

Histamine H₃ receptor blockade alleviates neuropathic pain through the regulation of glial cells activation

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ABSTRACT

Neuropathic pain is a disorder affecting the somatosensory nervous system. However, this condition is also characterized by significant neuroinflammation, primarily involving CNS-resident non-neuronal cells. A promising target for developing new analgesics is histamine H₃ receptor (H₃R); thus, we aimed to determine the influence of a novel H₃R antagonist/inverse agonist, E-98 (1-(7-(4-chlorophenoxy)heptyl)-3-methylpiperidine), on pain symptoms and glia activation in model of neuropathic pain in male mice (chronic constriction injury to the sciatic nerve). We investigated the effects of single intraperitoneal (i.p.) (1, 5, 10, 20 mg/kg) and intrathecal (i.th.) (10, 30, 60 µg/5 µL) E-98-injections on mechanical (von Frey) and thermal (cold plate, tail flick) stimuli. The effect of chronic E-98 (10 mg/kg, i.p.) treatment and its influence on glia activation within the lumbar spinal cord was investigated. The anti-inflammatory properties of E-98 (10 µM) were screened in primary microglial and astroglial cell cultures. We assessed the presence of H₃R within the spinal cord of control and neuropathic animals and in cell cultures. E-98 attenuated nociceptive responses in a dose- and time-dependent manner, and this effect is correlated with reduced microglia and increased astroglia activation. *In vitro* studies showed a decreased pro-inflammatory IL-6 level in both cell cultures. We observed co-localization of H₃R with spinal neurons, microglia, and astrocytes and in primary glial cell cultures. We suggest that an analgesic effect of E-98 is partially due to the modulation of glial activation. We explore a new mechanism of H₃R antagonists/inverse agonists analgesic action, bringing the potential benefits in pain management strategies.

1. Introduction

All of the functional constituents of the nociceptive transmission pathways work in concert to provide protection to the organism. While acute pain is a normal sensation that alerts individuals to a possible injury, chronic pain lasting more than 12 weeks loses its protective function. A lesion or a disease of the somatosensory nervous system may generate neuropathic pain, which is much more challenging to treat than other types of chronic pain. Pharmacological treatment of this type of pain is often ineffective, resulting in increased healthcare utilization and medication prescriptions. Despite the availability of treatment

regimens, a large group of patients show only partial alleviation of pain or experience no relief whatsoever [1,2]. The inadequate response to various drug therapies in neuropathic pain pathology constitutes a substantial unmet need, and all of those difficulties prompt researchers to seek new targets for creating effective and safe neuropathic pain therapy.

A growing body of evidence indicates that the histaminergic system is a therapeutic target for the management of neuropathic pain [3–6]. Histamine is an endogenous biogenic amine acting as a peripheral and central modulator. In the central nervous system (CNS), it acts as a neurotransmitter and contributes to the modulation of a variety of

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functions, such as arousal, circadian rhythms, learning, and memory, feeding, energy balance, and pain [4,7]. The effects of histamine are mediated by the activation of four receptor subtypes (H₁R-H₄R), which belong to the G-protein-coupled receptors (GPCRs) family. Due to the role of this amine in a multiplicity of physiological and pathological processes, its receptors are viable drug targets. Our recent studies [3,8] indicate that H₃R is a promising target for research into new analgesic drugs and for use as a co-adjuvant in pain therapy. A range of H₃R antagonists/inverse agonists have been discovered and advanced into clinical trials, among which pitolisant was approved in 2016 for the treatment of narcolepsy [9,10]. Consequently, the interest in the clinical application of novel H₃R antagonists/inverse agonists has increased.

We have already described a series of chlorophenoxyalkylamine derivatives with a promising pharmacological profile and sub-micromolar affinity for hH₃R [11]. The most promising compound, E-98 (1-(7-(4-chlorophenoxy)heptyl)-3-methylpiperidine), was tested in the Antiepileptic Screening Program (ASP) at the National Institute of Neurological Disorders and Stroke (USA). According to ASP, E-98 has a robust protective effect in the maximal electroshock (MES) test, a well-known model for generalized tonic-clonic seizures. Moreover, the tested compound did not show any neurological deficit indicated by ataxia, loss of placing response, and muscle tone in the Neurotoxicity (TOX) test. Those promising data prompt us to explore further effects of this compound.

Neuropathic pain is a complex disease strongly modulated by neuro-immune interactions [12–14]. The development and progression of this pathology are accompanied by profound central inflammation, which involves the CNS-resident non-neuronal cells, mainly microglia and astrocytes. For many years, the role of glial cells was associated with neurotrophic support and immune protection. However, recent studies, including those provided by us, have shown that glial cells are not just silent elements of the nervous system but active players capable of directly altering the nociceptive transmission [12,15,16]. Undoubtedly, the histaminergic system is a potent regulator of immune responses, however the role of H₃R in glial cells activation remains unknown. Notably, several studies, also provided by us, demonstrated the potential analgesic effect of H₃R antagonist/inverse agonist in neuropathic pain models [3,5,6,17–19].

Importantly, pharmacological treatment of neuropathic pain is often ineffective, resulting in increased healthcare utilization and medication prescriptions. Despite the availability of a broad spectrum of treatments, many patients experience only partial or no pain alleviation, and pain relief for most analgesic reach a modest overall outcome [20–22]. Therefore, further research in this field is needed to identify new targets and develop more effective and targeted therapies for neuropathic pain therapy.

Answering those challenges, in our present study, we investigated the effect of the intraperitoneal (i.p.) and intrathecal (i.th.) administration of a newly synthesized antagonist/inverse agonist of H₃R (E-98) in a preclinical model of neuropathic pain (chronic constriction injury, CCI) in mice after a single injection, as well as in chronic treatments schedule. Here, we have also analyzed the influence of the novel H₃R antagonist/inverse agonist on glial cells activation at the lumbar spinal cord level under neuropathic pain conditions. Moreover, we performed immunofluorescence studies to determine the cellular localization of H₃R in the spinal cord of naïve and neuropathic animals. Additionally, we tested our hypothesis using *in vitro* studies, investigating the direct impact of the tested compound on the inflammatory response of microglia and astrocytes in primary cell cultures. Here, we performed a set of ADME tests, together with screening for compound toxicity, to build a pharmacological profile of the new H₃R antagonist/ inverse agonist.

2. Materials and methods

2.1. *In Vitro* pharmacology

2.1.1. Human H₁R, H₂R, H₃R and H₄R binding assays

Radioligand binding was performed using membranes from CHO-K1 cells stably transfected with the human H₁R (PerkinElmer). All assays were carried out in duplicates. 50 µL working solution of the tested compounds, 50 µL [³H]-Pyrilamine (final concentration 1.5 nM) and 150 µL diluted membranes (5 µg protein per well) prepared in assay buffer (50 mM Tris, pH 7.4, 5 mM MgCl₂) were transferred to polypropylene 96-well microplate using 96-wells pipetting station Rainin Liquidator (Mettler-Toledo). Mepyramine (10 µM) was used to define nonspecific binding. The microplate was covered with sealing tape, mixed, and incubated for 60 min at 27°C. The reaction was terminated by rapid filtration through GF/B filter mate presoaked with 0.5 % polyethyleneimine for 30 minutes. Ten rapid washes with 200 µL 50 mM Tris buffer (4°C, pH 7.4) were performed using a 96-well FilterMate harvester (PerkinElmer, USA). The filter mates were dried at 37°C in a forced air fan incubator, and then solid scintillator MeltiLex was melted on filter mates at 90°C for 4 min. The radioactivity on the filter was measured in MicroBeta TriLux 1450 scintillation counter (PerkinElmer, USA). Data were fitted to a one-site curve-fitting equation with Prism 6 (GraphPad Software) and K_i values were estimated from the Cheng–Prusoff equation. The [¹²⁵I]-iodoaminopotentidine binding assay was performed with CHO-K1 cells stably transfected with the hH₂R. [³H]-N^α-methylhistamine binding assay performed with HEK293 cells stably transfected with the hH₃R (data from [10]). Radioligand binding studies utilizing [³H]histamine and membrane preparations of Sf9 cells transiently expressing the human H₄R (hH₄R), co-expressed with G- protein Gα₁₂ and Gβ_{1γ2} subunits were used as described previously [23].

2.1.2. H₃R intrinsic activity

Intracellular cAMP accumulation was measured with homogenous TR-FRET immunoassay, using LANCE Ultra cAMP kit (PerkinElmer) and HEK293 cells, stably expressing human H₃R. An antagonist dose-response experiments were performed in a total assay volume of 20 µl in white 384-well plates, using 750 cells/well. (R)(-)-α-Methylhistamine (30 nM) and forskolin (10 µM) were added simultaneously to cell suspension together with tested compounds in appropriate concentrations (in range 0.0003–10 µM). Cell stimulation was performed for 30 min at room temperature. After the incubation period, five microliters of europium (Eu) chelate-labeled cAMP tracer, and 5 µl of ULIGHT-labeled anti-cAMP mAb working solutions were added, mixed, and incubated for 1 h. TR-FRET signal was read on the Spark microplate reader (Tecan Life Sciences). Measured TR-FRET, which was inversely proportional to cAMP quantities in the sample, was then normalized and presented as % of maximal cAMP accumulation in the assay determined for the forskolin alone-stimulated cells. Sigmoidal dose-response curve fitting was performed (GraphPad Software v8.4.3), and IC₅₀ values were extracted from the fitting. K_b values of the compounds were estimated according to the Leff-Dougall variant of the Cheng-Prusoff equation $K_b = IC_{50} / ((2 + ([Ag]/[EC_{50}])^n)^{1/n} - 1)$, where IC₅₀ is the concentration of antagonist that inhibits agonist response by a 50 %; [Ag] is the concentration of agonist employed in the assay, [EC₅₀] is the agonist EC₅₀ value in the assay and n is the Hill slope of the concentration-response curve of the agonist [24]. The results represent the mean of three separate experiments, each performed in triplicates.

2.1.3. Metabolic stability

The metabolic stability *in vitro* was estimated using mouse liver microsomes (MLMs, Sigma-Aldrich, St. Louis, MO, USA). The evaluation of the metabolic pathway was performed by the prolonged (180 min) incubation, followed by the *in silico* prediction of the most probable sites of metabolism by MetaSite 6.0.1 software (Molecular Discovery Ltd., Hertfordshire, UK).

2.2. Animals

Adult male Albino-Swiss CD-1 mice initially weighing 20–22 g were purchased from Charles River Laboratories (Hamburg, Germany). The animals were housed in groups of 6–8 under controlled conditions (temperature $21 \pm 2^\circ\text{C}$; 12-h light/dark cycle – lights on at 6 am) with *ad libitum* food and water. All experiments were carried out according to the recommendations of the International Association for the Study of Pain [25] and the NIH Guide for the Care and Use of Laboratory Animals, and were approved by the II Local Ethics Committee branch of the National Ethics Committee for Experiments on Animals based at the Maj Institute of Pharmacology, Polish Academy of Sciences, Krakow, Poland (24/2022, 18/2024). Care was taken to minimize animal suffering and minimize the number of animals used (3R policy).

2.2.1. Sciatic nerve surgery

Chronic constriction injury (CCI) to the sciatic nerve was performed to model neuropathic pain according to Bennett and Xie [26] and modified for mice [3]. The procedure was performed under isoflurane anesthesia (2 % isoflurane (Baxter, Mississauga, Ontario, Canada) in 100% oxygen with a flow of 1.5 L/min). The right sciatic nerve was exposed, and three ligatures (4/0 silk thread) were tied around the nerve with 1-mm spacing until a brief twitch was observed. All mice developed hypersensitivity to mechanical and thermal stimuli. No procedure was conducted on the control animals.

2.2.2. Drug administration

The compound used in the present study (E-98 (1-(7-(4-chlorophenoxy)heptyl)-3-methylpiperidine), was synthesized in the Department of Technology and Biotechnology of Drugs (Jagiellonian University Medical College, Krakow, Poland), as described previously [11]. Chemical structure and *in vitro* H₃R data of E-98 are presented in Fig. 1. The evaluation of selected ADMET parameters of E-98 is presented in supplementary material, section S2, while the summary is presented in Table 1. E-98 was dissolved in 10 %DMSO/10 % (2-Hydroxypropyl)- β -cyclodextrin/water and administered intraperitoneally (i.p.) at doses 1, 5, 10, and 20 mg/kg (in volume 10 ml/kg of body weight) or intrathecally (i.th.) at doses 10, 30, and 60 μl / 5 μl in single administration scheme (Scheme 1). In the chronic administration scheme, E-98 (10 mg/kg) was injected twice daily (starting from day 7th after nerve injury) for 7 following days (Scheme 1). The control group received the vehicle (10 %DMSO/10 %(2-Hydroxypropyl)- β -cyclodextrin/water) according to the same schedule.

2.2.3. Behavioral tests

All behavioral experiments were performed from 8 to 14 a.m. on naïve and neuropathic animals (14 days after CCI) according to the schedule presented in Scheme 1. Evaluations were performed blindly on drug administration. For the behavioral studies, mice were assessed before (pretest) experiments (data not shown) to evaluate the level of nociceptive responses. There were no significant baseline differences between the experimental groups. Experiments were conducted 30, 90, 180 min (von Frey test), 35, 95, 185 min (cold plate test), and 40, 100, 190 min (tail-flick test), after the compound administration (Fig. 3A-C; 4A-C), and 90 min (von Frey test), 95 min (cold plate test), and 100 min (tail-flick test) after morning E-98 injection at day 2nd, 5th and 7th (after the first injection) in the chronic treatment scheme experiment (Fig. 5A-C). The results from a single i.p. injection of E-98 (10 mg/kg) on pain symptoms in the acute pain model in mice are presented in Supplementary materials, Figure S8.

2.2.3.1. Von Frey test. The von Frey test was used to assess mechanical hypersensitivity [3]. Mice were placed in plastic cages with a wire net floor 5 min before the experiment for habituation. Mechanical sensitivity to non-noxious stimuli was measured by applying a set of

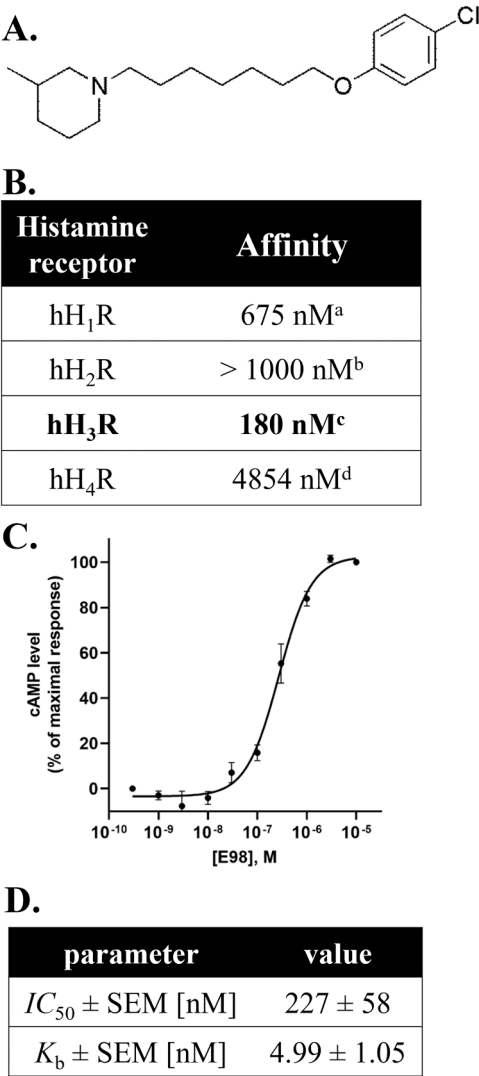
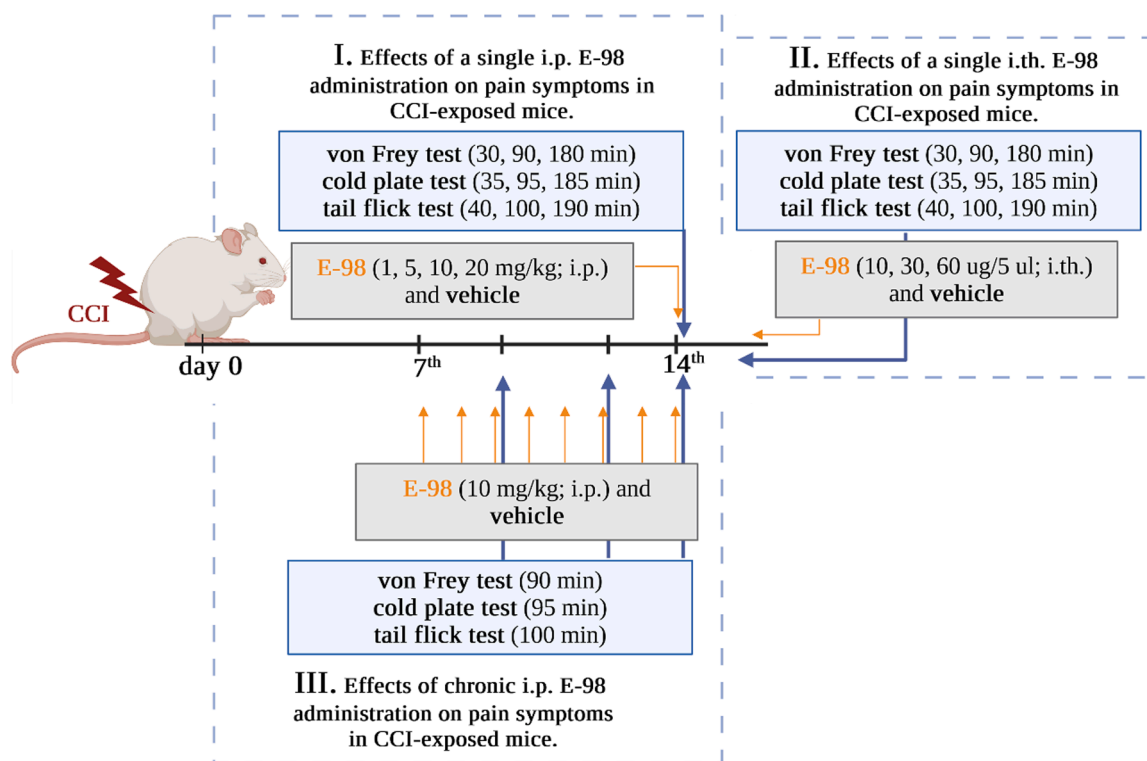


Fig. 1. Structure of E-98 (A), and the affinity at the human histamine receptors (B) and H₃R intrinsic activity (C,D). Structure of E-98 ((1-(7-(4-chlorophenoxy)heptyl)-3-methylpiperidine)) (A). ^a[³H]-pyrilamine binding assay performed with CHO-K1 cells stably transfected with the hH₁R; ^b[¹²⁵I]-iodoaminopotentidine binding assay performed with CHO-K1 cells stably transfected with the hH₂R. ^c[³H]-N^α-methylhistamine binding assay performed with HEK293 cells stably transfected with the hH₃R; data from [11]; ^d[³H]-histamine displacement assay with membrane preparation of Sf9 cells expressing hH₄R, co-expressed with G protein G_{oi2} and G_{β1γ2} subunits (B). cAMP accumulation studies in HEK293 cells stable expressing human H₃R (hH₃R), co-treated with forskolin, (R)(-)-α-methylhistamine and E-98 (C, D).

Table 1
Selected ADMET parameters of E-98.

PARAMETER	RESULTS
Permeability PAMPA assay (10 ⁻⁶ cm/s) ± SD	2.37 ± 0.52
Metabolic stability (% remaining after 180 min of incubation with MLMs)	92.4
CYP3A4 activity (% of control ± SD at 10 μM)	95.75 ± 5.8
HepG2 viability (% of control ± SD at 10 μM)	102.7 ± 24.3
BV–2 viability (MTT assay) (% of control ± SEM at 10 μM)	88.55 ± 3.9
RotaRod test (% MPE)	no effects
Open Field test (distance, speed, entries to inner zone, time in inner zone)	no effects



Scheme 1. Behavioral tests schedule [27].

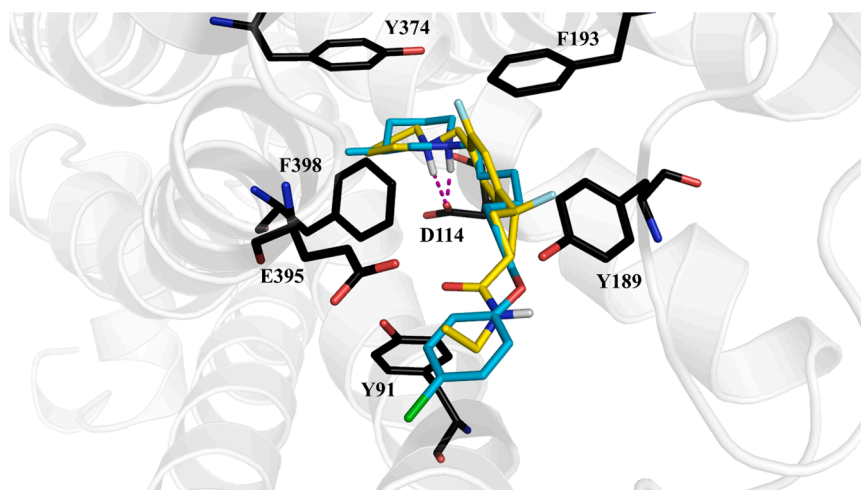


Fig. 2. Molecular modeling. Superposition of the binding modes of E-98 (cyan) and PF03654746 (yellow) in the H₃R(B) binding site. The salt bridge is marked in magenta.

calibrated nylon monofilaments (0.6–6 g; Stoelting) in serial increments on the mice's ipsilateral and contralateral hinds paw mid-plantar surface until a behavioral response (including paw withdrawal, shaking, and licking) was observed. The contralateral paw was not affected by compound treatment in CCI-exposed animals (data not shown). In the von Frey test, results are expressed as pressure [g] applied with the filament.

2.2.3.2. Cold plate test. Sensitivity to noxious thermal stimuli was assessed using the cold plate test (Cold/Hot Plate Analgesia Meter, Columbus Instruments), as previously described [3]. The mice were placed on the plate after it had been cooled to 2°C, and the cut-off latency was 30 s. The time taken to evoke nociceptive behavior in the mice (lifting, shaking, licking of the injured paw) was recorded as the response time

[s].

2.2.3.3. Tail flick test. The tail-flick test was used to measure spinal nociceptive responses to thermally induced pain using a tail-flick analgesia meter (Tail-Flick; Ugo Basile, Italy) as presented previously [3]. A focused beam of light was applied at two-thirds of the tail length to measure tail-flick latency. The time interval between the thermal stimulus's onset and the tail's withdrawal from the radiant heat was recorded. The cut-off latency was 9 s to prevent damage to a tail.

2.3. Primary microglial and astroglial cell cultures

The *in vitro* studies were performed using primary microglial and

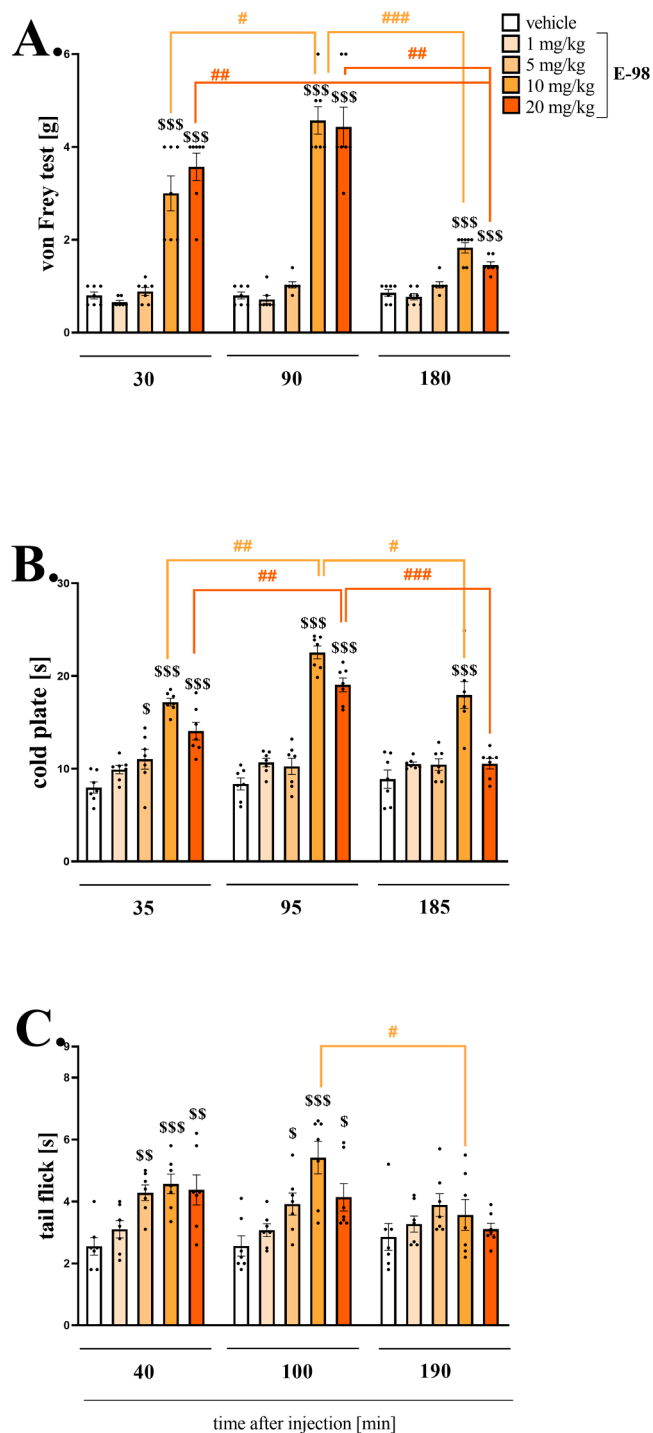


Fig. 3. Effects of a single E-98 injection on pain symptoms in CCI-exposed mice. The effects of single i.p. administration of E-98 (1, 5, 10, and 20 mg/kg) on mechanical (A, von Frey) and thermal (B, cold plate; C, tail-flick) stimulus on day 14th after nerve injury, were evaluated. The data are presented as the mean $\pm$ SEM. The one-way ANOVA ($^{\circ}$ vs. veh-CCI symbol) and repeated measures ANOVA ($^{\#}$ symbol) were performed. The differences between the groups were further analyzed using Bonferroni's *post-hoc* test. $p < 0.05$ was considered statistically significant.

astroglial cell cultures prepared from the cerebral cortex obtained from newborn Albino Swiss mice [28,29]. Only the minimal essential number of animals was used, and all of the procedures were performed according to the recommendations of the NIH Guide for the Care and Use of Laboratory Animals. The cells were seeded at a density of 3×10^5 cells/cm²

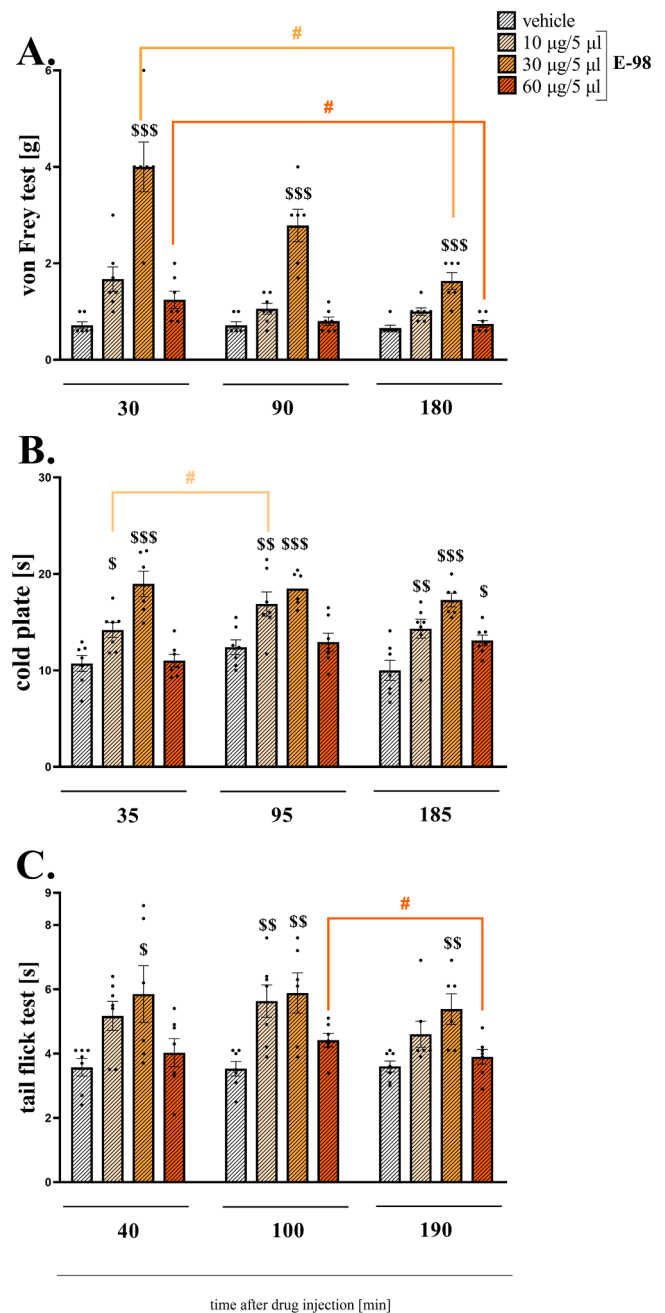


Fig. 4. Effects of a single E-98 i.th. injection on pain symptoms in CCI-exposed mice. The effects of single i.th. administration of E-98 (10, 30, and 60 µg/ 5 µl) on mechanical (A, von Frey) and thermal (B, cold plate; C, tail-flick) stimulus on day 14th after nerve injury were evaluated. The data are presented as the mean $\pm$ SEM. The one-way ANOVA ($^{\circ}$ vs. veh-CCI symbol) and repeated measures ANOVA ($^{\#}$ symbol) were performed. The differences between the groups were further analyzed using Bonferroni's *post-hoc* test. $p < 0.05$ was considered statistically significant.

in a culture medium consisting of high-glucose GlutaMAX™ DMEM supplemented with 10 % heat-inactivated fetal bovine serum, 0.1 mg/ml streptomycin and 100 U/ml penicillin (Gibco, New York, USA) in 0.025 mg/ml poly-L-lysine-coated 75-cm² culture flasks. The cells were grown in a 37°C incubator with a humidified atmosphere of 5 % CO₂ in the air. The culture medium was replaced after 3 and 8 days. On day 14, the microglial cells, loosely attached to the monolayer, were harvested by gentle shaking (70 rpm for 1.5 h) and centrifugation (800 rpm for 10 min). The fresh medium was added to the same culture

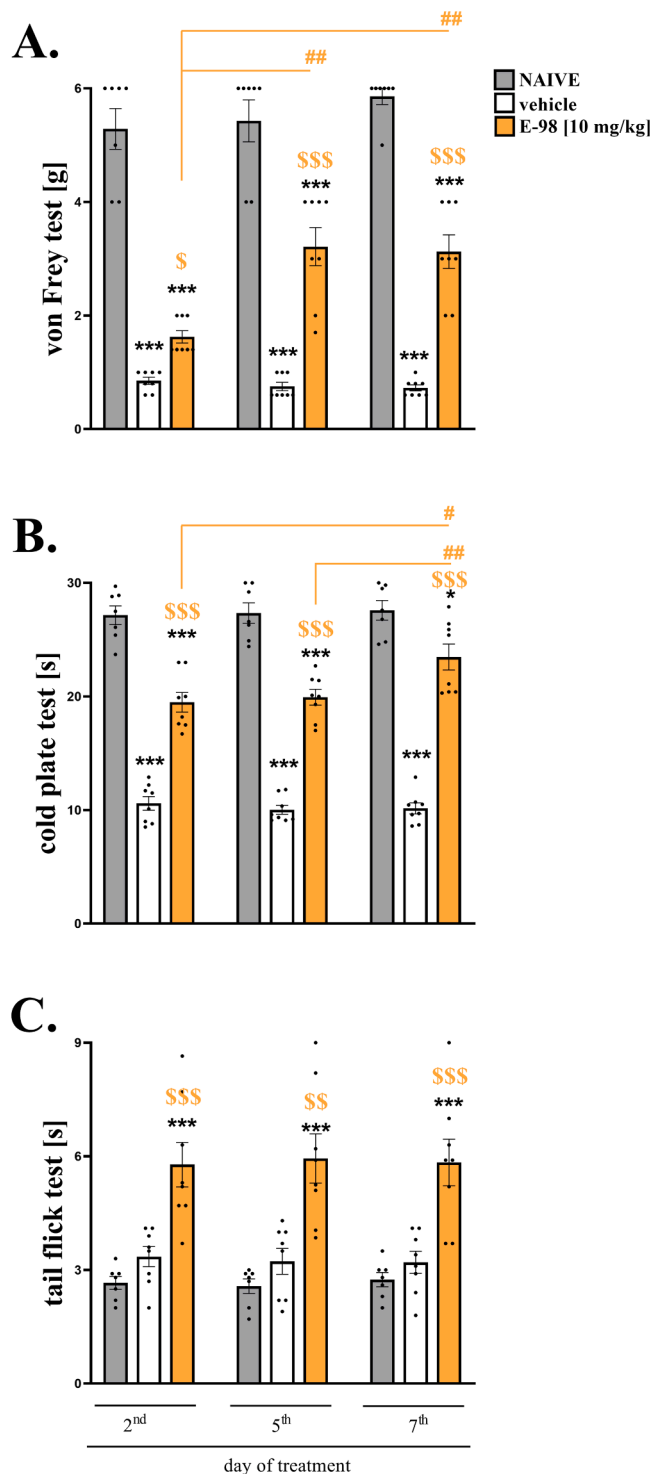


Fig. 5. Effects of repeated E-98 injections on pain symptoms in CCI-exposed mice. The impact of repeated i.p. administration of E-98 (10 mg/kg; injected twice daily (starting from day 7th after nerve injury) for 7 following days) on mechanical (A, von Frey) and thermal (B, cold plate; C, tail-flick) stimulus were evaluated. The behavioral tests were performed 90 min (A, von Frey), 95 min (B, cold plate), and 100 min (C, tail-flick) after morning E-98 injection on days 2nd, 5th, and 7th. The data are presented as the mean $\pm$ SEM. The one-way ANOVA (*vs. Naive, and § vs. veh-CCI symbols) and repeated measures ANOVA (# symbol) were performed. The differences between the groups were further analyzed using Bonferroni's *post-hoc* test. $p < 0.05$ was considered statistically significant.

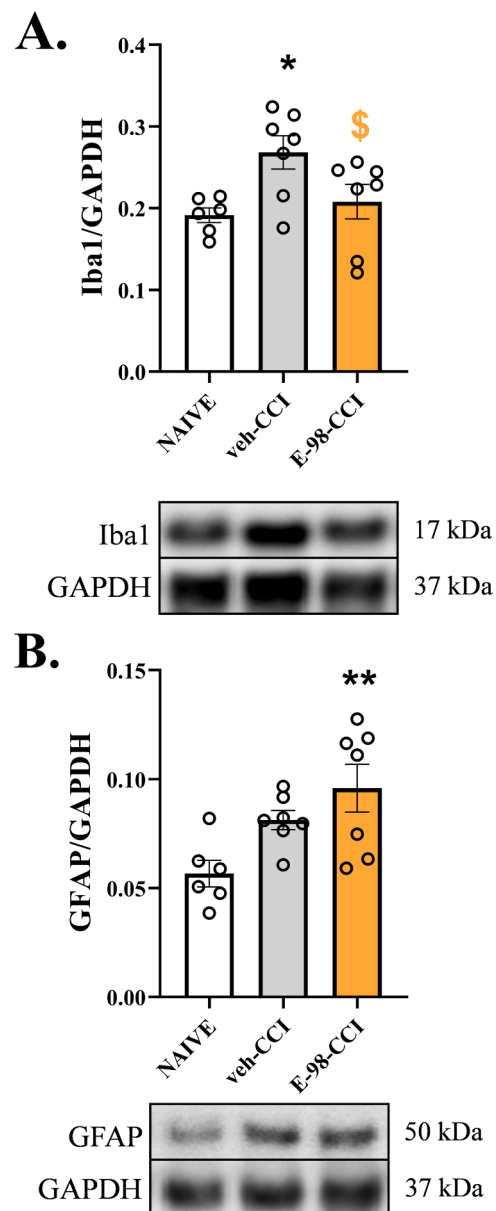


Fig. 6. The influence of chronic E-98 treatment on glial cell activation at the lumbar spinal cord. The impact of repeated i.p. administration of E-98 (10 mg/kg; injected twice daily (starting from day 7th after nerve injury) for 7 following days) on microglia (Iba1, A) and astrocytes (GFAP, B) activation. The data are presented as the mean $\pm$ SEM. The one-way ANOVA (*vs. Naive, and § vs. veh-CCI symbols) was performed. The differences between the groups were further analyzed using Bonferroni's *post-hoc* test. $p < 0.05$ was considered statistically significant.

bottles. On day 16th, the astroglial cells were prepared by shaking the flasks for 4 h and trypsinization using a 0.25 % trypsin-EDTA solution (Sigma-Aldrich, Saint Louis, USA). For the protein analysis, microglia and astroglia were seeded at a density of 1.8×10^5 cells/well in 24-well plates and incubated for 48 h before further experiments. The cells were treated with E-98 (10 μ M) for 30 min and then with LPS (lipopolysaccharide from *Escherichia coli* 0111:B4; Sigma-Aldrich, St. Louis, USA), which is a widely used inflammatory factor causing strong immunological responses) or solvent (PBS) as a control. The dose (100 ng/ml) of LPS and time point (24 h) were chosen based on our previous studies [30,31]. The dose of E-98 (10 μ M) was selected based on our *in vitro* studies (Supplementary materials, section S2).

2.4. Biochemical assays

2.4.1. Western blot analysis

The ipsilateral part of the lumbar spinal cord was collected from naïve and CCI-exposed after treatments. The tissues were at -80°C until the experiment. The tissue was placed in RIPA buffer supplemented with a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, USA), and then samples were centrifuged ($14,000 \times g$, 30 min, 4°C). The total protein concentration was measured using the bicinchoninic acid (BCA) method Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific Inc., Waltham, USA). The protein samples (20 μg) were heated for 8 min at 70°C in a loading buffer ($4 \times$ Laemmli Buffer, Bio-Rad, Warsaw, Poland), and then the samples were loaded in 4–15 % Criterion TGX precast polyacrylamide gels (Bio-Rad, Warsaw, Poland). The proteins were transferred from the gel to Immune-Blot PVDF membranes (Sigma-Aldrich, Mannheim, Germany) using a semidry transfer system (30 min, 25 V; Bio-Rad, Warsaw, Poland). The membranes were blocked with 5 % non-fat milk in TBST for 1 h, then washed with TBST (4×5 min), and incubated overnight at 4°C with the following commercially available primary antibodies: mouse polyclonal anti-GAPDH (1:5000; Merck, Darmstadt, Germany), rabbit polyclonal anti-Iba1 (1:500; Novus Biologicals, Centennial, USA), mouse monoclonal anti-GFAP (1:20000, Merck, Darmstadt, Germany). The membranes were washed in TBST (4×5 min) and then incubated in horseradish peroxidase-conjugated anti-rabbit (1:4000) or anti-mouse (1:3000) secondary antibodies (Vector Laboratories, Burlingame, USA) for 1 h at room temperature. The SignalBoost Immunoreaction Enhancer Kit (Merck, Darmstadt, Germany) dissolves the primary and secondary antibodies. The membranes were washed in TBST (4×5 min). The immune complexes were detected by the Pierce™ ECL Western Blotting Substrate (Thermo Fisher Scientific Inc., Waltham, USA) and visualized using the Fujifilm LAS-4000 Fluor Imager system. The relative levels of the immunoreactive bands were quantified using Fujifilm Multi Gauge. The full western blot images are presented in the [Supplementary material \(Figure S9\)](#).

2.4.2. ELISA assay

The cell culture supernatants were used for enzyme-linked immunosorbent assays (ELISAs) according to the manufacturer's recommendations. The levels of IL-1 β (IL-1 beta Mouse Uncoated ELISA Kit; Thermo Fisher Scientific Inc., Waltham, USA), IL-6 (IL-6 Mouse Uncoated ELISA Kit; Thermo Fisher Scientific Inc., Waltham, USA), and IL-10 (IL-10 Mouse Uncoated ELISA Kit; Thermo Fisher Scientific Inc., Waltham, USA) were measured using the Tecan Infinite M200 Pro M Plex plate reader (TECAN, Männedorf, Switzerland).

2.4.3. Immunofluorescence assays

The mice were sacrificed on day 14th after CCI, and the spinal cords were removed, postfixed in 4 % paraformaldehyde (PFA) overnight at 4°C , dehydrated in ethanol gradient, immersed in xylene, then embedded in paraffin and cut into 7 μm -thick sections on a microtome (Leica Microsystems CMS GmbH, Mannheim, Germany). Adjacent coronal sections from corresponding regions of the lumbar (L4 to L6) spinal cords of naïve ([Supplementary material, Figures S10–S12](#)) and CCI-exposed mice were deparaffinized in xylene and rehydrated in ethanol gradient. Antigen retrieval was performed by incubating sections in citrate buffer pH 6.0 (Vector Laboratories, Burlingame, USA) with microwave heating for 10 min. Sections were then cooled to RT, washed with distilled water, and blocked for 1 h at RT with 5 % normal swine serum (Vector Laboratories, Burlingame, USA) in PBS. After blocking, sections were washed with PBS and incubated overnight at 4°C with primary antibodies (listed below) dissolved in blocking solution. On the following day, after washing with PBS, sections were incubated with secondary antibodies in PBS for 1 h at RT, washed with PBS, and incubated in Hoechst 33342 solution (1:20000, ThermoFisher Scientific, Waltham, United States) in PBS for 10 min. Finally, sections were mounted using ProLong Glass Antifade Mountant (ThermoFisher

Scientific, Waltham, USA). The ipsilateral part of the lumbar (L4 to L6) spinal cord is presented in the representative images ([Figs. 7–9](#)).

Primary microglia and astrocytes were seeded at 3.0×10^4 cells/well on 0.025 mg/ml poly-L-ornithine (Sigma-Aldrich, Saint Louis, USA) coated 96-well plates (Phenoplate™, PerkinElmer, Waltham, USA). After 48 h incubation, the cell culture medium was aspirated, cells were washed twice with warm PBS, then fixed with 4 % PFA solution for 20 min at RT. PFA was then removed, and cells were washed twice with PBS. Permeabilization was performed by incubating cells in 100 μl of 0.2 % Triton X-100 solution in PBS for 15 min. Triton-X was then discarded, and 40 μl of blocking solution (0.2 % Triton-X, 5 % normal swine serum in PBS) was added to the wells. After 1 h incubation at RT, the blocking solution was removed, and 40 μl of primary antibodies diluted in the blocking solution were added. After overnight incubation at 4°C , cells were washed with PBS thrice, then 40 μl of secondary antibodies solution was added (diluted in blocking solution), and cells were incubated for 1 h at RT. Cells were then washed thrice with PBS, and 40 μl of 1:20000 solution of Hoechst 33342 in PBS was added; then, cells were incubated for 10 min at RT. After incubation, cells were washed three times with PBS and left in PBS at 4°C until imaging.

Primary antibodies used were as follows: rabbit anti-H₃R (1:600, Lifespan BioSciences, Lynnwood, United States), rat anti-Iba1 (1:1000, Abcam Inc., Cambridge, United Kingdom), chicken anti-GFAP (1:2000, Sigma-Aldrich, St. Louis, USA), mouse anti-NeuN (1:500, Abcam Inc., Cambridge, United Kingdom). Secondary antibodies: Alexa Fluor 488 conjugated donkey anti-rabbit IgG, Alexa Fluor 594 conjugated donkey anti-rat IgG, goat anti-chicken IgG, donkey anti-mouse IgG (ThermoFisher Scientific, Waltham, USA). Images were recorded using Leica Thunder Imager Live Cell and Leica TCS SP8 WLL confocal fluorescence microscope (Leica Microsystems CMS GmbH, Mannheim, Germany).

2.5. Molecular modeling

Initially, the three-dimensional structure of the H₃R (PDB ID: 7F71) [32] was retrieved from the Protein Data Bank (PDB) [33], providing a detailed framework for the analysis. The 3-dimensional ligand structures were processed using LigPrep v3.6 with pH-adjusted ionization states determined by Epik v3.4 in the Schrodinger Maestro suit [34,35]. The Protein Preparation Wizard facilitated bond order assignments, amino acid ionization state settings, and steric clash checks in protein structure. A receptor grid centered on the ligand PF03654746 was created using the OPLS4 force field. Glide [36,37] enabled automated, flexible docking at the SP level, generating up to ten poses.

2.6. Data analysis

The data are presented as the mean $\pm$ standard error of the mean (SEM). The one-way ANOVA and repeated measures ANOVA were performed. The differences between the groups were further analyzed using Bonferroni's *post-hoc* test. $p < 0.05$ was considered statistically significant. The analysis and charts were prepared using GraphPad Prism v.9.1.2 (GraphPad Software, USA).

3. Results

3.1. H₃R intrinsic activity and affinity of E-98 at the H₁R, H₂R, H₃R, and H₄R

As previously published, E-98 has a good affinity for the human histamine H₃ receptor (hH₃R) with K_i of 180 nM [11]. Further studies revealed that the compound has moderate affinity for the human histamine H₁ receptor (hH₁R) ($K_i = 675$ nM), very weak affinity for the human histamine H₄ receptor (hH₄R) ($K_i = 4854$ nM), and no affinity for human histamine H₂ receptor (hH₂R) receptors ($K_i > 10,000$ nM). These results indicated a selectivity profile of E-98 towards hH₃R, in particular, compared to hH₂R and hH₄R. Moreover, E-98 was able to counteract

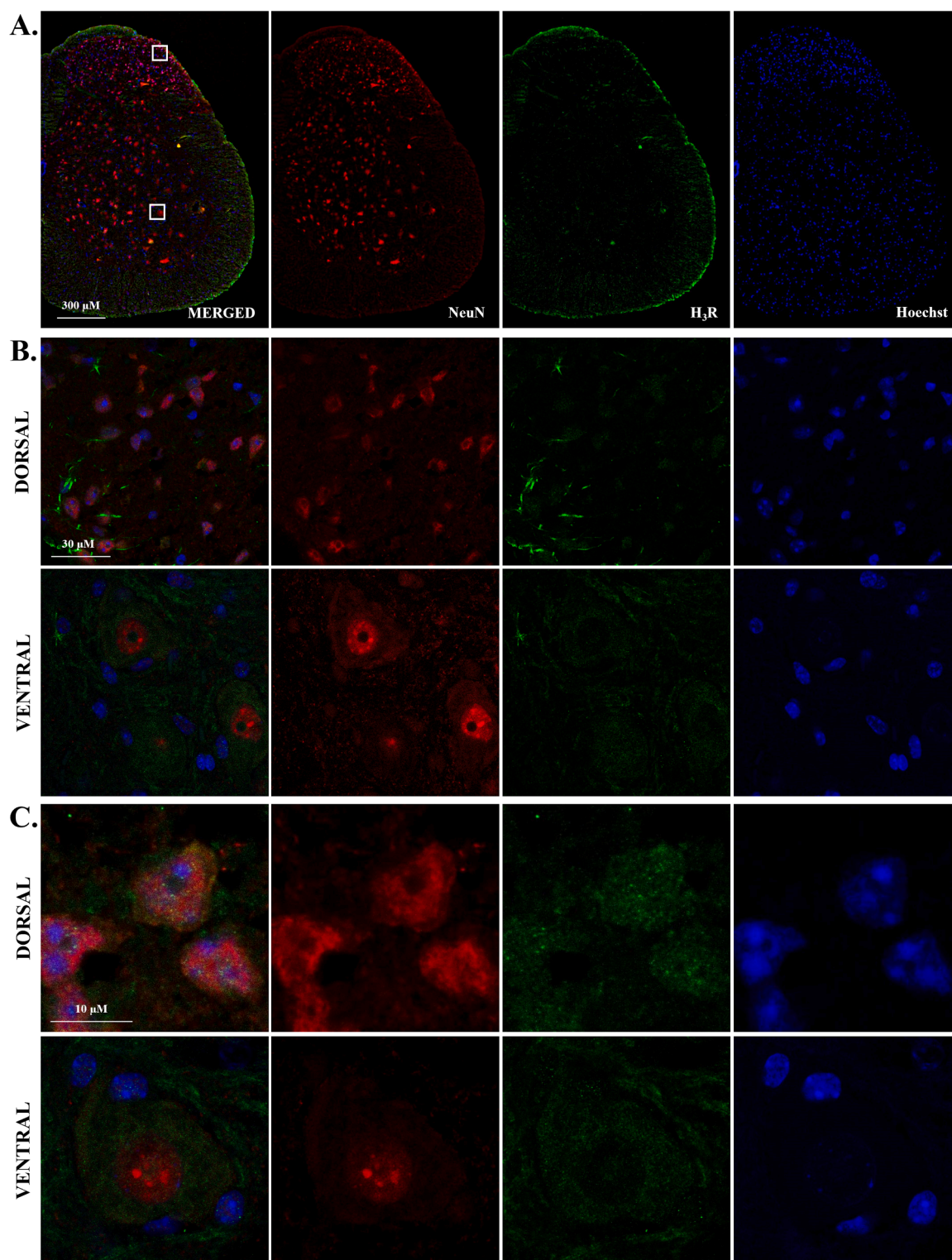


Fig. 7. Immunofluorescence analysis of H₃R localization with neuronal marker (NeuN) in the lumbar (L4-L6) spinal cord 14 days after CCI in the mice. Representative immunofluorescence images from colocalization analysis performed on the spinal cord (A-C), paraffin-embedded 7 μ M microtome slices: H₃R (green) with a neuronal marker (NeuN, neuronal nucleus; red), nuclei were marked with Hoechst 33342 (blue)). The lumbar spinal cord's dorsal and ventral parts (B-C) were shown as approximate fragments of selected images. Scale bars: 300 μ m (A), 30 μ m (B), 10 μ m (C).

(R)-(-)- α -methyl histamine-driven inhibition of cAMP production in forskolin-stimulated cells, which was indicative of its classification as an H₃R antagonist/inverse agonist. *In vitro* affinity data for the E-98 are summarized in Fig. 1.

3.2. Molecular modeling

In this study, molecular modeling was employed to investigate the interaction of compound E-98 with the H₃R binding pocket (PDB ID: 7F71, Fig. 2) [32]. The results revealed a substantial similarity in the

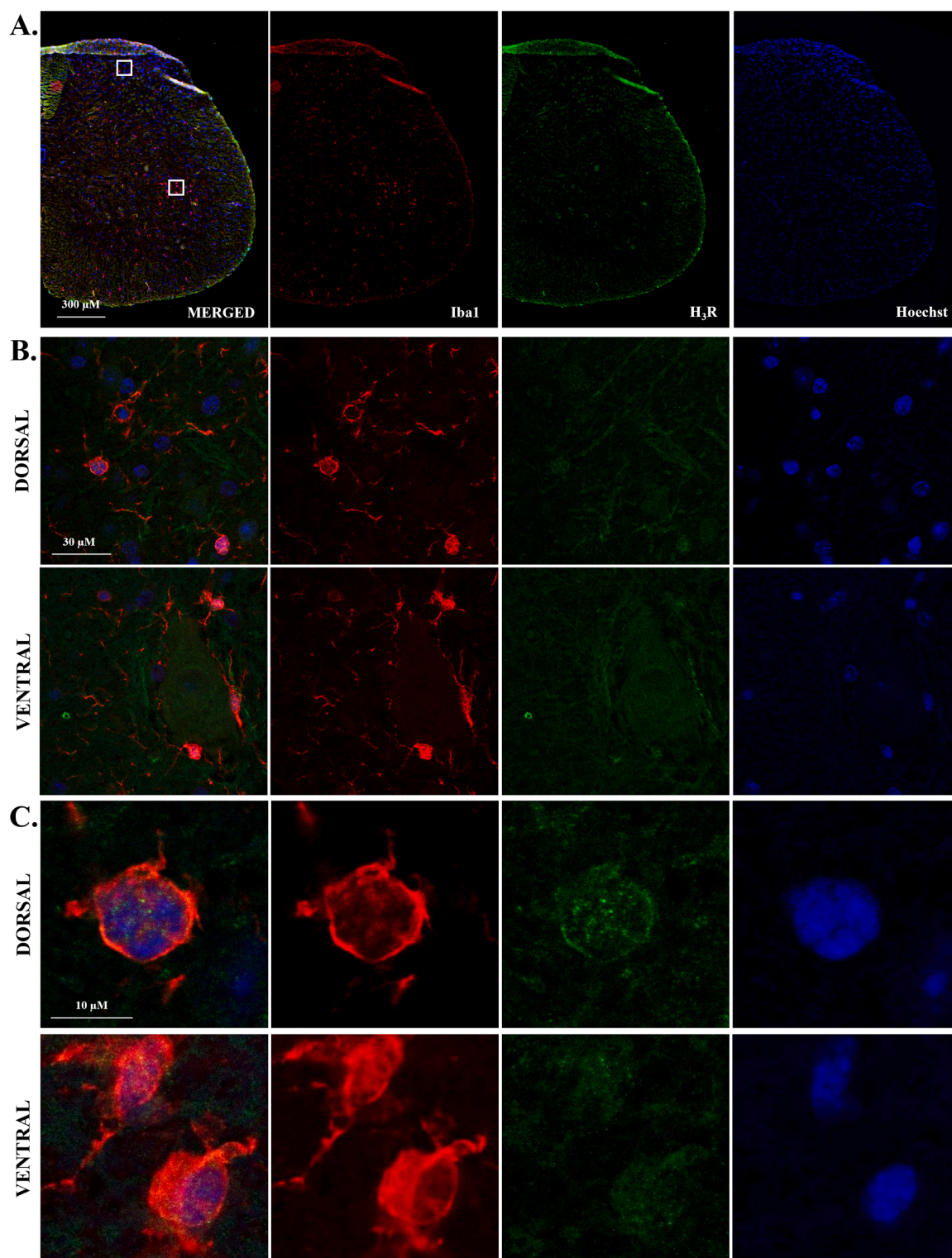


Fig. 8. Immunofluorescence analysis of H₃R localization with microglial cells marker (Iba1) in the lumbar (L4-L6) spinal cord 14 days after CCI in the mice. Representative immunofluorescence images from co-localization analysis performed on the spinal cord (A-C), paraffin-embedded 7 μM microtome slices: H₃R (green) with the microglial marker (Iba1, ionized calcium-binding adaptor molecule 1; red), nuclei were marked with Hoechst 33342 (blue)). The lumbar spinal cord's dorsal and ventral parts (B-C) were shown as approximate fragments of selected images. Scale bars: 300 μm (A), 30 μm (B), 10 μm (C).

binding mode of E-98 compared to the co-crystallized ligand PF03654746. Both compounds, E-98 and PF03654746, established a salt bridge (double charge-assisted hydrogen bond) with the D114 residue. Moreover, the spatial occupancy within the binding site by E-98 closely mirrors that observed for PF03654746. The p-chlorophenoxy fragment

of E-98 notably extends toward the extracellular exit of the receptor. In addition, the oxygen atom of this fragment engages in an O-H...O HB with the Y189 residue, contributing to the stabilization of the compound within the receptor.

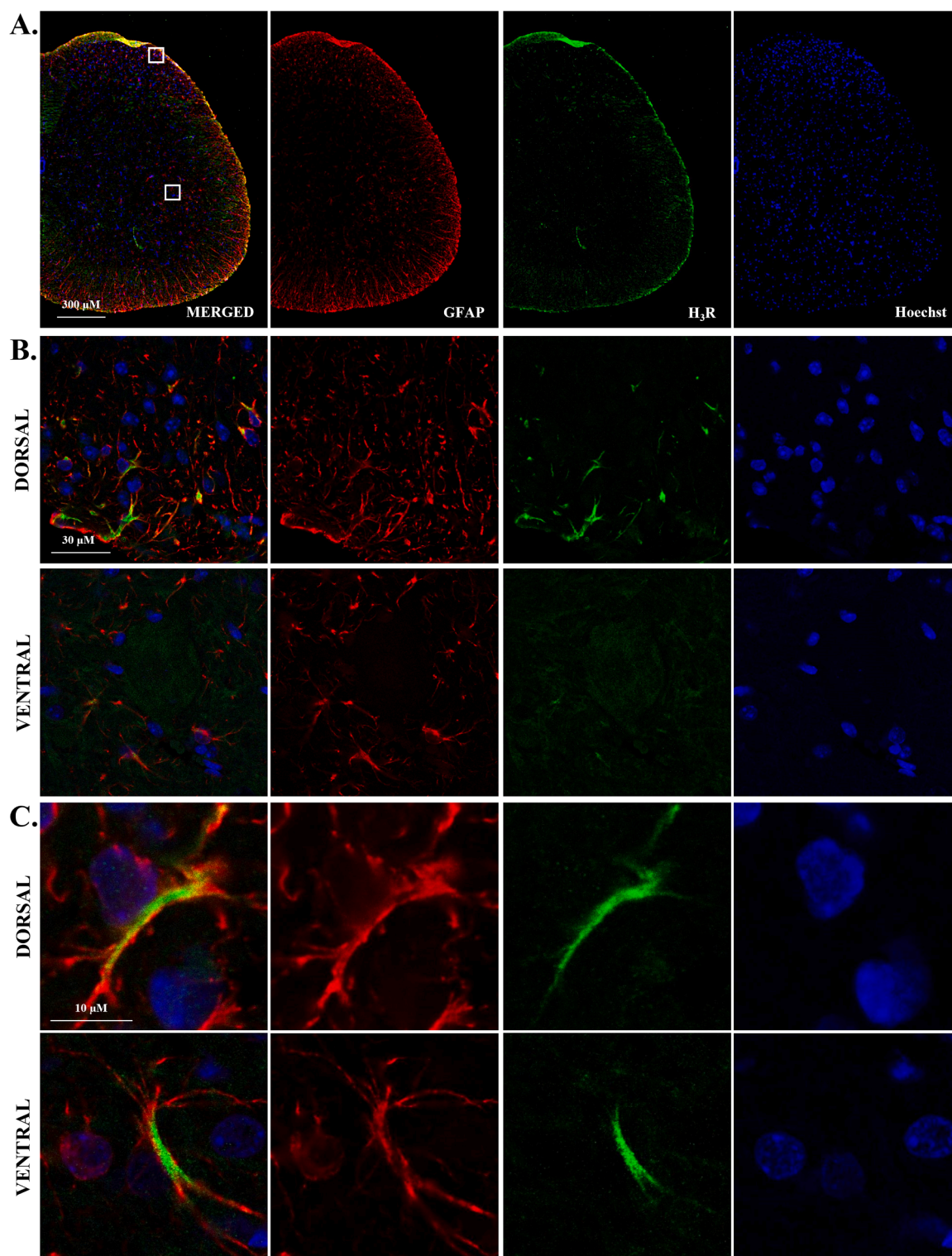


Fig. 9. Immunofluorescence analysis of H₃R localization with astroglial cells marker (GFAP) in the lumbar (L4-L6) spinal cord 14 days after CCI in the mice. Representative immunofluorescence images from colocalization analysis performed on the spinal cord (A-C), paraffin-embedded 7 μ M microtome slices: H₃R (green) with the astroglial marker (GFAP, glial fibrillary acidic protein; red), nuclei were marked with Hoechst 33342 (blue)). The lumbar spinal cord's dorsal and ventral parts (B-C) were shown as approximate fragments of selected images. Scale bars: 300 μ m (A), 30 μ m (B), 10 μ m (C).

3.3. Effects of single intraperitoneal E-98 administration on pain symptoms in CCI-exposed mice

Animals were randomly assigned to 5 treatment groups: vehicle-treated (i.p.) and E-98-treated (1, 5, 10, and 20 mg/kg, i.p.) (Scheme 1). The one-way ANOVA analysis performed at each time point showed

that a single injection of E-98 significantly increased the withdrawal thresholds in the von Frey test at doses 10 and 20 mg/kg (both $p < 0.0001$) 30 min, 90 min, and 180 min after injection as compared to the vehicle-treated group. Moreover, repeated measurements analysis showed a significant difference between 30 min vs. 90 min ($p = 0.0160$) and 90 min vs. 180 min ($p = 0.0004$) for a 10 mg/kg dose. We observed

significant differences between 30 min vs. 180 min ($p = 0.0016$) and 90 min vs. 180 min ($p = 0.0013$) for the highest tested dose (Fig. 3 A). The highest analgesic effect for both doses was observed 90 min after drug injection (Fig. 3 A).

An antinociceptive effect of E-98 was observed 35 min after administration for doses 5 mg/kg ($p = 0.0295$), 10 mg/kg, and 20 mg/kg (both $p < 0.0001$) compared to vehicle-treated mice in the cold plate test (Fig. 3B). E-98-induced analgesia was still observed 95 min after injection for doses 10 and 20 mg/kg (both $p < 0.0001$) and 185 min, however, only for dose 10 mg/kg ($p < 0.0001$) (Fig. 3B). The repeated measurements analysis showed a significant effect of E-98, similar to the Frey test, between 35 min vs. 95 min ($p = 0.0014$) and 95 min vs. 185 min ($p = 0.0101$) for dose 10 mg/kg (Fig. 3B) with the highest analgesic effect at 95 min after injection. In the case of dose 20 mg/kg, we observed significant differences between 35 min vs. 95 min ($p = 0.0038$) and 95 min vs. 185 min ($p < 0.0001$) (Fig. 3B).

In the tail-flick test, we also observed a significant effect of treatment with a novel H₃R antagonist/inverse agonist. Here, we observed significantly increased tail withdrawal latency after a single E-98 injection for doses 5, 10, and 20 mg/kg, 40 min ($p = 0.0039$, $p = 0.0008$, and $p = 0.0023$, respectively) and for doses 10 and 20 mg/kg, 100 min ($p < 0.0001$, and $p = 0.0293$, respectively) after administration as compared to the vehicle-treated group (Fig. 3C). None of the tested doses were effective at the last measured time point (190 min). The repeated measurements ANOVA analysis also revealed a significant effect of treatment; however, we observed differences only between 100 min vs. 190 min for dose 10 mg/kg ($p = 0.0412$) (Fig. 3C).

Based on these data, we chose a 10 mg/kg dose and a second time point to further analyse E-98's analgesic potency.

3.4. Effects of single intrathecal E-98 administration on pain symptoms in CCI-exposed mice

To study the effects of an H₃R antagonist/inverse agonist on pain processing specifically in the spinal cord, we administrated E-98 directly to the site of interest via lumbar puncture between L5 and L6. Animals were assigned to 4 treatment groups: vehicle-treated (i.th.) and E-98-treated (10, 30, and 60 µg/5 µl i.th.) (Scheme 1).

The single i.th. injection of E-98 significantly increased the withdrawal thresholds in the von Frey test only at a dose of 30 µg/5 µl ($p < 0.0001$) at all tested time points as compared to the vehicle-treated animals (Fig. 4A). Interestingly, the lower and higher doses (10 and 60 µg/5 µl, respectively) did not produce an analgesic effect, suggesting an inverted U-shaped dose-response curve at each tested time point. The repeated measures analysis showed a significant difference between 30 min and 180 min for 30 µg/5 µl ($p = 0.0115$) and 60 µg/5 µl dose ($p = 0.0113$) doses (Fig. 4A).

Reduced thermal hypersensitivity was observed 35 minutes after administration for doses 10 and 30 µg/5 µl at all tested time points (35 min: $p = 0.0306$, and $p < 0.0001$; 95 min: $p = 0.0079$, and $p = 0.0006$; 185 min: $p = 0.0041$, and $p < 0.0001$, respectively) compared to vehicle-treated mice in the cold plate test (Fig. 4B). Moreover, repeated measurements analysis showed the differences between 35 min vs. 95 min for a dose of 10 µg/5 µl ($p = 0.0349$). We observed a slight but significant analgesic effect of E-98 at a dose of 60 µg/5 µl ($p = 0.0468$) 185 min after injection (Fig. 4B).

We observed a significant increase in tail withdrawal latency after a single i.th. E-98 injection for dose 30 µg/5 µl 40 min ($p = 0.0193$), 100 min ($p = 0.0019$), and 190 min ($p = 0.0034$) after administration as compared to the vehicle-treated group (Fig. 4C). The E-98 at dose 10 µg/5 µl was effective only 95 min after injection ($p = 0.0038$). The repeated measurements ANOVA analysis revealed a significant effect of treatment; however, we observed differences only between 100 min vs. 190 min for dose 60 µg/5 µl ($p = 0.0412$) (Fig. 4C).

3.5. Effects of chronic E-98 administration on pain symptoms in CCI-exposed mice

Animals were randomly assigned into 3 groups: naïve, vehicle- (i.p.), and E-98-treated (10 mg/kg, i.p.) CCI-exposed. The compounds were injected into neuropathic animals 7 days after nerve injury (twice daily for 7 days). Behavioral tests were performed on days 2nd, 5th, and 7th after the morning drug administration (Scheme 1).

In the von Frey test, we observed significant differences between naïve and CCI-exposed animals at all tested time points ($p < 0.0001$). Two days of treatment with E-98 increased the paw withdrawal threshold compared to CCI-exposed vehicle-treated mice ($p = 0.0365$), and this effect was also observed on day 5th ($p < 0.0001$) and 7th ($p < 0.0001$) (Fig. 5A). Moreover, the repeated measurements ANOVA analysis showed significant differences between days 2nd and 5th ($p = 0.0020$) and 2nd and 7th ($p = 0.0018$) treatments (Fig. 5A).

The CCI-exposed mice exhibited strong hypersensitivity to thermal stimuli compared to the naïve as measured by the cold plate test ($p < 0.0001$). However, this effect was lower in the group of mice, which received E-98 on day 7 ($p = 0.0112$) (Fig. 5B). Moreover, we have shown reduced hypersensitivity in mice receiving E-98 ($p < 0.0001$), compared with vehicle-treated control at all tested days. The analysis of time-dependent differences revealed a robust analgesic effect on day 7 of drug injection compared to previously tested time points (day 2 y: $p = 0.0160$; day 5: $p = 0.0060$) (Fig. 5B).

In the tail-flick test, E-98 exhibited significant analgesic effects compared to naïve animals in all tested time points (day 2: $p < 0.0001$; day 5: $p = 0.0001$; day 7: $p = 0.0002$) (Fig. 5C). Similarly, a novel H₃R antagonist/inverse agonist enormously increased tail withdrawal latency compared to vehicle-treated CCI-exposed mice on day 2 ($p = 0.0009$), 5 ($p = 0.0010$), and 7 ($p = 0.0006$). The E-98-induced analgesia did not differ in time (Fig. 5C).

3.6. The influence of chronic E-98 treatment on glial cell activation at the lumbar spinal cord

The effect of chronic E-98 (10 mg/kg, i.p.) treatment on glial cells, microglia, and astrocytes activation at the ipsilateral lumbar spinal cord was assessed by Western blot assay (Fig. 6 A,B). We observed an elevated level of Iba1, a marker of microglial cells activation, following nerve injury ($p = 0.0205$), which was significantly reduced after treatments ($p = 0.0304$) (Fig. 6A). In the case of astrocyte activation, we did not observe significant changes in the protein level of GFAP (an astrocyte marker) after nerve injury; however, chronic E-98 treatment increased the level of this cellular marker ($p = 0.0029$) (Fig. 6B).

3.7. The cellular localization of H₃R in the lumbar spinal cord of naïve animals and 14th days after nerve injury in CCI-exposed mice

The results of the immunofluorescence analysis from CCI-exposed mice are shown in Fig. 7A-C; 8A-C; 9A-C, and from naïve animals in the Supplementary Materials (Figures S10-12). Here, we visualized two independent corresponding lumbar spinal cord regions (ventral and dorsal part) from naïve (Figures S10-12)) and CCI-exposed (Fig. 7A-C; 8A-C; 9A-C) mice.

The fluorescence immunohistochemical staining revealed clear colocalization of H₃R with NeuN-positive cells in both tested regions in neuropathic (Fig. 7A-C) as well as naïve (Figure S10A,B) animals. Our immunostaining studies showed that a subgroup of large cells (identified as motor neurons) in lamina IX contains H₃R (Fig. 7A-C; Figure S10A, B). Although the observed presence of the tested receptor is noticeable in neurons, its greater expression (by fluorescence intensity) was observed in glia.

Immunofluorescence analysis revealed strong activation of microglial cells in the ipsilateral part of the spinal cord (Fig. 8A-C) induced by nerve injury as compared to the corresponding region in naïve animals

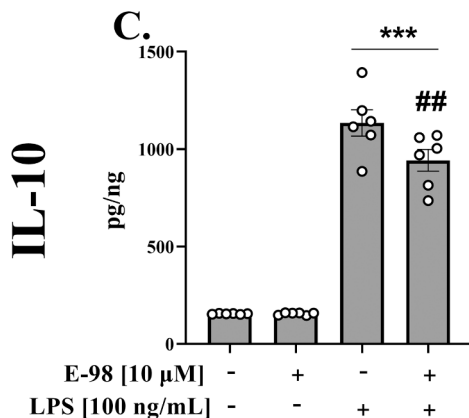
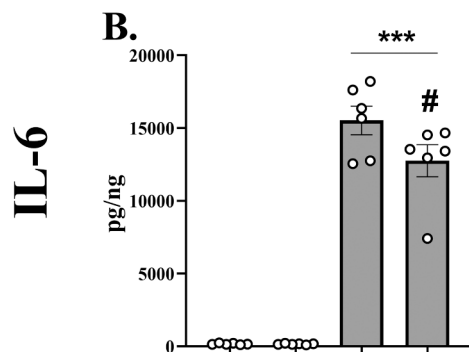
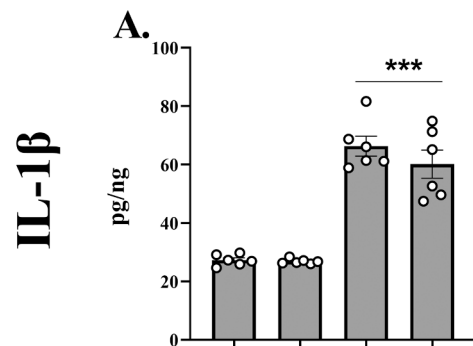
(Figures S11A, B). Importantly, we observed the co-localization of H₃R with microglia (Iba1-positive cells) in CCI-exposed mice (Fig. 8A-C) in both analyzed spinal cord regions. The co-localization of H₃R with Iba1 marker was also observed in naïve animals (Figures S11A, B).

Here, we have also investigated the expression of H₃R within the astroglial cells in the L4-L6 region of naïve (Figure S12A, B) and neuropathic animals (Fig. 9A-C). Our immunohistochemical staining showed a high level of GFAP-positive cells (astrocyte marker) at the ipsilateral side in the spinal cord of neuropathic mice (Fig. 9A) as compared to naïve control (Figures S12A, B). Moreover, we observed the co-localization of H₃R and GFAP in the spinal cord's dorsal and ventral parts (Fig. 9A, Figures S12A, B).

3.8. The influence of E-98 treatment on cytokines secretion in primary glial cell cultures

Finally, to better understand the effects of E-98 treatment on glial activation, we focused on the production of the proinflammatory interleukin-1 β (IL-1 β), modulatory interleukin-6 (IL-6), and anti-inflammatory interleukin-10 (IL-10) in primary glial cell cultures (Fig. 10A-F). The protein analysis demonstrated a significantly increased level of IL-1 β after LPS treatment compared to controls in both microglial and astroglial cell cultures ($p < 0.0001$; Fig. 10A, D). E-98 [10 μ M] did not influence the protein levels of this cytokine in microglia (Fig. 10A); however, in astrocytes, the IL-1 β level was up-regulated ($p = 0.0032$). The 24-hour treatment with LPS also elevated IL-6 level

Primary microglial cell cultures



Primary astroglial cell cultures

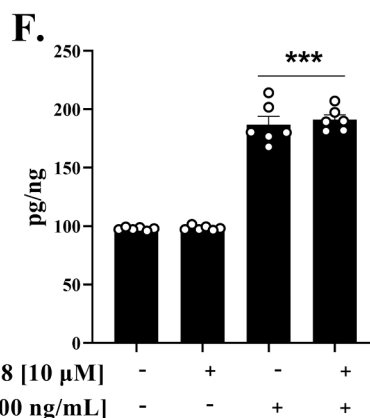
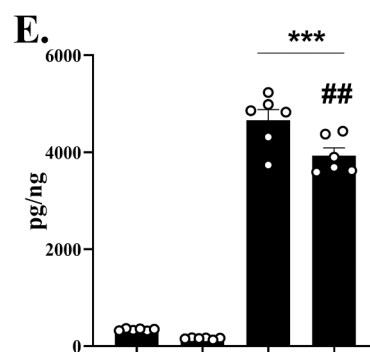
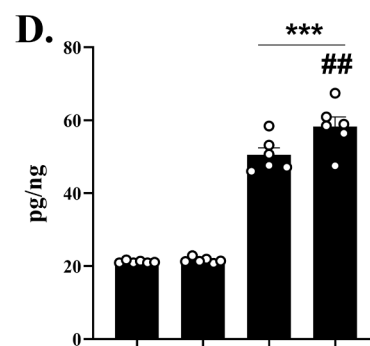


Fig. 10. The influence of E-98 treatment on cytokines (IL-1 β , IL-6, IL-10) secretion in primary glial cell cultures. Microglial and astroglial cells were treated with E-98 [10 μ M] for 30 min and then with LPS [100 ng/ml] for 24 h. The level of IL-1 β (A,D), IL-6 (B,E), and IL-10 (C,F) was analyzed by ELISA assay. The influence of The data are presented as the mean $\pm$ SEM. of 6 independent experiments. The one-way ANOVA (*vs. vehicle-, and #vs. LPS-treated) was performed. The differences between the groups were further analyzed using Bonferroni's *post-hoc* test. $p < 0.05$ was considered statistically significant.

($p < 0.0001$), while E-98 diminished it in both microglia ($p = 0.0151$) and astrocytes ($p = 0.0011$) cell cultures (Fig. 10B, E). Here, we also analyzed the effect of E-98 on anti-inflammatory IL-10. The level of this cytokine was up-regulated in both cell glial cell cultures after LPS treatment ($p < 0.0001$; Fig. 10C, F), but only in microglia, E-98 was able to reduce this effect ($p = 0.0057$; Fig. 10C).

3.9. The localization of H₃R in the murine primary microglial and astroglial cell cultures

Using confocal imaging of double immunofluorescence staining, we analyzed the expression of H₃R in primary microglial and astroglial cell cultures. Our studies revealed the co-localization of H₃R with both Iba1 (Fig. 11A) and GFAP (Fig. 11B) markers.

4. Discussion

This study was designed and implemented to identify the cellular mechanisms underlying H₃R antagonist/inverse agonist analgesic effects under neuropathic pain. Our present studies demonstrated the analgesic effects of the novel H₃R antagonist/inverse agonist (E-98) in a preclinical model of neuropathic pain. The results indicated a substantial correlation between H₃R antagonist/inverse agonist action with reduced activation of spinal microglia and potentiated activation of astrocytes. Additionally, our *in vitro* studies demonstrated the direct influence of tested antagonist/inverse agonist on pro- and anti-inflammatory cytokines secreted by microglia and astrocytes in primary murine cell cultures. Moreover, we have shown, for the first time, the expression of H₃R within the lumbar spinal cord of neuropathic mice co-expressed with neurons, microglia, and astrocytes. Thus, our data suggest that modulation of glial cell activation by H₃R antagonist/inverse agonist might underpin its potential analgesic efficacy.

The last years brought a wild spectrum of novel H₃R antagonists/inverse agonists. One of them, pitolisant (Wakix®, Ozawade®), is now the first marketed H₃R antagonist/inverse agonist, successfully used in human therapy for adults suffering from narcolepsy and Obstructive Sleep Apnoea (OSA) [39]. Recently, in February 2023, pitolisant received its first approval in adolescents and children for the treatment of narcolepsy in the EU [38]. Consequently, the clinical application of

novel H₃R antagonists/inverse agonists has increased. The role of H₃R in pain processing has been demonstrated, and the latest studies, also performed by us, outlined the analgesic effect of H₃R antagonists/inverse agonists in chronic pain [3,5,6]. However, discrepancies in the effectiveness of H₃R ligands arose due to variations in the pain models, drug administration routes, or the nature of the nociceptive stimulus [39]. Moreover, the selectivity of the tested compounds, such as thioperamide, which is both an H₃R and H₄R inverse agonist, is also a critically limiting factor [40]. Several lines of evidence have shown that agonists of H₃R have pain-relieving and anti-inflammatory effects [41, 42]. The antinociceptive profile of H₃R agonists was both modality- and intensity-specific [42]. Those discrepancies in H₃R ligands could be due to an internalization of the H₃Rs observed after pharmacological stimulation and, consequently, reduced activity and selectivity [43].

Therefore, effective and selective pharmacological tools are needed to study the role of the histaminergic system. Our present studies investigate the analgesic effect of a novel H₃R antagonist/inverse agonist with a favorable pharmacological profile. Our results indicated a selectivity profile of E-98 towards hH₃R, particularly compared to hH₂R and hH₄R; however, it has a moderate affinity for hH₁R. Compounds with a high affinity for the H₁R (such as the first generation of H₁R antagonists) show a sedative effect. Here, we performed two behavioral tests (RotaRod and Open Field tests) to investigate whether our new compound can impair motor functions and the exploratory behaviors of the experimental animals. No differences were observed in the traveled distance, mean speed, number of entries to the inner zone, time spent in the inner zone, and motor coordination on an accelerated rotarod in E-98-treated mice compared to control animals. Moreover, independent experiments performed at the National Institute of Neurological Disorders and Stroke, National Institutes of Health (Rockville, USA) within the Anticonvulsant Screening Program showed no neurological deficit (indicated by ataxia, loss of placing response, and muscle tone) in the rats after treatment with E-98 [11]. Those data strongly indicate that novel H₃R antagonist/inverse agonist has no sedative effect. In the present studies, we performed experiments expanding the E-98 pharmacological profile. Moreover, in the cAMP accumulation assay, we characterized E-98 as a potent H₃R antagonist. Our molecular modeling studies revealed that E-98 established a salt bridge (double charge-assisted hydrogen bond) with the D114 residue, which is

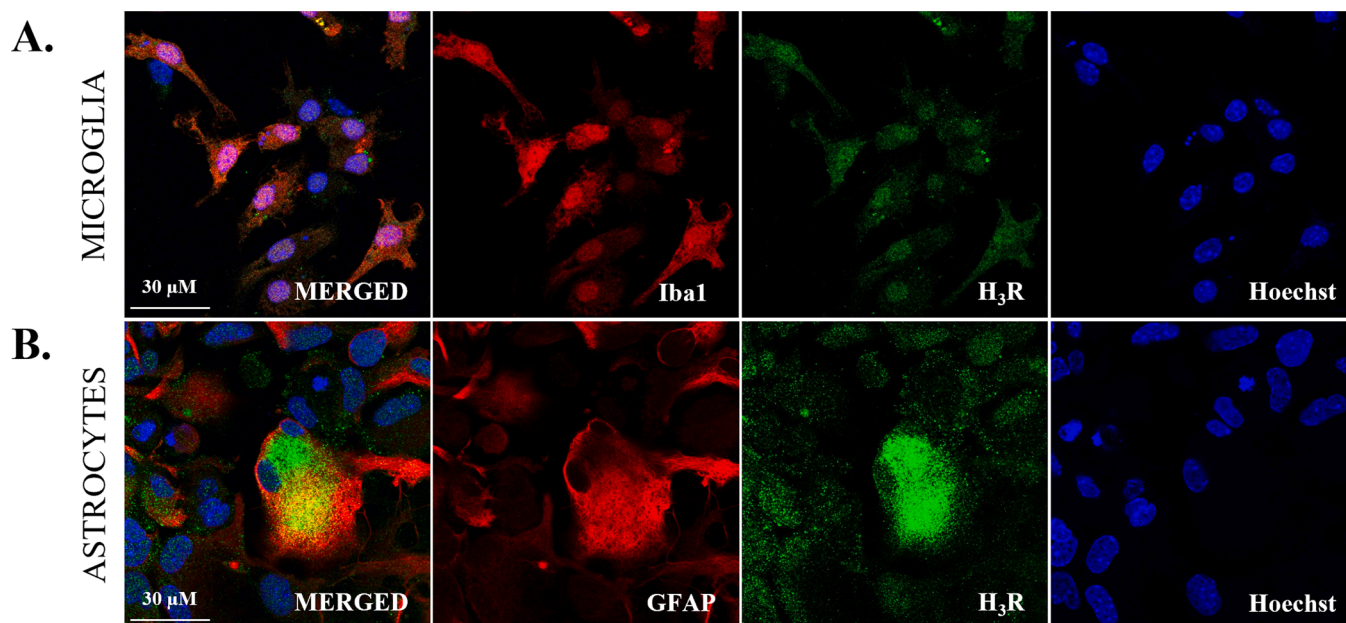


Fig. 11. Immunofluorescence analysis of H₃R localization in primary microglial and astroglial cells cultures. Representative immunofluorescence images from primary glial cell cultures showing the co-localization of the H₃R (green) with the microglial (Iba1, ionized calcium-binding adaptor molecule 1; red) and astroglial (GFAP, glial fibrillary acidic protein; red) markers; nuclei were marked with Hoechst 33342 (blue). Scale bars: 30 μm (A,B).

recognized as one of the strongest in medicinal chemistry, playing a crucial role in binding ligands to aminergic receptors of G protein-coupled receptors (GPCRs). The presence of such a salt bridge is a significant factor in GPCR ligand binding, often dictating the orientation and stability of the ligand within the binding pocket. Our data clearly indicate that E-98 can be considered a highly stable ligand, with weak inhibition of CYP3A4 and no effects on motor disturbances.

Our present data revealed that systemic administration of a novel H₃R antagonist/inverse agonist, reduced neuropathic pain symptoms. Here, we have shown that a single i.p. injection of E-98 time- and dose-dependently reduced nociceptive responses to mechanical stimuli (von Frey) as well as to heat (tail flick) and cold (cold plate). It was previously demonstrated, also by us, that a single i.p. administration of H₃R antagonist/inverse agonist reduced mechanical and cold hypersensitivity associated with neuropathic pain [3,5,6,19]. Moreover, we have demonstrated, for the first time, that direct administration of an H₃R antagonist/inverse agonist to the spinal cord is also analgesic. However, this effect was not observed for the antagonist/inverse agonist in the higher dose, suggesting an inverted U-shaped response. The cause of the inverted U-shaped response observed with E-98 after i.t. injection may reflect the fact that H₃R antagonists/inverse agonists can increase the release of multiple neurotransmitters, such as histamine, acetylcholine, dopamine, serotonin, and noradrenaline [44–48], or it may be a consequence of the existence of numerous H₃R splice variants [49]. Moreover, the inverted U-shaped dose-response curve was already observed for the H₃R antagonist/inverse agonist, both in clinical [50] and preclinical [51] studies regarding the role of H₃R in learning and memory consolidation. The appearance of a bell-shaped response curve may imply a relatively narrow therapeutic window of a potential drug; however, in the case of E-98, this effect was only observed after i.t. injection in the high dose. Moreover, we have also performed studies with repeated i.p. E-98 injections to mimic potential clinical dosing regimens more closely. There is still a paucity of information on the influence of chronic H₃R antagonist/inverse agonist treatments on pain symptoms and the molecular mechanism underlying those effects. The studies revealed that repeated, orally-derived H₃R antagonists/inverse agonists (GSK189254, GSK334429, S38093, and A-960656) reduced paw withdrawal threshold to mechanical stimuli [18,52,53]. Here, we have shown that our new compound not only reduced mechanical but also thermal hypersensitivity.

The expression of H₃R was observed in the spinal cord [54,55], but also in the periphery within the A σ and A β fibers [56]. The potential site of H₃R antagonist/inverse agonist action was evaluated in autoradiography studies, showing dense H₃R labeling at the dorsal horn of the rat spinal cord [53]. Notably, the authors demonstrated a similar expression pattern in human spinal cord sections. Those data strongly indicate the involvement of H₃R in pain processing. However, the mentioned studies were performed only in naïve animals, which brings limitations in translating it into the pathological condition of neuropathic pain. Here, we have demonstrated for the first time the expression of H₃R in the spinal cord of neuropathic animals. Our immunofluorescence studies show the expression of H₃R within the lumbar spinal cord of neuropathic mice, both dorsal and ventral parts. This strongly suggests that the H₃R could have implications for modulating nociceptive information during pathological pain processing. Moreover, our immunostaining studies showed that a subgroup of large cells, identified as motor neurons, in lamina IX contains H₃R, which may partially explain the motor behavior following pharmacological manipulation, as well as data from H₃R-deficient mice [55,56]. Moreover, the electrophysiological experiments with TTX indicated the presence of H₃Rs on motoneuron membranes, and cresyl violet staining visualized the distribution of H₃Rs expressed on the membrane of 35 % of the large diameter (> 15 μ m) cells in lamina IX [57], which strongly supports our data.

In recent years, there has been strong evidence supporting the involvement of non-neuronal cells in developing and maintaining neuropathic pain. Glial cells are the immune cell population in the

central nervous system (CNS) that responds to various pain-related neuromodulators. Moreover, they are also capable of directly altering the nociceptive pathways, as they release specific pronociceptive mediators to drive central sensitization, which is manifested as pain hypersensitivity [12,13,30]. Therefore, neuropathic pain is considered a neuroimmune disorder. Microglial cells are a type of specialized macrophages that are found in the CNS; they provide innate immunity, regulate neuronal activity, and protect the CNS from invaders, microbes, and demyelination by removing defective cells. Microglial cells are highly responsive to injury in the nervous system (both peripheral and central), which may lead to changes in their function. Activated microglia release proinflammatory factors, thereby initiating the pain process and perpetuating the neuroinflammation that triggers pain and leads to a chronic neuropathic pain condition. Nerve injury also affects astrocytes, the most abundant and versatile glial cell type. Astrocytes maintain the homeostasis of ions and neurotransmitters, improve synaptic connections within the CNS, and ensure the proper neurons metabolism. Astrocytes undergo structural and functional modifications after nerve injury and switch their phenotype to an active state, proliferate, and release many pro-nociceptive factors (cytokines, enzymes, NO, ATP, PGs) [12,13]. Understanding the role of glial cells in chronic pain is crucial for unraveling the process of pain sensitization and identifying potential new targets for drug development.

Here, we have demonstrated for the first time the co-localization of H₃R with microglia and astrocytes in control and neuropathic animals, as well as in murine microglial and astroglial cells in primary cultures, which suggests the new immunomodulatory role of this receptor. However, the participation of glial cells in H₃R-mediated actions remains unresolved. Additionally, our biochemical studies revealed that chronic treatment with an H₃R antagonist/inverse agonist diminished the level of activated microglial cells, while up-regulated the level of astrocytes in the ipsilateral lumbar spinal cords of neuropathic mice. Using primary microglial cell cultures, we observed down-regulation of inflammatory cytokines IL-6 and IL-10, which suggests a reduced activation level of those cells after E-98 treatment. Our newest published data has shown that novel H₃R antagonist/inverse agonist attenuated the inflammatory process in the BV-2 cell line (a murine microglia cell line) by down-regulating pro-inflammatory cytokines (IL-1 β , IL-6, TNF- α) [58], which is in agreement with our present data from primary cultures.

There is still a paucity of information concerning the role of H₃R antagonists/inverse agonists in astrocytes. Surprisingly, we observed an up-regulated level of GFAP (an astrocyte marker) after treatment with E-98 in neuropathic mice, which revealed a different influence of H₃R antagonist/inverse agonist on astrocytes. It is well known that astrocytes promote histamine clearance, which is an essential process for balanced histaminergic activity [59]. In 2018, Xu et al. [60] presented that histamine upregulates astrocyte activation but simultaneously diminishes the secretion of the TNF- α and IL-1 β . The H₃R antagonist/inverse agonist alone did not affect cytokine production in rat primary astrocytes. Surprisingly, the co-administration of histamine and the H₃R antagonist/inverse agonist strongly enhances the secretion of both cytokines. Although the authors suggested the neuroprotective role of histamine in astrocytes, the participation of H₃R and its antagonists/inverse agonists in this effect is unclear. Our studies in primary murine astrocyte cultures have also shown that novel H₃R antagonist/inverse agonist alone did not influence the expression of any tested cytokine (IL-1 β , IL-6, IL-10). However, we observed that E-98 in LPS-stimulated cells elevated the level of proinflammatory IL-1 β while diminishing the level of IL-6.

Many studies, also published by us, demonstrated that reducing microglial cells activation is correlated with diminished neuroinflammation and neuropathic pain [12,13,61–63]. Here, we have demonstrated that the novel H₃R antagonist/inverse agonist modulates the activation of glial cells at the spinal cord level, which might be partially correlated with its analgesic effects. Our present study provides the first evidence of a potential opportunity to interrogate glial function

and neuropathic pain treatment by targeting H₃R.

Sex is recognized as a major factor influencing chronic pain. A growing body of evidence, both from clinical and preclinical studies, highlights essential differences between men and women in the prevalence of chronic pain. Epidemiological reports demonstrate a higher prevalence of pain-related disorders (such as migraine, fibromyalgia, and arthritis) in women, which is associated with more frequent analgesic use [64,65]. Gender-related differences have also been observed in sensitivity to histamine. Unlike males, female rats exhibit histamine-induced depolarization not only in unmyelinated C-type nerve fibers but also in myelinated A-type nerve fibers [66]. We were the first to demonstrate sex-related differences in the analgesic effects of H₃R antagonists, observing a stronger analgesic effect in neuropathic female mice [3]. However, this phenomenon requires further investigation. We suggest those differences may appear due to a different glial cell activation between sexes. During neuropathic pain, neuro-immune cross-talk may represent an essential point of the observed gender-related dissimilarities [15,67,68]. The analysis of glial markers in neuropathic animals revealed that astrocytes and microglia are differently activated in males and females [15]. Females have a late activation of glial cells, which may lead to hypersensitivity and prolonged pain sensations. Another study revealed that glial inhibitors reduce hypersensitivity in neuropathic male mice but not females [67]. Our present studies revealed that the analgesic effect of a novel H₃R antagonist/inverse agonist was correlated with the modulation of glial activation in male neuropathic mice. Further studies are needed to investigate the mechanism of this compound in neuropathic females.

5. Conclusion

The obtained results indicate that a novel H₃R antagonist/inverse agonist, E-98, has the potential to alleviate sciatic nerve injury-induced neuropathic pain. The results of our studies, which were provided by a series of *in vitro* and *ex vivo* experiments complemented with computation methods, improve the effectiveness and efficiency of a novel H₃R antagonist/inverse agonist. Our data demonstrated that E-98 has favorable pharmacological, pharmacokinetic, and toxicological profiles and might be considered a future pharmacological tool or drug candidate, especially since its structural analog, pitolisant (Wakix™), is already used in the clinic. Moreover, our immunofluorescence studies provide valuable data showing the co-localization of H₃R not only with neurons but also with glial cells, revealing its immunomodulatory role. According to our studies, the analgesic action of H₃R antagonist/inverse agonist might be partially mediated by modulation of spinal glia activation. This crucial study presents the initial evidence of a promising opportunity to explore the function of glial cells in treating neuropathic pain by focusing on H₃R.

CRedit authorship contribution statement

Maciej Degutis: Writing – original draft, Visualization, Investigation, Formal analysis, Data curation, Conceptualization. **Dorota Łażewska:** Writing – review & editing, Methodology, Investigation, Funding acquisition, Formal analysis. **Justyna Barut:** Investigation, Formal analysis. **Magdalena Białoń:** Writing – review & editing, Visualization, Investigation, Formal analysis. **Gniewomir Latacz:** Writing – review & editing, Visualization, Investigation, Formal analysis. **Katarzyna Szczepańska:** Writing – review & editing, Methodology, Investigation, Funding acquisition, Formal analysis. **Wojciech Pietruś:** Investigation. **Tobias Werner:** Investigation. **Tadeusz Karcz:** Investigation. **Holger Stark:** Writing – review & editing, Methodology, Conceptualization. **Grzegorz Kreiner:** Writing – review & editing. **Katarzyna Kieć-Kononowicz:** Writing – review & editing, Methodology, Conceptualization. **Katarzyna Starowicz:** Writing – review & editing. **Katarzyna Popiolek-Barczyk:** Writing – review & editing, Writing – original draft, Supervision, Project administration,

Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work the authors didn't used AI and AI-assisted technologies.

Declaration of Competing Interest

In accordance with the Elsevier policy on disclosure of conflicts of interest, the authors of the manuscript entitled *Histamine H₃ receptor blockade alleviates neuropathic pain through the regulation of glial cells activation*, declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. The authors confirm that all financial and non-financial relationships have been disclosed fully in this declaration.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2025.117850.

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