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Neuroprotective and anti-inflammatory activity of DAT inhibitor R-phenylpiracetam in experimental models of inflammation in male mice

Liga Zvejniece[1,*], Baiba Zvejniece[1, 2], Melita Videja[1, 3], Gundega Stelfa[1, 4], Edijs Vavers[1], Solveiga Grinberga[1], Baiba Svalbe[1] and Maija Dambrova[1, 3]

[1]Latvian Institute of Organic Synthesis, Riga, Latvia,

[2]Latvian University Faculty of Medicine, Riga, Latvia,

[3]Riga Stradins University, Riga, Latvia,

[4]Latvia University of Life Sciences and Technologies, Jelgava, Latvia

*Correspondence to: L Zvejniece, Latvian Institute of Organic Synthesis, 21 Aizkraukles Street, Riga, LV-1006, Latvia. E-mail: liga@farm.osi.lv

Running title R-phenylpiracetam possesses anti-inflammatory activity

Keywords Inflammation; R-phenylpiracetam; LPS-induced endotoxaemia model; Carrageenan-induced paw oedema; Formalin-induced paw licking test

Acknowledgments

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Abbreviations

R-PhP - R-phenylpiracetam (R-PhP, (4*R*)-2-(4-phenyl-2-oxopyrrolidin-1-yl)acetamide)

DAT - Dopamine transporter

LPS – lipopolysaccharide

I.p. - intraperitoneal

P.o. - peroral

TNF-α - tumour necrosis factor-α

IL-1β - interleukin 1 beta

iNOS - inducible nitric oxide synthase

DA - dopamine

S-PhP - S-phenylpiracetam

UPLC/MS/MS - ultra-performance liquid chromatography-tandem mass spectrometry

PCR - polymerase chain reaction

SEM - standard error of the mean

ANOVA - analysis of variance

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Abstract

R-phenylpiracetam (R-PhP, (4*R*)-2-(4-phenyl-2-oxopyrrolidin-1-yl)acetamide) is an optical isomer of phenotropil, a clinically used nootropic drug that improves the physical condition and cognition. Recently, R-PhP was shown to bind to the dopamine transporter (DAT). Since growing evidence suggests that dysfunction of the dopaminergic system is associated with persistent neuroinflammation, the aim of this study was to determine whether R-PhP, an inhibitor of DAT, has neuroprotective and anti-inflammatory effects in male mice.

The pharmacokinetic profiles of R-PhP in mouse plasma and its bioavailability in brain tissue were assessed. To study possible molecular mechanisms involved in the anti-inflammatory activity of R-PhP, target profiling was performed using radioligand binding and enzymatic activity assays. To clarify the neuroprotective and anti-inflammatory effects of R-PhP, we used a lipopolysaccharide (LPS)-induced endotoxaemia model characterized by reduced body temperature and overexpression of inflammatory genes in the brain. In addition, the antinociceptive and anti-inflammatory effects of R-PhP were tested using carrageenan-induced paw oedema and formalin-induced paw licking tests.

R-PhP (50 mg/kg) reached the brain tissue 15 min after intraperitoneal (i.p.) and peroral (p.o.) injections. The maximal concentration of R-PhP in the brain tissues was 28 µg/g and 18 µg/g tissue after i.p. and p.o. administration, respectively. In radioligand binding assays DAT was the only significant molecular target found for R-PhP. A single i.p. injection of R-PhP significantly attenuated the LPS-induced body temperature reduction and the overexpression of inflammatory genes, such as tumour necrosis factor-α (TNF-α), interleukin 1 beta (IL-1β) and inducible nitric oxide synthase (iNOS). Seven-day p.o. pretreatment with R-PhP dose-dependently reduced paw oedema and the antinociceptive response, as shown by the carrageenan-induced paw oedema test. In addition, R-PhP decreased the nociceptive response during the inflammatory phase in the formalin-induced paw licking test.

Our study showed that R-PhP possesses neuroprotective and anti-inflammatory effects, demonstrating the potential of DAT inhibitors as effective therapeutics.

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Introduction

R-PhP is the optically pure enantiomer of the clinically used drug phenotopil ((R,S)-2-(2-oxo-4-phenylpyrrolidin-1-yl)acetamide), also known as carphedon, phenylpiracetam. At the end of 90ies phenotropil was included into the list of the banned substances by the International Olympic Committee due to concerns that it could be abused as a performance enhancer by athletes (Kim et al., 1999). Despite this, phenotropil is clinically used for more than 30 years as a nootropic drug that improves physical condition, mood and cognition (Malykh and Sadaie, 2010). Phenotropil induces stimulatory effects: increased concentration,attention performance (both physical and mental) and boosted motivation. Phenotropil is available as a prescription drug in Belarus, Kazakhstan, Russia and Ukraine. It has been shown that phenotropil helps to restore neurological function and daily living activities after stroke (Koval'chuk et al., 2010), it is beneficial to people who develop cognitive deficits and/or depression after encephalopathy and brain injuries (Savchenko et al., 2005) and it could be used to treat asthenia and chronic fatigue syndromes (Sazanov et al., 2006). A most common side effect of phenotopil is insomnia, while less observed are impulsivity, irritability, and anxious mood. In our previous experiments, we showed that the antidepressant effects and the increased locomotor activity induced by phenotropil were observed for both isomers and were more pronounced with R-PhP (Zvejniece et al, 2011). R-PhP inhibits dopamine (DA) uptake and reuptake (Sommer et al., 2014), and R-PhP is 3.5 times more active than S-phenylpiracetam (S-PhP) in *in vitro* assays of DAT binding (Zvejniece et al., 2017).

DA neurotransmission in the central nervous system is implicated in the regulation of motor activity, learning, stress responsiveness, reward and motivational state, while dysfunction of DA signalling contributes to various psychiatric and neurological disorders (Giros and Caron, 1993; Berk et al., 2007). Pharmacological blockade of DAT results in an increase in extracellular DA levels, which augments DA receptor stimulation (Horn, 1990; Moron et al., 2003). In recent years, increasing evidence has shown that inflammation triggers dopaminergic dysfunction and vice versa. Inflammatory cytokines were reported to increase the expression and function of the reuptake transporters for DA, norepinephrine and serotonin, thus increasing the intracellular monoamine concentration (Miller et al., 2013; Miller and Raison, 2016), and treatment with the cytokine interferon-alpha induces depression in humans (Capuron and Miller, 2004). Phenotropil possesses not only nootropic and mood-enhancing effects but also immunomodulatory and neuroregenerative activity (Samotrueva et al., 2011; Tyurenkov et al., 2015; Koval'chuk et al., 2010). Treatment with phenotropil at a dose of 25 mg/kg prevented the development of anxiety, fear, and sluggishness in rats that were treated with 100 μg/kg LPS (Samotrueva et al., 2011). Moreover, phenotropil attenuated the LPS- and cyclophosphamide-induced increases in proinflammatory cytokines in rats (Tyurenkov et al., 2015).

These data prompted us to focus on models of inflammation to test whether R-PhP had neuroprotective and anti-inflammatory effects. Therefore, we detected the bioavailability of R-PhP in brain tissue and performed target profiling using radioligand binding and enzyme assays. An LPS-induced endotoxaemia model to determine whether R-PhP can inhibit the decreased body temperature and inflammatory gene overexpression in the brain was

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conducted. In addition, formalin-induced paw licking and carrageenan-induced paw oedema tests were used to assess local inflammation. S-PhP was used to gain insight into the DAT-related activity of phenylpiracetam isomers.

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Materials and Methods

Ethics statement

The experimental procedures were performed in accordance with the guidelines reported by the EU Directive 2010/63/EU and in accordance with the local laws and policies; all of the procedures were approved by the Latvian Animal Protection Ethical Committee of Food and Veterinary Service of Riga, Latvia. All studies involving animals are reported in accordance with the ARRIVE guidelines (Kilkenny et al., 2010; McGrath et al., 2010).

Animals

Two hundred and forty-six ICR mice (Laboratory Animal Breeding Facility, Riga Stradins University, Latvia) aged 8-10 weeks and weighing 23–25 g were used. All animals were housed under standard conditions (21-23 °C, 12 h light-dark cycle) with unlimited access to standard food (Lactamin AB, Mjölby, Sweden) and water in individually ventilated cage housing system (Allentown Inc., Allentown, New Jersey, USA). Each cage contained bedding of EcoPure ™ Shavings wood chips (Datesand, Cheshire, UK), nesting material and wooden block from TAPVEI (TAPVEI, Paekna, Estonia). For the enrichment transparent tinted (red) non-toxic durable polycarbonate safe harbor mouse retreat (Animalab, Poznan, Poland) was used. The mice were housed with up to 5 mice per standard cage (38 x 19 x 13 cm).

Doses of R-PhP were selected based on our previous results, where it was shown that R-phenotropil at doses of 10 and 50 mg/kg can significantly increase locomotor activity (Zvejniece et al., 2011). All mice were randomly allocated to the treatment groups. The investigators performing the procedures and behavioural testing were blinded to the study group assignment. Animals were excluded from the study if carrageenan or formalin leaked out of the hind paw after intraplantar injection.

Chemicals

R-PhP and S-PhP were obtained from Olainfarm (Olaine, Latvia). Acetonitrile and methanol (HPLC grade) were purchased from Merck (Darmstadt, Germany), and 98% formic acid (LC/MS grade) was obtained from Fluka (Buchs, Switzerland). Physiological saline (0.9%) was purchased from Fresenius Kabi (Warszawa, Poland). Lambda carrageenan, indomethacin and LPS from *Escherichia coli* 055:B5 were purchased from Sigma-Aldrich (Steinheim, Germany). Formaldehyde (ACS reagent, 37 wt% sol., stab. 10-15% methanol) was obtained from Acros Organic (Geel, Belgium).

Experimental procedures

Determination of R-PhP in the plasma and brain tissue after p.o. and i.p. administration

The concentrations of R-PhP in the brain tissue extracts and plasma were measured by ultra-performance liquid chromatography-tandem mass spectrometry (UPLC/MS/MS). To determine the concentration of R-PhP in the plasma and brain, mice received an i.p. and p.o. administration of R-PhP at a dose of 50 mg/kg 15 and 30 min and 1, 2, 4, 6 and 24 h before the plasma and brain tissue collection.

The animals were decapitated, and the blood and brain samples were collected. Blood was collected in heparin-coated tubes after mouse decapitation and centrifuged at 3000 rpm at 4 ºC for 10 minutes to separate the plasma. Each group consisted of 4 animals (56 mice in total). The brain tissue was gently removed and divided into the right

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and left hemispheres; thus, two samples were obtained from one animal. Then, the brain tissues were homogenized with a Cole Parmer 130-Watt ultrasonic processor set at 35 kHz for 35 s each in ice-cold Milli-Q water at a w/v ratio of 1:5. The obtained homogenate was centrifuged at 16500 rpm for 10 min at 4 ºC. The supernatant was then decanted, but the pellet was homogenized in the same volume of Milli-Q water as before. The obtained homogenate was centrifuged at 16500 rpm for 10 min at 4 ºC. The supernatants were combined and stored frozen (-80 ºC) until analysis.

Sample preparation was performed by deproteinization with an acetonitrile/formic acid mixture. Brain tissue extract or blood plasma sample (100 µl) was mixed with 500 µl of 0.1% formic acid solution in acetonitrile (v/v), vortexed and centrifuged at 10000 rpm for 20 min. The supernatant was transferred to UPLC vials and used for UPLC/MS/MS analysis. UPLC was carried out using a Waters Acquity UPLC system equipped with an Acquity BEH Shield RP18 column (2.1 x 100 mm, 1.7 µm). MassLynx 4.1. software with a QuanLynx 4.1. module (Waters, Milford, USA) was used for data acquisition and processing.

Radioligand binding assays

R-PhP was profiled in a commercially available panel of 103 radioligand-binding assays on several G-protein coupled receptors, ion channels and transporters and 30 enzymes (Eirofins, Poitiers, France). A specific list of the assays performed including details regarding the methods used to conduct each assay is available at https://www.eurofinsdiscoveryservices.com/. Results showing an inhibition (or stimulation for assays run in basal conditions) higher than 50% are considered to represent significant effects of the test compounds.

LPS-induced inflammation in mice

Six hours before brain tissue sampling, inflammatory gene expression was stimulated by a single i.p. injection of LPS (20 mg/kg). The control animals received an i.p. injection of saline (0.9% NaCl). R-PhP and S-PhP at a dose of 50 mg/kg or saline (0.9% NaCl) were injected i.p. simultaneously with LPS. The compounds and LPS were injected in the opposite sides. The rectal temperature was measured before and 6 h after the LPS injection using a thermometer (Thermalert TH-5, USA).

Quantitative polymerase chain reaction (PCR) analysis

The brain tissue samples were immediately frozen in liquid nitrogen and stored at -80 °C. Total RNA from the brain tissue was isolated using TRI Reagent (Sigma, USA) according to the manufacturer's protocol. The samples were diluted with water at a v/v ratio of 1:20, and the quality and quantity of the extracted total RNA were examined by measuring the absorbance at 230, 260 and 280 nm with a µQuant™ (BioTek) spectrophotometer. The samples were diluted with water to reach a concentration of 0.5 µg/ml, and first-strand cDNA was synthesized using a High Capacity cDNA Reverse Transcription Kit (Applied BiosystemsTM, USA) following the manufacturer's instructions. Quantitative PCR analysis of interleukin 1 beta (IL-1β), tumour necrosis factor-α (TNF-α) and inducible nitric oxide synthase (iNOS) was performed by mixing synthesized cDNA (diluted with water at v/v ratio of 1:10), appropriate primers, and SYBR® Green Master Mix (Applied BiosystemsTM, USA) and run in an Applied Biosystems Prism 7500 system according to the manufacturer's protocol. The transcript levels of the constitutive housekeeping gene product β-actin were quantitatively measured for each sample, and the PCR data are reported as the number of transcripts per number of β-actin mRNA molecules. The primer sequences used in this study were as

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follows: IL-1β (NM_008361.4), 5'-GGG CCT CAA AGG AAA GAA TC-3' (forward) and 5'-TTG CTT GGG ATC CAC ACT CT-3' (reverse); TNF-α (NM_013693.3), 5'-CCC TCA CAC TCA GAT CAT CTT CT-3' (forward) and 5'-GCT ACG ACG TGG GCT ACA G-3' (reverse); iNOS (NM_010927.4), 5'-GTT CTC AGC CCA ACA ATA CAA GA-3' (forward) and 5'-GTG GAC GGG TCG ATG TCA C-3' (reverse).

Carrageenan test

Mouse paw oedema was induced by intraplantar injection of carrageenan using a previously described method (Posadas et al., 2004) with some modifications. Before the test, all of the animals received p.o. administration of saline, R-PhP (10, 25 and 50 mg/kg) or indomethacin (10 mg/kg) for 7 days. In the second setup of carrageenan test, we compared the anti-inflammatory effects of both isomers of PhP. R-PhP and S-PhP were administered via a p.o. injection at a dose of 50 mg/kg for 7 days. On day 7, the animals received saline or the drug 60 min prior to injection of carrageenan. Each group of animals received intraplantar (right paw) administration of 40 µl of saline or 40 µl of 2% carrageenan (w/v) in saline. A 2% carrageenan solution was prepared 24 h before use. The volume was measured by using a plethysmometer (IITC's Paw Volume Meter, IITC Life Science, California, USA) before intraplantar injection (Vo) and 2, 4, 6 and 24 h thereafter (Vt). The right hind paw was marked to ensure it was immersed to the same extent in the measurement chamber, and the paw volume was always measured by the same experimenter blinded to the treatment groups. The percentage increase in paw volume was calculated by using the following formula: paw oedema (%) = ((Vt – Vo)/Vo)*100).

Electronic von Frey test

Mechanical allodynia was assessed in the mice by measuring the withdrawal threshold of the right hind paw, where the carrageenan was injected, in response to a mechanical stimulus using an electronic von Frey anaesthesiometer (model 2391C; IITC Life Science, Inc., Woodland Hills, CA, USA) (Hara et al., 2014). The test was assessed in the mice 7 h after carrageenan injection. During the tests, the mice were placed on a metallic grid floor in an individual plastic observation chamber and allowed to acclimate to the environment for 10 min prior to session. The von Frey filament was applied to the midplantar surface of the right hind paw. The withdrawal threshold was defined as the average force (g) required to cause withdrawal of the stimulated paw over three trials.

Formalin-induced paw-licking test

The test was performed as described previously (Zvejniece et al., 2015). Before the test, all of the animals received p.o. administration of saline (control), R-PhP (10 and 50 mg/kg) or S-PhP (50 mg/kg) for 7 days. On day 7, the animals received saline or the drug 60 min prior to injection of formalin. The mice were gently restrained, and 30 µl of formalin solution (1.5% in saline) was injected intraplantarly into the right hind paw using a microsyringe with a 27-gauge needle. Each mouse was then placed in an individual clear Plexiglas observation chamber (42 x 18 x 26 cm), and the total licking time of the hind paw of each mouse was registered with a stopwatch and quantified in subsequent 5-min intervals for 60 min. Recording of the time spent licking started immediately (the first phase) and lasted for 5 min. The late phase (the second phase) started approximately 15–20 min after formalin injection and lasted up to 35–40 min.

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Statistical analysis

All results are expressed as the mean ± standard error of the mean (SEM). The Shapiro-Wilk test was used to examine the distribution of the data. Statistical analysis was evaluated using repeated measures two-way or one-way analysis of variance (ANOVA) followed by Tukey's and Dunnett's tests, respectively. The Kruskal-Wallis test followed by Dunn's test was used for non-normally distributed data sets. A post hoc test was performed if ANOVA or the Kruskal-Wallis test indicated significant differences. The statistical calculations were performed using the GraphPad Prism software package (GraphPad Software, Inc., La Jolla, California, USA). In all cases, $p < 0.05$ was considered to be statistically significant.

Sample size calculations were based on the effects of R-PhP in LPS-induced inflammation model in mice. It was concluded that both R-PhP and S-PhP demonstrate very-large anti-inflammatory effect (the calculated Cohen's d value was 2.5 and 1.9, respectively). Through a power calculation (using G-power software) for a two-way ANOVA test (five group comparison, 5 measurements per group (0, 2, 4, 6 and 24 h)) with $\alpha = 0.05$, a power of 80 %, and a standardized effect size Cohen's $d = 0.7$, a total sample size of 10 mice per group for carrageenan test was deemed sufficient. The same numbers of animals per group were used also in the formalin-induced paw-licking test.

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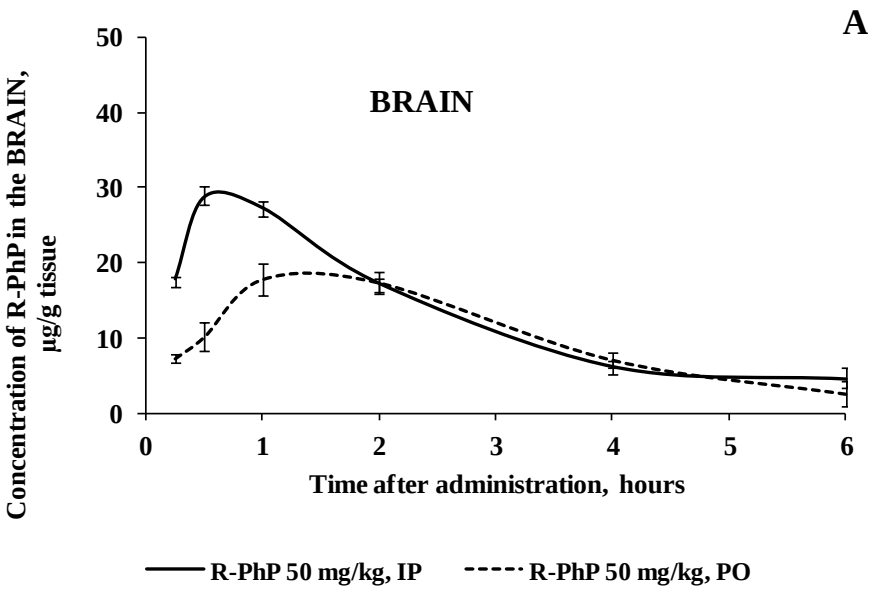
Results

R-PhP crosses the blood-brain barrier

R-PhP in the brain tissue extracts was detected 15 min after a single p.o. and i.p. injection (Fig. 1A, B). The maximal concentrations of R-PhP in the brain tissues were observed 30 - 60 min after i.p. injection and 60 - 120 min after p.o. administration. The maximal concentrations of R-PhP in the brain tissues were 28 μg/g and 18 μg/g tissue after the i.p. and p.o. injections, respectively (Fig. 1A). R-PhP in the brain tissues was not detected 24 h after both the p.o. and i.p. injections.

As shown in Fig. 1B, R-PhP in plasma could be detected 15 min after a single p.o. and i.p. injection. The maximal concentrations of R-PhP in the plasma were observed 15-30 min after the i.p. injection and 60 min after the p.o. administration (Fig. 1B). The maximal concentration of R-PhP in the plasma after the i.p. injection was 45 μg/ml; at the same time, the maximal concentration of R-PhP in the plasma after the p.o. injection was 24 μg/ml (see Fig. 1A). R-PhP in the plasma was 0.05 μg/ml at 24 h after the p.o. and i.p. injections.



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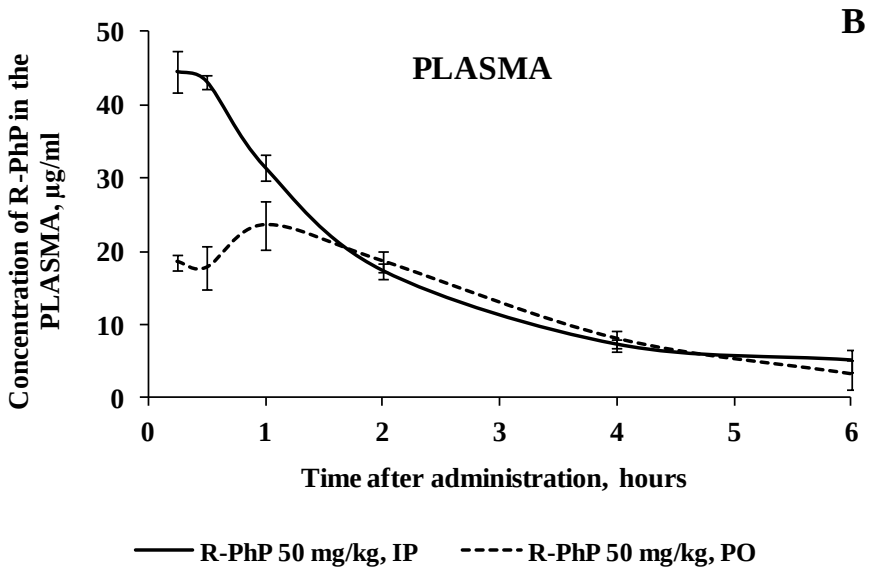

Fig. 1 The concentration of R-PhP in the mouse brain tissue and plasma after a single administration. Mice received an i.p. and p.o. injection of R-PhP at a dose of 50 mg/kg. The amount of compound in the brain tissue extracts **(A)** and plasma **(B)** was measured 15 and 30 min and 1, 2, 4 and 6 h after R-PhP administration. Values are represented as the mean ± SEM.

Radioligand binding data

Radioligand binding experiments were performed to determine possible molecular targets that could be involved in anti-inflammatory effects of R-PhP. R-PhP at a concentration of 10 µM showed significant competition with dopamine reuptake inhibitor BTCP for binding to DAT and R-PhP demonstrated 61 % inhibition of [^{3}H]BTCP binding (Supplementary Table 1). Competitive binding of R-PhP to other investigated molecular targets demonstrated non-significant activity, which was presented as inhibition value lower than 50 % (Supplementary Table 1).

R-PhP inhibits the LPS-induced hypothermia and inflammatory gene overexpression

The body temperature in all experimental groups was similar before LPS injection (data not shown). The body temperature was significantly decreased 6 h after LPS injection compared with that of the saline-treated animals, and pretreatment with R-PhP and S-PhP significantly restored the LPS-induced decrease in body temperature (Fig. 2).

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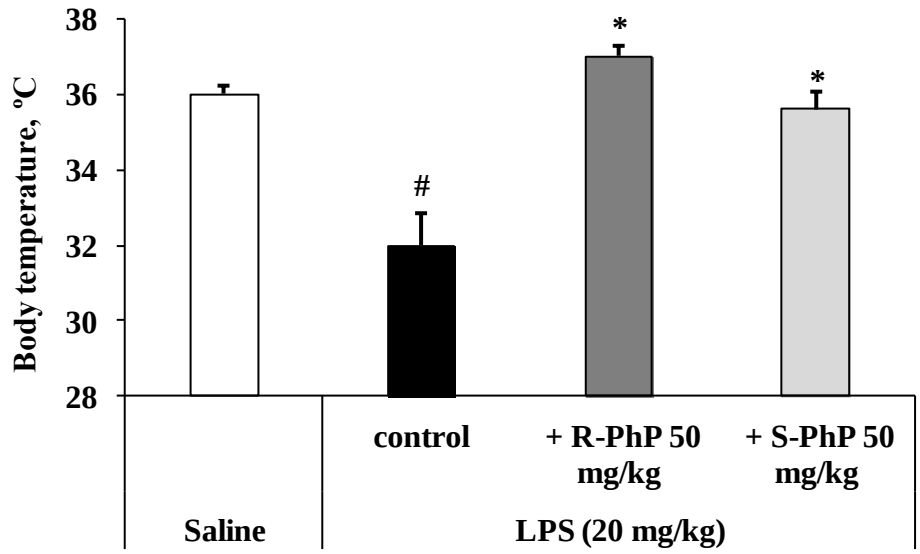

Fig. 2 Effects of R-PhP and S-PhP on the LPS-induced decrease in body temperature. The temperature was measured 6 h after LPS (20 mg/kg) i.p. administration. R-PhP and S-PhP at 50 mg/kg were injected (i.p.) simultaneously with LPS. Data are expressed as the mean ± SEM (n = 10). $^{\#}p$ <0.05 saline control vs. the LPS control group, * p <0.05 vs. the LPS control group (one-way ANOVA followed by Dunnett's test).

As shown in Fig. 3, i.p. administration of LPS significantly increased the expression of the Il-1β, TNF-α and iNOS genes by approximately 8-, 19- and 12-fold, respectively. Pretreatment with R-PhP decreased the Il-1β, TNF-α and iNOS gene levels by 75%, 73% and 65%, respectively. The inflammation-related gene expression in the R-PhP group was not significantly different from that in the saline control group. Pretreatment with S-PhP resulted in slightly reduced Il-1β and TNF-α gene expression compared to that of the LPS control group, but gene expression in the S-PhP group was significantly higher than that in the saline-treated animals. There was a significant difference between the R-PhP- and S-PhP-treated animals in LPS-induced iNOS gene overexpression (Fig. 3).

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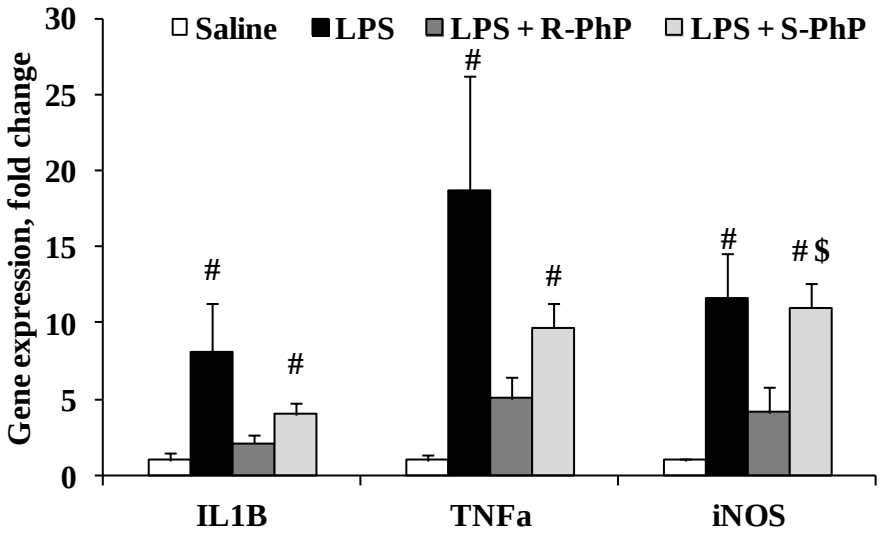

Fig. 3 Quantitative RT-PCR analysis of inflammatory gene expression in the brain tissue. LPS (20 mg/kg) was administered (i.p.) simultaneously with R-PhP (50 mg/kg), S-PhP (50 mg/kg) or saline 6 h before brain tissue sampling. Saline i.p. injection was used as a control. Data are expressed as the mean ± SEM (n = 10). $^{\#}p < 0.05$ vs. the saline control, $^{\$}p < 0.05$ the R-PhP group vs. the S-PhP group (Kruskal-Wallis followed by Dunn's test).

R-PhP attenuates carrageenan-induced oedema formation and mechanical sensitivity

The inflammation of the paw was indicated by an increase in paw size in mice due to oedema caused by subcutaneous injection of carrageenan. The carrageenan injection significantly increased paw oedema in a time-dependent manner compared with that of the saline group (Fig. 4A, $p < 0.001$). Two-way repeated-measures ANOVA showed that the compounds time-dependently attenuated the carrageenan-induced oedema formation in the mice (Fig. 4A, main effects of time ($F(4,208) = 97.72$, $p < 0.0001$) and group ($F(5,52) = 17.73$, $p < 0.0001$) and interaction between group and time ($F(20, 208) = 6.149$, $p < 0.0001$)). R-PhP at a dose of 50 mg/kg significantly reduced the paw oedema 2, 4 and 6 h after the carrageenan injection by approximately 60%, 40% and 45%, respectively, compared with that in the carrageenan group (Fig. 4, $p < 0.05$). At doses of 10 and 25 mg/kg, R-PhP did not reduce the paw volume. Indomethacin at a dose of 10 mg/kg significantly reduced the paw oedema 4 and 6 h after the carrageenan injection by approximately 36% and 45%, respectively, compared with that of the carrageenan group (Fig. 4, $p < 0.05$). As demonstrated by the data expressed as areas under the curves (AUCs, from 2 to 6 h) (Fig. 4B), R-PhP (50 mg/kg) and indomethacin (10 mg/kg) significantly attenuated the carrageenan-induced oedema formation ($F_{(5,52)} = 12.91$, $p < 0.0001$) in the mouse paw.

The effective dose (ED_{50}) for the anti-inflammatory activity of R-PhP was calculated from AUC values. The ED_{50} of R-PhP was 37 mg/kg to reduce the inflammation of the paw.

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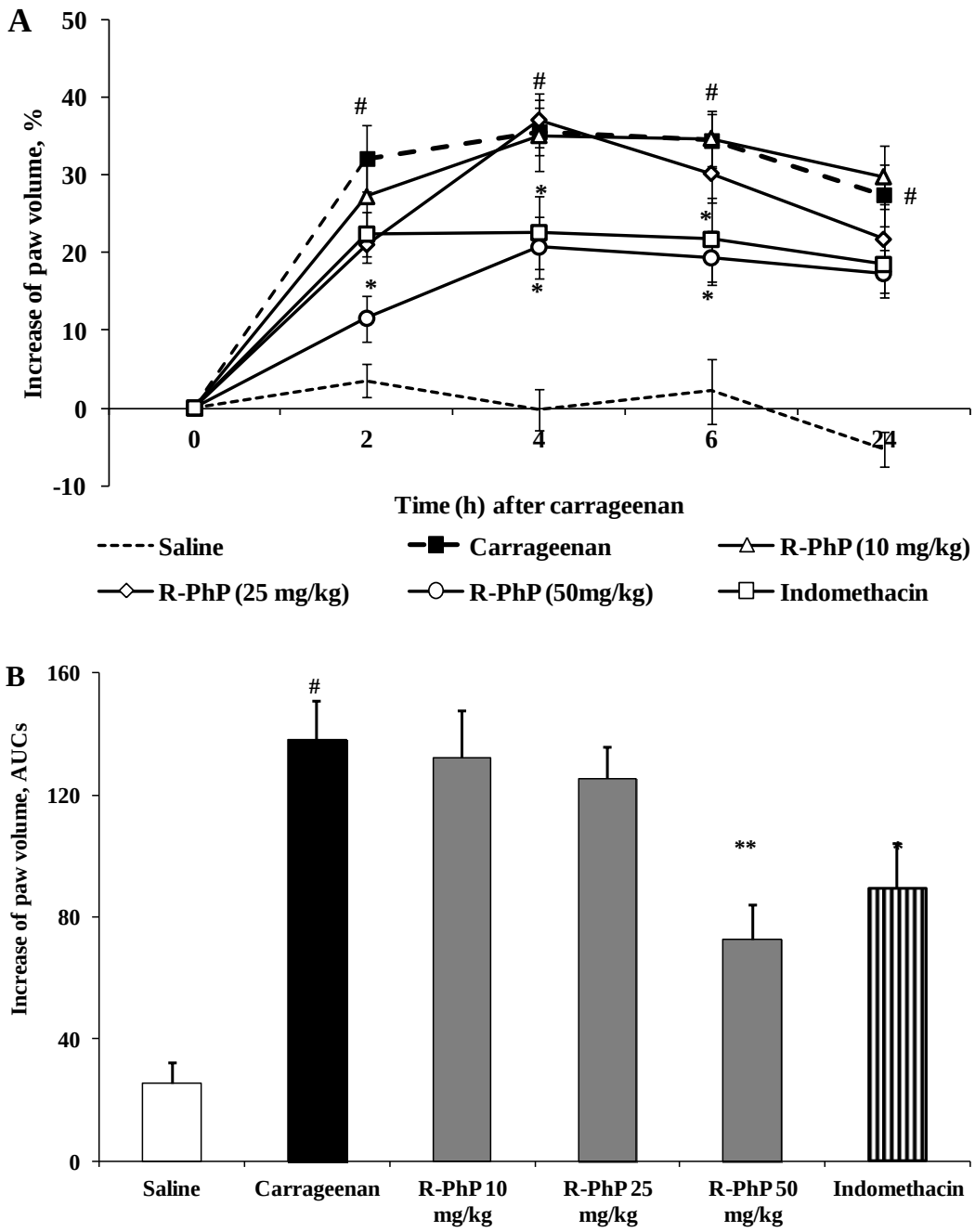

Fig. 4 The effects of R-PhP on the carrageenan-induced mouse paw oedema. The paw volume was measured before and 2, 4, 6 and 24 h after the carrageenan injection. R-PhP was administered (p.o.) for 7 days at doses of 10; 25 and 50 mg/kg and on the day of the experiment 60 min before the test. Indomethacin was administered at a dose of 10 mg/kg. Carrageenan (2%; 40 µL) was injected into the plantar surfaces of the right hind paw of the mice. (**A**) The increase in paw volume during the 24 h period. $^{\#}p < 0.05$ the saline group vs the carrageenan group, $^{*}p < 0.05$ vs. the carrageenan group (two-way ANOVA followed by Tukey's test). (**B**) The AUC shows an increase in the paw oedema during the 2-6 h interval. Data are expressed as the mean ± SEM (n = 9-10). $^{*}p < 0.05$ vs. the carrageenan group, $^{**}p < 0.01$ vs. the carrageenan group (one-way ANOVA followed by Dunnett's test).

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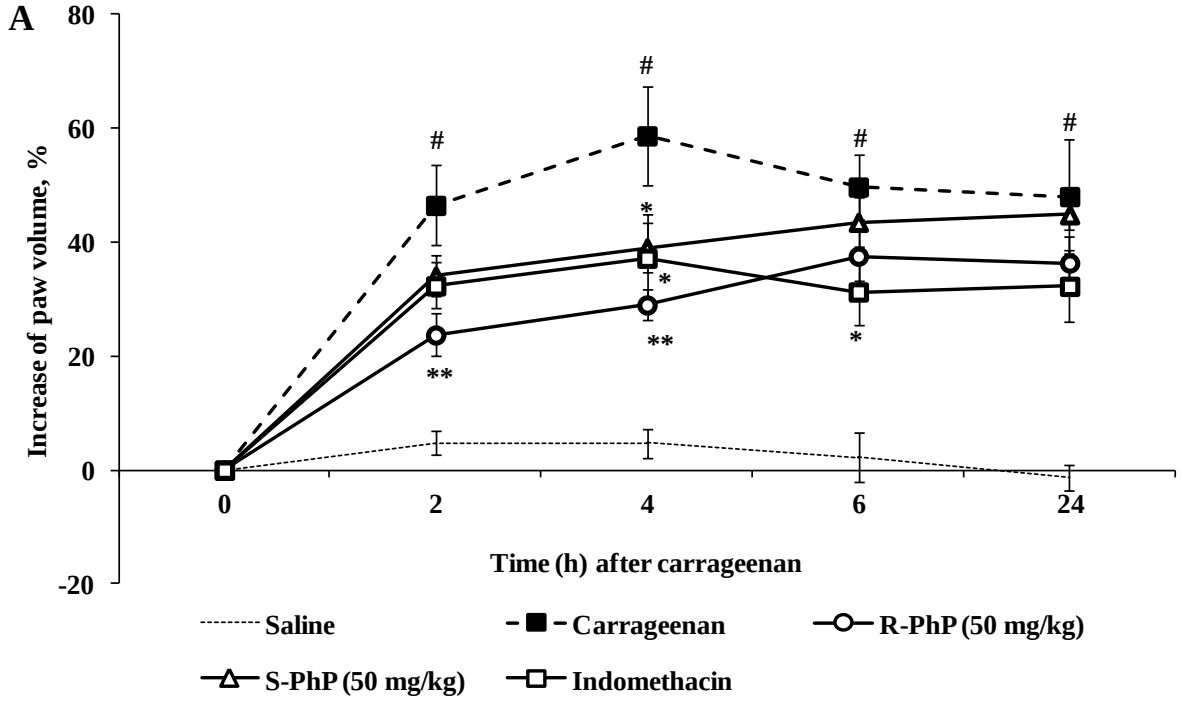
Two-way repeated-measures ANOVA showed that chronic administration of R-PhP and S-PhP at 50 mg/kg and indomethacin reduced the carrageenan-induced oedema formation in the mice (Fig. 5A, main effects of time ($F_{(4,176)}$ = 98.13, $p < 0.0001$) and group ($F_{(4,44)}$ = 16.34, $p < 0.0001$) and interaction between group and time ($F_{(16, 176)}$ = 7.573, $p < 0.0001$)). Pretreatment with R-PhP significantly decreased the paw oedema 2 and 4 h after the carrageenan injection by approximately 50%, that with S-PhP significantly decreased the paw oedema 4 h after the carrageenan injection by 34% and that with indomethacin significantly decreased the paw oedema 4 and 6 h after the carrageenan injection by 37% (Fig. 5A, $p < 0.05$). As demonstrated by the data expressed as the AUC from 2 to 6 h (Fig. 5B), R-PhP and indomethacin significantly attenuated the carrageenan-induced oedema formation ($F_{(3,35)}$ = 3.894, $p < 0.05$) in the mice. Pretreatment with S-PhP slightly reduced the carrageenan-induced oedema formation during the 2-6 h interval, as shown by the AUCs (Fig. 5B), but the effect was not significant.

The electronic von Frey filament test was used for the determination of the mechanical sensitivity threshold in the rodents. The mice showed a significant reduction in the withdrawal threshold 7 h after the carrageenan injection ($F_{(4,43)}$ = 17.17, $p < 0.001$) compared with an intraplantar injection of saline (Fig. 5C). The p.o. administration of R-PhP (50 mg/kg) and indomethacin (10 mg/kg) 60 min before carrageenan injection significantly reduced the carrageenan-induced mechanical sensitivity by 35% and 40%, respectively (Fig. 5C, $p < 0.05$). S-PhP at a dose of 50 mg/kg did not affect the carrageenan-induced mechanical sensitivity.



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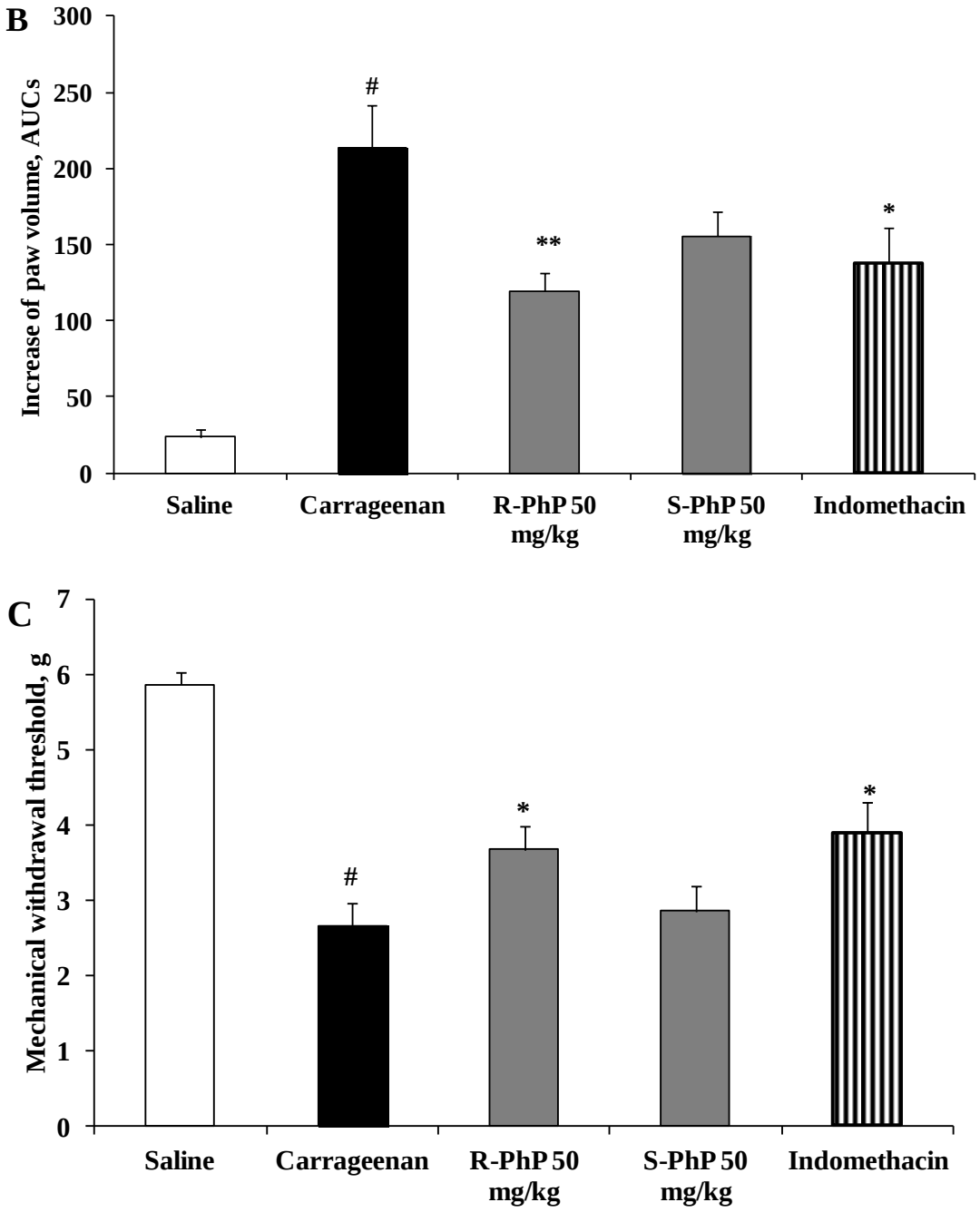

Fig. 5 The effects of R-PhP and S-PhP on the carrageenan-induced mouse paw oedema. The paw volume was measured before and 2, 4, 6 and 24 h after the carrageenan injection. Indomethacin was administered at a dose of 10 mg/kg. (**A**) The increase in paw volume during the 24 h period. $^{\#}p$ <0.05 the saline group vs the carrageenan group, $*p < 0.05$ vs. the carrageenan group, $**p < 0.01$ vs. the carrageenan group (two-way ANOVA followed by Tukey's test). (**B**) The AUC shows an increase in paw oedema during the 2-6 h interval. $*p < 0.05$ vs. the carrageenan group, $**p < 0.01$ vs. the carrageenan group (one-way ANOVA followed by Dunnett's test). (**C**) Mechanical sensitivity was assessed in the mice 7 h after the carrageenan injection using the electronic von Frey test. The data are

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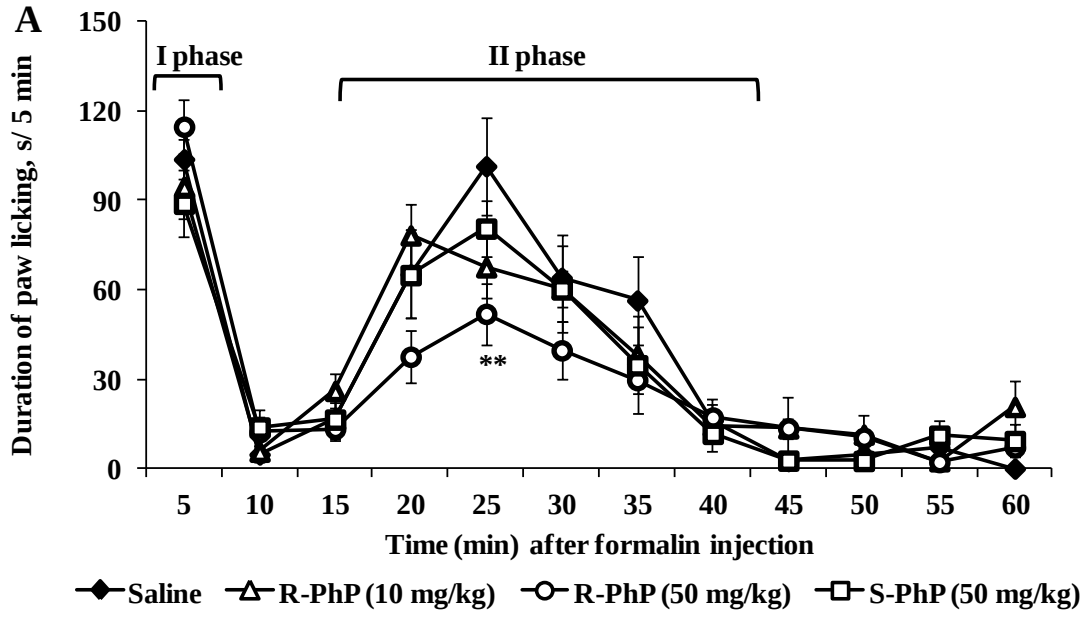
expressed as the threshold in grams. *$p < 0.05$ vs. the carrageenan group (one-way ANOVA followed by Dunnett's test). Data are expressed as the mean ± SEM (n = 9-10).

R-PhP reduces the inflammation-related pain behaviour in the formalin-induced paw licking test

The antinociceptive effects of R-PhP and S-PhP were studied in the formalin-induced paw licking test of the mice (Fig. 6A, B). As shown in Fig. 1, formalin injection in the saline-treated mice caused an acute, immediate nociceptive response, which included the licking and shaking of the injected paw and lasted for 5 min (the first phase). The second phase of nociceptive behaviour began at 15–20 min after the formalin injection in the saline-treated mice and lasted for an additional 35–40 min (Fig. 6A, B). Repeated measures ANOVA indicated that there was a time effect ($F_{(11, 330)} = 45.94$, $p < 0.0001$) and interaction between the group and time effect ($F_{(33, 330)} = 1.493$, $p < 0.05$) but not a group effect ($F_{(3, 30)} = 1.224$, $p > 0.05$). As shown in Fig. 6B, R-PhP (10 and 50 mg/kg, p.o.) dose-dependently reduced the formalin-induced nociceptive behaviour during the second phase. During this phase, R-PhP at a dose of 50 mg/kg significantly reduced the duration of paw licking by approximately 50% ($p < 0.05$). However, S-PhP at a dose of 50 mg/kg did not affect the duration of paw licking in the first and second phases (Fig. 6A, B).



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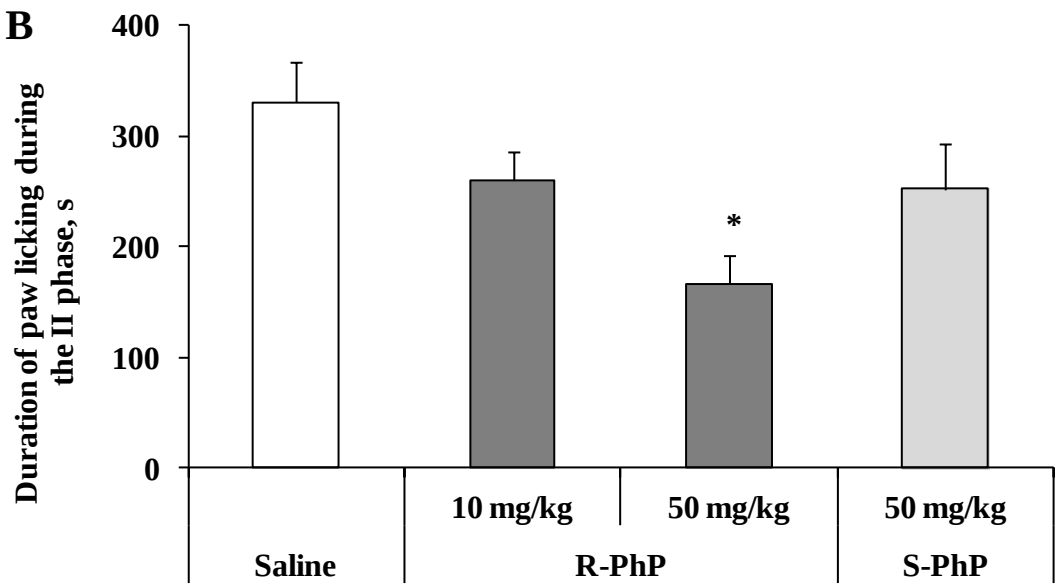

Fig. 6 Effects of R-PhP and S-PhP on the antinociceptive behaviours in the formalin-induced paw licking test of the mice. R-PhP and S-PhP were administered (p.o.) for 7 days and on the day of the experiment 60 min before the test. Formalin (1.5%; 30 μL) was injected s.c. into the plantar surfaces of the right hind paws of the mice. **(A)** Licking of the injected paw was recorded as the total time (s) spent licking within each of twelve 5-min segments, for a total duration of 60 min. $^{**}p < 0.01$ vs. the saline group (two-way ANOVA followed by Tukey's test). **(B)** The II phase was from 15 to 40 min after the injection of formalin. The data are expressed as the mean ± SEM (n = 8-10). $^{*}p < 0.05$ vs. the saline group (one-way ANOVA followed by Dunnett's test).

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Discussion

We investigated the neuroprotective and anti-inflammatory effects of the DAT inhibitor R-PhP using LPS-induced endotoxaemia, carrageenan-induced paw oedema and formalin-induced paw licking models. In the LPS-induced endotoxaemia model, a single R-PhP injection at a dose of 50 mg/kg significantly inhibited the LPS-induced decrease in body temperature and IL-1β, TNF-α and iNOS gene overexpression. Our results demonstrated that chronic p.o. pretreatment with R-PhP dose-dependently reduced the paw oedema and mechanical sensitivity induced by carrageenan. Moreover, R-PhP reduced the formalin-induced nociceptive behaviour during the inflammatory phase. S-PhP, a weaker DAT inhibitor than R-PhP, at the same doses attenuated the LPS-induced body temperature reduction but not the LPS-induced inflammatory gene expression. In addition, S-PhP reduced the carrageenan-induced paw oedema in the first 6 h but did not affect the carrageenan-induced mechanical sensitivity, thus exhibiting similar but weaker neuroprotective and anti-inflammatory effects compared to R-PhP. The only significant molecular target of R-PhP in target profiling screening was DAT. Our results indicate that the binding activity of R-PhP and S-PhP to DAT correlates with their neuroprotective and anti-inflammatory activity.

For the first time, we showed that reasonable amount of R-PhP could be detected in the brain 15 min after a single p.o. administration while and about 20 % of maximal concentration (estimated brain concentration about at 10 μM) was present in the brain even after 6 hours. The target profiling screening pointed at DAT as the only significant molecular target for R-PhP at 10 μM was DAT, which is consistent with previously published results that R-PhP binds to DAT with 16 μM calculated Ki value (Zvejniece at al., 2017). Taken together, R-PhP at a dose of 50 mg/kg can reach brain tissue in concentrations, that are sufficient to target DAT even 6 hours after administration.

Previous studies showed that treatment with phenotropil restored the normal levels of IL-1β and IL-6 and increased the level of interleukin-4 after immune system stimulation with LPS (100 mg/kg) in rats (Tyurenkov et al., 2015). In the present study we found that R-PhP simultaneously administered with LPS (20 mg/kg) possess anti-inflammatory effect and restores LPS-induced body temperature reduction in mice. In addition, pretreatment with R-PhP impeded the LPS-induced increase in Il-1β, TNF-α and iNOS gene expression levels in mice brain, however, no direct effect of R-PhP on TNF-α or iNOS was observed in the target profiling assays. In comparison to R-PhP, the activity of S-PhP was less pronounced in LPS-induced inflammation test, and it correlated with the lower binding activity to DAT. The role of DAT in the immune system and inflammatory processes has been increasingly recognized (Mackie et al., 2018; Felger and Miller, 2012). For example, DAT is highly expressed in lymphocytes and human monocyte-derived macrophages (Arreola et al., 2016). It has been shown how dysfunctional DAT in brain can alter peripheral DAT and immune function (Mackie et al., 2018). A significant link between peripheral immune response and CNS disorders also has been demonstrated previously. Administration of cytokines or agents (e.g., endotoxin or LPS) that induce the proinflammatory cytokine cascade were shown to cause the syndrome of sickness behaviour, including psychomotor slowing, cognitive dysfunction, anhedonia and depressive-like behaviour (Frenois et al., 2007). Furthermore, patients with major depression were shown to exhibit increased biomarkers of inflammation, and psychosocial stressors were reported to activate innate immune signalling, such as nuclear factor kappa B (NF-κB) and interleukin-6 (Capuron and Miller, 2004; Bierhaus et al., 2003). For example, R-PhP was shown to possess

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antidepressant, increased locomotor and memory-improving activity in naive animals (Zvejniece et al., 2011). In addition, the LPS-stimulated immune response manifested in the reduction of locomotor function, exploratory activities, orientation, and increased anxiety, and pretreatment with phenotropil (25 mg/kg) for 5 days exhibited an immunomodulatory effect: increased locomotion, exploratory behaviour and inhibition of increased fear behaviour (Samotrueva et al., 2011). To date, binding to DAT is the only significant molecular target of R-PhP, which can be associated with its anti-depressant, locomotor stimulating and anti-inflammatory activities.

The anti-inflammatory and antinociceptive activity of R-PhP was also tested in carrageenan-induced paw oedema and formalin-induced paw licking tests, which demonstrated significant dose-dependent effects of R-PhP on reduction of oedema formation and mitigation of pain behaviour. Also previously it was shown that DAT inhibitors such as cocaine and LPM580098 can reduce formalin-induced nociceptive behaviour in rodents (Lin et al., 1989; Li et al., 2019). Interestingly, it was demonstrated that antinociceptive effect of cocaine in formalin test could not be blocked with naloxone, and central effect on monoaminergic synaptic transmission, especially dopaminergic, was suggested to explain the activity of the drug (Lin et al., 1989). In addition, other centrally acting compounds have been shown to be effective in models of peripheral inflammation and nociception. For example, gabapentin and amitriptyline, which are clinically used for the treatment of epilepsy and depression, respectively, were shown to treat peripheral neuropathies and inflammation (Gustafsson et al., 2003; Sawynok et al., 2001). Gabapentin and amitriptyline have shown antinociceptive activity in the second (inflammatory) phase of the formalin-induced paw licking test (Gustafsson et al., 2003; Sawynok et al., 2001) and an anti-inflammatory effect in the carrageenan-induced paw oedema test (Kilic et al., 2018; Hajhashemi et al., 2010). Recently it was shown that dopamine neuron activation in ventrolateral periaqueductal gray was sufficient to inhibit the persistent nociception caused by carrageenan-induced inflammation (Taylor et al., 2019). This supports the evidence that anti-inflammatory activity of R-PhP could include central inhibition of DAT, which would result in increased dopamine level and subsequent dopamine receptor activation.

For the first time, we showed that DAT inhibitors could be used to treat peripheral neuropathies and inflammation. Thus, R-PhP is neuroprotective and reduces inflammation-related pain behaviour and carrageenan-induced oedema formation, thus demonstrating that inhibiting DAT function is a unique therapeutic strategy to suppress inflammation.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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