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Trigeminal neuropathic pain development and maintenance in rats are suppressed by a positive modulator of α6 $GABA_A$ receptors

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Abstract

γ-Aminobutyric acid type A ($GABA_A$) receptors containing the α6 subunit are located in trigeminal ganglia, and their reduction by small interfering RNA increases inflammatory temporomandibular and myofascial pain in rats. We thus hypothesized that enhancing their activity may help in neuropathic syndromes originating from the trigeminal system. Here, we performed a detailed electrophysiological and pharmacokinetic analysis of two recently developed deuterated structurally similar pyrazoloquinolinone compounds. DK-I-56-1 at concentrations below 1 μM enhanced γ-aminobutyric acid (GABA) currents at recombinant rat α6β3γ2, α6β3δ and α6β3 receptors, whereas it was inactive at most $GABA_A$ receptor subtypes containing other α subunits. DK-I-87-1 at concentrations below 1 μM was inactive at α6-containing receptors and only weakly modulated other $GABA_A$ receptors investigated. Both plasma and brain tissue kinetics of DK-I-56-1 were relatively slow, with half-lives of 6 and 13 hr, respectively, enabling the persistence of estimated free brain concentrations in the range 10–300 nM throughout a 24-hr period. Results obtained in two protocols of chronic constriction injury of the infraorbital nerve in rats dosed intraperitoneally with DK-I-56-1 during 14 days after surgery or with DK-I-56-1 or DK-I-87-1 during 14 days after trigeminal neuropathy were already established, demonstrated that DK-I-56-1 but not DK-I-87-1 significantly reduced the hypersensitivity response to von Frey filaments.

Significance: Neuropathic pain induced by trigeminal nerve damage is poorly controlled by current treatments. DK-I-56-1 that positively modulates α6 $GABA_A$ receptors is appropriate for repeated administration and thus may represent a novel treatment option against the development and maintenance of trigeminal neuropathic pain.

For this article, a commentary is available at the Wiley Online Library

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1 | BACKGROUND

Trigeminal neuropathic pain is a continuous and burning pain that involves the orofacial region, and is accompanied by paroxysms that can be instantly provoked by touch or other mild stimuli. It is commonly induced by a direct trauma of the trigeminal nerve and thus is also known as painful traumatic trigeminal neuropathy (Napeñas & Zakrzewska, 2011). Treatment of trigeminal neuropathy relies on off-label drug use (Benoliel, Kahn, & Eliav, 2012; Benoliel, Teich, & Eliav, 2016). However, in a recent open study only 11% of patients with painful traumatic trigeminal neuropathy had significant pain reduction in response to the available pharmacotherapy (Haviv, Zadik, Sharav, & Benoliel, 2014). Thus, there is a clinical need for new treatment options for trigeminal neuropathy.

γ-Aminobutyric acid type A ($GABA_A$) receptors are GABA-gated chloride channels composed of five protein subunits that belong to eight different subunit classes with 19 distinct subunits (6α, 3β, 3γ, δ, ε, π, θ, 3ρ). Most $GABA_A$ receptors are composed of two α, two β and one γ2 subunit. The classical benzodiazepines allosterically modulate GABA-induced currents at αβγ2 receptors containing α1, α2, α3 or α5, but not α4 or α6 subunits (Olsen & Sieghart, 2008). Positive allosteric modulators (PAMs) that preferentially modulate α2βγ2 $GABA_A$ receptor subtypes are able to elicit a systemic analgesic effect (Knabl et al., 2008; Rudolph & Knoflach, 2011). However, it is questionable to what extent it is possible to separate the antihyperalgesic actions of α2-receptor-preferring benzodiazepine-like compounds from their potential to induce sedation (Ralvenius et al., 2016).

$GABA_A$ receptors containing the α6 subunit are abundant populations of inhibitory receptors in the cerebellar and cochlear nucleus granule cells and are also present in the olfactory bulb, spinal cord and retina (Gutiérrez, Khan, & Blas, 1996; Pirker, Schwarzer, Wieselthaler, Sieghart, & Sperk, 2000; Wisden, Laurie, Monyer, & Seeburg, 1992). In addition, they have been identified in two major types of cells in the trigeminal ganglia (TG), namely in neurons and satellite glia (Hayasaki et al., 2006; Puri et al., 2012). Reducing α6 expression by infusing small interfering RNA into TG increased nociceptive responses in myofascial pain and inflammatory temporomandibular joint pain models (Kramer & Bellinger, 2013; Puri et al., 2012). We therefore hypothesized that positive modulation of α6-containing receptors may influence trigeminal neuropathic pain.

Pyrazoloquinolinones have been investigated as potential anxiolytics with an exceptionally low liability for sedative-like effects (Williams et al., 1989). Recently, pyrazoloquinolinones with specific substitution patterns were identified as the first ligands functionally selective for α6β2/3γ2 receptors; one of them was PZ-II-029 (presented as compound 6 in Varagic et al., 2013). Furthermore, LAU165, an inactive compound with similar chemical properties, was also identified (Treven et al., 2018).

To investigate whether positive modulation of α6-containing receptors may reduce trigeminal neuropathic pain, here we used two recently published deuterated, and thus metabolically more stable, pyrazoloquinolinone compounds: the PZ-II-029 analogue DK-I-56 and the LAU165 analogue DK-I-87-1 (presented as 8b and 8 m, respectively, in Knutson et al., 2018). DK-I-87-1 was inactive, while DK-I-56-1 inhibited the development and reduced the established trigeminal neuropathic pain in a rat model of chronic constriction injury of the infraorbital branch of the trigeminal nerve (Deseure & Hans, 2015).

2 | METHODS

2.1 | Ligands

DK-I-56-1 (7-methoxy-2-(4-(methoxy-d3)phenyl)-2,5-dihydro-3H-pyrazolo[4,3-c]quinolin-3-one) and DK-I-87-1 (8-chloro-2-(2-(methoxy-d3)phenyl)-2,5-dihydro-3H-pyrazolo[4,3-c]quinolin-3-one) were synthesized at the Department of Chemistry and Biochemistry, University of Wisconsin—Milwaukee, USA (Knutson et al., 2018). For stock solutions, compounds were dissolved in 100% DMSO.

2.2 | Two-electrode voltage clamp electrophysiology

Preparation of mRNA for rat α1, α2, α3, α4, α5, α6, β3, γ2 and δ subunits and electrophysiological experiments with *Xenopus laevis* oocytes were performed as described previously (Forkuo et al., 2016). Mature female *Xenopus laevis* (Nasco, Fort Atkinson, WI, USA) were anaesthetized in a bath of ice-cold 0.17% tricaine (Ethyl-m-aminobenzoate, Sigma-Aldrich, St. Louis, MO, USA) before decapitation and transfer of the frog's ovary to ND96 medium (96 mM NaCl, 2 mM KCl, 1 mM MgCl2, 5 mM HEPES; pH 7.5), in full accordance with the rules of the Austrian animal protection law. Following incubation in 1 mg/ml collagenase (Sigma-Aldrich, St. Louis, MO, USA) for 30 min, stage 5–6 oocytes were singled out of the ovary and defolliculated using a platinum wire loop or glass Pasteur pipette. Oocytes were stored and incubated at 18°C in NDE medium (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 5 mM HEPES, 1.8 mM $CaCl_2$; pH 7.5) that was supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin and 2.5 mM pyruvate. Oocytes were injected with an aqueous solution of mRNA. A total of 2.5 ng of mRNA per oocyte was injected. Subunit ratio was 1:1:5 for αxβ3γ2 ($x = 1, 2, 3, 5$), 3:1:5 for α4/α6β3γ2 and α6β3δ, and 1:1 for α6β3 receptors. Injected oocytes were incubated for at least 36 hr before electrophysiological recordings. Oocytes were placed on a nylon grid in a bath of NDE medium. For

current measurements, oocytes were impaled with two microelectrodes, which were filled with 2 M KCl and had a resistance of 2–3 MΩ. The oocytes were constantly washed by a flow of 6 ml/min NDE that could be switched to NDE containing GABA and/or drugs. Drugs were diluted into NDE from DMSO solutions resulting in a final concentration of 0.1% DMSO. To test for modulation of GABA-induced currents by compounds, a GABA concentration was titrated to trigger a specific fraction X of the respective maximum GABA-elicited current of the individual oocyte (ECX, e.g. EC3–6 or EC90) and was applied to the cell together with various concentrations of tested compounds. All recordings were performed at room temperature at a holding potential of −60 mV using a Warner OC-725C two-electrode voltage clamp (TEV) (Warner Instrument, Hamden, CT, USA) or a Dagan CA-1B Oocyte Clamp or a Dagan TEV-200A TEV amplifier (Dagan Corporation, Minneapolis, MN, USA). Data were digitized using a Digidata 1322A or 1550 data acquisition system (Axon Instruments, Union City, CA, USA), recorded using Clampex 10.5 software (Molecular Devices, Sunnyvale, CA, USA) and analysed using Clampfit 10.5 and GraphPad Prism 6.0 software (La Jolla, CA, USA). Concentration–response data were fitted using the Hill equation. Data are given as mean ± *SEM* from at least three oocytes of two batches.

2.3 | In vivo studies

2.3.1 | Animals and preparation

Experiments were carried out on eight-week-old male outbred Wistar albino rats weighing 160–180 g at arrival (88 rats for the neuropathic pain model and 60 for pharmacokinetic studies), supplied by Military Farm, Belgrade, Serbia. Animals were housed individually, except for pharmacokinetic studies (3–4 per cage), in Makrolon type III cages and had free access to food and water. The temperature of the animal room was 22 ± 1°C, relative humidity 40%–70% and illumination 120 lx, with a 12-hr light/dark cycle (lights on at 06:00 hr). All experiments took place during the light phase of the diurnal cycle (09:00–16:00 hr). All procedures were conducted according to the National Institutes of Health Animal Care and Use Committee guidelines and were approved by the Ethical Committee on Animal Experimentation of the Faculty of Pharmacy in Belgrade. Throughout the study, only experimentally naïve animals were used.

In a procedure slightly modified from Đorđević et al. (2015), biocompatible nanoemulsions of DK-I-56-1 and DK-I-87-1 were prepared by high-pressure homogenization at 50°C to a compound concentration of 2 mg/ml. They were composed of 13.33% medium-chain triglycerides, 6.67% castor oil, 2% soybean lecithin, 0.03% sodium oleate, 2% polyoxyethylensorbitanmonooleate, 0.05% butylhydroxytoluene, 2% DMSO and 73.67% ultrapure water.

2.4 | Pharmacokinetic studies

A detailed description of pharmacokinetic studies in rats is given in the Supporting Information Appendix S1. The studies with DK-I-56-1 included assessment of pharmacokinetic behaviour of 2, 5, 10 and 20 mg/kg doses measured one hour after application (in order to choose the dose for repeated-dose studies), determination of the plasma and brain pharmacokinetic profile after a single 10 mg/kg dose measured at various time points after application, and measurement of plasma and brain concentrations after six or eleven daily doses of 10 mg/kg DK-I-56-1. In the latter case, the measurements were performed 20 min and 24 hr after the final dose. Due to the ethical constraints of performing an elaborate analysis of a compound expected to be biologically inactive, the pharmacokinetic studies with DK-I-87-1 were carried out only with the 10 mg/kg dose (demonstrated to be devoid of behavioural effects in Knutson et al., 2018). In a separate in vitro experiment, the rapid equilibrium dialysis assay was used to determine the free fraction of DK-I-56-1 in rat plasma and brain tissue (Obradović et al., 2014).

2.5 | Chronic constriction injury of infraorbital nerve (IoN-CCI)

The unilateral ligation of the infraorbital nerve (IoN) as an animal model of neuropathic pain was validated by Vos, Strassman, & Maciewicz, 1994. The present study was performed in a randomized and blinded manner. Rats were anaesthetized intraperitoneally with a combination of ketamine and xylazine (60 mg/kg + 10 mg/kg). A midline scalp incision was made exposing skull and nasal bone. The infraorbital part of the IoN was exposed using a surgical procedure similar to that described by Deseure & Hans, 2015 and Vos et al., 1994. Specifically, the edge of the orbit, formed by the maxillary, frontal, lacrimal and zygomatic bones, was uncovered and the IoN was dissected free at its most rostral extent in the orbital cavity, just caudal to the infraorbital foramen. Two chromic catgut ligatures (5–0) were loosely tied around the IoN (2 mm apart). To obtain the desired degree of constriction, a criterion proposed by Bennett and Xie (1988) was applied. The ligatures reduced the diameter of the nerve by a just noticeable amount and retarded, but did not interrupt the circulation through the superficial vasculature. The scalp incision was closed using silk sutures (5–0), and the rat was allowed to recover. In sham-operated rats, the IoN was exposed using the same procedure, but it was not ligated. To minimize variability, all surgeries were performed by a single investigator.

2.6 | Study design

In Experiment 1 (Figure 3a), rats ($n = 46$) were randomly assigned to one of the four groups: rats subjected to IoN surgery and treated with DK-I-56-1, rats subjected to IoN surgery and treated with placebo, sham-operated rats treated with DK-I-56-1 and sham-operated rats treated with placebo. For studying the effects of subchronic prophylactic treatment with the nanoemulsion formulation of DK-I-56-1 in IoN-CCI rats, DK-I-56-1 (10 mg/kg) or its vehicle (placebo nanoemulsion) was injected i.p. daily, for 14 consecutive days starting one day after surgery. Sham-operated rats were treated in parallel.

In Experiment 2 (Figure 3a), all rats ($n = 42$) were subjected to IoN surgery. After assessment of the neuropathy on day 14 (the criterion of increased response score to von Frey filament stimulation compared to baseline value), the animals that met the criterion were randomly assigned to one of the three groups: DK-I-56-1, DK-I-87-1 or placebo. The respective treatment was administered i.p. once daily for 14 days, starting on day 15. In both experiments, treatment was administered at 3:00 p.m., up to 6 hr after finishing the behavioural testing (9:00–12:00 a.m.) or handling/habituation for the given day.

2.7 | Mechanical stimulation testing

After arrival, rats were acclimated to the housing facility for two days prior to subjecting to habituation to the test procedure. Rats were transported from the housing room to the test room (1-min trip) and placed into transparent glass cages ($L \times W \times H$: 250 × 150 × 165 mm) with a metal lid. Habituation and testing were conducted in a darkened room (indirect white neon tubes fixed on the wall), with no background noise. Rats were habituated to von Frey filaments every day for 15 min, starting seven days before baseline data were obtained until the end of the experiment. Every 30 s, the researcher opened the lid and gently touched the wall of the cage with a plastic rod.

In Experiment 1, the response scores to the application of a graded series of three von Frey filaments in ascending order (1, 2 and 6 g) (Aesthesio® von Frey Kit, Touch-Test Sensory Evaluator, Ugo Basile, Varese, Italy) onto the ipsilateral vibrissal pad were measured one day before surgery ("pre") and 7, 14, 21 and 28 days after IoN-CCI. Each animal was stimulated 3–5 times per filament to determine the response score for all three filaments. The response was categorized as score 0 (a complete lack of response), 1 (a stimulus detection), 2 (a withdrawal reaction), 3 (an escape/attack response) or 4 (asymmetric face grooming) (Vos et al., 1994). This categorization is widely used and accepted in the field. The grooming signed as four does not represent any regular grooming behaviour expected from the animals, but a series of three or more asymmetric grooming movements towards the injured region, and was validated as a reliable and specific measure of nociceptive response (Vos, Hans, & Adriaensen, 1998). For each rat, the mean of the assessed response to three filaments was calculated as the score used in statistical analysis (Deseure, Koek, Adriaensen, & Colpaert, 2003). Lower scores indicated a weak responsiveness to mechanical stimulation, while higher scores indicated a strong, hypersensitivity responsiveness. The criterion for exclusion of animals from statistical analyses was predefined for IoN-CCI–placebo group (consistently decreased reactivity in post-surgery period when compared to baseline values), and for sham–placebo group (consistently increased reactivity in post-surgery period when compared to the baseline values, the mean difference being more than or equal to 1.25 score points). The animals treated with DK-I-56-1 could not have been excluded.

Throughout Experiment 2, a slightly different series of von Frey filaments (0.6, 1.4 and 4 g) was applied to the ipsilateral vibrissal pad. Since the number of disposable filaments was limited, it was decided in advance to choose two series of similar filaments in ascending order to be used in Experiment 1 and Experiment 2. The response data were obtained one day before surgery ("pre") and 7, 14, 21, 28 and 35 days after surgery; the latter three measurements were conducted only in those animals that met the criterion of neuropathy on day 14 and were, thus, subjected to treatment. The scoring system used to assess the rat's reaction to the stimulus was the same as described above.

2.8 | Face grooming recordings

In Experiment 1, face grooming as a spontaneous activity was recorded. Face grooming during body grooming represents a reliable ethological measure to control for possible non-specific treatment effects, such as sedation or motor impairment (Deseure & Hans, 2015). Behaviour was videotaped for 10 min two days before surgery and 2, 4, 8 and 15 days after surgery (i.e. approximately 18 hr after 1, 3, 7 and 14 applications of treatment). The amount of time spent on face grooming was determined using a stopwatch. Videotaped behaviour was analysed by two experimenters who were blind to the experimental group of rats.

2.9 | Statistical analysis

All data are expressed as mean ± *SEM*. Pharmacokinetic parameters were calculated using PK Functions for Microsoft Excel software (by Joel Usansky, Atul Desai and Diane Tang-Liuwere). In Experiments 1 and 2, von Frey response score data were analysed using one-way analysis of variance (ANOVA) for the given day, with treatment as between-subject factor. Post hoc comparisons, where

applicable, were performed using SNK test. The parametric analysis of the response to von Frey filaments, as an ordinal variable, was well validated as statistically acceptable and provides improved coherence between the statistical output and graphical representation of data obtained in IoN-CCI (Deseure et al., 2003). Face grooming during body grooming data in Experiment 1 were analysed non-parametrically (Kruskal–Wallis test and Friedman test). Differences were considered significant when $p < 0.05$. Statistical analysis was performed using SigmaPlot 11 software (Systat Software Inc., Richmond, USA).

3 | RESULTS

3.1 | In vitro activity of DK-I-56-1 and DK-I-87-1 on $GABA_A$ receptors

In a set of experiments with a panel of subunit combinations expressed in *X. laevis* oocytes, we extended our investigation (Knutson et al., 2018) of the α6-selectivity of DK-I-56-1 and DK-I-87-1 to eight $GABA_A$ receptor subtypes, and confirmed the $GABA_A$ receptor subtype selectivity of DK-I-56-1, the deuterated version of PZ-II-029. In the panel of receptors investigated, we did not only include α6β3δ, but also the binary α6β3 receptors, because the latter receptors likely represent a fraction of receptors in oocytes, possibly contaminating current measurements at ternary receptors, and because they conceivably could also be present in TG. While α6β3γ2-injected oocytes have highly variable GABAmax currents (Figure 1b), α6β3- and α6β3δ-injected oocytes display more homogenous characteristics. However, GABA-induced currents of these receptors were quite low, likely due to the fact that GABA is a partial agonist at these receptors (Sieghart & Savić, 2018). Variability in the maximum currents results from a variability of the total number of receptors formed as well as from a variability of the receptor composition in the oocytes, despite tightly controlled expression conditions. A variable extent of α6β3 receptors formed in α6β3γ2-injected oocytes thus causes a larger variability in the maximum current than in α6β3δ-injected oocytes. In α6β3γ2, DK-I-56-1 induced a left shift of the GABA concentration–response curve, thus increasing GABA potency, and a weak increase in the maximal GABA-induced currents (data shown in Supporting Information Figure S1a). Due to the very low GABA-induced currents in α6β3- and α6β3δ-injected

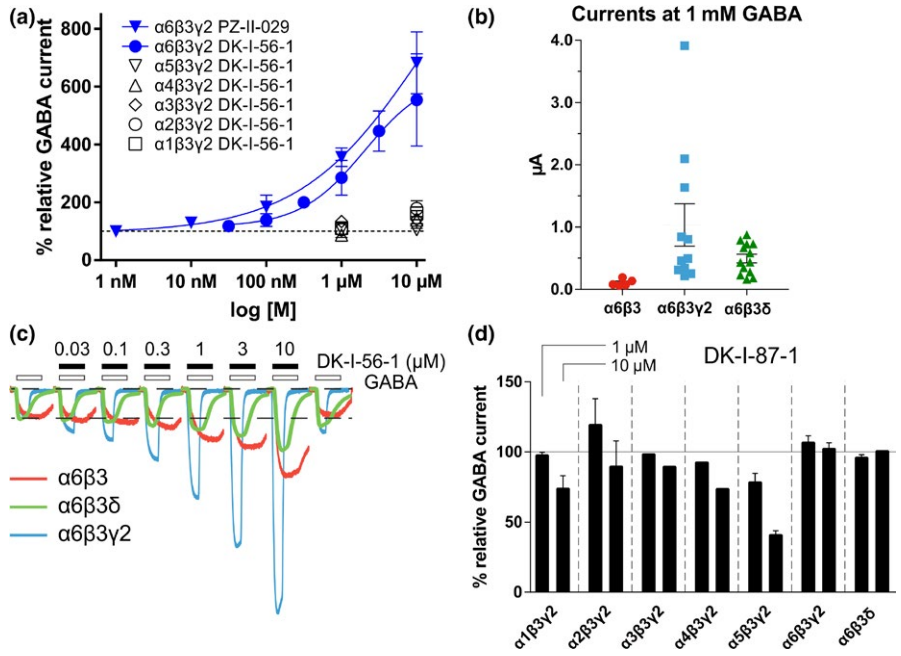

FIGURE 1 (a) Concentration–response curves of the modulation of GABA EC3–6 (100%) currents by increasing concentrations of PZ-II-029 and DK-I-56–1 in the α6β3γ2 receptor. Concentration–response curves for PZ-II-029 were taken from Varagic et al. (2013). Other receptor subtypes were investigated only at 1 and 10 µM DK-I-56-1, because this compound did not significantly modulate GABA EC3–6 at these receptors below 1 µM concentration. (b) Currents elicited by 1 mM GABA in α6β3γ2-, α6β3δ- and α6β3-injected oocytes. (c) Representative traces of the cells expressing α6β3γ2, α6β3δ and α6β3 receptors, while applying GABA at a fixed concentration (0.3 µM for α6β3γ2, 10 µM for α6β3 and 100 µM for α6β3δ) and an increasing concentration of DK-I-56-1. The traces are scaled to display the reference traces (GABA alone at EC3–6 for α6β3γ2 receptor and ~EC90 for α6β3δ- and α6β3-injected cells) at the same size (see panel 1B for the current levels of the respective subunit combinations) so that the modulatory efficacy can be readily compared. (D) Screening receptor subtypes for modulatory effects of DK-I-87-1. Bars per subunit combination represent compound concentrations of 1 and 10 µM, respectively. Data are presented relative to reference currents elicited by GABA alone (~ GABA EC3-6 for all αβ3γ2 receptors, ~ EC90 for α6β3δ receptors)

oocytes, currents could not be measured at GABA EC3–6. However, in both α6β3 and α6β3δ receptors, DK-I-56-1 did not change potency but enhanced GABA efficacy only, but to a much stronger extent than at α6β3γ2 receptors (see Supporting Information Figure S1c,b, respectively). It was thus possible to assess the modulation of α6β3 and α6β3δ receptors at GABA EC90. Recently summarized evidence indicated that at the upper limit of the estimated extrasynaptic GABA concentration in the brain (2.5 μM), the GABA-induced currents of the highly GABA-sensitive α6β3δ (and, if they exist physiologically, α6β3) receptors are 80% of their respective maximal GABA-induced currents and above (Mortensen, Patel, & Smart, 2012; Sieghart & Savić, 2018). Measuring the effects of drugs at GABA EC90 at these receptors is thus physiologically relevant. Supporting Information Figure S1d exemplifies the currents elicited by various GABA concentrations at the three $GABA_A$ receptor subtypes.

We confirmed that PZ-II-029 and DK-I-56-1 have comparable receptor subtype selectivity. Both compounds did not or only weakly modulated GABA-induced currents at αxβ3γ2 $GABA_A$ receptors containing α1, α2, α3, α4 or α5 subunits below 1 μM concentration (Figure 1a and Varagic et al., 2013) but substantially enhanced GABA-induced chloride flux at α6β3γ2, α6β3δ or α6β3 receptors (Figure 1a,c) already at sub-μM or low μM concentrations. Within methodological precision, and as expected, the deuterated analogue behaves pharmacologically as the parent compound. Example current recordings for α6β3γ2, α6β3δ or α6β3 receptors are shown in Figure 1c and in Supporting Information Figure S1. In contrast to DK-I-56-1, DK-I-87-1 did not significantly modulate GABA-induced currents at α6 receptors and only weakly modulated other receptor subtypes investigated at 1 μM concentration (Figure 1d). It thus represents an appropriate control compound for behavioural studies.

3.2 | Pharmacokinetic studies

The concentration–time profiles of DK-I-56-1 in rat plasma and brain following intraperitoneal administration of a 10 mg/kg dose in the nanoemulsion, with the calculated pharmacokinetic parameters, were similar to those given in Knutson et al. (2018) that were obtained with a suspension formulation, and given in Supporting Information Figure S2. The kinetic curves of DK-I-56-1, with maximum concentrations above 10 μM in plasma and above 1 μM in brain, confirmed its ability to be absorbed and distributed in brain, as previously demonstrated (Knutson et al., 2018). Both plasma and brain tissue kinetics were relatively slow with half-lives being approximately 6 and 13 hr, respectively. While the free fraction of DK-I-56-1 determined by rapid equilibrium dialysis in blood was exceptionally low (below the limit of quantification), the free fraction in brain tissue equalled 19.45%.

The concentrations of DK-I-56-1 in rat plasma and brain tissue 1 hr following i.p. administration of Wisden 2, 5, 10 and 20 mg/kg doses are shown in Supporting Information Table S1. The 10 mg/kg dose resulted in concentrations in plasma and brain in the micromolar, or close to micromolar range, respectively, and was thus selected for further study. Supporting Information Table S1 also shows the concentrations in plasma and brain obtained following subacute (6 days) or subchronic (11 days) DK-I-56-1 dosing. The concentrations attainable in repeated-dose studies were in general agreement with those expected on the basis of the pharmacokinetic profile presented in Supporting Information Figure S2, meaning that administration of multiple doses of DK-I-56-1 may only result in slight quantitative changes in elimination pathways, and enabling the persistence of estimated free brain concentrations in the minimum to maximum range 10–300 nM throughout the 24-hr period.

The results presented in Supporting Information Table S2 show an exceptionally stable plasma level that is maintained at about 1 μM as long as 24 hr following the last repeated dose of DK-I-87-1. On the contrary, the concentrations obtainable in brain tissue were relatively low in general and particularly low following 11 doses, suggesting that some qualitative changes, possibly in brain permeability and/or metabolism, occurred during repeated dosing.

3.3 | Behavioural studies

3.3.1 | Experiment 1: Prophylactic antinociceptive effect of DK-I-56-1 in the neuropathic pain model

In this experiment, animals were treated either with daily injections of 10 mg/kg DK-I-56-1 or placebo on days 1 to 14 after surgery, or sham surgery, respectively, to assess a prophylactic effect of the pyrazoloquinolinone ligand on the development of neuropathic pain (the hypersensitivity response). Figure 2a summarizes the timeline of the experiment.

The results of the assessment of the hypersensitivity response in Experiment 1 are summarized in Figure 2b (response score to von Frey filament stimulation for all four groups, measurements were performed in steady state before the next injection of drug or placebo). Three rats were not included in statistical analysis due to exclusion criteria; all raw data are presented in Supporting Information Table S3.

As shown in Figure 2b, basal response scores ("pre") were similar among all four groups ($F(3,39) = 1.72$; $p = 0.18$); moreover, the scores of the two IoN-CCI groups (at about 1.7 points) were virtually identical. Although the response to von Frey filaments in the IoN-CCI–placebo group assessed

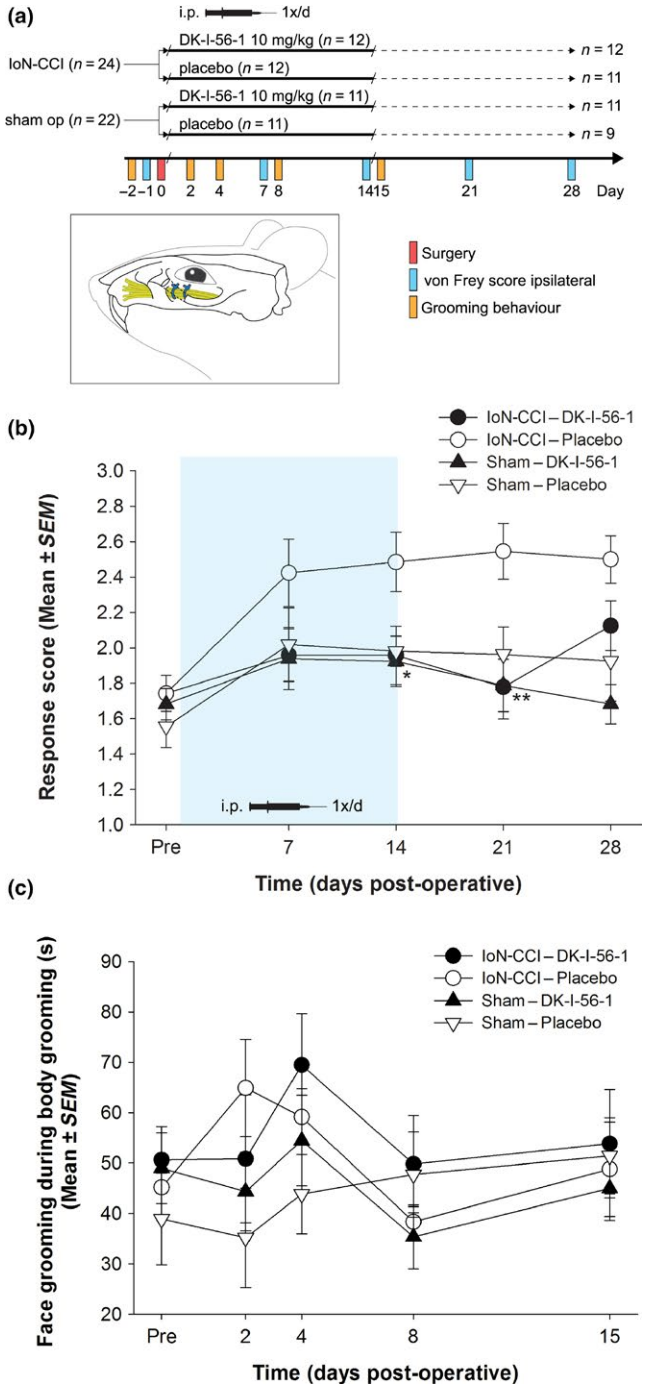

FIGURE 2 (a) Treatment group timeline of Experiment 1. (b) Response score to von Frey filament stimulation for all four groups. Response to the application of a graded series of three von Frey filaments onto ipsilateral (left) vibrissal pad of IoN-CCI and sham-operated animals was measured one day before surgery (pre) and on post-operative days 7, 14, 21 and 28. Treatment (10 mg/kg DK-I-56-1 or placebo) was administered during 14 days, started one day after operation. Experimental groups: IoN-CCI–DK-I-56-1, IoN-CCI–placebo, sham–DK-I-56-1 and sham–placebo consisted of 12, 11, 11 and 9 animals, respectively. Values are presented as mean ± *SEM*. Data were analysed using one-way ANOVA and SNK post hoc test. Significant differences versus placebo are indicated by * = $p < 0.05$. (c) Face grooming during body grooming recordings were taken two days before surgery (pre) and 2, 4, 8 and 15 days after surgery. See b for treatment groups. Values are presented as mean ± *SEM*

seven days after surgery reached a 2.4 score points, overall statistical difference among the four groups was not found at this time point ($F(3,39) = 1.67$; $p = 0.19$).

Significant differences between the four groups on post-operative days 14 ($F(3,39) = 3.2$; $p < 0.05$), 21 ($F(3,39) = 5.07$; $p < 0.05$) and 28 ($F(3,39) = 5.24$; $p < 0.05$) were revealed by one-way ANOVA applied on the response scores. Post hoc tests demonstrated significant differences between IoN-CCI–placebo and sham–placebo groups on all three time points, suggesting that the performed IoN-CCI induced a consistent neuropathic-like response. Post hoc comparisons also showed that repeated treatment with DK-I-56-1 can provide a preventive effect on the development of neuropathic pain, based on a significant difference between IoN-CCI–DK-I-56-1 and IoN-CCI–placebo groups on post-operative days 14 and 21. On post-operative day 28, i.e. 14 days after the last dose of DK-I-56-1, the significance of the effect of DK-I-56-1 disappeared. Thus, DK-I-56-1 demonstrated a capability to suppress the development of neuropathic pain related to the constriction of branches of the trigeminal nerve during treatment administration and for seven days after discontinuing treatment.

The results of the assessment of face grooming during body grooming, as a physiological manifestation of the rat's behaviour on which IoN-CCI is expected to have little or no effect, are summarized in Figure 2c. The level of activity was distinct on each of the five days monitored, and no significant differences among groups were detected. This parameter, characterized by a substantial variability, demonstrated the lack of sedation-like or motor-impairing influences of DK-I-56-1, at least at the time of behavioural testing.

3.3.2 | Experiment 2: Therapeutic effect of DK-I-56-1 in the neuropathic pain model

The affirmative results of Experiment 1 ethically justified the use, in the next experiment, of a suitable compound of the same chemotype (DK-I-87-1) as a negative control for the α6 $GABA_A$ receptor-mediated mechanism(s). In this experiment, animals were treated with daily injections of 10 mg/kg DK-I-56–1, or 10 mg/kg DK-I-87-1, or placebo, on days 15 to 28 after surgery, respectively, to assess a therapeutic effect of the pyrazoloquinolinone ligands on the fully developed neuropathic hypersensitivity. Figure 3a summarizes the timeline of the experiment.

Among the tested animals, five rats were not included in statistical analysis due to exclusion criteria, while one rat was highly aggressive from the beginning of treatment

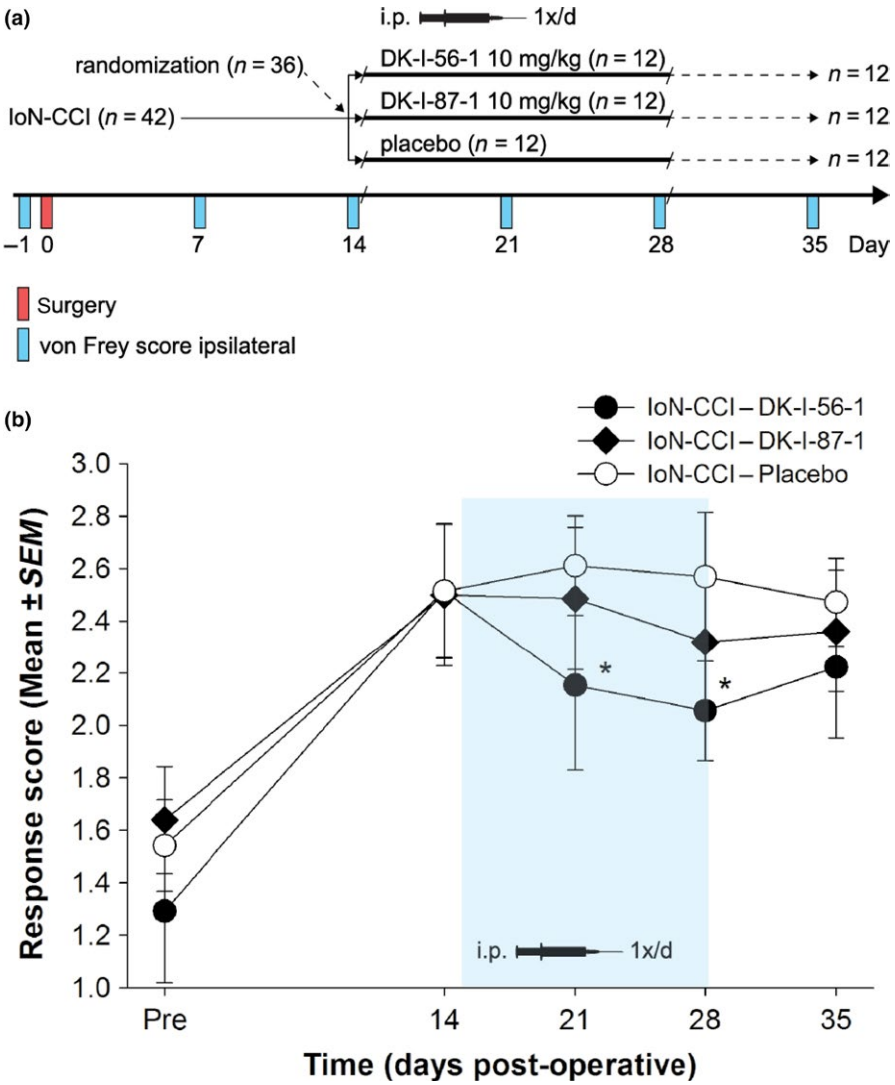

FIGURE 3 (a) Treatment group timeline. (b) Response score to von Frey filament stimulation for three groups. Response to the application of a graded series of three von Frey filaments onto ipsilateral (left) vibrissal pad of IoN-CCI animals was measured one day before surgery (pre) and on post-operative days 14, 21, 28 and 35. Treatment (DK-I-56-1, DK-I-87-1 or placebo) was administered after assessment of mechanical hypersensitivity on day 15. Animals were treated during 14 consecutive days. Twelve animals were in each treatment group (DK-I-56-1, DK-I-87-1 and placebo). Values are presented as mean ± *SEM*. Data were analysed using one-way ANOVA and SNK post hoc test. Significant differences versus placebo are indicated by * = $p < 0.05$

and was hence discontinued; all raw data are presented in Supporting Information Table S4. Similar to Experiment 1, IoN-CCI resulted in a mean response score of about 2.5 on post-operative day 14 (Figure 3b). One-way ANOVA showed a significant difference between groups on days 21 ($F(2,33) = 3.50$; $p < 0.05$) and 28 ($F(2,33) = 4,84$; $p < 0.05$), but not on day 35 ($F(2,33) = 1,22$; $p = 0.31$). Post hoc tests revealed that repeated doses of DK-I-56-1 from day 15 to day 28 resulted on days 21 and 28 in a statistically significant decrease in von Frey scores when compared to rats treated with placebo. The rats treated with DK-I-87-1 did not differ significantly compared to either placebo- or DK-I-56-1-treated rats.

4 | DISCUSSION

Chronic constriction injury of the infraorbital branch of the trigeminal nerve (IoN-CCI), with elicited hyperresponsiveness to mechanical stimulation of the whisker pad skin (i.e. hypersensitivity, cf. Hansson & Bouhassira, 2015) that is not accompanied by a latency or refractory period, has been validated as a model of trigeminal neuropathic pain (Deseure & Hans, 2015; Iwata, Imamura, Honda, & Shinoda, 2011). Using this model, we tested the hypothesis that positive modulation of α6-containing receptors with an appropriate ligand recently discovered by our group (Knutson et al., 2018) may prevent the development and/or suppress maintenance

of trigeminal neuropathic pain. Both parts of the hypothesis were confirmed with a 10 mg/kg dose of DK-I-56-1 that was repeatedly administered throughout the first or the second 14-day period after surgery and thus during development or maintenance of neuropathic pain, respectively (Deseure & Hans, 2015). Notably, the 10 mg/kg dose of DK-I-56-1 did neither acutely affect anxiety level, muscle strength and basic sensorimotor capabilities of rats as assessed in the elevated plus maze, grip strength test and cued water-maze training, respectively (Knutson et al., 2018), nor affect, in the present experiment 1 of its repeated administration, face grooming during body grooming, as a part of general body grooming behaviour, non-specific to pain (Deseure & Hans, 2015).

Effect of DK-I-56-1 treatment on neuropathic pain development could still be observed at day 21, and thus, 7 days after treatment was terminated, but not thereafter. Analogous results were presented for a selective agonist at 5-HT_{1A} receptors, in a similar IoN-CCI protocol (Deseure et al., 2003). In the experiment investigating the effect of DK-I-56-1 on already established neuropathic pain, treatment effects were present after 6 as well 13 daily doses, and declined within one week afterwards. The effects are thus not prolonged, but determined by its plasma or brain levels that fade away after treatment termination. The prolonged effect in the first experiment might then have reflected the time needed for the pathological process to flare up after initial suppression by treatment. It must be emphasized that, in contrast to previous studies with other compounds (Deseure et al., 2003; Gris et al., 2016; Michot, Bourgoin, Viguier, Hamon, & Kayser, 2012), the actions of DK-I-56-1, DK-I-87-1 or placebo on the development or maintenance of neuropathic pain were investigated under steady-state conditions. In this vein, mild hyperlocomotion noticed in both rats and mice acutely treated with DK-I-56-1 but not with DK-I-87-1 (Knutson et al., 2018), while reflecting a subtle increase in motivational drive possibly applicable in depressive conditions (Bewernick, Urbach, Broder, Kayser, & Schlaepfer, 2017), could not have affected behavioural measurements in the present study.

The α6 subunit has been most extensively studied in the cerebellum, where it is known to form synaptic α6β3γ2 receptors and extrasynaptic α6β3γ2 and α6β3δ receptors (Jechlinger, Pelz, Tretter, Klausberger, & Sieghart, 1998; Nusser, Sieghart, & Somogyi, 1998). In TG, immunohistochemical studies in 2- to 3-week-old rats indicated that the majority of neurons express α1, α3, α4, α5, β2/3 and γ1-3 subunits in their cell bodies, whereas α6 and δ subunits were detected only in cell bodies of a subset of small neurons (Hayasaki et al., 2006). However, in a subsequent immunofluorescent study performed in adult rats (Puri et al., 2012), 86%, 74% and 74%, of all small, medium and large TG neurons, respectively, expressed α6-containing $GABA_A$ receptors. Age differences in the animals investigated might have contributed to the different abundance of α6-containing $GABA_A$ receptors in these studies. The subunit composition of α6-containing $GABA_A$ receptors within the TG neurons is currently unclear, but both α6β2/3γ2 and α6β2/3δ receptors might be present in these neurons (Hayasaki et al., 2006). The present electrophysiological analysis demonstrated that in contrast to DK-I-87-1, that is inactive at α6-receptors and slightly active at some of the other $GABA_A$ receptors at 1 μM concentrations, DK-I-56-1 concentration-dependently enhances the action of GABA at α6β3γ2, α6β3δ and α6β3 receptors at 0.1 μM and above, but requires concentrations of 1 μM and above for starting the modulation of most of the other $GABA_A$ receptor subtypes investigated. The functional selectivity of DK-I-56-1 for α6-containing receptors is further accentuated by the low-to-moderate free brain concentrations (10–300 nM) achieved by this compound, as indicated in the present and a previous study (Knutson et al., 2018). Such concentrations do not allow modulation of GABA-induced currents of other $GABA_A$ receptor subtypes possibly present at the TG neurons.

Rapid firing of TG neurons mediates neuropathic pain evoked by the IoN-CCI protocol (Vos et al., 1994). Although cell bodies of mammalian sensory ganglia are generally devoid of synaptic contacts, exogenous GABA can induce Cl^- currents in all TG neurons examined. Thus, all $GABA_A$ receptors present at these cell bodies are extrasynaptic (Hayasaki et al., 2006). In TG, GABA is synthesized and released by 70% of all neurons, but can be accumulated only by their surrounding satellite cells. It has been hypothesized that frequently firing sensory neurons can lead to elevated extracellular K^+ concentrations during repolarization after action potentials, which might induce GABA release from satellite cells and/or neurons, providing an inhibitory feedback to sensory neurons (Hayasaki et al., 2006). Subsequent activation of the highly GABA-sensitive α6-containing receptors (Karim et al., 2013; Mortensen et al., 2012) on the cell bodies of TG neurons might then have inhibited the firing of the respective TG neurons and thus also the synaptic transmission at the central terminals of the trigeminal nucleus caudalis. DK-I-56-1 might have enhanced the effect of endogenous GABA and by that sufficiently reduced synaptic transmission at the respective terminals of the trigeminal nucleus caudalis to suppress pain perception.

Kinetic profiles of DK-I-56-1 and DK-I-87-1, formulated into biocompatible nanoemulsions with high content of water, were sufficiently slow that even once-daily dosing has been appropriate for reaching effective concentrations during most of the inter-dose interval. In particular, incorporation of 20% of oil phase in the nanoemulsion formulation of DK-I-56-1 was shown to result in substantial (threefold vs. intravenous, and fivefold vs. intraperitoneal route) increase in the brain half-life when compared to the suspension formulation (Knutson et al., 2018), which is concordant with the formulation effects previously seen with diazepam (Đorđević

et al., 2015). Remarkably, the free DK-I-56-1 concentration in plasma was extremely low and non-calculable but still resulted in significant concentrations of the compound in the brain. Due to the lipophilicity of DK-I-56-1, brain uptake presumably was unimpaired. A similar observation, that brain uptake is not markedly influenced by low free plasma fraction of a ligand, has been made with a series of lipophilic N-benzyl-substituted phenethylamines (Ettrup et al., 2011). Interestingly, the trigeminal ganglion not only is rich in lipids, but also is located outside the blood–brain barrier (Eftekhari et al., 2015). However, the pharmacological targets of DK-I-56-1 may also be located more rostrally, such as in the synaptic terminals of α6-containing TG neurons in the spinal trigeminal subnucleus caudalis, or anywhere else in the trigeminal pain pathway, and thus behind the blood–brain barrier (Shibuta et al., 2012). Independently of where the α6-containing receptors are located that mediate the effects of DK-I-56-1, its pharmacologically effective concentration is expected to be related to the brain concentration; namely, it has recently been demonstrated that the unbound fraction in brain tissue homogenates of a set of several low molecular weight drugs highly correlates with that in the dorsal root ganglion, which can be taken as a more general surrogate for the situation in peripheral nerve tissue (Liu et al., 2018).

Although DK-I-56-1 is a highly selective positive modulator of α6-containing $GABA_A$ receptors, we cannot exclude alternative sites of action of this compound, which is a clear limitation of the present study; namely, its action might also be in part mediated by interactions with some of the high-affinity benzodiazepine binding site subtypes of $GABA_A$ receptors (Hulse et al., 2015), at which DK-I-56-1 acts as a high-affinity, electrophysiologically silent ligand (functional benzodiazepine antagonist; Knutson et al., 2018; Varagic et al., 2013). Furthermore, although it has been demonstrated that DK-I-56-1 displayed negligible displacement in a panel of radioligand binding assays for 46 receptors, transporters and channels, except for the benzodiazepine site of $GABA_A$ receptors and very weak binding at $5\text{-}HT_7$ and μ-opioid receptors (Knutson et al., 2018), we also cannot exclude a possible functional modulation by this ligand of these and other targets. To investigate whether the effects of DK-I-56-1 on neuropathic pain could be blocked by an antagonist at the proposed site of its action, the α6+β3- interface of $GABA_A$ receptors would be the method of choice to confirm its site of action. Unfortunately, such an antagonist currently is not available (Sieghart & Savić, 2018). The competitive non-selective GABA site antagonist bicuculline was sometimes used intrathecally or intradermally in acute pain models (e.g. Nadeson & Goodchild, 2000; Pathirathna et al., 2005) to probe for the involvement of GABAergic neurotransmission. In our chronic pain model, continuous intrathecal infusion of bicuculline would be required to block the action of DK-I-56-1 under steady-state conditions. However, this would result in a complex, unpredictable outcome, including the possible expression of the proconvulsant properties of bicuculline. Further experiments will thus have to be performed to identify the actual target of action of DK-I-56-1 against neuropathic orofacial pain and its location in the peripheral or central part of the trigeminal system.

In summary, we demonstrated that DK-I-56-1, a functionally selective PAM at $GABA_A$ receptors containing the α6 subunit, significantly reduced both the development and the maintenance of trigeminal neuropathic pain. These effects were assessed in the steady state and thereby were devoid of any behaviourally non-specific influences. These data for the first time demonstrate that positive modulators of α6-containing $GABA_A$ receptors might represent a mechanistically novel treatment option for trigeminal neuropathic pain, with promising potential for clinical development, as underlined by their lack of benzodiazepine-like pharmacological properties (Knutson et al., 2018).

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CONFLICT OF INTEREST

Authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Sieghart, Savić, Cook, Scholze and Ernst participated in research design. Vasović, Divović, Brković, Savić, Steudle, Treven, Fabjan, Knutson and Scholze conducted experiments. Vasović, Divović, Steudle and Treven performed data analysis. Vasović, Divović, Savić, Treven, Obradović, Ernst, Sieghart, Cook and Knutson wrote or contributed to the writing of the manuscript.

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SUPPORTING INFORMATION

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