sensitive receptors is not able to alleviate migraine-like symptoms.

Since pyrazoloquinolinones, such as Compound 6 and DK-I-56-1, modulate $\alpha 6$ GABA_ARs by binding at the $\alpha 6 + \beta$ -interface [17], they not only modulate $\alpha 6\beta\gamma 2$ but also $\alpha 6\beta\delta$ receptors, although to a smaller extent [52, 68, 69]. And probably they will also modulate other $\alpha 6$ GABA_ARs containing an $\alpha 6 + \beta$ -interface. A pharmacological identification of the $\alpha 6$ GABA_ARs mediating the “anti-migraine” effects of Compound 6 and DK-I-56-1 thus requires further studies with compounds that can distinguish different subtypes of $\alpha 6$ GABA_ARs.

$\alpha 6$ GABA_ARs Located on Glia Cell Might Contribute to the Effects of Compound 6 and DK-I-56-1 in Migraine

The current finding that $\alpha 6$ GABA_ARs are also highly expressed in SGCs of TG is interesting. Glia cells activated by K^+ that is extruded from the axons at the end of action potentials not only can release GABA but also can cause the production and release of a variety of neuroexcitatory substances including nitric oxide, excitatory amino acids, and proinflammatory cytokines, which are known to be related to migraine pathology [3]. It has been suggested that drugs that are able to block SGC activation and the subsequent release of neuroexcitatory substances could alleviate migraine-related symptoms [70]. $\alpha 6$ GABA_ARs on SGCs might thus represent a feedback mechanism that limits the potassium ion-evoked release of GABA as well as of neuroexcitatory substances from glia cells. Modulation of the abundantly expressed $\alpha 6$ GABA_ARs in TG SGCs may thus be a second

Fig. 8 Schematic diagrams illustrating the trigeminovascular system (TGVS) and proposed changes in trigeminal ganglia (TG) after migraine induced by repeated nitroglycerin (NTG) injections. (A) The TGVS consisting of peripheral dura mater, TG and central trigeminal nucleus caudalis, is involved in migraine. (B) Repeated NTG injections may lead to impaired neurotransmission mediated by GABA and $\alpha 6$ subunit-containing GABA_A receptors ($\alpha 6$ GABA_ARs), resulting from less GABA in TG due to a downregulation of 65 kDa glutamate decarboxylase (GAD65), the synthesizing enzyme of GABA, in TG neurons. However, the expressions of $\alpha 6$ GABA_ARs and GABA

transporter 1 (GAT1) are not affected by repeated NTG injections. (C) Possible anti-migraine mechanisms of $\alpha 6$ GABA_AR PAMs. As impaired GABA- $\alpha 6$ GABA_AR neurotransmission in TG might contribute to the pathogenesis of migraine, the unaffected TG $\alpha 6$ GABA_AR level during migraine may be exploited as the therapeutic target. $\alpha 6$ GABA_AR PAMs may enhance the activity of $\alpha 6$ GABA_ARs, thus restoring the TG GABA- $\alpha 6$ GABA_AR neurotransmission which was impaired due to decreased GABA synthesis. CGRP: Calcitonin gene-related peptide; CGRPR: Calcitonin gene-related peptide receptor

mechanism contributing to the anti-migraine effects of Compound 6 and DK-I-56-1.

In summary, the present study indicated that DK-I-56-1 might have the potential to be developed as a clinically used anti-migraine agent. Previous studies have indicated that DK-I-56-1 was non-toxic even at 400 μ M *in vitro*, and devoid of sedation and motor incoordination effects *in vivo* [23]. The absence of conditioned preference after repeated administrations further supports DK-I-56-1 to be a good drug candidate for both abortive and preventive treatments of migraine without abuse potential.

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Authors' Contributions HRT and MTL conducted experiments, analyzed data, and wrote the paper; PCF and WS contributed to data interpretation, data analysis, result discussion, and paper writing; DEK and JC synthesized and provided compounds; LCC provided supervision, designed experiments, analyzed data, and wrote the paper.

Compliance with Ethical Standards

Conflict of Interest HRT, MTL, and THL declare no conflict of interest. A patent application has been jointly filed by PCF, DEK, JC, WS, and LCC as inventors for the composition and use patents of $\alpha 6$ GABA_AR-modulating pyrazoloquinolinone compounds for multiple indications. The patent right is co-owned by the inventors' institutions.

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